Conference
Responses to DNA damage:
from molecule to disease
Conference
Responses to DNA damage: from molecule to disease
Egmond aan Zee, The Netherlands, April 17-22, 2016

ABSTRACTS
of lectures and poster presentations
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MARIE CURIE ACTIONS

SEVENTH FRAMEWORK PROGRAMME

EMBO Young Investigator

EMBO excellence in life sciences

EACR European Association for Cancer Research

AstraZeneca

MISSION Therapeutics

ELSEVIER

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Keynote addresses
Sunday, April 17, 16:00 – 17:50 h
Mismatch repair (MMR) is a complex pathway of DNA metabolism that has evolved to increase the fidelity of DNA replication by removing misincorporated nucleotides from the newly-synthesized strand. The importance of this pathway to human health is highlighted by the fact that loss of mismatch repair, either through mutation or transcriptional silencing of MMR genes, predisposes to cancer of the colon, endometrium and ovary. However, it could be shown already many years ago that MMR proteins are involved also in the control of prokaryotic and eukaryotic recombination processes, and more recent findings implicate MMR proteins also in DNA damage signalling, somatic hypermutation and class switch recombination of immunoglobulin genes, as well as in the processing of oxidative DNA damage. In an attempt to understand how MMR proteins participate in such diverse pathways of DNA metabolism, we carried out high-throughput assays designed to identify their interacting partners (1, 2).

In a yeast genetic screen (1) we identified ELG1 (ATAD5 in human), an alternative large subunit of replication factor C. In proteomic screens (2) we identified the chromatin-assembly factor 1 (CAF1), FANCJ, a 5’ to 3’ DNA helicase and FAN1, a 5’ flap endonuclease. In this presentation, I shall focus on some of our most recent findings in the above research projects.


Plenary session  
Presenter: Maria Jasin  

Protecting the genome by homologous recombination: Role of the BRCA2 tumor suppressor  

Elizabeth Kass, Weiran Feng, Pei Xin Lim, Carla Abreu, Maria Jasin  
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Mutations in multiple genes involved in homology-directed repair (HDR), considered a particularly accurate pathway for repairing DNA double-strand breaks (DSBs), are linked to breast cancer susceptibility, including BRCA1 and BRCA2. The mammary gland undergoes significant proliferation after birth, but little is known about how developmental changes impact DSB repair. We generated DR-GFP mice that inducibly express I-SceI endonuclease to analyze HDR within primary cells and tissues. We observe that HDR is robust in mammary tissue during puberty and pregnancy and is higher than in other tissues analyzed, suggesting that hormone-dependent proliferative stages of mammary gland development have greater utilization of HDR for DSB repair. Brca2 mutation led to HDR defects in mammary tissue during puberty and pregnancy, including in different epithelial lineages, which were similar in other proliferative tissues. Our results suggest that, rather than a tissue-specific defect in HDR per se, the greater reliance on HDR during mammary gland proliferation may magnify the effect of BRCA2 mutation, making the mammary epithelium more susceptible to tumorigenesis.  
In addition to its role in repairing DNA damage, we have also shown that HDR proteins like BRCA2 protect DNA from undergoing DNA damage. In particular, when replication forks are stalled by treating cells with hydroxyurea, BRCA2 protects nascent strands from degradation, at least in part by protecting from the Mre11 complex. We will discuss the relative contributions of BRCA2 deficiency in each of these two roles, i.e., protecting DNA from damage and repairing DNA damage once it occurs.
Plenary session: 'Replication Stress'
Monday, April 18, 9:00 – 12:40 h
Plenary session

Replication Stress

Presenter: Thomas Kunkel

Studies of Leading and Lagging Strand DNA Replication Fidelity in Yeast

*Thomas A. Kunkel*
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Coordinated replication of the two undamaged DNA strands of the eukaryotic nuclear genome is primarily catalyzed by DNA polymerases alpha, delta and epsilon. These replicases are related, but they differ in protein partnerships and physical and biochemical properties, including fidelity. This talk will focus on their roles in leading and lagging strand DNA replication fidelity in budding yeast. Emphasis will be on recent studies in which deep sequence analysis has been used to define the genome-wide rates at which leading and lagging strand replication errors are generated and corrected, and on the consequences of defects in these processes.
Plenary session

Replication Stress

Presenter: Helle Ulrich

Dealing with DNA damage during replication

Ronald Won, Néstor García Rodríguez, Helle D. Ulrich
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DNA is susceptible to a variety of insults from exogenous and endogenous sources. Dealing with damage during replication is particularly important because the replication machinery cannot cope with defective templates. In order to avoid a permanent arrest in this situation, cells have developed mechanisms of damage bypass. In contrast to DNA repair systems, which usually rely on the excision and subsequent re-synthesis of the damaged region to restore the original sequence information, DNA damage bypass mechanisms allow the replication machinery to tolerate lesions without their actual removal. They ensure the completion of DNA replication on damaged templates and are therefore essential for survival of a cell in the presence of genotoxic agents. As lesion bypass is often associated with damage-induced mutations, however, the pathway is also a potential source of genome instability in itself and therefore needs to be tightly controlled. In eukaryotic cells, DNA damage bypass is modulated via posttranslational modification of the essential replication factor PCNA with mono- and polyubiquitin. I will discuss our efforts to develop tools for the real-time monitoring of damage bypass events and their temporal and spatial regulation in vivo.
Accurate completion of replication relies on the ability of cells to activate error-free recombination-mediated DNA damage-bypass at sites of perturbed replication. However, as anti-recombinase activities are also recruited to replication forks, how recombination-mediated damage-bypass is enabled at replication stress sites remained puzzling. The most elucidated mechanism for counteracting replication-associated recombination is mediated by SUMOylated PCNA, a PCNA modification that occurs coincidently with replication. This modification acts to recruit the Srs2 anti-recombinase to replication forks and to suppress recombination. We uncovered that the conserved SUMO-like domains-containing "Saccharomyces cerevisiae" protein, Esc2, facilitates recombination-mediated DNA damage tolerance by allowing optimal recruitment of the Rad51 recombinase specifically at sites of perturbed replication. Mechanistically, Esc2 binds stalled replication forks and counteracts the anti-recombinase Srs2 helicase via a two-faceted mechanism involving chromatin recruitment and turnover of Srs2. Importantly, point mutations in the SUMO-like domains of Esc2 that reduce its interaction with Srs2 cause suboptimal levels of Rad51 recruitment at damaged replication forks. Our results reveal how recombination-mediated DNA damage tolerance is locally enabled at sites of replication stress, while globally prevented at undamaged replicating chromosomes.
Plenary session

Replication Stress

Presenter: Antony Carr

Mechanisms of replication-associated genome rearrangement

Izumi Miyabe, Yasukazu Daigaku, Andrea Keszthelyi, Johanne M. Murray, Antony M. Carr
Genome Damage and Stability Centre, School of Life Sciences, University of Sussex, Brighton, UK

When the progress of DNA replication is arrested, the replisome remains associated with the DNA fork structure (paused/stalled fork) and is protected by the ATR-dependent inter-S phase checkpoint. Stalled forks efficiently resume when the stress is relieved. However, if the replisome dissociates from the fork (collapsed fork), or the fork structure breaks (broken fork), replication restart can proceed by either homologous recombination (HR) or microhomology-primed re-initiation (FoSTeS/MMBIR).

Using the fission yeast model we examined the consequences of replicating a region of DNA with a fork that has been correctly restarted by HR. We identified a novel mechanism of chromosomal rearrangement: HR-restarted forks show an exceptionally high propensity to execute a U-turn at small inverted repeats. Such HR-restarted forks also demonstrate an increase in replication slippage at microhomology. Thus, HR-restarted replication forks are highly error prone. To investigate the nature of the HR-restarted replication machine we examined which DNA polymerases were used for DNA replication.

Using DNA polymerase mutants that incorporate excess ribonucleotides we have shown that, following replication restart by HR-dependent mechanisms within S phase, both strands are synthesised by polymerase delta. Density substitution experiments further demonstrated that, unlike BIR in S. cerevisiae, HR-restarted replication within S phase is semi-conservative. We have developed a protocol (Pu-Seq) to map the Polymerase usage of delta and epsilon genome-wide. Our data plot, at very high resolution, the location of the replication origins and identify regions of the genome where there is increased usage of polymerase delta to replicate the DNA duplex. Such sites may represent regions prone to genome instability.
Plenary session                                                                 Replication Stress

Presenter: Meindert Lamers

Cryo-EM structures of the E. coli replicative DNA polymerase in DNA synthesis and DNA editing mode

Rafael Fernandez-Leiro, Julian Conrad, Sjors Scheres, Meindert H. Lamers
MRC - Laboratory of Molecular Biology, Cambridge, Francis Crick Avenue, UK

The E. coli replicative DNA polymerase is a highly efficient molecular machine that synthesizes DNA with speeds up 1000 nt/s. During DNA replication, the polymerase needs to quickly respond to different signals in the DNA such as mis-incorporated nucleotides, modified bases, or the Okazaki fragment termini on the lagging strand. How the polymerase can respond to these different signals on the DNA is poorly understood. We have determined the cryo-EM structures of the DNA polymerase in complex with sliding clamp and exonuclease in a DNA-free state, a DNA synthesis state, and a DNA editing state. The three structures reveal how the polymerase discriminates between the different signals and undergoes large conformational changes to switch between the different states. The structures furthermore reveal that both binding pockets of the dimeric DNA sliding clamp β are bound by the polymerase and exonuclease. This suggests that repair pathways such as translesion DNA synthesis and DNA mismatch repair cannot co-localize with the replicative DNA polymerase, but instead compete for access to the clamp at the site of a DNA lesion.

A new mechanism of DNA interstrand cross-link repair

Johannes Walter
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DNA interstrand cross-links (ICLs) prevent unwinding of the double helix and are therefore extremely cytotoxic. In all known ICL repair pathways, cross-link resolution (“unhooking”) involves dual nucleolytic incisions surrounding the lesion. When replication forks collide with ICLs in S phase, the FANCI-FANCD2 complex promotes dual incisions, creating a double-strand break (DSB) intermediate. In this talk, I will describe a new, replication-coupled ICL repair pathway that does not require DNA incisions or FANCI-FANCD2.
Plenary session

Replication Stress

Presenter: Marco Foiani

An integrated ATR, ATM and mTOR-mechanical network controlling nuclear plasticity

Gururaj Kidyoor(1), Giulia Bastianello(1), Qinseng Li(1), Martin Kosar(1), Amit Kumar(2,3), Galina V. Beznoussenko(1), Alexandre A. Mironov(1), Dario Parazzoli(1), G.V. Shivashankar(4), Jiri Bartek(5), Michele Mazzanti(6), Giorgio Scita(1,6), Marco Foiani(1,6)

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ATR and ATM control chromosome integrity, chromatin dynamics and cell cycle events. mTOR exhibits similarities to ATR and ATM and coordinates nutrient sensing pathways and cytoskeleton dynamics.

We recently found (A.Kumar et al. Cell, 2014) that ATR, ATRIP and Chk1 associate to the nuclear envelope during S phase and prophase, and in response to mechanical stimulation of the plasma membrane. The ATR-mediated mechanical response occurs within the range of physiological forces, recovers rapidly, and is not influenced by RPA or DNA damage. ATR defective cells exhibit aberrant chromatin condensation and nuclear envelope breakdown.

We found that this pathway is influenced by mTOR, actin dynamics and calcium levels. We used electron microscopy to visualize the nucleus morphology of the nucleus in ATR and CHK1-defective cells and found aberrant condensation events and nuclear envelope anomalies that may contribute to micronuclei formation and chromosome fragmentation.

Using mechanobiology approaches we measured the stiffness of wild type, ATR, ATM, CHK1 and mTOR defective cells and found significant differences that influence cell plasticity and interstitial migration. These and other observations implicate ATR, ATM and mTOR in the control of genome integrity, nuclear dynamics and cell plasticity and suggest the existence of an integrated mechanical network involving different PI3-kinases.
PLenary session

Replication Stress

Presenter: Pierre-Henri Gaillard

SLX4 and EME1 struggle with SUMO to maintain genome stability

Pierre-Henri Gaillard
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The SLX4 protein was identified as a scaffold that interacts with many genome maintenance factors, including the XPF-ERCC1, MUS81-EME1 and SLX1 structure-specific endonucleases, the mismatch repair MSH2-MSH3 complex, the telomere maintenance protein TRF2 as well as with the cell-cycle control PLK1 kinase. This puts SLX4 at the cross-roads of several genome maintenance mechanisms with recently confirmed key functions in DNA interstrand crosslink (ICL) repair, the resolution of Holliday junctions during homologous recombination and the regulation of telomere homeostasis. An unexpected role of SLX4 in the control of HIV infection and the innate immune response has also been reported.

We and others recently brought new insight into how SLX4 might control and channel the action of its partners into various DNA repair pathways with the realization that it has SUMO binding properties and appears as an essential component of a SUMO E3 ligase that SUMOylates SLX4 itself as well as the XPF subunit of the XPF-ERCC1 structure-specific nuclease. Interestingly, while SLX4 also has ubiquitin binding properties that are essential for ICL repair, its SUMO-related functions are not. Instead they channel the SLX4 complex down another route necessary for the maintenance of common fragile sites. I will present some of our latest work on the SUMO-related functions of SLX4 and put them in perspective with our recent findings on the contribution of SUMO in the control of Mus81-Eme1 in fission yeast.
Plenary session  
Replication Stress

Presenter: Simone Sabbioneda

**UBR5 protects DNA replication from DNA polymerase η; toxicity mediated by ubiquitylated H2A**

_Lina Cipolla(1), Chih-Chao Liang(2), Alan R. Lehmann(3), Martin A. Cohn(2), Simone Sabbioneda(1)_

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Accurate replication of the DNA is critical for the maintenance of genome integrity and cellular survival. Cancer-associated alterations often involve key players of DNA replication and of the DNA damage-signalling cascade. Post-translational modifications play a fundamental role in coordinating replication and repair and central among them is Ubiquitylation. The E3 ligase UBR5 has been found overexpressed or mutated in several tumours. We show that downregulation of UBR5 results in S phase accumulation and slower S phase progression. This is due to a reduction in replication fork speed followed by an accumulation of single strand DNA (ssDNA). After knockdown of UBR5 we also detect increased ubiquitylated PCNA, a key regulator of DNA damage tolerance mechanisms such as DNA translesion synthesis. The effect of UBR5 knockdown is related to a mis-regulation in the pathway that controls the ubiquitylation of histone H2A (uH2A) and blocking this modification is sufficient to rescue the cells from the replication defects. Finally, we show that the presence of polymerase eta is the main cause of cell death when UBR5 is silenced, indicating a novel role of uH2A in the control of polymerase eta as a central player of the DNA damage tolerance response.
Plenary session

Replication Stress

Presenter: Sasa Svikovic

R-loops potentiate structured DNA-associated epigenetic instability

Sasa Svikovic, Julian E. Sale
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The accurate propagation of histone marks during replication is proposed to be an important mechanism by which cells maintain gene expression states across cell division. In order to maintain histone marks in register with original sequence, histones must be rapidly deposited on newly synthesized DNA strands during replication. Defective replication of G-quadruplex DNA can cause perturbation of histone deposition ahead of replication fork, leading to stochastic loss of epigenetic memory, a phenomenon termed epigenetic instability (1).

Using a previously described assay for G-quadruplex induced epigenetic instability, the BU-1 locus in chicken DT40 cells (2,3), we show that trinucleotide repeats can also generate stochastic epigenetic instability during replication in a length and orientation-dependent manner. We show that, as for G-quadruplexes (4), loss of the recently discovered primase/polymerase (PrimPol) accelerates trinucleotide repeat-induced epigenetic instability for given repeat lengths, suggesting that PrimPols ability to reprime close to replication-blocking secondary structures limits single strand DNA exposure.

DNA secondary structures have been associated with the formation of stable transcription-associated RNA:DNA hybrids (R-loops) (5) but how the formation of one influences the other has been unclear. We show that DNA secondary structure formation in the BU-1 locus promotes R-loop formation. R-loop formation is potentiated by the absence of PrimPol both in the BU-1 locus and genome wide. However, overexpression of RNaseHI (which degrades RNA:DNA hybrids in vivo) significantly reduced extent of epigenetic instability suggesting that R-loops also stabilise the formation of DNA secondary structures.

Together, our data suggest that DNA secondary structures and R-loops potentiate each others formation and that both are enhanced by the formation of excessive single stranded DNA at a stalled replication fork, an event that can be mitigated on the leading strand with close-coupled repriming by PrimPol. Since the loss of the previously active transcriptional state of BU-1 in this context is followed by its repression, these results have potential implications for understanding the mechanism by which trinucleotide repeats induce gene silencing.

Plenary session: 'Chromosome Stability'
Monday, April 18, 14:30 – 16:30 h
Genome-wide analysis of DNA end resection during yeast meiosis

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In most sexually reproducing organisms, homologous recombination initiated by DNA double-strand breaks (DSBs) lies at the heart of meiosis by promoting proper segregation of homologous chromosomes. During recombination, the 5’-terminal strands of each DSB are processed (resected) by endo- and exonucleases to reveal the 3’-ssDNA tails that are substrates for the strand exchange proteins Dmc1 and Rad51. DSBs are introduced by the transesterase Spo11 non-randomly along chromosomes, mostly concentrated in small (~100-250 bp) regions called hotspots. Spo11 forms a covalent complex with DSB ends as part of the cleavage reaction, and is then endonucleolytically released as the initial step of end resection. DNA removed by DSB processing is restored by new DNA synthesis, which copies genetic information from the intact homologous template. The coordinated degradation and re-synthesis of DNA dictate homology usage and determine repair product configuration and, although they reside in the heart of the recombination pathway, are not well understood. We developed a new assay to study DNA end resection genome-wide at high spatial resolution during meiotic recombination in S. cerevisiae. The assay relies on the fact that removal of the ssDNA tails of resected DSBs marks the position where resection stopped. Molecular features of resection are revealed by sequencing of these ssDNA-to-dsDNA junctions and comparison to high-resolution DSB maps made by sequencing of short DNA oligonucleotides covalently bound to Spo11. Bioinformatic analysis of the sequencing results and construction of individual and genome-wide resection maps is exposing unforeseen features of this fundamental and highly conserved process. Our analysis extends to hypo- and hyper-resection mutants in our attempt to obtain mechanistic insights. Mathematical modeling of a simple two-step resection process as described from genetic studies is not enough to predict the patterns we derive from our maps. Based on our results we propose that end-resection is heavily context dependent and that chromosomal features such as chromatin structure shape the resection landscape.
Plenary session  

Chromosome Stability

Presenter: Matt Neale

Spatial patterning of meiotic recombination by DNA damage checkpoint kinases

Marg Crawford(1), Tim Cooper(1), Valerie Garcia(1), Dominic Johnson(1), Rachal Allison(1), Bertrand Llorente(2), Marie-Claude Marsolier(3), Matthew J Neale(1)

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Formation of programmed DNA double-strand breaks (DSBs) is a distinctive feature of meiotic prophase in most sexually reproducing organisms. Subsequent interhomologue-directed DSB repair facilitates the pairing of homologous chromosomes, the reassortment of chromosomal segments, and accurate reductional chromosome segregation—a suite of processes that collectively generate unique, recombinant, haploid genomes suitable for sexual reproduction.

For many decades it has been known that the sites of chromosomal exchange (crossovers) are distributed nonrandomly across the genome—being more spaced out than expected by chance—yet the molecular steps that govern this process remain poorly understood. We recently demonstrated that the distribution of DSBs—the precursors of crossovers—is also nonrandom, being influenced by the activity of the evolutionarily conserved DNA damage response (DDR) checkpoint protein Tel1 (ATM in mammals). Specifically, Tel1 mediates distance-dependent suppression of coincident DSB formation over regions spanning ~100 kb—a phenomenon we term “DSB interference”. Yet it remains to be determined what impact the activity of Tel1 and other DDR pathways elicit on the subsequent distribution of observable noncrossover and crossover recombination events.

To extend our conclusions, we have built a computer model capable of simulating both DSB interference and a second phenomenon, DSB clustering (negative interference). We have complemented this in silico work with an analysis of the genome-wide distribution of all observable recombination events resulting from the meiosis of a hybrid S. cerevisiae strain containing approximately 70,000 single-nucleotide polymorphisms. By comparison with computer models of random deposition versus models that include simulated interference windows, we are investigating the relative influence that various DDR factors exert over the spatial patterning of meiotic recombination events. Our preliminary results suggest that classical crossover interference is dependent on Rad24, the loader of the 9-1-1 DDR checkpoint clamp, yet is only moderately dependent on the Mec1(ATR) kinase itself. By contrast, Tel1(ATM) exerts its impact on crossover interference more locally.

Overall, our observations add to the complex set of overlaying factors that collectively govern the spatial patterning of genetic change that arises within the germline of individuals, and by extension, within the population at large.
Plenary session

Presenter: David Pellman

Mechanisms leading to chromothripsis

David Pellman
Dana-Farber Cancer Institute, Harvard Medical School, USA

Our group recently defined a mechanism that generates chromothripsis, a mutational process where only one or a few chromosomes are subject to massive rearrangement. Using a combination of live cell imaging and single cell genome sequencing, we found that the abrupt disruption of the envelope of abnormal nuclear structures called micronuclei can trigger chromothripsis. I will discuss our recent progress in defining detailed mechanisms underlying this phenomenon, with particular emphasis on experiments to determine the basis for nuclear envelope fragility of micronuclei and chromosome bridges.
Intrinsic molecular limits of genome surveillance

Jiri Lukas
The Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Denmark

Our laboratory is interested in how are proteins that guard the integrity of the human genome wired into functional pathways, and how are these pathways organized in the three-dimensional space of the cell nucleus. We are addressing these issues by high-content imaging of DNA damage responses in living cells, with a particular focus on endogenous chromosomal lesions induced by errors during DNA replication.

In our view, the genome integrity field has now reached the stage when we need to ask not only ‘how DNA repair and signalling works’ but also ‘what are the physiological limits of the underlying biochemical reactions’. An outstanding question in the field is how much damage cells can endure while still being able to chose a proper repair pathway to guard against cancer-predisposing mutations and at the same time shield healthy parts of their genomes against untimely or excessive DNA and chromatin transactions.

I will provide evidence that spatial and temporal limits of genome surveillance indeed exist and that they can be explained by evolutionary constraints on genome maintenance. I will present a new high-content imaging technique, and which enables us to interrogate repair pathway choices at the cell population level, and which has been instrumental for us to identify several rate-limiting genome caretakers both on damaged DNA and the flanking chromatin. I will focus this analysis on DNA double strand breaks (DSBs) and show that the dynamic range of DSB repair pathway choice is surprisingly narrow and that its saturation undermines genome integrity by enforcing illegitimate and highly mutagenic repair reactions. I will discuss these results in a framework of an emerging concept that while cancer cells initially benefit from imperfections in genome surveillance, they do it on the cost of ‘living at the edge’. I will argue that understanding these limits can help rationalise strategies for how to selectively eliminate cancer cells.
Plenary session

Chromosome Stability

Presenter: Serena Nik-Zainal

Recent advances in mutational signatures identified in human somatic cells
Serena Nik-Zainal
Wellcome Trust Sanger Institute, Genome Campus Hinxton, Cambridge, UK

Mutational signatures are the imprints of the biological processes that have gone awry in human cells. We previously outlined the methods for identifying and quantifying base substitution mutational signatures present in primary human cancers (http://cancer.sanger.ac.uk/cosmic/signatures). Here, using a highly-curated cohort of 560 whole genome sequenced breast cancers, we extend the understanding of mutational signatures to include six novel rearrangement signatures. These signatures distinguish clinical cohorts in breast cancer and have intriguing genomic properties with potential for clinical application as a biomarker.
Inborn defects in genome maintenance pathways are associated with complex developmental disorders whose causal mechanisms are poorly understood. Using an in vivo tagging approach in mice and high-throughput proteomics strategies, we show that the nucleotide excision repair (NER) structure-specific endonuclease ERCC1-XPF complex interacts with protein factors involved in chromatin architecture and organization during mammalian development. Loss of Ercc1 or exposure to various genotoxins triggers the aberrant localization of chromatin regulators, altered histone marks and the aberrant expression of selected gene targets. The response is cell-autonomous and requires functional DDR. We propose that persistent DNA damage signaling triggers chromatin changes that affect gene expression programs associated with NER developmental disorders.
Plenary session: 'Repair Mechanisms-I'
Tuesday, April 19, 9:00 – 12:40 h
Telomere length maintenance is a requisite feature of cellular immortalization and a hallmark of human cancer. While most human cancers express telomerase activity, approximately 10-15% employ a recombination-dependent telomere maintenance pathway known as Alternative Lengthening of Telomeres (ALT), an incompletely understood process that is characterized by multi-telomere clusters. We have recently shown that a DNA double-strand break (DSB) response at ALT telomeres triggers long-range movement and clustering between chromosome termini, resulting in homology-directed telomere synthesis (Cho et al. Cell 2014). Damaged telomeres initiate increased random surveillance of nuclear volumes before displaying rapid directional movement and association with recipient telomeres over micron-range distances. This phenomenon required Rad51 and the Hop2-Mnd1 heterodimer, implicating a specialized homology searching mechanism that exhibits some similarities to meiotic recombination in ALT dependent telomere maintenance. This presentation will describe new data that provides insights into signaling events that promote homology searches and mechanisms underlying homology directed telomere synthesis during ALT. We have recently developed novel methodologies to quantify break induced ALT telomere replication. Data derived from this approach reveals noncanonical mechanisms of homology directed DNA synthesis that occur specifically at ALT telomeres. These data define an ordered series of steps necessary for ALT telomere synthesis and have implications for one-sided break repair at other genomic sites.

This work was supported by NIH grants GM101149, CA138835, and CA17494, and funds from the Abramson Family Cancer Research Institute and Basser Research Center for BRCA.
Snapshots of DNA mismatch repair: trapping transient states

Flora S. Groothuizen(1), Ines Winkler(2), Michele Cristóvão(2), Alexander Fish(1), Herrie H.K. Winterwerp(1), Annet Reumer(1), Andreas D. Marx(2), Nicolaas Hermans(3), Robert A. Nicholls(4), Garib N. Murshudov(4), Joyce H.G. Lebbink(3), Peter Friedhoff(2), Titia K. Sixma(1)

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To avoid mutations in the genome, DNA replication is followed by DNA mismatch repair (MMR). This process starts when a MutS homolog recognizes a mismatch and undergoes an ATP-dependent transformation to an elusive sliding clamp state. How this transient state promotes MutL homolog recruitment and activation of repair is unclear. Here we present crystal structures of MutS and the MutS/MutL complex where we trap transient states, by making use of highly specific mutants and single-cysteine crosslinking. The resulting structures have surprisingly large conformational changes that were validated by FRET, binding studies and mutagenesis and interpreted in terms of the MMR cycle. The structures capture the MutS conformational changes from searching for a DNA mismatch to sliding clamp formation. In the MutS/MutL complex we observe the sliding clamp conformation, where tilting of the MutS subunits across each other pushes DNA into a new channel, and reorientation of the connector domain creates an interface for MutL with both MutS subunits. Our work explains how the sliding clamp promotes loading of MutL onto DNA, to activate downstream effectors. We thus elucidate a crucial mechanism that ensures that MMR is initiated only after detection of a DNA mismatch.
Pivotal roles of MutL homologs in mismatch repair and meiosis: insights from structural studies

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Eukaryotic MutL homologs play central roles in mismatch repair and in meiosis (Guarné & Charbonnier, 2015, Prog Biophys Mol Biol). In particular, MutLa (Mlh1-Pms1 in S. cerevisiae and MLH1-PMS2 in human) and MutLγ (Mlh1-Mlh3) are considered respectively as the main endonuclease activity in MMR and as the main resolvase activity for cross-over formation in meiosis. Dysfunctions of these factors are associated with colorectal cancers called Lynch syndrome for MutLa and sterility and chromosome translocations for MutLγ. We previously reported the first crystal structure of the C-terminal region of Mlh1-Pms1 and showed an unexpected role of the extreme C-terminus of Mlh1 in Pms1 endonuclease site (Gueneau & al, 2013, Nat Struct Mol Biol). More recently, we solved the first crystal structure and SAXS analysis of a eukaryotic MutLγ C-terminal region. This allowed us to compare the resolvase and MMR endonuclease heterodimer structure and endonuclease active sites (J Dai & al, in preparation). The MutLγ heterodimer presents a similar overall MutL fold with key differences: (i) some motifs essential for MutLa formation and stability are missing in MutLγ, (ii) the regulatory domain of MutLγ adopts a different position thus redefining the electrostatic surface close to the endonuclease site, (iii) contrary to MutLa, the C-terminal domain of MutL specifically binds Holliday junctions. Finally though genetic studies, we characterized separation of function mutants of Mlh1 and proved a central role of the conserved C-terminus of Mlh1 in both MMR and meiosis.
Plenary session  Repair Mechanisms-I

Presenter: Samuel Wilson

Step-to-step coordination in mammalian base excision repair

*Samuel H. Wilson*
Genome Integrity and Structural Biology Laboratory, National Institutes of Health-NIEHS, USA

DNA lesions arise from many endogenous and environmental agents and can promote deleterious events leading to genomic instability if not repaired. Base excision repair (BER) is an important DNA repair pathway involved in repairing single strand breaks, base lesions and abasic sites in mammalian cells. During the multi-step BER process, DNA repair intermediates can be channeled from one step to the next in a sequential fashion so that release of toxic repair intermediates is minimized. This includes handoff of the product of gap-filling DNA synthesis by DNA polymerase β to the final step of BER, DNA ligation. The conformational changes in polymerase β associated with nucleotide insertion can influence channeling of the repair intermediate to the DNA ligation step. In addition, post-insertion conformational events after polymerase β gap-filling synthesis may influence 5’-excision by flap endonuclease 1, extension off the newly inserted nucleotide, and 3’-excision by the enzyme’s pyrophosphorolysis activity. Implications of the various structure-based post-insertion decisions by DNA polymerase β will be discussed.
Neil DNA glycosylases coordinate substrate hand-over during oxidative DNA demethylation

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In vertebrates, cytosines within CpG dinucleotides can be methylated at carbon 5. DNA cytosine methylation is an epigenetic mark which plays important roles in development and disease. It is now widely accepted that DNA methylation is dynamic and is subject to enzymatic demethylation. Yet, propositions of repair-based demethylation mechanisms have met with skepticism because the danger of genomic instability for cells is considered too high. Thus, a key question is what are the biochemical mechanisms which ensure that DNA demethylation proceeds as an orderly controlled process. In the Tet/Tdg-mediated demethylation pathway, methylated cytosine is iteratively oxidized by ten-eleven translocation (Tet) dioxygenases and unmodified cytosine is restored via thymine DNA glycosylase (Tdg) and base excision repair. The most vulnerable intermediate in Tet/Tdg demethylation is the abasic (AP) site which, if unprotected, is unstable and cytotoxic. Here we show that the human nei endonuclease VIII-like bifunctional DNA glycosylases/AP lyases NEIL1 and NEIL2 coordinate AP site processing during TET/TDG DNA demethylation. Instead of processing cytosine derivatives directly as DNA glycosylases, NEILs cooperate with TDG. After base excision, TDG occupies the AP site and is displaced by NEILs which further process the baseless sugar, thereby stimulating TDG substrate turnover. In early Xenopus embryos Neil2 cooperates with Tdg to remove oxidized methylcytosines and to specify neural crest development together with Tet3. Thus, Neils function as AP lyases in the coordinated substrate hand-over during oxidative DNA demethylation.
The regulation of CRL4(DDB2) activity by the Cop9 Signalosome

Simone Cavadini(1), Eric Fischer(2), Richard Bunker(1), Alessandro Cavadini(1), Julius Rabl(1), Nicolas H. Thomä(1)

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The cullin-RING ubiquitin E3 ligase (CRL) family comprises over 200 members in humans. The COP9 signalosome complex (CSN) regulates CRLs, by removing their ubiquitin-like activator NEDD8. CRL4ADDB2 monitors the genome for UV-induced DNA damage. CRL4ADDB2 is inactive in absence of damaged DNA and requires CSN to regulate the repair process. The structural basis of CSN binding to CRL4ADDB2 and principles of CSN activation are poorly understood. Here we present cryo-electron microscopy (cryo-EM) structures for CSN in complex with neddylated CRL4A ligases to 6.4 Å resolution. The CSN conformers defined by cryo-EM and a novel apo-CSN crystal structure detail an induced-fit mechanism that drives CSN activation by neddylated CRLs. We find that CSN and a substrate cannot bind to a CRL4A simultaneously, favouring a deneddylated, inactive state for substrate-free CRL4s. These architectural and regulatory principles appear conserved across CRL families, allowing global regulation by CSN.
Plenary session  Repair Mechanisms-I

Presenter: Kaoru Sugasawa

Dissection of the DNA damage recognition machinery in mammalian nucleotide excision repair

Kaoru Sugasawa
Biosignal Research Center, and Graduate School of Science, Kobe University, Japan

Nucleotide excision repair (NER) is a versatile DNA repair pathway, which can remove an extremely broad range of base lesions from the genome. In mammalian global genomic NER, the heterotrimeric complex containing the xeroderma pigmentosum group C (XPC) protein (XPC-RAD23-CETN2) initiates the repair reaction by recognizing sites of DNA damage, and this depends on detection of oscillating intact bases, rather than lesions themselves, within the DNA duplex. Most DNA lesions processed by NER associate with a relatively large helix distortion that locally destabilizes base pairs; however, UV-induced cyclobutane pyrimidine dimers (CPDs) retain hydrogen-bonding with opposite purines, and thereby easily escape direct detection by XPC.

The highly defined cell-free NER assay reconstituted with six purified recombinant NER factors (XPC, TFIIH, XPA, RPA, XPG, ERCC1-XPF) revealed that the presence of mismatched bases in 5' side of CPD substantially enhances repair efficiencies of the photolesions. Given the asymmetric fashion of the XPC-DNA interaction, this corroborates the model that XPC first needs to interact with unpaired normal bases within the 'undamaged' strand and then loads the XPD helicase in TFIIH onto the 'damaged' strand. Loading of XPC in a wrong orientation does not culminate productive dual incisions, indicating strict strand selectivity of this system. The presence and location of a lesion is finally verified by XPD, when it translocates along the DNA strand in 5'-3' direction and stalls at sites with abnormal DNA chemistry. Notably, the helicase activity and the damage verification function of TFIIH are markedly enhanced by the presence of XPA. Thus the tripartite XPC-TFIIH-XPA complex would be responsible for a final decision to proceed with the repair process toward dual incisions.

Another key issue that still remains to be addressed is involvement of chromatin structures in initiation of NER. If a UV-induced photolesion is present within the nucleosome core, accessibility to XPC, but not to UV-DDB (the DDB1-DDB2 heterodimer), is severely impaired. This suggests that UV-DDB bound to a damage-containing nucleosome induces chromatin remodeling by dissolving and/or sliding of the histone octamer. Based on the structures of reconstituted nucleosomes containing defined lesions, the possible underlying mechanism will be discussed, by which UV-DDB senses the presence of DNA damage within the nucleosome core.

Tripartite DNA Lesion Recognition and Verification by XPC, TFIIH and XPA in Nucleotide Excision Repair

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Transcription factor IIH (TFIIH) is essential for both transcription and nucleotide excision repair (NER). DNA lesions are initially detected by either NER factors XPC and XPE or stalled RNA polymerases, but only bulky lesions are preferentially repaired by NER. To elucidate substrate specificity in NER, we have prepared homogeneous human 10-subunit TFIIH and its 7-subunit core (Core7) without the CAK module and show that bulky lesions in DNA inhibit the ATPase and helicase activities of both XPB and XPD in Core7 to promote NER, whereas non-genuine NER substrates have no such effect. Moreover, the NER factor XPA activates unwinding of normal DNA by Core7, but inhibits the Core7 helicase activity in the presence of bulky lesions. Finally, the CAK module inhibits DNA binding by TFIIH and thereby enhances XPC-dependent specific recruitment of TFIIH. Our results support a tripartite lesion verification mechanism involving XPC, TFIIH and XPA for efficient NER.
Plenary session

Presenter: Robert Fuchs

Processing opposing lesions during Nucleotide Excision Repair triggers UV mutagenesis in E. coli

Regine Janel-Bintz (2), Rita L Napolitano (1), Asako Isogawa (1), Shingo Fujii (1), Robert P Fuchs (1)

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Point mutations are known to occur when damaged DNA is replicated. However, here we provide genetic evidence for a replication-independent mutation pathway that involves processing of opposing lesions by Nucleotide Excision Repair (NER). This pathway, referred to as Nucleotide Excision Repair-induced Mutagenesis (NERiM), exhibits several characteristics distinct from replication-induced mutagenesis: i) following UV irradiation, NER-induced mutations are fixed much more rapidly (t ½ ≈ 30 min) than replication dependent mutations (t ½ ≈ 80-100 min) ii) NERiM specifically requires DNA Pol IV in addition to Pol V iii) NERiM exhibits a two-hit dose-response curve that strongly implies processing of opposing lesions. A mathematical model let us define the structure of the opposing lesion intermediate generated by NER. This critical intermediate requires Pol IV / Pol II for repair, it is either lethal if left un-repaired or mutation-prone when repaired. Finally, NERiM is found to operate in stationary phase cells providing an intriguing possibility for ongoing evolution in the absence of replication.
How Global Genome–NER is organized in the yeast genome

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The rates at which lesions are removed by DNA repair can vary widely throughout the genome with important implications for genomic stability. We measured the distribution of nucleotide excision repair (NER) rates for UV induced lesions throughout the yeast genome. By plotting these repair rates in relation to all ORFs and their associated flanking sequences, we reveal that in normal cells, genomic repair rates display a distinctive pattern, suggesting that DNA repair is highly organised within the genome. We compared genome-wide DNA repair rates in WT and in RAD16 deleted cells, which are defective in the global genome-NER (GG-NER) sub-pathway, demonstrating how this alters the normal pattern of NER rates throughout the genome. By examining the genomic distribution of global genome NER factor binding in chromatin before and after UV irradiation, we reveal that GG-NER is organized and initiated from specific locations within the yeast genome. The GG-NER complex regulates the histone H3 acetylation status and chromatin structure in the vicinity of these genomic sites to promote the efficient removal of UV induced lesions. This demonstrates that chromatin remodeling during the GG-NER process is organized in the genome. Importantly, we demonstrate that deleting the histone modifier GCN5, which acetylates histone H3 in response to UV radiation, and is an accessory factor necessary for chromatin remodeling during GG-NER, significantly alters the genomic distribution of NER rates. These observations may have important implications for the effect of histone and chromatin modifiers on the distribution of genomic mutations acquired throughout the genome.
Plenary session: 'Transcription Stress'
Tuesday, April 19, 14:30 – 16:25 h
The transcription-related DNA damage response

Laura Williamson, Stefan Boeing, Marco Saponaro, Jesper Q Svejstrup
Mechanisms of Transcription Laboratory, The Francis Crick Institute, Clare Hall Laboratories, South Mimms, UK

The cellular response to DNA damage remains poorly understood. UV-irradiation triggers a dramatic reduction in RNA synthesis and changes to mRNA splicing, but the underlying factors, mechanisms and cellular consequences are poorly understood. In order to facilitate identification of new factors and pathways, we performed several descriptive proteomic screens and a functional genomics screen in parallel. Any single, low-stringency screen in itself yielded innumerable candidates of uncertain value. In contrast, numerous factors could be identified with higher confidence when the screen results were superimposed and interpreted together, incorporating biological knowledge. Besides uncovering roles in the damage response for numerous proteins and complexes, including Integrator, Cohesin, PHF3, ASC complex, and SCAF4/-8/-11, we also uncovered a new role for a poorly studied, melanoma-associated factor.

We also analyzed the transcription-related DNA damage response on a genome-wide scale. Our data indicate a dramatic slowdown of transcript elongation upon UV treatment, followed by recovery over the next 24-48 hours. Remarkably, elongation slow-down is associated with a shift from expression of long mRNAs to shorter transcript isoforms, incorporating alternative last exons (ALEs) that are more proximal to the transcription start site. Notably, this includes a shift from a protein-coding ASCC3 mRNA to a shorter transcript isoform of which the 3’-untranslated region, rather than the encoded protein, is critical for transcription recovery after DNA damage. These data uncover a UV-induced transcription- and splicing-response, involving messenger- and non-coding RNA expressing genes, which is important for the cellular response to DNA damage.
Plenary session

Transcription Stress

Presenter: Andrés Aguilera

Role of Chromatin and the DNA damage response in transcription-associated genome instability

*Irene Salas-Armentero, Rosa Luna, Andrés Aguilera*

University of Seville – CABIMER, Spain

Coordination of DNA replication with DNA-damage sensing, repair and cell cycle progression ensures with high probability genome integrity during cell divisions, thus preventing mutations and DNA rearrangements. Such events may be harmful for the cell and the organism, and are usually associated with pathological disorders. One important type of genome instability is that associated with transcription. R-loops are transcriptional by-products that can be formed naturally as key intermediates in specific cellular processes, but they are also a major source of genome instability.

Specific RNA processing factors have been shown to play a role in preventing R-loop and transcription-associated genome instability. The first one identified with this role was the yeast THO complex, a conserved factor working at the interface between transcription and RNA export. However, other proteins directly or indirectly related with a function in RNA processing, such as human SRSF2 and AQR or yeast Trf4 or Npl3 among others, also prevent RNA-DNA hybrids. The working hypothesis suggests that in cells defective in such factors a suboptimal nascent mRNA-protein particle is formed, enhancing the probability that the nascent RNA interacts with the DNA. However, it is unclear how this mechanism occurs.

To explore further the mechanism by which RNA processing factors control genome integrity, we have screened a human library for proteins that physically interact with components of the human THO complex using the yeast two-hybrid system. Further confirmation of this interaction via co-immunoprecipitation and Proximity Ligation Assay, has permitted us to identify a chromatin remodeling complex. Functional analyses of this interaction and of the effect of depleting cells from these factors has permitted us to propose a new model to explain how cells prevent co-transcriptional RNA-DNA hybrid formation and transcription-associated genome instability. Our work opens new perspectives to understand the different mechanisms used by the cells to prevent the accumulation of DNA structures that compromise genome integrity.
Our genomes are constantly threatened by endogenous and exogenous sources of DNA damage, and a growing body of evidence implicates R-loop structures as an important endogenous source of genomic instability. R-loops are three-stranded nucleic acid structures consisting of an RNA-DNA hybrid and displaced single-stranded DNA. They are thought to form during transcription when the nascent RNA transcript hybridizes with the DNA template, and various physiological processes are regulated by these structures on chromatin. However, unscheduled or increased levels of R-loops, which can arise when factors that normally regulate their formation are perturbed, can lead to the accumulation of DNA damage. In this talk, we will summarize recent findings about the processes driving R-loop-induced DNA damage and genome instability. In particular, we will discuss a new system we have developed and are using to study the impact of replication on genomic stability when active forks collide with transcription-related obstacles, including R-loops and transcription complexes.
Transcription Coupled Nucleotide Excision Repair, focusing on RNA Polymerase 2

Barbara Steurer, Roel Janssens, Franziska Wienholz, Marit Geijer, Maria Tresini, Wim Vermeulen, Jurgen Marteijn
Erasmus MC, Department of Molecular Genetics, Rotterdam, The Netherlands

Transcription blocking DNA lesions cause cellular dysfunction, senescence and apoptosis, finally resulting in DNA damage-induced aging. These deleterious effects are counteracted by transcription-coupled repair (TCR), a dedicated DNA repair system that specifically removes these cytotoxic lesions from the transcribed strand of actively transcribed gene. The severe progeroid symptoms associated with inherited TCR defects as in Cockayne syndrome (CS) patients, underscore its importance to health. Despite a similar cellular TCR defect in response to UV-induced DNA damage in UV-sensitive syndrome (UVSS) cells as in CS cell, both TCR-syndromes exhibit strikingly distinct symptoms; CS patients display severe developmental and premature aging features, while UVSS patients only feature UV-hypersensitivity.

To understand the TCR response and to dissect the molecular mechanism how cells cope with transcription blocking DNA lesions we study the spatio-temporal behavior of different TCR factors in living cells in relation to DNA damage-induced changes in interactions and post-translational modifications of the TCR machinery. Recently, we have generated a CRISPR/CAS9-mediated GFP-tagged RNA polymerase 2 knock-in cell line. This cell line appeared ideally suited for a combined analysis by live cell imaging and quantitative proteomics to study the molecular consequence and fate of RNAP2 when stalled at different types of DNA lesions. This approach allows us to determine how transcription is inhibited and subsequently restarted upon repair. Together, this will significantly advance our understanding of the molecular mechanism of TCR and result in a better comprehension of the biological impact of TCR and its role in DNA-damage induced aging.
**Plenary session**

**Presenter:** Julie Soutourina

**Novel Mediator function connecting transcription and nucleotide excision DNA repair in eukaryotes**

Diivavarshini Gopaul(1), Adrien Georges(1), Thomas Eychenne(1), Olivier Alibert(2), Michel Werner(1), Julie Soutourina(1)

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Maintenance of genome integrity and transcription are two key functions of the cell, allowing the transmission and expression of the genetic material. How these processes are coordinated in vivo in eukaryotes remains a key biological question that relates to serious human diseases.

Mediator is a large multisubunit complex conserved in all eukaryotes, which plays a crucial role in transcription activation. We have discovered a novel role of Mediator as a link between transcription and DNA repair via a direct contact between the Med17 Mediator subunit and Rad2/XPG DNA repair protein (Eyboulet et al. 2013 Genes & Development). Rad2/XPG endonuclease is involved in nucleotide excision DNA repair (NER) and mutations in human XPG gene give rise to severe diseases, xeroderma pigmentosum (XP) associated with Cockayne syndrome (CS). Genome-wide location analyses revealed that Rad2 is associated with Pol II-transcribed genes in the absence of exogenous genotoxic stress in yeast and that Rad2 occupancy of class II gene promoters is highly correlated with that of Mediator. Furthermore, Mediator med17 mutants that are defective in Mediator-Rad2 interaction are UV-sensitivity in a global-genome repair deficient background and are epistatic with a transcription-coupled repair deficient mutant. This UV sensitivity of Mediator mutants is correlated with reduced Rad2 occupancy of class II genes. Our results strongly suggest that Mediator is involved in transcription-coupled DNA repair by facilitating Rad2 recruitment to transcribed genes (Soutourina & Werner 2014 Cell Cycle). Now we are addressing the molecular mechanisms governing the Mediator link with DNA repair machinery. We are analysing an interaction interface and investigating the functional link between Mediator and NER factors, including TFIIH, Rad1/XPF-Rad10/ERCC1, Rad26/CSB, in the absence and in the presence of UV exposure. To determine the genomic distribution of Mediator and NER factors related to DNA damages, we develop advanced functional genomics approaches. Considering the conservation of Mediator and DNA repair proteins, the molecular events governing the Mediator link to DNA repair likely exist in all eukaryotes. We are directly addressing the conservation of these mechanisms in human cells. We showed that Mediator interacts with XPG protein in unmodified HeLa cells and primary fibroblasts. The analysis of Mediator role in NER in human cell lines derived from XP/CS patients is in progress.

In conclusion, our work will contribute to new concepts of the functional interplay between transcription and DNA repair and might give insights into our understanding of human diseases like XP/CS syndromes.


Plenary session

Presenter: Chit Fang Cheok

Transcription Stress

p53 promotes genomic stability by preventing interference between transcription and replication

Chit Fang Cheok
IFOM, the FIRC Institute of Molecular Oncology, Singapore

p53 tumor suppressor maintains genomic stability, typically acting through cell cycle arrest, senescence and apoptosis. We discover a function of p53 in preventing conflicts between transcription and replication, independent of its canonical roles. p53 deficiency sensitizes cells to Topoisomerase II(TOP2) inhibitors, resulting in DNA damage arising spontaneously during replication. TOP2A-DNA complexes preferentially accumulate in isogenic p53 mutant or knockout cells, reflecting an increased recruitment of TOP2A to regulate DNA topology. We propose that p53 acts to prevent DNA topological conflict originating from transcription during S phase, and therefore promotes normal replication fork progression. Consequently, replication fork progression is impaired in the absence of p53, which is reversed by transcription inhibition. Pharmacologic inhibition of transcription also attenuates DNA damage and decreases TOP2-DNA complexes, restoring cell viability in p53-deficient cells. Together, our results demonstrate a previously unrecognized function of p53 which may underlie its role in tumor suppression.
Plenary session: 'Repair Mechanisms-II'

Wednesday, April 20, 9:00 – 12:40 h
New players in the repair of DNA inter-strand crosslinks

John Rouse(1), Devtlev Schindler(2), Laura Feeney(1)

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DNA inter–strand crosslinks (ICLs) are highly toxic largely because they block the progression of replisomes. Much of what we know about the repair of inter-strand crosslinks in intact mammalian cells has come from studying the products of the genes mutated in Fanconi anemia. FA is a rare, recessive disorder characterized by a heterogeneous range of abnormalities including developmental problems, aplastic anemia and cancer predisposition. Here we describe a new FA gene, and present data describing the biological roles of the relevant gene product, which has not previously been implicated in the repair of DNA inter-strand crosslinks.
Plenary session

Presenter: Marcel Tijsterman

Repair Mechanisms-II

Genome maintenance and diversification by polymerase theta-mediated end joining

Marcel Tijsterman
Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands

Faithful DNA replication is vital to prevent disease-causing mutations, chromosomal aberrations and malignant transformation. Accuracy, however, conflicts with pace and flexibility, and cells rely on specialized polymerases and helicases to ensure effective and timely replication of genomes that contain DNA lesions or secondary structures. How cells deal with permanent barriers to replication is unknown. We have recently shown that a single unresolved G-quadruplexed DNA structure can persist through multiple mitotic divisions without changing conformation. Failed replication across a G-quadruplex causes single-strand DNA gaps that give rise to DNA double-strand breaks in subsequent cell divisions, which are processed by polymerase theta (POLQ)-mediated alternative end-joining. Alternative end-joining thus enables cells to proliferate in the presence of mitotically inherited replication blocks. Using whole genome sequencing of propagated populations we have been able to shown that POLQ-mediated alternative end-joining is a major driver of inheritable genomic alterations in C. elegans. New mechanistic insights into how pol theta mediated end-joining processes DNA breaks resulting from replicating damaged or alternatively structured DNA will be discussed.
Plenary session

Presenter: Jacqueline Jacobs

Repair Mechanisms-II

EMBO Young Investigator Lecture

Mechanisms underlying genome instability upon DNA repair at telomeres

Zeliha Yalcin, Inge de Krijger, Vera Boersma, Nathalie Moatti, Marieke H. Peuscher, Jaco van der Torre, Sandra Segura-Bayona, Marco Simonetta, Brigitte Wevers, Jacqueline J.L. Jacobs
Division of Molecular Oncology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Telomeres are specialized nucleoprotein structures that help maintain genome integrity by protecting natural chromosome ends from being recognized and handled as broken DNA. Telomeres in human somatic cells progressively shorten with every cell division, eventually causing telomere dysfunction. Loss of telomere protection activates a DNA damage-like response that initiates cell death or senescence, thereby contributing to aging while preventing outgrowth of potentially cancerous cells. However, deprotected chromosome ends are also processed by DNA repair factors, causing chromosome end-to-end fusions by non-homologous end-joining. If cells with chromosomes fused at their telomeres escape from apoptosis and senescence due to insufficient DNA damage checkpoint activity, continuation of cell division results in missegregation of chromosomes and unbalanced chromosomal rearrangements. This compromises cell viability but can also promote cancer development by causing genetic diversification and selective outgrowth of variant cell clones. Similarly, the inaccurate or inappropriate repair of DNA lesions can contribute to aging or tumorigenesis by fueling genetic alteration. The mechanisms underlying the control of DNA damage responses and repair activities are not completely understood. Therefore, our laboratory aims to identify the genes and activities that play important roles in the telomere damage response and telomere-driven genomic instability. Through functional genetic screening in mammalian cells we identified several factors, without a previously recognized role at telomeres, that promote ligation of telomere-deprotected chromosome ends by non-homologous end-joining and thereby contribute to genomic instability upon telomere uncapping. These factors are known to participate in diverse activities, including posttranslational modification by ubiquitylation, histone methylation and DNA damage tolerance. Through mechanistic characterization of the activities of these factors at telomeres and at DNA DSBs we aim for a better understanding of how cells respond to telomere dysfunction and to DNA damage.
Tumor suppressors in action: Cellular and biochemical arrangements of BRCA2 - RAD51 complexes

Claire Wyman
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Genome maintenance processes such as homologous recombination involve the coordinated action of many proteins. To understand how proteins function in complex interaction networks details of their in vivo behavior need to be defined and quantified. For this purpose we analyzed the mobility of BRCA2 in live cells at the single-molecule level using both single-particle tracking and fluorescence correlation spectroscopy, supported by simulations. We exploited cell lines engineered to express functional tagged proteins from endogenous loci and developed imaging and analysis tools that combined allow quantitative analysis of the movement and composition of individual BRCA2 complexes in live cells. This approach revealed several novel aspects of BRCA2 behavior: it exists in oligomeric complexes that include all detectable nuclear RAD51 and these complexes show diffusive behavior characterized by frequent transient immobility. In response to DNA damage induction BRCA2 was less mobile, both the frequency and duration of immobility increased.

We also analyzed the arrangement and composition of BRCA2-RAD51 complexes formed in vitro by combined fluorescence and scanning force microscopy. Purified BRCA2 also oligomeric and appears elongated in variable arrangements. This apparent architectural flexibility changes dramatically upon interaction with RAD51. Loading RAD51 onto ssDNA was stimulated by BRCA2, with apparent release of BRCA2 if RPA was present. In the nucleus BRCA2 and RAD51 are at separated locations at sites of DNA repair as determined by super resolution microscopy. Our results indicate that although RAD51 and BRCA2 form a complex and diffuse together in the nucleus they separate at the site of action on DNA. These results define quantitative aspects of tumor suppressor function and suggest new ideas about BRCA2 structure and how this could be affected by mutations.
Structural basis of BRCA1A function in DNA repair

Julius Rabl(1), Richard Bunker(1), Simone Cavadini(1), Andreas Schenk(1), Martijn Luijsterburg(2), Haico van Attikum(2), Tewis Bouwmeester(3), Nicolas Thoma(1)
1. FMI, Basel, Switzerland; 2. LUMC, Leiden, the Netherlands; 3. NIBR, Basel, Switzerland

Recruitment of BRCA1 to DNA damage foci by the large, nuclear, K63-linkage specific deubiquitinase (DUB) complex BRCA1A is essential for DNA repair. BRCA1A shares three subunits, including the active DUB, with BRISC, a cytoplasmic DUB complex involved in regulation of inflammation and the immune response. BRCA1A is recruited to sites of DNA damage via adaptor protein Rap80, while BRISC binds to metabolic enzyme SHMT2 in the cytosol. In light of the substantial degree of identity between BRCA1A and BRISC, it is currently not understood how the markedly different biological functions arise. We have determined the crystal structure of mammalian BRCA1A complex and gained insight into the structural basis of Rap80 recruitment. We found that adaptor proteins Rap80 and SHMT2 bind to their respective complexes in substantially different manner. Our structure explains how BRCA1A was repurposed for its function in DNA repair and suggests a structural basis for the differing biological functions of BRCA1A and BRISC.
Signaling mechanisms in DNA double-strand break repair

Niels Mailand
The Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Denmark

DNA double-strand breaks (DSBs) are highly cytotoxic DNA lesions, whose faulty repair may alter the content and organization of cellular genomes. To counteract this threat, a large number of signaling and repair proteins are recruited hierarchically to the chromatin areas surrounding DSBs to facilitate faithful repair of the lesions and restoration of genome integrity. Using proteomics-driven strategies to systematically monitor the assembly and disassembly of protein complexes at DSB-containing chromatin, we have discovered and functionally characterized a range of new components of DNA damage signaling and repair pathways in vertebrate cells. My presentation will focus on the specific roles of some of these new factors in promoting genome stability maintenance following genotoxic insults.
Plenary session

Presenter: Steve Jackson

Cellular responses to DNA double-strand breaks

Steve Jackson
The Gurdon Institute and Department of Biochemistry, University of Cambridge, UK

Work in my laboratory aims to decipher the mechanisms by which cells respond to DNA damage, particularly DNA double-strand breaks (DSBs). Much of our work addresses the functions of proteins that mediate such processes, and how the activities of these proteins are controlled by post-translational modifications (PTMs). In this talk, I will highlight some of our recent work showing how PTMs control DSB repair, for example by promoting molecular transitions in DDR-protein complex assembly and disassembly. I will also explain how this and related work is providing opportunities for developing novel anti-cancer agents.

Review articles:
Plenary session  Repair Mechanisms-II

Presenter: Stephen West

Activation of MUS81 endonuclease by formation of the SMX trinuclease complex

Stephen C. West, Haley Wyatt, Gary Chan, Kasper Fugger
Francis Crick Institute, UK

All living organisms feature DNA repair pathways that safeguard the integrity of the genome, and mutations in proteins that mediate key events in DNA repair have been linked to genome instability and tumorigenesis. One particularly dangerous form of DNA damage occurs when the chromosomes suffer double-strand breaks and these need to be repaired efficiently in order to avoid DNA translocations or partial chromosome loss.

Homologous recombination repair plays a key role in maintaining genome stability after double-strand break formation. BRCA2 and RAD51 play important roles in driving interactions between homologous DNA molecules (usually sister chromatids) leading to the formation of recombination intermediates that need to be resolved by structure-selective endonucleases that ensure chromosome segregation at mitosis. The primary nucleases necessary for the resolution of recombination intermediates are MUS81-EME1 and GEN1, and mutations in these proteins result in synthetic lethality associated with an inability to promote the separation of sister chromatids at mitosis.

At the prometaphase stage of the cell cycle, MUS81-EME1 is activated by association with the SLX4 scaffold, an event that occurs in response to CDK1/PLK1-mediated phosphorylation. Remarkably, SLX4 serves as a scaffold for the assembly of a novel trinuclease complex comprising SLX1-SLX4, MUS81-EME1 and XPF-ERCC1, which we define as the SMX complex. The human SMX complex has been purified following over-expression in insect cells, and its biochemical properties have been investigated. We find that SMX is significantly more active than the individual component nucleases, and cleaves a broad range of secondary structures in DNA, including those that mimic replication forks, cross-linked forks, 3’- and 5’-flap structures, and Holliday junction recombination intermediates. However, the primary nucleolytic activity within the complex resides in MUS81-EME1, which is activated by association with SLX1-SLX4 and XPF-ERCC1. Our results define a role for SMX in the resolution of all types of secondary structures present in DNA prior to chromosome segregation and mitotic division.
DNA interstrand cross-links (ICLs) are highly toxic DNA lesions as they prevent DNA strand separation. ICL repair requires several classes of repair enzymes including translesion DNA polymerases, structure-specific endonucleases, recombinases, and Fanconi anemia (FA) proteins. Mutation in any one of the 16 currently known FA genes leads to the cancer predisposition disorder Fanconi anemia. However, it is still largely unclear how the FA proteins and the other repair factors collaborate to repair ICLs.

We study the molecular mechanism of ICL repair using a Xenopus egg extract-based system that recapitulates replication-dependent ICL repair in vitro. Previously we have shown that activation of the FA pathway by ubiquitylation of the FANCI-FANCD2 (ID) complex is important for a specific step in ICL repair, namely the incisions that unhook the lesion from one of the DNA strands. We next demonstrated that binding of this activated ID complex to the crosslink promotes the recruitment of the incision-complex, composed of the adapter protein SLX4 and the structure specific endonuclease XPF-ERCC1. Both XPF and SLX4 have recently been identified as FA genes highlighting their importance in ICL repair. Although ICL unhooking appears to be a major function of the FA pathway the biochemical details of this process are still unclear. We will present our latest findings regarding the role of XPF-ERCC1 and SLX4 in ICL repair.
The activation of the XPF-ERCC1 nuclease during interstrand crosslink repair by RPA

Ummi Abdullah(1), Joanna F. McGouran(2), Tom Brown(2), Sook Y. Lee 1,2), Peter J. McHugh(1)
1. Department of Oncology, Weatherall Institute of Molecular Medicine, University of Oxford, UK; 2. Department of Chemistry, University of Oxford, UK

The human XPF (ERCC4, FANCQ) and ERCC1 proteins form a heterodimeric structure-selective endonuclease that plays a critical role in maintaining genome stability. Mutations in the XPF gene cause several heritable disorders including Fanconi anemia (FA), a condition associated with defects in the repair of DNA interstrand cross-links (ICLs).

ICL repair is triggered when the nascent leading strand of a replication fork collides with an ICL, where XPF-ERCC1 catalyses the incisions that initiate ICL processing. Consistent with previous studies, in biochemical reconstitution reactions we found that XPF-ERCC1 incises simple model fork structures containing ICLs within the duplex DNA region (5' to the junction). However, we also observed that the presence of a nascent leading strand on these model forks, mimicking the effects of replication arrest at ICLs, completely eliminates the activity of XPF-ERCC1. Strikingly, addition of the XPF-interacting factor replication protein A (RPA) restores XPF-ERCC1 activity on such structures, and permits quantitative processing of structures that model replication forks stalled at ICLs. We found that the stimulation of XPF by RPA requires both protein-protein interactions between XPF and RPA, as well as the presence of a free, single-stranded DNA region on the lagging-strand template.

SNM1A is a 5'-3' exonuclease capable of processively digesting DNA substrates containing a wide variety of abnormal DNA structures and lesions, including ICLs. Additional reconstitution studies demonstrated that SNM1A is able to load onto model fork structures from XPF-ERCC1-RPA induced incisions and digest past the ICL. This in vitro reaction is extremely efficient, producing a near-quantitative processing and, therefore, 'unhooking' of ICL-containing fork substrates.

We postulate that during replication-coupled ICL repair, the arrest of nascent leading strands by ICLs produces a substrate that is inhibitory to XPF-ERCC1. This inhibition can be overcome through the dramatic stimulation of XPF-ERCC1 by RPA. XPF-ERCC1-RPA-induced incision enables SNM1A to digest past the ICL, which unhooks the ICL from the duplex DNA, enabling subsequent fork repair by translesion synthesis and homologous recombination to occur.
Plenary session: 'Chromatin Stress'
Thursday, April 21, 9:00 – 12:40 h
Plenary session  

Presenter: Aaron Goodarzi

CHD6 and the mammalian response to DNA damage induced by oxidative stress

Shaun Moore(1), Rami Abou-Zeinab(1), Martijn Luijsterburg(2), Shujuan Fang(1), Haico Van Attikum(2), Aaron A. Goodarzi(1)

1. Robson DNA Sciences Centre, Arnie Charbonneau Cancer Institute, Departments of Biochemistry & Molecular Biology and Oncology, Cumming School of Medicine, University of Calgary, Alberta, Canada; 2. Department of Human Genetics, Leiden University Medical Center, The Netherlands

Genome damage is a constant threat to the health and survival of a cell. The DNA double strand break (DSB) is one of the most lethal types of DNA damage, and a significant proportion of DSBs, as well as single strand breaks (SSBs), arise as a consequence of oxidative stress caused by an imbalance between reactive oxygen species (ROS) and cellular antioxidant defences. Exogenous ionizing radiation exposure also generates SSBs, DSBs and oxidative stress, eliciting robust DNA damage responses. The DNA damage response often demands chromatin modification via the manipulation of nucleosome spacing by ATP-dependent chromatin remodeling enzymes, which are able to displace, exchange or remove nucleosomes. The Chromodomain, Helicase, DNA-binding (CHD) class of chromatin remodelling enzymes are one of the major families of chromatin remodelers, with several of these enzymes having defined roles within DNA damage signalling and/or repair. We will present evidence of a novel role for the class III CHD chromatin remodelling enzyme CHD6 in the mammalian response to DNA damage induced by oxidative stress. We demonstrate that CHD6 relocates rapidly to 355 nm microlaser or oxidatively-induced DNA damage sites. CHD6 recruitment to DNA damage sites requires both PARP activity and functional CHD6 chromodomains, and conforms to the typical recruitment and dispersal kinetics for XRCC1. In response to oxidative stress, including ionizing radiation exposure, CHD6 protein levels are stabilized rapidly via reduced proteolytic degradation. Ablation of CHD6 leads to elevated DNA single strand breakage. Our ongoing studies will be discussed.
Plenary session  

Chromatin Stress

Presenter: Evi Soutoglou

Temporal and spatial uncoupling of DNA Double Strand Break repair pathways within mammalian heterochromatin.

Katerina Tsouroula(1,2,3,4), Melanie Rogier(1,2,3,4), Vincent Heyer(1,2,3,4), Audrey Furst(1,2,3,4), Anne Maglott-Roth(1,2,3,4), Bernardo Reina-San-Martin(1,2,3,4), Evi Soutoglou(1,2,3,4)

1. Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch, France; 2. Institut National de la Santé et de la Recherche Médicale (INSERM), U964, Illkirch, France; 3. Centre National de Recherche Scientifique (CNRS), UMR7104, Illkirch, France; 4. Université de Strasbourg, Illkirch, France

Repetitive DNA is packaged into heterochromatin structures to maintain its integrity. Here, we use CRISPR/Cas9 to induce DSBs in heterochromatin and demonstrate that in pericentric heterochromatin, DSBs arising in G1 are repaired at the core of the domain by NHEJ. In G2, end-resection occurs at the core but resected DNA ends relocate to the periphery to be repaired by HR. DSBs that fail to relocate are repaired in situ through NHEJ or SSA. Mechanistically, DSB-relocalization requires end resection and exclusion of RAD51 from the core. We propose that the spatial disconnection between end-resection and homology search prevent the activation of mutagenic pathways and illegitimate recombination. Interestingly, we also show that DSBs in centromeric heterochromatin activate both NHEJ and HR throughout the cell cycle. Our results highlight striking differences between pericentric and centromeric heterochromatin repair and reveal that the DNA repair pathway regulates the position of the breaks within heterochromatin structures.
Probing the genetic architecture of the response to DNA damage in human cells

Michal Zimmermann(1), Olga Murina(2), Traver Hart(3), Michael Aregger(3), Stephane Angers(4), Jason Moffat(3), Andrew P. Jackson(2), Daniel Durocher(1,5)

1. The Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Canada; 2. MRC Human Genetics Unit, IGMM, University of Edinburgh, UK; 3. Donnelly Centre, University of Toronto, Canada; 4. Leslie Dan Faculty of Pharmacy, University of Toronto, Canada; 5. University of Toronto, Canada

Exactly how the DNA damage response network is wired in human cells is poorly understood largely due to the relative lack of genetic tools to systematically probe the genetic architecture of the response to DNA damage in vertebrates. However, with the advent of CRISPR/Cas9-mediated mutagenesis, we have now the unprecedented opportunity to undertake unbiased genetic screens in human cells. Our group has initiated a large effort to map gene-drug and gene-gene interaction networks that relate to the DNA damage signalling and repair. In my presentation, I will present how we use high-resolution pooled CRISPR/Cas9 screens and I will focus on the response to poly(ADP-ribose) polymerase (PARP) inhibitors to highlight how these screens enable the discovery of unsuspected relationships between repair pathways.
Histone H3 lysine 36 trimethylation (H3K36me3) plays a central role in both orchestrating the DNA damage response and in suppressing tumorigenesis. Accordingly SETD2-dependent H3K36me3 is frequently lost in a number of cancer types, including high-grade pediatric gliomas (>50%), and metastatic renal carcinomas (~60%), for which prognosis is poor. These findings identify loss of this histone mark to be an important potential therapeutic target. We have identified an evolutionarily conserved synthetic lethal interaction between histone H3K36me3 deficiency and inhibition of the cell cycle regulator WEE1. We show that H3K36me3-deficient human cells are selectively killed with the WEE1 inhibitor, AZD1775. Cell death is associated with replication stress, DNA damage and apoptosis. An in vivo experiment in SETD2-deficient A498 xenografts showed that treatment with AZD1775 regressed tumours, producing a marked reduction in tumour size compared with vehicle-treated control. We have also developed a biomarker to detect H3K36me3 loss in patient tissue microarrays (TMAs). As AZD1775 is already in Phase II clinical trials, we anticipate these findings will be of clinical relevance.
Regulation of global-genome DNA nucleotide excision repair by KMT2H histone methyltransferase

Chiara Balbo Pogliano, Peter Rüthemann, Hanspeter Naegeli
Institute of Pharmacology and Toxicology, University of Zurich-Vetsuisse, Switzerland

Nucleotide excision repair (NER) is specialized on the removal of bulky lesions caused by the UV radiation of sunlight, chemical carcinogens, certain drugs and oxygen radicals. Although the global-genome NER (GG-NER) reaction has been fully reconstituted using naked substrates, the so far deduced mechanism is not representative for the condensed mammalian genome where chromatin modifiers are thought to guide DNA repair factors to nucleosome arrays. To test the contribution of histone methylation in the mammalian GG-NER reaction, we depleted human cells of the 332-kDa histone methyltransferase KMT2H (also known as ASH1L). This KMT2H down regulation impairs the methylation of histone H3 at position Lys4, causes hypersensitivity to UV-C light and slows down the excision of cyclobutane pyrimidine dimers (CPDs) but not (6-4) photoproducts. Instead, depletion of SETD2, a well-studied histone methyltransferase with an established role in other DNA repair pathways, does not affect CPD excision. The depletion of KMT2H also slows down the UV-induced degradation of DDB2 and Immunofluorescence analyses (involving formaldehyde fixation of cells) demonstrate that the reduced KMT2H level results in a prolonged residence of a subset of early NER factors (XPC, DDB2, p62 and XPA) on nuclear spots of UV lesions. This abnormal retention of some NER subunits interferes with the recruitment of XPD (the DNA helicase required for damage verification) and ERCC1 (a subunit of the endonuclease that makes the first DNA incision at damaged sites). Chromatin-binding assays carried out without any formaldehyde fixation show that the depletion of KMT2H weakens the ability of XPC (the initial damage sensor in the GG-NER pathway) to form tight complexes with CPD sites. Our findings indicate that KMT2H activity coordinates the initial docking of the XPC sensor complex on histone-associated CPD sites, which in turn is essential for the efficiency of downstream DNA damage verification and incision processes.
Function of chromatin during DNA Double Strand Break repair

F. Aymard(1), E. Guillou(1), BM. Javierre(2), M. Aguirrebengoa(1), T. Clouaire(1), B. Bugler(1), P. Fraser(2), G. Legube(1)

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DNA double-strand breaks (DSBs) are highly toxic lesions that are rapidly repaired by two main pathways, namely Homologous Recombination (HR) and Non Homologous End Joining (NHEJ), and the decision between these pathways partly relies on the chromatin environment where the break occur (1). Using a cell line, called DIvA (for DSB Inducible via AsiSI), where multiples breaks can be induced at annotated positions throughout the human genome, we recently found that some DSBs induced on the genome undergo clustering in an ATM dependent manner (2). Using high throughput microscopy and genome wide studies, we are now characterizing the chromatin and chromosome dynamics triggered by DSBs as well as their mechanisms and function in DSB repair.

This project DIvA has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement No 647344)

Plenary session

Presenter: Haico van Attikum

Regulation of DNA repair in a chromatin context


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The response to DNA double-strand breaks (DSBs) requires alterations in chromatin structure to promote the assembly of repair complexes on broken chromosomes. Non-homologous end-joining (NHEJ) is the dominant DSB repair pathway in human cells, but our understanding of how it operates in chromatin is limited. Here, we define a mechanism that plays a crucial role in regulating NHEJ in chromatin. This mechanism is initiated by DNA damage-associated poly(ADP-ribose) polymerase 1 (PARP1), which recruits the chromatin remodeler CHD2 through a poly(ADP-ribose)-binding domain. CHD2 in turn triggers rapid chromatin expansion and the deposition of histone variant H3.3 at sites of DNA damage. Importantly, we find that PARP1, CHD2, and H3.3 regulate the assembly of NHEJ complexes at broken chromosomes to promote efficient DNA repair. Together, these findings reveal a PARP1-dependent process that couples ATP-dependent chromatin remodeling with histone variant deposition at DSBs to facilitate NHEJ and safeguard genomic stability.
The PBAF chromatin remodelling complex and maintenance of genome stability

_Peter M Brownlee, Cornelia Meisenberg, Penny A Jeggo, Jessica A Downs_

Genome Damage and Stability Centre, University of Sussex, UK

In eukaryotes, genomic DNA is packaged into the nucleus primarily by association with histone proteins to form chromatin. This structure, while necessary for compaction and chromosome segregation, is inhibitory to most processes that require access to DNA, such as transcription, replication and repair. For this reason, cells have two powerful mechanisms for manipulating the structure of chromatin; covalent modification of histones and ATP-dependent chromatin remodelling activities. Multiple chromatin modifying activities are involved in preventing genome instability by functioning to signal and repair damaged DNA, as well as to promote faithful chromosome segregation. The PBAF chromatin remodelling complex, one of two SWI/SNF complexes in mammalian cells, plays multiple roles that contribute to genome stability. PBAF contributes to sister chromatid cohesion and centromeres, and as a consequence impacts on chromosome segregation and aneuploidy. In addition, we found PBAF helps to repress transcription in the vicinity of DNA double strand breaks. We are currently investigating the molecular mechanisms by which PBAF promotes these cellular activities.
Plenary session

Chromatin Stress

Presenter: Dipanjan Chowdhury

DNA damage induced alternative polyadenylation of DNA repair gene transcripts influences the DNA damage response

Marie-Eve Brault, Khyati Meghani, Pascal Drane, Dipanjan Chowdhury
Department of Radiation Oncology, Division of Genomic Stability and DNA Repair, Dana-Farber Cancer Institute, Harvard Medical School, Boston, USA

The optimum level of DNA repair proteins is critical for an efficient DNA damage response. However, global transcription is repressed upon DNA damage potentially limiting the production DNA repair factors. Therefore the cell must rely on other mechanisms to ensure the availability of DNA repair proteins. In recent years, alternative polyadenylation has emerged as an important co-transcriptional player in gene regulation. We performed 3’RNA-seq analysis of 103 DNA repair genes and found that about 40% of them display alternative polyadenylation (APA) events. We focused on Mre11, an important factor in the DNA damage response, and found that Mre11 3’UTR length is highly regulated by DNA damage. Exposure to ionizing radiation (IR) in G1 phase of the cell induced Mre11 3’UTR switching to longer 3’UTR transcripts while IR in S phase favored shorter Mre11 3’UTR isoforms. Importantly, reporter assays reveal that Mre11 transcripts with shorter 3’UTR displayed increased protein levels compared to the longer 3’UTR variant. The IR induced APA event regulating Mre11 3’UTR length is mediated by ATM. ATM phosphorylates at least two important factors of the cleavage and polyadenylation machinery. We observe that forced expression of Mre11 long 3’UTR using CRISPR knockout of the proximal polyadenylation cleavage site resulted in ~50% decrease in cellular Mre11. From the functional standpoint these cells are significantly more sensitive to IR. Overall this phenotype is reminiscent of ataxia-telangiectasia-like disorder (ATLD), a disease caused by mutation in Mre11 and characterized by lower levels of Mre11 protein and radiosensitivity. Together, our results highlight for the first time that ATM-mediated DNA damage signaling regulates alternative polyadenylation of DNA repair genes and that this regulation is important for DNA repair.
The BRCA1:BARD1 Ubiquitin ligase activity counters chromatin barriers to DNA resection

Jo Morris
Birmingham Centre for Genome Biology and Institute of Cancer and Genomic Sciences, College of Medical and Dental Schools, University of Birmingham, UK

Optimal DNA repair suppresses genomic instability which is a driver of cancer and ageing. A role, if any, of the intrinsic activity of the BRCA1 protein as an E3 ubiquitin ligase in DNA repair responses has been controversial. Through mutation of a novel element within the dimeric RING domains of the BRCA1:BARD1 complex we demonstrate the ligase activity is required for subset of BRCA1 functions. Further we demonstrate that the ubiquitin ligase activity of the BRCA1:BARD1 complex is a key determinant of how BRCA1 counters the inhibitor influence of 53BP1 on DNA resection and homologous recombination (HR). A model of how this is achieved will be presented.
Plenary session: 'Clinical Aspects-I'
Thursday, April 21, 14:30 – 16:30 h
Plenary session

Presenter: Peter McKinnon

New insights into base excision repair and the prevention of nervous system disease

Peter J. McKinnon
Dept Genetics, St Jude Children's Hospital, Memphis, USA

Genome stability is a prerequisite for the development and function of the nervous system. Multiple DNA damage response pathways ensure that DNA lesions resulting from replication stress and other types of damage such as oxidative damage do not impact neural homeostasis. The DNA damage response is especially critical during early neurogenesis when rapid proliferation and progenitor expansion and differentiation generates cellular diversity in the nervous system. For example, numerous congenital human neurologic syndromes are associated with defective DNA damage signaling and compromised genome integrity. These syndromes arise from inactivation of key DNA damage response factors, and can involve diverse neuropathology, including neurodegeneration, neurodevelopmental defects and brain tumors, highlighting the varied tissue-specific needs for neural genome stability. Data from these syndromes and from genetically engineered mouse models have been critical for understanding the physiologic context for different DNA repair pathways. Recent findings from our work using mouse models of Base Excision Repair deficiency in the nervous system will be discussed. These data will emphasis genome stability mechanisms that maintain neural homeostasis to prevent neurologic diseases.
Plenary session

Clinical Aspects-I

Presenter: Leona Samson

Biological responses to alkyllation and inflammation

Leona D. Samson
Biological Engineering Department, Biology Department, Center for Environmental Health Sciences, Koch Institute for Integrative Cancer Research, MIT, Cambridge, MA 02318, USA

DNA damage has been clearly linked to a variety of human diseases, namely cancer, neurodegeneration and premature aging. We have studied the processing of DNA alkylation damage via several DNA repair pathways, including direct reversal pathways and base excision repair (BER). The biological role of the repair pathways has been studied in various mouse models. In particular we have examined the repair of DNA damage induced by model alkylating agents or by the reactive chemical species produced during an inflammatory response. In some circumstances the action of DNA repair can alleviate the detrimental effects induced by the DNA damaging agents, as one would normally expect, but in other circumstances the action of DNA repair can induce cell death and extensive tissue damage. The role of various DNA repair pathways in suppressing or causing disease will be discussed in the context of the large inter-individual differences in DNA repair capacity in human populations.
Plenary session

Presenter: KJ Patel

Alcohol derived endogenous aldehydes mutate the DNA of haematopoietic stem cells

Juan Garraycochea, Gerry Crossan, KJ Patel
MRC Cambridge, UK

A two tier protection system consisting of the enzyme Aldh2 in combination with the Fanconi anaemia DNA repair pathway are essential for the production of blood in mice and humans. Without this protection endogenous aldehydes produced from metabolism damage blood stem cells causing their loss. In my talk I shall present new data that show that the loss of these stem cells occurs through a p53 dependent mechanism. Using single stem cell reconstituted mice we obtained the genomic sequence of two tier deficient stem cells damaged by aldehydes. I will reveal the mutational landscape that aldehydes cause in blood stem cells, and discuss how this may lead to their dysfunction and death.
Plenary session  Clinical Aspects-I

Presenter: Tomoo Ogi

**Molecular cloning and characterisation of new human DNA repair genes**

*Tomoo Ogi, Yasuyoshi Oka, Chaowan Guo, Kiyonobu Karata, Yuka Nakazawa*
Department of Genetics, Research Institute of Environmental Medicine (RIeM), Nagoya University, Japan

Cells are continuously exposed to many types of DNA damaging activities; consequently, all living organisms have developed efficient DNA repair mechanisms to maintain the integrity of genetic information. We are focusing on the molecular mechanisms of human DNA damage checkpoint and DNA repair pathways of UV-induced photolesions. Nucleotide excision repair (NER), is the most versatile DNA repair system, which removes the major UV-photolesions from cellular DNA. In mammals, compromised NER activity is the cause of several hereditary diseases including xeroderma pigmentosum (XP), Cockayne syndrome (CS), as well as trichothiodystrophy (TTD). We have established a rapid and efficient screening system for measuring DNA repair activities including NER; based on the system, we are running diagnostic services for genetic disorders associated with DNA repair-deficiencies. Last five years of our activities, we totally diagnosed ~700 patients who displayed clinical features associated with DNA repair-deficiencies. We extracted patients whose genetic causes have yet to be identified. These cases were further analysed their genomes to determine the pathogenic mutations by next generation DNA sequencing (NGS) platforms. From the cellular and the genetic combinatorial screenings, we have identified new pathogenic mutations in the following genes: WDR4 and XRCC4 from CS-like patients; UVSSA from two Japanese UV-sensitive syndrome cases; ATRIP from a Seckel syndrome family; ERCC1 and XPF from Cockayne syndrome patients. Detailed molecular pathogenesis for these diseases have been studied.
Regulation and Assembly of DNA Strand Break Repair Protein Complexes Associated with Human Genetic Disease

Keith W. Caldecott
Genome Damage and Stability Centre, School of Life Sciences, University of Sussex, UK

The repair of DNA single-strand and double-strand breaks is regulated and facilitated by protein ADP-ribosylation, via activation of one or more of the ADP-ribosyl transferases PARP1, PARP2, and PARP3. PARP enzymes promote DNA single-strand break repair (SSBR) and DNA double-strand break repair (DSBR) by promoting the recruitment of protein scaffolds at sites of chromosome breakage and thereby enabling the assembly of enzyme complexes that process and repair DNA breaks. New data identifying the mechanism/s by which PARP enzymes achieve this, and by which SSBR and DSBR protein complexes are assembled, will be presented.
Plenary session

Presenter: Ross Chapman

53BP1 tunes p53-dependent transactivation events independently of the DNA damage response

Raquel Cuella-Martín(1), Catarina Oliveira(1), Helen Lockstone(2), Natalia Gromulsova(1), J. Ross Chapman(1)

1. Chromatin and Genome Integrity Laboratory, Wellcome Trust Centre for Human Genetics, University of Oxford, UK; 2. Bioinformatics and Statistical Genetics, Wellcome Trust Centre for Human Genetics, University of Oxford, UK

The tumour suppressor protein 53BP1 was first identified as a p53-interacting protein over two decades ago, however its direct contribution to p53-dependent cellular activities remains enigmatic. We have reinvestigated the link between 53BP1 and p53 and demonstrate 53BP1 plays an important role in directly regulating p53-dependent transcription in response to multiple stimuli. Specifically, we find p53-dependent transactivation events are significantly impaired in the absence of 53BP1, and will provide mechanistic insight into the source of this defect. Moreover, we have fine-mapped the domains in 53BP1 that modulate p53 activity and reveal it requires multiple features that include tandem-BRCT domain-mediated interactions with p53. Lastly, we have identified a novel and essential component of the p53-53BP1 axis that directly interacts with 53BP1. These interactions are essential for supporting an optimal p53-dependent response, yet are dispensable for 53BP1’s canonical DNA repair activities. Collectively, our data provides the first mechanistic insight into 53BP1’s direct regulation of the p53-dependent transcriptional programme, and reveals this activity to be distinct and separable from its better-characterised DNA repair activities. Our study therefore defines important and novel functions for 53BP1 in enforcing a vital tumour suppressor pathway that are likely to contribute to tumour suppression.
Keynote address

Friday, April 22, 9:00 – 9:45 h
Plenary session  
Presenter: Tomas Lindahl

The intrinsic fragility of DNA  

Tomas Lindahl  
Francis Crick Institute, Clare Hall Laboratory, Hertfordshire, United Kingdom

Tomas Lindahl received the Nobel Prize in Chemistry 2015 for mechanistic studies of DNA repair.

Living cells have DNA molecules that carry an organism's genes. For the organism to live and develop, its DNA cannot change. DNA molecules are not completely stable, and they can be damaged. Damaged sites in the chromosomal DNA can result in cell death or cancer, but may be corrected by DNA repair enzymes prior to phenotypic expression.

From the mid-1970s, through studies of bacteria, Tomas Lindahl showed how certain protein molecules, repair enzymes, remove and replace damaged parts of DNA. These discoveries have increased our understanding of how the living cell works, the causes of cancer and aging processes.

Plenary session: 'Clinical Aspects-II
Friday, April 22, 9:45 – 12:05 h
Plenary session

Presenter: Kristijan Ramadan

The molecular mechanism of SPRTN (DVC1) in Ruijs-Aalfs or SPARTAN syndrom

Kristijan Ramadan
Cancer Research UK/MRC Oxford Institute for Radiation Oncology, University of Oxford, UK

We have demonstrated that the central element of the ubiquitin-proteasome system, AAA+ ATPase p97 (known as VCP in mammals or Cdc48 in lower eukaryotes), plays an essential role in chromatin-associated proteostasis and genome stability by removing ubiquitinated substrates from chromatin. Recent discovery of a new human syndrome (Ruijs-Aalfs or Spartan syndrome) caused by biallelic and monogenic mutations in p97 cofactor SPRTN (also known as DVC1) further supports our model of chromatin-associated proteostasis and its role in genomic stability. Ruijs-Aalfs or Spartan syndrome is characterised by genomic instability, premature ageing and early-onset hepatocellular carcinoma. We are now able to prove that SPRTN possesses an intrinsic metalloprotease activity that removes chromatin-bound proteins and thus maintains chromatin proteostasis and genomic stability. I will discuss our unpublished results on the molecular mechanism of the SPRTN metalloprotease activity in chromatin-associated proteostasis and genome stability, and why SPRTN-patient mutations are pathogenic in Ruijs-Aalfs or Spartan Syndrome.
Brca1- and Brca2-deficient cells have reduced capacity to repair DNA double strand breaks (DSBs) and consequently are hypersensitive to DNA damaging agents such as platinum salts and poly(ADP-ribose) polymerase (PARP) inhibitors. While these agents are clinically effective, the majority of Brca1/2-mutant carcinomas acquire resistance to these drugs3. Besides reduced uptake and increased efflux of drugs, the most well described mechanism that drives chemotherapeutic resistance in Brca1/2-deficient tumors is through the restoration of homologous recombination (HR). Here we show that loss of the MLL3/4 complex protein PTIP protects Brca1/2-deficient cells from DNA damaging agents and rescues the lethality of Brca2-deficient embryonic stem cells. However, PTIP deficiency does not restore HR activity at DSBs. Instead, its absence inhibits the recruitment of the MRE11 nuclease to stalled replication forks, which in turn, protects nascent DNA strands from extensive degradation. More generally, acquisition of PARPi and cisplatin resistance is strongly associated with replication fork (RF) protection in Brca2-deficient tumor cells that do not develop secondary reversion mutations. Disruption of multiple proteins including PARP1 (ARTD1) and CHD4 leads to the same end point of RF protection, highlighting the complexities by which tumor cells evade chemotherapeutic interventions and thus acquire drug resistance.
Chromatin regulates genome targeting by cisplatin

Emmanouil Zacharioudakis(1), Poonam Agarwal(2), Alexandra Bartoli(1), Nathan Abell(2), Blerta Xhemalce(2), Raphaël Rodriguez(1), Kyle M. Miller(2)

1. Institut Curie Research Center, Organic Synthesis and Cell Biology Group, Paris Cedex 05, France; 2. Department of Molecular Biosciences, Institute for Cellular and Molecular Biology, University of Texas at Austin, USA

Cisplatin treatment damages DNA and is extensively used to treat several cancers including ovarian and testicular. The cellular response to cisplatin is pleiotropic and inherently complex, making it challenging to identify drug mechanisms of cisplatin for the use of this drug as an anticancer treatment and during drug resistance, a major limitation of cisplatin use in the clinic. As a means to overcome these obstacles to provide new insights into cisplatin mechanisms of action, we have developed a cisplatin derivative that can be fluorescently labeled or tagged in cells. The chemical labeling of our cisplatin probe in cells enabled the visual detection of platinated DNA lesions for the first time. Pharmacological inhibition of histone deactylases (HDACs) promoted focal drug accumulation into visible drug foci that co-localized with the translesion synthesis (TLS) regulator RAD18. The TLS pathway normally bypasses cisplatin lesions to allow damage tolerance, a mechanism involved in resistance to cisplatin. However, we observed that cisplatin and HDACi co-treatment synergistically activated apoptosis in cancer cells. These results suggest that formation of clustered platinated DNA adducts by HDACi co-treatment blocks the “DNA damage tolerance” function of TLS. These findings have important implications in cisplatin drug resistance cells that utilize TLS. Our results suggest that HDACi treatment can resensitize cisplatin-resistant cells by creating local clusters of platinated lesions that are inhibitory towards “bypass” mechanisms afforded by TLS pathways. The ability to identify platinated lesions in cells allowed for the unbiased screening of small molecule modulators of genome targeting with platinum drugs and provided unprecedented insights into how pharmacological alterations of chromatin can sensitize cancer cells and drug resistant cells to this class of chemotherapeutic compounds.
Genetic dissection of tumor development, therapy response and resistance mechanisms in mouse models of BRCA1-deficient breast cancer

Jos Jonkers
Division of Molecular Pathology, Netherlands Cancer Institute, Amsterdam, The Netherlands

BRCA1-deficient cancers are defective in DNA double-strand break repair via homologous recombination and therefore hypersensitive to DNA-damaging agents, including platinum drugs and PARP inhibitors. However, these treatments do not result in tumor eradication and eventually resistance develops. To maximize therapeutic efficacy of these drugs and achieve durable remissions, it is important to identify new vulnerabilities of BRCA1-deficient cancers, and to unravel the mechanisms by which these tumors acquire resistance to platinum drugs and PARP inhibitors, in order to develop combination therapies that prevent development of resistance or re-sensitize resistant tumors.

We have established several genetically engineered mouse models (GEMMs) and patient-derived tumor xenograft (PDX) models for BRCA1-deficient breast cancer. These mice develop mammary tumors that are characterized by genomic instability and hypersensitivity to DNA-damaging agents and PARP inhibitors. We have used these mammary tumor models for preclinical evaluation of therapy response and elucidation of mechanisms of acquired drug resistance. Using functional genetic screens, reverse genetics and genomic analysis of therapy-resistant tumors, we found that therapy response and resistance of BRCA1-deficient mammary tumors to cisplatin and the clinical PARP inhibitor olaparib is affected by several factors, including drug efflux transporter activity, type of BRCA1 founder mutation and 53BP1 or REV7 status. Also BRCA1 re-activation via genetic or epigenetic mechanisms contributes to acquired therapy resistance in PDX models of BRCA1-deficient breast cancer. Additional resistance mechanisms are under investigation and will be discussed.
Plenary session  Clinical Aspects-II

Presenter: Lenka Oplustil O’Connor

A nanoparticle topoisomerase I inhibitor combination with DDR agents provides a novel approach to increasing therapeutic index

Lenka Oplustil O’Connor(1), Anderson Wang(1), David Jones(1), Rajesh Odedra(1), Michael Spreadborough(1), Jamini Reens(1), Scott Eliasof(2), Andres Tellez(2), Claire Sadler(1), Mark O’Connor(1)

1. AstraZeneca, UK; 2. Cerulean Pharma, USA

Topoisomerase I inhibitors are used as standard-of-care chemotherapy in many types of cancer but are associated with significant toxicities. There is potential to improve their efficacy further by combining with inhibitors of the DNA damage response, such as the PARP inhibitor olaparib. However, while preclinical data highlight the improved efficacy of this combination, subsequent clinical trials have struggled due to dose limiting myelotoxicity.

CRLX101 is an investigational nanoparticle-drug conjugate (NDC) containing the payload camptothecin (the most potent topoisomerase I inhibitor known). This agent is preferentially targeted to tumours and demonstrated a favourable toxicity profile in the clinic.

Here, we explored the molecular mechanism and therapeutic potential of combining CRLX101 with either olaparib or the WEE1 inhibitor AZD1775, by testing both efficacy and safety in preclinical models. In vitro studies using NCI-H417a small cell lung cancer (SCLC) cells demonstrated that combination with both olaparib and AZD1775 potentiated the efficacy of CRLX101 although by different mechanisms. Cellular analyses revealed that CRLX101 treatment alone predominantly activated ATM-mediated DNA damage response and resulted in late S/G2 cell cycle arrest. Combination with a PARP inhibitor further enhanced the CRLX101-induced DNA damage response and prolonged cell cycle arrest in late S/G2 phase. In contrast, WEE1 inhibition abrogated late S/G2 cell cycle arrest induced by CRLX101, resulting in aberrant mitotic entry and enhanced cell death.

Our in vivo studies using wild type Wistar rat model showed that CRLX101, olaparib and AZD1775, are well tolerated as single agents. However, concurrent combination of CRLX101 with either olaparib or AZD1775 resulted in a dose-dependent decrease in haematological parameters. We investigated sequenced schedules and demonstrated that at a 24h delay between the CRLX101 and olaparib mitigates much of the combined bone marrow toxicity, while improving the efficacy above CRLX101 alone in xenograft tumours from NCI-H417a cells.

Collectively, these preclinical data demonstrate increased anti-tumour efficacy of CRLX101 when combined with DDR inhibitors. The combination schedule for CRLX101 and olaparib identified in our preclinical models as providing an increased therapeutic index has been used to develop protocols to test this combination in a relapsed (2nd line) SCLC human clinical trial (in collaboration with NCI).

(1) Safety and tolerability of the PARP inhibitor olaparib (AZD2281) in combination with topotecan: a phase I study, Samol et al., Invest New Drugs 2012
(2) CRLX101 – a novel nanopharmaceutical of camptothecin in clinical development, Young et al., Current Bioactive Compounds 2011
Exploiting Cancer’s Addiction to Deregulated G1/S Transcription

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Oncogene-induced replication stress is a crucial driver of genomic instability and one of the key events contributing to the onset of cancer. Despite its proven role in cancer-initiation and development, the mechanisms underlying oncogene-induced replicative stress and the ability of cancer cells to tolerate these high levels of replication stress remain poorly understood. Insight into this will help to define the best therapeutic window in which to target pathways that enable cancer cells to cope with high levels of replication stress.

To prevent replication stress-induced DNA damage cells have evolved a cellular response named the DNA replication stress checkpoint. Previously work from our lab has shown that the DNA replication stress checkpoint maintains E2F-dependent G1/S cell cycle transcription. We now show that sustained E2F-dependent transcription is a key mechanism in the DNA replication stress checkpoint response. Our work shows that sustained E2F-dependent transcription is both required and sufficient for many essential functions of the checkpoint response, including fork stalling, stabilisation and resolution.

Importantly we find that also in the context of oncogene-induced replication stress, where increased E2F activity is thought to lie at the basis of causing replication stress, E2F-dependent transcription is required to limit DNA damage levels. The increased reliance on sustained E2F-dependent transcription creates a potentially large therapeutic window for damaging cancer cells without affecting normal cells. In line with this, our work in yeast shows that many G1/S target genes acquire essential functions after pathological deregulated G1/S transcription. These G1/S target genes, regulated by E2F in human cells, may represent therapeutic targets to selectively kill cancers with high levels of replication stress.

Oncogenes induce E2F-dependent transcription during the G1 phase of the cell cycle to drive proliferation. This causes premature entry into S phase, thought to be at the basis of oncogene-induced replication stress. Our data indicates that inappropriate E2F activity during S phase increases replication fork speed, activating the DNA replication checkpoint. These data suggest that deregulation of E2F-dependent transcription during different phases of the cell cycle has the potential to induce replication stress by different mechanisms. Linking distinct deregulation of E2F-dependent transcription to specific cancer-associated mutations will aid in the diagnosis and treatment of human cancers.

Our work highlights a far greater role than previously suspected for G1/S transcription in determining the outcome of DNA replication stress. Gaining a comprehensive understanding of the mechanisms by which E2F-dependent transcription contributes both to induction of oncogene-induce replication stress and tolerance to it will therefore provide potential new strategies for cancer treatment.
Keynote address
Friday, April 22, 12:05 – 12:40
Maintaining Nature’s perfection: the impact of DNA repair on sustaining health

W. Vermeij(1), M. Dollé(2), E. Reiling(1,2), D. Jaarsma(3), C. Payan-Gomez(1), C. Bombardieri(1), A. Roks(4), R. Brandt(1), S. Barnhoorn(1), Á. Gyenis(1), J. Pothof(1), H. van Steeg(2), and J. Hoeijmakers(1)

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The molecular basis underlying ageing and ageing-related diseases is one of the main unsolved questions in biology. Ageing in various model organisms appears remarkably plastic: e.g. suppressing insulin signalling extends lifespan in worms, flies and mice. On the other hand, virtually all premature aging syndromes in man provide a link with genome instability. We have generated mouse models which strikingly mimic human DNA repair deficiency syndromes and display wide-spread accelerated aging. For instance, DNA repair-deficient Ercc1Δ/- mice defective in 3 or more repair pathways show numerous accelerated aging features limiting lifespan to 4-6 month. Simultaneously they exhibit an anti-aging ‘survival response’, which suppresses growth and enhances maintenance, resembling the longevity response induced by dietary restriction (DR). Interestingly, subjecting these progeroid, dwarf mutants to actual DR resulted in the largest lifespan increase recorded in mammals. Thirty percent DR tripled median and maximal remaining lifespan, and drastically retarded numerous aspects of accelerated aging, e.g. DR animals retained 50% more neurons and maintained full motoric function. Repair-deficient XpgΔ/- mice also showing many premature aging symptoms responded similarly to DR, extending this observation beyond Ercc1. The DR response in Ercc1Δ/- mice resembled DR in wild type animals including reduced insulin signaling. Interestingly, ad libitum Ercc1Δ/- liver expression profiles showed gradual preferential extinction of expression of long genes, consistent with genome-wide accumulation of stochastic, transcription-blocking lesions, which affect long genes more than short ones. DR largely prevented this decline of transcriptional output, indicating that DR prolongs genome function. Phenotypes of conditional DNA repair models targeting aging to selected organs will be presented exhibiting striking parallels with Alzheimer’s disease. Our findings strengthen the link between DNA damage and aging, establish Ercc1Δ/- mice as powerful model for identifying interventions to promote healthy aging, reveal untapped potential for reducing endogenous damage, provide new venues for understanding the molecular mechanism of DR, and suggest a counterintuitive DR-like therapy for human progeroid genome instability syndromes and DR-like interventions for preventing neurodegenerative diseases.
POSTER SESSION A1: 'Base Excision Repair'

Poster viewing: Sunday, April 17, 20:00 – 22:00 h

Discussion: Monday, April 18, 16:55 – 18:25 h
Regulation of ER stress by the DNA repair enzyme Alkyl Adenine Glycosylase (AAG)

Clara Forrer Charlier(1), Diana Bordin(1), Abdullah Aljohani(1), Axel Nohturfft(2), Leona Samson(3), Lisiane Meira(4)

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Dysregulation of essential stress response pathways, such as the DNA repair response and ER stress, underpin the pathogenesis of several chronic diseases associated with ageing. We propose the DNA repair enzyme Alkyl Adenine Glycosylase (AAG) is a critical linchpin mediating the crosstalk between the DNA repair response and the ER stress response pathways. To investigate the mammalian response to alkylation, and the role played by DNA damage and repair in the process, we examined the global transcriptional response mounted by the mouse liver to the alkylating agent methyl methane sulfonate (MMS) in mice deficient in Aag compared to wild type animals. Our results indicate that the DNA repair process itself, initiated by Aag, is linked to a robust transcriptional response and this response is profoundly altered in the absence of Aag. Importantly, transcripts related to endoplasmic reticulum (ER) stress/unfolded protein response (UPR) and the acute phase response are up-regulated after MMS treatment, but only in wild type liver, suggesting either that Aag-dependent DNA damage detection and/or processing drives cellular systemic stress responses or that Aag has repair independent functions necessary for the induction of the unfolded protein response. To confirm these results, we performed qPCR for markers of ER stress (ATF6 and CHOP) in MMS-treated liver tissue of wild type and Aag null mice. We find that CHOP expression is significantly induced after MMS treatment in the wild-type liver (p<0.0001), but, importantly, Aag knock-out leads to a significant reduction in CHOP induction (p=0.0052). Finally, to confirm that ER stress can be induced in response to MMS in a human cell model, we used a luciferase reporter gene assay, consisting of a construct where a promoter containing multiple ATF6 binding sites drives the transcription of the luciferase reporter gene luc2P in response to ER stress. These constructs were used to transfect both ARPE-19 (human retinal pigment epithelium) and liver HepG2 cells that were then treated with MMS or with the classical ER stress inducer thapsigargin. As expected, thapsigargin treatment induced luciferase expression. Importantly, luciferase expression was also induced in an ATF6-specific and dose dependent manner after MMS treatment, confirming that alkylation treatment can trigger the ER stress response. Luciferase expression in response to MMS will also be examined in our recently developed ARPE-19 AAG knock-out cell system to confirm AAG is driving alkylation-induced ER stress. Taken together, our data strongly suggests a new role for AAG in driving ER stress in response to alkylation.
DNA polymerase β (Polβ) is essential for base excision/single strand break repair (BER/SSBR) of lesions such as those induced by methylating agents and radiation. We found that Polβ protein expression, as determined by IHC analyses, is frequently and significantly overexpressed in prostate tumors and glioblastoma, as compared to matched normal tissue. We previously demonstrated that cellular Polβ and XRCC1 levels are tightly regulated and stability is governed by Hsp90-mediated and Polβ/XRCC1 binding dependent degradation processes. The cause and cellular consequence of tumor associated overexpression is not known. Considering its frequent occurrence in multiple tumor types, it is however also not clear whether overexpression of Polβ affects repair or therapeutic response. While Polβ overexpression results in increased cellular resistance to methylating agents, here, we show that Polβ overexpression (Polβ-ov) sensitizes to radiation. Radiosensitization is remarkably strong and is comparable to XRCC1 or Polβ loss by knockdown. Sensitization was robust and observed in multiple tumor cell lines of different origin. This radiation phenotype contrasts to the increased resistance observed after methylating agents, suggesting a damage dependent interference in BER activity or other DNA damage repair processes.

Due to its potential impact in cancer treatment response, we further analyzed this potential interference in DNA repair. Consistent with the decreased survival after radiation exposure and an impact on DNA repair, Polβ-ov results in increased Rad51 foci, γH2AX foci and PAR formation after radiation. However, fiber assays also demonstrated interference in replication and shows an elevation in stalled replication forks in Polβ-ov cells after radiation. Inhibition of ATM, ATR and HSP90, but not PARP, resulted in greater radiosensitization in Polβ-ov cells, indicating an ATM- and ATR-independent DNA damage repair defect that is further amplified by DSB repair inhibition. Using mutant Polβ-ov models, we show that the apparent Polβ-ov radiation repair defect did require Polβ’s dRP-lyase activity but not the Polβ/XRCC1 interaction site.

Overall, our results suggest that increased expression or stabilization of Polβ protein, such as found in several tumor types, results in interference in DNA repair and replication processes, revealing Polβ as a potential target for therapeutic strategies.
Inhibition of single strand break repair (SSBR) in tumour cells that have defective double-strand break repair (DSB) has proved effective in the clinic and Poly-ADP ribose polymerase (PARP) inhibitors have been approved for the treatment of cancer in patients with mutations in the homologous recombination repair genes BRCA1 and BRCA2. Poly-ADP ribose glycohydrolase (PARG) degrades the polymers of ADP ribose (PAR) that are built up on PARP and is instrumental in the correct functioning of SSBR. Inhibition of PARG may overcome potential resistance mechanisms arising from functional redundancy within the PARP/ARTD family. Alternatively, there may be a completely distinct patient population, as yet unidentified, that would benefit from treatment with PARG inhibitors. Previous efforts to develop PARG inhibitors have generally been hampered by poor physicochemical properties, off-target pharmacology and a lack of cell permeability. We have carried out a high throughput screen (HTS) and through computational design and focussed medicinal chemistry we have developed a series of novel PARG inhibitors which display drug-like properties and attractive pharmacokinetic parameters. Resultant compounds show selectivity against PARP and a close glycohydrolase homologue ARH3. More importantly, the compounds demonstrate inhibition of PARG cellular activity. Furthermore, these derivatives have allowed us to explore if the phenotypes resulting from genetic reduction of PARG are reproduced with small molecule inhibition.

We have dosed cervical and breast cancer cell lines with methyl methane sulphonate (MMS) to promote SSBR activity and show that the addition of PARG inhibitors prevents hydrolysis of the resulting PAR chains. In addition, we have determined the effect of PARG inhibition on HeLa cell proliferation and show that in this 2D setting PARG inhibition greatly increased the growth inhibitory effect of MMS. We have also investigated the consequence of PARG inhibition on gamma-H2AX levels in breast cancer cells. Our results show that PARG inhibitors may be important tools to discover new therapeutic strategies and uncover the complex mechanisms of DNA damage repair.
Cells deficient in base-excision repair reveal cancer hallmarks originating from adjustments to genetic instability

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Genetic instability, provoked by exogenous mutagens, is well linked to initiation of cancer. However, even in unstressed cells, DNA undergoes a plethora of spontaneous alterations provoked by its inherent chemical instability and the intracellular milieu. Base excision repair (BER) is the major cellular pathway responsible for repair of these lesions, and as deficiency in BER activity results in DNA damage it has been proposed that it may trigger the development of sporadic cancers. Nevertheless, experimental evidence for this model remains inconsistent and elusive. Here, we performed a proteomic analysis of BER deficient human cells using stable isotope labelling with amino acids in cell culture (SILAC), and demonstrate that BER deficiency, which induces genetic instability, results in dramatic changes in gene expression, resembling changes found in many cancers. We observed profound alterations in tissue homeostasis, serine biosynthesis, and one-carbon- and amino acid metabolism, all of which have been identified as cancer cell ‘hallmarks’. For the first time, this study describes gene expression changes characteristic for cells deficient in repair of endogenous DNA lesions by BER. These expression changes resemble those observed in cancer cells, suggesting that genetically unstable BER deficient cells may be a source of pre-cancerous cells.

Investigating MUTYH promoter function: The setup of a minigene model

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Inactivating germline mutations in the human DNA repair gene MUTYH are associated with colorectal polyposis and cancer. This recessively heritable disease is called MUTYH-associated polyposis (MAP). The MUTYH protein is involved in the correction of mismatches resulting from a faulty base pairing of the oxidized base 8-hydroxyguanine (8-oxoG) with adenine. This, in turn, can give rise to a transversion mutation during the following replication. For that reason tumors occurring in MAP patients bear distinctive somatic GC>TA transversions e.g. in the APC or KRAS target genes.

The MUTYH gene shows strong alternative splicing, especially in exon 1 and 3. Plotz et al. previously described that the alternative first exons α, β and γ show distinct expression patterns in different tissues. Nuclear-targeted proteins from the β and γ transcripts are mostly expressed in highly proliferative tissue, while the α form (whose protein is targeted to the mitochondria) is predominantly expressed in muscle tissue. But how this tissue specific expression and splicing is being regulated is still unknown. It is also not known to what extent single nucleotide alterations found in the promotor region in polyposis patients affect the correct alternative transcription of the gene.

For studying this regulation and for investigating the effects of substitutions, we constructed a minigene which contains a large DNA segment comprising the putative promotor regions and the transcriptional start positions of the alternative MUTYH transcripts. We introduced specific alterations to distinguish transcripts of the minigene from endogenous MUTYH transcripts and tested the efficiency of transcription and splicing by transient transfection in cell culture and subsequent transcript analysis. We present our initial results of this novel minigene model for alternative transcription of the MUTYH gene.

Formation of irreversible complexes with abasic sites in DNA is a new putative function of GAPDH under oxidative stress

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an evolutionary conservative and abundant protein widely known as a glycolytic enzyme. Active GAPDH is a tetramer consisting of identical 37 kDa-subunits. Interestingly, GAPDH has many additional putative functions: it takes part in membrane transport, microtubule bundling, signal transduction and many other processes. GAPDH is known to interact with DNA and RNA, some types of DNA damages and DNA repair enzymes (APE1, HMGB1).

Abasic (AP) sites are among the most common genomic DNA lesions. The loss of DNA bases and the subsequent formation of AP sites occur as a result of the spontaneous hydrolysis of the N-glycosidic bond or the removal of the damaged bases at the early stage of base excision repair, which is catalyzed by DNA glycosylases. Unrepaired AP sites are mutagenic and cytotoxic.

Certain proteins can interact with the deoxyribose of an AP site to form a Schiff base, which can be stabilized by borohydride treatment. Several types of AP DNA were used to trap proteins in human cell extracts by this method. In the case of single-stranded AP DNA and the AP DNA duplex with both 5’ and 3’ protruding ends, the major crosslinking product had an apparent molecular mass of 45 kDa. Using peptide mass mapping based on mass-spectrometry data, we identified the protein forming this adduct as an isoform of GAPDH. Next, we have studied interaction of GAPDH purified from HeLa cells with different AP DNAs. Intriguingly, about 30% of GAPDH–AP DNA adducts were borohydride-independent. These data indicate that GAPDH, at least partially, may be covalently linked with an AP site by a mechanism other than the Schiff base formation. Indeed, GAPDH was shown to lose the ability to form adducts with AP DNA after disulfide bond reduction. NAD+ also inhibited GAPDH–AP DNA adduct formation. GAPDH was proven to crosslink preferentially to AP DNA cleaved via the β-elimination mechanism (spontaneously or by AP lyases), but it did not display the AP lyase activity. Moreover, we used AP DNA as a probe for GAPDH detection in cell extracts. GAPDH formed the adducts with AP DNA with the highest intensities in the extracts of the lymphoid cells.

One can assume that under oxidative stress the following scenario may be realized. GAPDH undergoes disulfide bond formation that results in the enhancement of its DNA-binding capacity and translocates to the nucleus. At the same time, oxidative DNA damages lead to the activation of PARP-1, which synthesizes poly(ADP-ribose) using NAD+. As a result, the pool of NAD+ is exhausted and the NAD+-binding site of GAPDH is empty. Thus, the enzyme acquires the ability to bind DNA and, if the unrepaired AP site is present, GAPDH can be trapped in the stable covalent complex that would hamper DNA repair. This may be a suicidal event in the case of multiple DNA damage that could be one of the factors leading to cell death.

This work was supported by the RSF grant no. 14-14-00501.
**Base excision repair - A1**

Presenter: Luis Polo

**New structural insight of PARP3 as a DNA-damaged sensor**

*Luis M. Polo(1), Gabrielle Grundy(1), Stuart Rulten(1), Yingqi Xu(2), Pathompong Paomephan(1), Steve Sweet(1), Stephen Matthews(2), Antony Oliver(1), Keith Caldecott(1), Laurence Pearl(1)

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ADP-ribosylation of chromatin is an important signal of DNA damage in animal cells. In mammals, this reaction is performed by specialised members of the PARP — poly-(ADP ribose) polymerase — family of enzymes, which are activated by a range of DNA strand nicks, breaks and distortions. The most recently characterised member of the family is PARP3, which participates in the repair of chromosomal DNA single-strand breaks. PARP3, like the structurally related PARP1 and PARP2 enzymes, contains two highly conserved domains — WGR (Trp-Gly-Arg) and Catalytic — connected together by a short 15 amino acid linker region. In addition, it also contains a largely uncharacterised 48 amino acid region at its N-terminus. 2D NMR experiments reveal that PARP3 employs a conserved DNA binding interface within the WGR domain to detect ligatable DNA nicks with canonical termini. It was described that PARP3 shows specificity for nicked DNA substrates with a 5’-phosphate adjacent to the DNA break. In vitro, nicks in naked DNA stimulate PARP3 autoribosylation, however we show here that nicks in mononucleosomes promote trans-ribosylation of this histone target. Furthermore, PARP3 accelerates the repair of chromosomal radiation-induced single-strand DNA breaks in cells and PARP3 deficiency sensitises them to radiation. These data identify the sensing mechanism of PARP3 of damaged DNA and establish it as a molecular sensor of nicked nucleosomes.
**Base excision repair - A1**

Presenter: Larissa Milano de Souza

**Elucidating the role of alkylation DNA damage repair in the response of glioblastoma cells to radiotherapy**

*Milano L.(1,2), Machado M.(2), Henriques J.(2), Lenz G.(3), Meira L.(1)*

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Glioblastoma multiforme (GM) is a highly malignant type of brain tumour that is presently incurable. Temozolomide (TMZ) is an oral alkylating agent and the only clinically approved adjuvant treatment for GM, along with surgery and radiation therapy. Expression of alkyl adenine DNA glycosylase (AAG), the enzyme that initiates Base Excision Repair (BER) for alkylation DNA damage, is an important determinant in GM’s patients overall survival. This project aims to explore the possibility that AAG is an important determinant in GM response to chemotherapeutic and radiation therapy. For this, we propose to study the response of human GM cells that are either knock-down or overexpressing AAG to treatment with TMZ in combination with radiotherapy, using cellular and biochemical assays to study survival, metabolism and repair. For silencing in T98G cells, was used a lentivirus system with shAAG and control plasmids. AAG protein and mRNA levels in GM cells T98G Wild Type and T98G NS (Non Silenced) as well as in transduced T98G cells shAAG1, shAAG2, shAAG3 and shAAG4 were analyzed. The shAAG’s lines showed lower AAG expression, corresponding to approximately 40 - 60% decrease in relation to NS levels. The biological characterization of AAG’s role in the response of T98G cells to alkylation is ongoing; however, our preliminary results show that AAG silencing at the levels mentioned above did not affect cell cycle and sensitivity to MMS. The analysis with AAG overexpressing cells is in progress.
Neil DNA glycosylases coordinate substrate hand-over during oxidative DNA demethylation

In vertebrates, cytosines within CpG dinucleotides can be methylated at carbon 5. DNA cytosine methylation is an epigenetic mark which plays important roles in development and disease. It is now widely accepted that DNA methylation is dynamic and is subject to enzymatic demethylation. Yet, propositions of repair-based demethylation mechanisms have met with skepticism because the danger of genomic instability for cells is considered too high. Thus, a key question is what are the biochemical mechanisms which ensure that DNA demethylation proceeds as an orderly controlled process. In the Tet/Tdg-mediated demethylation pathway, methylated cytosine is iteratively oxidized by ten-eleven translocation (Tet) dioxygenases and unmodified cytosine is restored via thymine DNA glycosylase (Tdg) and base excision repair. The most vulnerable intermediate in Tet/Tdg demethylation is the abasic (AP) site which, if unprotected, is unstable and cytotoxic. Here we show that the human nei endonuclease VIII-like bifunctional DNA glycosylases/AP lyases NEIL1 and NEIL2 coordinate AP site processing during TET/TDG DNA demethylation. Instead of processing cytosine derivatives directly as DNA glycosylases, NEILs cooperate with TDG. After base excision, TDG occupies the AP site and is displaced by NEILs which further process the baseless sugar, thereby stimulating TDG substrate turnover. In early Xenopus embryos Neil2 cooperates with Tdg to remove oxidized methylcytosines and to specify neural crest development together with Tet3. Thus, Neils function as AP lyases in the coordinated substrate hand-over during oxidative DNA demethylation.
Base Excision Repair (BER) is an efficient process to protect mammalian cells against the most common types of DNA damage. Multiple protein-protein interactions between BER enzymes and accessory proteins contribute to the regulation and coordination of the overall process. Most of them are transient making them difficult to detect by traditional biochemical approaches. Here we characterize quantitatively for the first time the strength of dynamic protein-protein interactions in a number of complexes formed by DNA polymerase β (Polβ), apurinic/apyrimidinic endonuclease 1 (APE1), poly(ADP-ribose) polymerase 1 (PARP1), X-ray repair cross-complementing protein 1 (XRCC1) and tyrosyl-DNA phosphodiesterase 1 (TDP1), using developed by us original fluorescence-based approaches. Binary complexes of APE1 with PARP1 and TDP1, and of Polβ with TDP1 as well as DNA-independent association of APE1 with Polβ have been revealed and characterized quantitatively for the first time. The oligomeric state and stability of several complexes are further explored by light scattering techniques. The combined results provide strong evidence that the most stable complex is formed between XRCC1 and Polβ. Rearrangements of most complexes induced by model DNA intermediates mimicking different steps of BER are detected by fluorescence resonance energy transfer (FRET) experiments. The strength of protein-protein interactions is modulated to different extents by the various DNA intermediates. We quantitatively characterized the interaction between APE1 and Polβ in the absence and presence of various BER intermediates under true equilibrium conditions. Distinct effects were produced by the various DNAs on the APE1-Polβ binding affinity and FRET efficiency, indicating that they are related to regulation and coordination of the enzymatic functions during BER. An unexpected finding is that Polβ interacts more strongly with APE1 in the presence of AP site-containing DNA than in the complex mimicking a step after the AP site incision. This result suggests that the APE1-incised BER intermediate is effectively channeled to Polβ immediately during the incision step. The efficiency of FRET within the APE1-Polβ complex appeared sensitive to the presence of XRCC1 and PARP1, indicating modulation of the APE1-Polbetaβ interaction by the formation of ternary protein–protein complexes. The coordination of enzymatic steps mediated by APE1 and Polβ is obviously facilitated by interactions of the enzymes with each other and with the regulatory proteins. Interestingly, rearrangement of the most stable XRCC1-Polβ complex induced by various DNA intermediates of BER was not accompanied by a detectable change in protein–protein binding affinity which is important for key role of this complex in BER. Our findings advance understanding of the molecular mechanisms underlying coordination and regulation of BER.

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Base excision repair - A1

Presenter: Elizaveta Alemasova

**Y-box-binding protein 1 as a non-canonical factor of DNA repair**

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An effective cellular response to DNA damage requires fine-tuned action of DNA repair enzymes at lesions. Tight regulation is achieved by posttranslational modifications and internal interactions of DNA damage response proteins and may be mediated by non-canonical accessory factors.

Y-box-binding protein 1 (YB-1) is a transcription factor involved in variety of cellular processes mainly those concerning nucleic acids metabolism. YB-1 possesses high multivalence as it can interact with DNA, RNA, poly(ADP-ribose) and plenty of proteins including enzymes from different DNA repair pathways. Its distinctive feature is also accumulation in the nucleus upon genotoxic stress. The most part of YB-1 structure is intrinsically disordered that is a hallmark of key regulatory proteins in the cell. However, its role in DNA repair is still open to question.

The aim of present research was to investigate YB-1 potential to participate in base excision repair (BER) pathway as an accessory regulatory protein. The data obtained demonstrated that YB-1 can directly interact with DNA lesions that are processed by BER machinery – abasic site and 5-formyluracil. By using fluorescence titration method, we established physical interactions of YB-1 with key BER proteins: APE1, NEIL1, pol β, PARP1, PARP2 and DNA-binding fragment of PARP1 – p24, with these interactions probably being non-specific. YB-1 was shown to inhibit APE1-dependent and to promote NEIL1-dependent cleavage of abasic site within model DNA oxidative damage cluster. We also observed inhibitory effect of YB-1 on dRP-lyase activity of pol β. We demonstrated for the first time a novel posttranslational modification of YB-1 – poly(ADP-ribose)ylation by PARP1 and PARP2 in the presence of damaged DNA. The covalent attachment of poly(ADP-ribose) polymer results in dramatic decrease in YB-1 affinity to DNA and eliminates the inhibitory impact of YB-1 on APE1 activity. Modulation of APE1 and NEIL1 activity as well as poly(ADP-ribose)ylation by PARP1 was also shown for truncated nuclear form of YB-1 – YB-1(1-219).

These results not only reveal YB-1 potential to facilitate BER, but also offer a challenge for future research as discovered poly(ADP-ribose)ylation of YB-1 can contribute to multiple functions of YB-1 in cell.

This work was supported by fellowship from President of RF and by grant from RSF (14-24-00038).
Inhibiting the DNA damage response (DDR) is an attractive therapeutic strategy in cancer therapy since i) resistance to many of the current standard-of-care genotoxic therapies has been associated with increased DDR signaling and ii) many cancers have defects in certain components of the DDR rendering them highly dependent on other DDR proteins/pathways for survival. Based on the latter, the concept of synthetic lethality as a targeting strategy, has gathered significant momentum in recent years. The incentive to develop this type of personalized therapy for cancer treatment is driven by the premise that it will increase therapeutic efficacy and reduce the toxicity associated with standard chemotherapeutic regimens. Understanding the underlying cellular and molecular basis of the disease has been extremely important in the design of these novel therapies; however, identifying new drug targets for personalized therapies remains problematic. Encouragingly though, there has been some progress in this area; PARP inhibitors are now demonstrating clinical benefit in BRCA1/2 patients. This has driven the search for other synthetic lethal (SL) interactions that could be exploited therapeutically. There are three common methods for identifying novel SL interactions in human cells. These include: (i) RNAi screens using isogenic or functionally relevant cell models (ii) high throughput screens with chemical libraries to identify compounds that kill cells in a genotype-specific manner and (iii) SL approaches using RNAi libraries in combination with chemical inhibitor.

In the drug discovery unit (DDU) at the Cancer Research UK Manchester Institute we are employing ‘hypothesis’ driven approaches both in-house and as collaborations with Pharma and academia to identify potentially clinically relevant SL interactions. Here we show our work confirming a previous report of XRCC1 deficiency in ~16% of breast cancer patient tissue microarrays (1) and our efforts to identify SL partners for X-ray repair cross-complementing protein 1 (XRCC1) and Poly (ADP-ribose) glycohydrolase (PARG), a DDR target for which we have an active drug discovery program. As part of this strategy we initiated a large cell panel screen (240 cancer cell lines) at Eurofins to test cell sensitivity following treatment with one of our PARG inhibitors. We will review the data obtained, the limitations of this approach and our follow up in-house validation studies. We will also elaborate on our discovery of a SL interaction between XRCC1 and PARG and discuss our plans to exploit this.

The over-expression of multifunctional DNA repair enzyme APE1 (also referred as Ref-1) in cancer contributes to enhanced tumor growth and anti-cancer therapy resistance. APE1 participates in both: repair of oxidative DNA damage and redox-dependent transcriptional regulation of oncogenes which may be linked to cellular proliferation. Number of transcription factors (TFs) contain redox-sensitive cysteine residues at their DNA-binding sites. Hence oxygen radicals induced thiol oxidation of these TFs strongly inhibits their DNA binding activities and transcription of target genes. In human cells APE1 stimulates the DNA binding activities of the oxidized TFs that regulate cell growth, differentiation, survival, and death including AP-1, NF-κB, HIF-1α, p53, Egr-1, c-Myb et cetera. It is thought that APE1 acts through thiol-mediated redox reaction using its Cysteine-65 residue to attack the disulfide bond in another protein. This reaction leads to formation of disulfide bond in APE1 between Cys65 and Cys93 and disruption of disulfide bond in the target protein. However, this model was challenged by number of contradictory findings. At present, the molecular mechanism underlying the non-DNA repair functions of APE1 remains unclear. Previously, we showed that the N-terminal redox domain of APE1 is essential for nucleotide incision repair (NIR) suggesting that these two functions share a common mechanism. In this work, we found that APE1 can bind to both regular and damaged DNA duplexes and induce specific conformational changes over entire length of DNA fragment. This APE1-induced DNA conformational change facilitates assembly of TFs on their DNA sites. We propose that APE1 acts as a DNA chaperone that binds to DNA in cooperative manner to introduce changes in helix conformation, this in turn enables DNA cleavage and facilitates binding of the sequence-specific nuclear proteins. Potential role of the APE1-catalyzed redox function in the organization of DNA/chromatin domains and the regulation of DNA transcription and replication in proliferating cells are discussed.
Chlorodeoxyuridine hypersensitizes cells to PARP inhibitors by promoting breakage of the DNA replication template strand and replication fork collapse

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Single-strand breaks (SSBs) are the most frequent form of DNA damage and are rapidly recognised and repaired by a predominantly PARP1 and XRCC1-dependent pathway termed single-strand break repair (SSBR). Loss of PARP1 or XRCC1 increases spontaneous rates of homologous recombination (HR), most likely because unrepaired SSBs induce DNA replication fork collapse and HR-dependent fork restart. PARP inhibitors are currently being trialled for treatment of HR-defective tumours, such as BRCA1/2-deficient breast and ovarian cancers. However, resistance mechanisms are emerging both clinically and in the laboratory, including the reactivation of HR-mediated repair, highlighting the need for better understanding of the mechanisms by which PARP inhibitors kill cells. Here, we report that the thymidine analogue chlorodeoxyuridine (CldU) profoundly increases PARP inhibitor cytotoxicity at concentrations that are otherwise non-toxic to normal cells, such that PARP inhibition is lethal even in HR-proficient cells. We find that CldU causes massive replication fork collapse, G2/M arrest, sister chromatid exchange, and anaphase bridges in SSBR-defective cells with depleted or deleted XRCC1 or PARP1, or in wild type cells in the presence of PARP inhibitor. We show that CldU incorporation during DNA replication sensitises cells to PARP inhibitor in the subsequent S phase, when CldU is present only in the template strands. We propose that CldU induces low levels of SSBs which, while non-toxic in normal cells, are lethal in SSBR-defective cells or in the presence of PARP inhibitor, due to a level of replication fork collapse that overwhelms cellular HR capacity.
Clustered damages (or multiple lesions), where oxidized bases, apurinic/apyrimidinic (abasic or AP) sites and strand breaks are situated within one or two turns of the DNA helix, appear under the action of ionizing radiation and radiomimetic drugs and present a significant problem for the cell. AP sites are among the most frequent endogenous lesions in cellular DNA and their repair is critical for genome stability and cell survival. The presence of AP sites within clusters as well as breaks in the opposite DNA strand increases the probability of double-strand break formation. The combined action of oxidative stress and genotoxic polycyclic aromatic hydrocarbons derivatives can lead to cluster-type DNA damage that includes both a modified nucleotide and a bulky lesion. The major enzyme in eukaryotic cells that catalyzes the cleavage of AP sites is apurinic/apyrimidinic endonuclease 1 (APE1) that cleaves the phospho-diester bond on the 5'-side of abasic sites. We found that the efficiency of AP site cleavage by APE1 was affected by the benzo[a]pyrenyl-DNA adduct (BPDE-dG) in the opposite strand; the position of AP sites influences APE1 efficiency to a greater extent than the isomeric form of the BPDE-dG adducts. AP sites directly opposite the modified dG or shifted to the 5' direction were hydrolyzed by APE1 with the efficiency similar to the AP site in the control DNA duplex whereas AP sites shifted to the 3' direction were hydrolyzed ~100 fold less effectively. For all DNA structures except DNA with the AP site shifted with 3 nucleotides to the 5' direction (AP(+3)-BP-DNA), hydrolysis was more effective in the case of (+)-trans-BPDE-dG. Using molecular dynamic simulation, we have shown that in AP(+3)-BP-DNA the BPDE-dG residue is located within the DNA bend induced by APE1 and contacts with amino acids in the enzyme catalytic center and with the catalytic metal ion. The geometry of APE1 active site is perturbed more significantly by the trans-isomer of BPDE-dG, which intercalates in the APE1-DNA complex near to the cleaved phosphodiester bond. Recently, we have shown that human tyrosyl-DNA phosphodiesterase 1 (TDP1) cleaves AP sites with the formation of the 3'- and 5'-phosphate termini, suggesting a novel APE-independent pathway of AP-site processing that can be involved in the repair of AP sites clustered with bulky or other DNA lesions. The efficiency of AP-site cleavage by TDP1 increased in the presence of an additional AP site in the opposite DNA strand depending on its position and was stimulated by poly(ADP-ribose)polymerase 1.

This work was supported by Russian Scientific Found, grant no. 14-14-00501.
Base excision repair - A1

Presenter: Pablo Radicella

Base Excision Repair initiation in the context of nuclear architecture: a role for Cohesin and Mediator complexes?

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Cellular DNA is continuously exposed to oxidative stress arising from both endogenous and exogenous sources. As a consequence, lesions such as modified bases, abasic (AP) sites, and single-strand breaks (SSBs) are generated. One of the major base lesions induced by oxidative stress is the mutagenic 8-oxoguanine (8-oxoG), which is recognised and excised by the DNA glycosylase OGG1, initiating the base excision repair (BER) pathway. The AP site produced by the OGG1 DNA glycosylase activity is then cleaved by the AP endonuclease APE1, resulting in a SSB. The subsequent synthesis and ligation steps are carried out by polymerase β (POLβ) and ligase 3 (LIG3), respectively, to restore an intact DNA molecule. How DNA repair machineries detect and access, within the context of chromatin, lesions inducing little or no distortion of the DNA structure is still poorly understood. We show that upon induction of 8-oxoguanine, OGG1, together with other proteins involved in BER, is recruited to euchromatin regions rich in RNA and RNA polymerase II and is completely excluded from heterochromatin (Amouroux et al., 2010; Campalans et al., 2013; Campalans et al., 2015). We have implemented a genome-wide siRNA screen to identify proteins potentially involved in the recruitment of OGG1 to chromatin after the induction of 8-oxoG into cellular DNA. Cellular imaging and biochemistry experiments reveal the essential role of the Cohesin and Mediator complexes in the recruitment of OGG1, suggesting a link between BER and transcription. We are currently characterising the role of those complexes in the cascade of events that leads to the initiation of the repair process for 8-oxoG.

The kinetics of DNA damage-induced loading of endogenous XRCC1 into chromatin in PARP1, PARP2 and PARP3 knockout human cells

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The repair of DNA single strand breaks is regulated by the activity of several poly(ADP-ribose) polymerases (PARPs). These enzymes catalyse the polymerisation of ADP-ribose on a diverse range of protein targets at the site of damage, initiating and coordinating multi-step repair events. Despite the progress that has been made in our understanding of the DNA damage-stimulated PARPs (PARP1, PARP2 and PARP3), the extent of their redundancy and importance for SSBR remains unclear. An early SSBR event is the recruitment of the scaffold protein XRCC1, which acts as a platform for subsequent DNA end-processing, gap filling and religation steps. Previous studies have largely relied on focal accumulation of over-expressed XRCC1 and non-physiological sources of DNA damage as a tool for studying XRCC1 recruitment. These approaches have been useful in identifying functional regions of XRCC1 but by their non-physiological nature may not be accurately representing the kinetics and PARP requirements exhibited by endogenous XRCC1. With the aid of a panel of CRISPR/CAS9 generated knockout human cell lines we have established a high-content fluorescence imaging approach for studying the dynamic chromatin-loading of endogenous XRCC1 in response to H2O2 and camptothecin. Data will be presented identifying the requirements of PARP1, PARP2 and PARP3 in these responses.
A dual role of Gadd45a in TET1 driven DNA demethylation

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Growth Arrest and DNA Damage 45- (GADD45) and Ten-Eleven-Translocation (TET) proteins both act in active DNA demethylation however their functional relationship is unresolved. Our data shows that GADD45a physically interacts- and functionally cooperates with TET1 in DNA demethylation. Moreover, TET1 requires GADD45a for reporter demethylation and for global 5mC oxidation. Synergistic gene activation by GADD45a/TET1 is accompanied by both: an increase in 5-hydroxymethylcytosine (5hmC) levels, and a reduction of 5-formyl- (5fC) and 5-carboxylcytosine (5caC). Our data suggest a dual role of GADD45a in active DNA demethylation process: it activates TET1 and simultaneously enhances the subsequent 5fC/5caC removal, thereby promoting gene specific DNA demethylation.
Misincorporation of ribonucleotides (rNMPs) during DNA replication and repair has recently been shown to be more frequent than previously reported, with an estimated two misincorporated rNMPs per kilobase of DNA due to high ratios of ribonucleotide triphosphate/deoxyribonucleotide triphosphate (rNTP/dNTP) pools. Under normal conditions, rNMPs are removed by the RNase H2 pathway. However, if topoisomerase I (Top1) binds to the rNMP before RNase H2 and if RNase H2 is defective, as in Aicardi-Goutières syndrome, Top1 converts rNMPs into nicks bearing 2',3'-cyclic phosphates (1,2).

We will present our recent findings demonstrating the molecular mechanisms and cellular evidence explaining how Top1-mediated DNA nicks at rNMPs give rise to a hypermutagenic phenotype with short base pair deletions (3). We will also present unpublished findings explaining the molecular mechanisms for the generation of toxic DNA double-strand breaks by Top1 in the presence of misincorporated ribonucleotides. Finally, we will discuss the potentially beneficial effects of Top1 as a mechanism to reverse rNMP-induced nicks and possibly initiate rNMP removal.

NRF2 and GSH as key mediators to temozolomide resistance in glioma and melanoma cells

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Cancer is one of main causes of death worldwide and the limiting factor in antitumoral therapy is resistance to chemotherapy. Several mechanisms command drug resistance and many of them can be tissue or drug specific. Thus, it is paramount to unveil the mechanisms involved in chemoresistance to achieve a better therapeutic efficacy. The alkylating agent temozolomide (TMZ) has been widely used to treat glioma, but with limited success due to drug resistance. In the present study, using TMZ sensitive and resistant glioma cell lines, we provide further insights on the role of glutathione (GSH) on TMZ resistance. TMZ induced lower oxidative stress on resistant cells compared to the sensitive ones, but the difference was abolished when BSO (GSH inhibitor) was added to the treatment. In addition, the resistant cells presented higher expression of NRF2, the main antioxidant transcription factor regulator, as well as its molecular targets such as GCML and GSTπ, genes involved in GSH synthesis and use. Interestingly, following TMZ treatment, we observed an induction of mRNA and protein levels of NFR2 and also a higher thiol concentration. It was demonstrated that NFR2 silencing greatly enhanced cell death upon TMZ treatment in vitro and in vivo. Also, we observed a higher DNA damage and cell death when BSO or GSTπ inhibitor was added to medium in combination to TMZ, when compared to TMZ as a single agent. In addition, using in vitro and in vivo model of human and murine melanoma, we showed that BSO potentiated TMZ-induced cell death, indicating that GSH has a decisive role on TMZ resistance. Thus, the therapeutic regimen of BSO and TMZ is also efficient in another tumour model, as devastating as glioma and configures an interesting alternative therapeutic for fighting approach for fighting glioma and melanoma.
Base excision repair - A1

Presenter: Giannis Ampatziadis-Michailidis

The role of transcription deregulation in the SCAN1 disease

Giannis Ampatziadis-Michailidis(1), Zoe Spyropoulou(1), Dimitris Konstantopoulos(1), Sherif El-Khamisy(2), Maria Fousteri(1)

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Patients suffering from SpinoCerebellar ataxia with Axonal Neuropathy 1 (SCAN1) carry mutations in the Tyrosyl-DNA phosphodiesterase 1 (TDP1) gene and their cells display reduced rate of repair of single-stranded DNA breaks arising from topoisomerase 1 (Top1) activity or oxidative stress. Our research focuses on the role of TDP1 in the molecular etiology of SCAN1 and the repair of Top1 cleavable complexes (Top1ccs). Employing Next Generation sequencing approaches we find that mutated TDP1 results in the differential expression of large subsets of genes (RNA-seq profiling of SCAN1 cells) that fall under specific Gene Ontology terms, indicating that their deregulation might be implicated in the disease phenotype. Interestingly, preferential binding of TDP1 in Normal cells in the promoter/transcription start sites of a subset of genes that are downregulated in SCAN1 cells (TDP1 ChIP-seq) implies a role of TDP1 in the expression of these genes. To gain more insights on the role of TDP1 in the repair of Top1ccs, we studied the effects of Camptothecin (CPT), a potent chemotherapeutic on nascent RNA synthesis (GRO-seq) as well as RNA polymerase II and Top1 genome wide occupancy in Normal and SCAN1 cells (RNAPII, Top1 ChIP-seq). Our studies identify a 5’ to 3’ shift of Top1 towards the gene body that highly correlates with active elongating RNAPII in response to CPT. Recovery of cells in CPT-free medium demonstrated that SCAN1 cells show only a partial delay in the recovery of RNAPII dynamics and nascent RNA synthesis in comparison to Normal cells, probably due to the redundant repair pathways of Top1ccs in cycling cells. Only a small number of genes were unable to recover CPT treatment in SCAN1 cells. In conclusion, our studies reveal an unanticipated role of TDP1 in the transcription of specific subsets of genes and underline the causative link between transcription irregularities and the etiology of SCAN1 disease.
Poly(ADP-ribose)polymerase-1 (PARP-1) is a key eukaryotic stress sensor that responds in seconds to DNA single-strand breaks (SSBs), the most frequent genomic damage. A burst of poly(ADP-ribose) synthesis links DNA damage signaling with a concerted modulation of chromatin structure, while inhibition of PARP-1 kills BRCA-deficient tumor cells selectively, providing the first anti-cancer therapy based on synthetic lethality. However, the mechanism underlying PARP-1 function remained obscure; inherent dynamics of SSBs and PARP-1 multi-domain architecture hindered structural studies. Here, we reveal the structural basis of SSB detection, and how multi-domain folding underlies the allosteric switch that determines PARP-1 signaling response. Two flexibly linked N-terminal zinc fingers recognize the extreme deformability of SSBs and drive co-operative, stepwise self-assembly of remaining PARP-1 domains to control activity of the C-terminal catalytic domain. Automodification in cis explains subsequent release of monomeric PARP-1 from DNA, allowing repair and replication to proceed. Our results provide a framework for understanding PARP inhibitor action, and more generally allosteric control of dynamic, multi-domain proteins.
Histone H3 lysine 36 methyltransferase coordinates proper response to alkylating damage in fission yeast.

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Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Post-translational modification imposed epigenetic regulation on the chromatin platform to control its compaction and recruitment of effectors in a timely and spatially-specific manner. One of the most important regulations is the coordination of the response to DNA damage. Recently, it is becoming clear that enzymes that catalyse the transfer of chemical moieties onto the histones intimately modulate the accessibility and precise repair of the DNA. The family of histone methyltransferase that methylate lysine 36 of histone H3 has been shown to be essential for the repair of DNA double-stranded break. To further investigate the involvement of this enzyme in the repair of other forms of DNA damage, we have tested both the null mutant of set2 in fission yeast as well as a point-mutant of histone H3 lysine 36 (H3K36R) to a range of DNA damaging agents and showed a prominent hypersensitivity towards the alkylating agent methyl methane sulfonate (MMS). Epistasis analyses showed that loss of set2 exacerbated the hypersensitivity of base excision repair factors and Ku70 protein, which participates in non-homologous end joining suggesting that Set2 act synergistically with these factors to repair MMS-induced damage. Conversely, Set2 acts in the same functional group as the nucleotide excision repair factors in response to MMS. We further show evidence that Set2 regulate the timely activation of the DNA damage checkpoint protein Chk1. Taken together, our analyses point to a multi-prong control of Set2 of multiple DNA damage response pathways to maintain genomic stability of a fission yeast cell challenged with alkylating damages.
Roles for SUMO in genome stability

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Small Ubiquitin-like Modifiers (SUMOs) are ubiquitin family members which are essential for eukaryotic life. They are predominantly located in the nucleus and have been implicated in orchestration of e.g. transcriptional regulation, chromatin remodelling, the DNA damage response and cell cycle progression. To understand SUMO signalling at the cell-wide level, we have developed novel methodology to purify and identify SUMO target proteins and the SUMO acceptor lysines in these target proteins. This has resulted in the identification of over 1,600 SUMO target proteins and over 4,300 SUMO acceptor lysines in these target proteins.

In response to methyl methanesulfonate treatment, SUMO orchestrates chromatin modifiers, including JARID1B/KDM5B, JARID1C/KDM5C, p300, CBP, PARP1, SetDB1, and MBD1. Whereas SUMOylated JARID1B was ubiquitylated by the SUMO-targeted ubiquitin ligase RNF4 and degraded by the proteasome in response to DNA damage, JARID1C was SUMOylated and recruited to the chromatin to demethylate histone H3K4. Currently, we are using our new methodology to study SUMOylation dynamics in cellular responses to different DNA damaging agents.
POSTER SESSION A2: 'Nucleotide Excision Repair'
IV Annual aDDResss meeting

Poster viewing: Monday, April 18, 20:00 – 22:00 h

Discussion: Tuesday, April 18, 16:55 – 18:25 h
Nucleotide excision repair—Annual aDDResS meeting - A2

Presenter: Cristina Ribeiro-Silva

BRM stimulates Nucleotide Excision Repair by stabilizing TFIIH and facilitating its loading on DNA damage

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Nucleotide Excision repair (NER) removes a wide range of structurally unrelated DNA injuries that distort the DNA double helix. NER is initiated either when RNA Polymerase II is stalled by a lesion, called Transcription-coupled NER (TC-NER), or by the concerted action of DDB2 and XPC which detect lesions over the entire genome, called Global Genome NER (GG-NER). Damage detection leads to the recruitment of transcription factor IIH (TFIIH) via its XPB subunit, which unwinds DNA and verifies the presence of the lesion via its XPD helicase subunit.

For optimal genome maintenance, NER should be capable of accessing lesions everywhere in the genome at any moment, regardless of the chromatin conformation. However, it is not precisely known how chromatin is modified to ensure optimal removal of lesions. Previously, we identified proteins of the SWI/SNF family of chromatin remodeling complexes as essential factors for optimal UV-survival in C. elegans. To understand the function of SWI/SNF complexes in NER, we focused on one of its two ATPases subunits, BRM. Our goal was to determine whether – and how – this protein facilitates the repair of UV-DNA damage by NER.

Here, we show that the mammalian SWI/SNF ATPase BRM has a critical role in regulating NER efficiency early after damage is induced, both in GG-NER and TC-NER. We found that BRM facilitates loading of TFIIH proteins and downstream repair factors on DNA damage. Additionally, BRM promotes the release of DDB2 from damage without affecting XPC recruitment or dissociation. These results strongly suggest that, upon damage detection by DDB2 and XPC, BRM facilitates the binding of TFIIH to XPC by promoting the dissociation of DDB2. Furthermore, our experiments showed that BRM is crucial for the stability of the TFIIH proteins, adding an additional layer of NER regulation by BRM.

Even though our results underscore the idea that NER is accompanied by complex chromatin remodeling events, our data also suggests that the involvement of chromatin remodelers in DNA repair goes beyond providing access of detection proteins to DNA damage. Moreover, as SWI/SNF is often found to be mutated in different types of cancer, our results suggest that such mutations may have serious consequences to DDR function in cancer cells.
Nucleotide excision repair–Annual aDDress meeting - A2

Presenter: Ana Henriques

Role of ERCC1 in Intestinal Inflammation and Tumorigenesis

Ana Henriques(1), Vasiliki Koliaraki(2), Niki Karagianni(1)

Cells are constantly exposed to endogenous and exogenous insults that introduce damage into our DNA. To counteract the harmful effects of DNA damage, DNA repair pathways are activated to remove the damage and promote genetic stability. When the DNA repair mechanisms are defective or incomplete, mutations are not excised, leading to genetic instability and risk of hereditary diseases or cancer (1). It remains yet unknown whether the presence of irreparable DNA lesions may induce chronic inflammation in vivo, thereby contributing both to tumor development and the premature appearance of age-related pathological features, such as those seen in animals carrying defects in the Nucleotide Excision Repair (NER) pathway. Excision repair cross-complementing group 1 (ERCC1) is a molecule that plays an essential role in the NER pathway and has been associated with various types of cancer (e.g. skin and lung cancer) (2, 3), while recently it was also linked to a chronic auto-inflammatory response triggered by DNA damage (4).

The aim of this study was to determine the role of the NER pathway, and more specifically of ERCC1 in intestinal inflammation and inflammation-driven tumorigenesis. For this reason, we generated mice carrying a deletion of ERCC1 in intestinal epithelial cells (IECs). ERCC1ΔIECko mice were viable and fertile, did not exhibit obvious phenotypic defects and showed an efficient and specific deletion of ERCC1 protein in IECs. To study the cell-specific role of ERCC1 in intestinal inflammation, we subjected ERCC1ΔIECko mice to both the acute and chronic models of DSS colitis. Phenotypic and histological analysis showed no difference in susceptibility to either acute or chronic DSS-induced colitis between ERCC1 conditional knockout mice and their littermate controls in terms of weight loss, inflammation and tissue damage histological scores. To further investigate the role of ERCC1 in inflammation-driven tumorigenesis, we used the well-established AOM/DSS model of colitis-associated cancer. ERCC1ΔIECko mice showed no statistical significant difference in incidence, number or size of colorectal tumors compared to controls. Interestingly, we found that in APCmin/+ mice, a mouse model of spontaneous intestinal tumorigenesis that is not induced by inflammation, ERCC1 expression was significantly upregulated in tumors in comparison to healthy tissue. Collectively, our results show that in IECs the NER pathway and in particular ERCC1 does not play a significant role in intestinal inflammation and inflammation-driven tumorigenesis. However, our preliminary data points to a significant increase of ERCC1 in spontaneous colorectal cancer, where its specific role still needs to be determined.

(2) Gregg SQ, et al., DNA Repair (Amst), 2011. 10(7): p. 781-91
Inspecting the Progression of Proliferative cells undergoing Senescence.

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In eukaryotes, it is well studied that senescence, an irreversible cell cycle arrest, is caused by the induction of oncogenes in the background of p53 expression. The most versatile activator of p53 is nutlin-3a. The stress activated transcription factor p53 is strong enough to induce apoptotic cell death. The p53 tumor suppressor also causes permanent cell cycle arrest. Alternative splicing is a regulated process during gene expression that enables a single gene to code for multiple proteins. Previously, many studies have shown that changes in splicing can be a major cause of human diseases, e.g. imbalances in altered splicing patterns can lead to the progression of cancer. In-vivo studies have shown an association of cancer and ageing with senescence.

In this study, we explore transcriptional and translational control in various conditions. We analysed data from RNA-seq and Ribo-seq of immortalised human primary BJ fibroblasts. Datasets for different conditions generated with both sequencing techniques were retrieved from “Loayza-Puch et al.”. We used genome-wide datasets from successive conditions reflecting proliferation and transformation processes. The sequences in ribosomal profiling data represent RNA fragments that are protected by ribosomes. We developed a pipeline for analysing Ribo-seq data. Moreover, we provide a workflow for elucidating the presence of alternatively spliced transcripts by taking advantage of the Ribo-seq data. The key finding of our study was that several genes differentially expressed under different conditions in the RNA-seq as well as the Ribo-seq data showed opposite effects, i.e. downregulation according to the Ribo-seq data but upregulation in the RNA-seq data.

We also found valuable clues to an important role of the regulation of splicing for senescence.

Nucleotide excision repair–Annual aDDReSS meeting - A2

Presenter: Aude Guénolé

Tip80, A novel genotoxic stress response factor

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We identified a new protein, Tip80 (Tat interacting protein 80kDa), which we found associated to the carbon catabolite-repression 4-Not (CCR4-NOT) complex. The CCR4-Not complex has been implicated in many processes that regulate gene expression from mRNA to proteins both in the nucleus and in the cytoplasm. Tip80 co-purified with all the nine CCR4-NOT complex subunits making Tip80 a new component of this complex.

Here we present data strongly suggesting that Tip80 play a role in the regulation of the DNA damage response (DDR), thus potentially bridging transcription and post-transcriptional control to the DDR.

First, we found that Tip80 relocalizes to the nucleus shortly after exposition to various genotoxic agents such as ultraviolet light (UV), Hydroxyurea (HU), Methylmetane sulfonate (MMS), or Etoposide.

In yeast, CCR4-NOT has been shown to be involved in the response to UV (1) and to replicative stress induced by HU (2). We show that depletion of Tip80 or CNOT8 (the main CCR4-NOT deadenylase) by siRNA induced cell cycle defect after UV stress suggesting that they work in the same pathway. RNA-seq from Tip80 depleted cells before and after UV is under way to uncover genes regulated by Tip80 in this process.

Next, we uncovered that Tip80 displayed a RING domain dependent ubiquitin E3 ligase activity in vitro and in vivo. And we further show that Tip80 E3 ligase activity is necessary for cell cycle progression in response to UV stress. Moreover some of our preliminary data suggest that CNOT8 is ubiquitilated by Tip80. Recent work by Cano et al. (3) shows that the mRNA binding E3 ligase (MEX-3C) regulates CNOT7 (another CCR4-NOT deadenylase subunit) deadenylase activity by ubiquitylation. These data thus suggest that Tip80 could be another E3 ligase modulating CCR4-NOT deadenylase activity.

Altogether, these data imply that Tip80 regulates gene expression in response to genotoxic stress through modulating CCR4-NOT activity. RNA-seq data and finding other substrates ubiquitilated by Tip80 will provide us more mechanistic details in this process.


(2) DNA damage and replication stress induced transcription of RNR genes is dependent on the Ccr4-Not complex. Mulder KW, Winkler GS, Timmers HT. Nucleic Acids Res. 2005;


Regulation of DNA methylation patterns is a crucial step for maintenance of cell homeostasis. Recently, a process for active DNA demethylation coupled with mechanisms associated to DNA repair have been related to the context of gene expression. Among the proteins associated to DNA repair, we have demonstrated the implication of the nucleotide excision repair (NER) factor XPG in transcription. In particular, we already showed that XPG, via its endonuclease activity, can promote DNA breaks formation and demethylation at CpG downstream of activated RARb2 gene transcription-start site, in absence of genotoxic attack. Nevertheless, our recent results indicate that the accomplishment of active DNA demethylation requires not only XPG but also his partner, the general transcription and NER factor TFIIH. Hence, this study proposes to dissect the role of XPG in DNA demethylation-coupled transcription by means of genome-wide approaches. In particular, we show that XPG is recruited to CG-rich genes promoters together with TFIIH. Moreover, the recruitment of XPG/TFIIH at gene promoters is at the basis of differential methylation patterns and the absence of XPG is related to dysregulation of gene expression. The determination of the roles played by this NER factor is crucial because mutations in the gene coding this protein originate several genetic disorders associated with cancer such as Xeroderma pigmentosum (XP), Cockayne syndrome (CS), for some cases combined in XP/CS phenotype. Thereby, the description of the mechanism dependent on XPG in a normal as well as pathological context, is crucial for a better understanding of the aetiology of NER-related and cancer-associated diseases.
Exploring the role of the SMC-5/6 complex in cancer, ageing and kidney disorders

Federica Schiavoni, Emilio Lecona, Ariana Jacome, Oscar Fernández-Capetillo
Genomic Instability Group, Spanish National Cancer Research Centre, Madrid, Spain

The SMC-5/6 complex is part of the Structural Maintenance of Chromosomes (SMC) family of proteins, which includes condensin and cohesin and whose main function is the regulation of chromosomal architecture and organization. Several roles have been proposed for SMC-5/6 in genome maintenance, but its specific function is still unclear. Using a collection of mouse models of a SUMO ligase named NSMCE2, which is an integral part of the SMC5/6 complex, we have now seen that NSMCE2 is essential for mammalian cells, and that the complex suppresses cancer and ageing in mice. In addition to overall the accelerated ageing that appears when NSMCE2 is deleted in adult mice, these animals have a distinct phenotype on their kidneys. Given the recent connections between DNA damage, replication stress and kidney disease, part of my PhD is now to explore the roles of NSMCE2, and the SMC5/6 complex, in the kidneys. An overview of our overall work on mouse models of the SMC5/6 complex, plus some early data on the connection between NSMCE2 and kidney disease will be discussed.
Nucleotide excision repair–Annual aDDRes meeting - A2

Presenter: Gururaj Rao Kidiyoor

Role of ATR in regulating nuclear plasticity and cell migration

Gururaj Rao Kidiyoor(2), Galina Beznusenko(1), Qingsen Li(1), Amit Kumar(2), Matthew Raab(3), Umberto Restuccia(1), Andrea Disanza(1), Andrea Palamidessi(1), Alexandre Mironov(1), Angela Bachi(1), Giorgio Scita(1), Matthieu Piel(3), Marco Foiani (1)

1. IFOM- FIRC Institute of Molecular Oncology, Milan, Italy; 2. Indian Institute of Toxicology Research (IITR), Lucknow, India; 3. Institut Curie/CNRS, Systems biology of cell polarity and cell division, Paris, France

ATR is key Kinase involved in maintaining genome integrity, in sensing DNA damage and initiating the DNA damage response. Complete depletion of ATR causes embryonic lethality in mice models and hypomorphic mutations in humans are associated with the autosomal recessive disorder called Seckel syndrome.

We have previously reported that ATR mediates a response to mechanical stress by relocating at the nuclear envelope and Nucleoli(Kumar et al. Cell 2014). We now show that depletion of ATR alters cellular and nuclear morphology and alters the overall cell stiffness. The nucleuses of ATR depleted cells, accumulated both type I and type II nuclear envelope invaginations. All these results of ATR depleted cells in nuclear and cell plasticity suggest that ATR has a role in regulating plasticity of cell. Further, ATR defective cells were assayed in variety of migratory tests including wound healing, invasion in collagen matrix and migration through constrictions. Results from these assays confirm that cells lacking ATR exhibit plasticity problems and are defective in migration.

The impact of DNA-damage driven transcriptional stress in development and disease

Garinis George, Agathangelou Kyriacos
IMBB-FORTH, Heraklion, Greece

DNA damage blocks transcription, affecting gene expression and directly influencing proper genomic administration. Transcription elongation factors, including TFII-S and its TCEA2 parologue, aid RNA polymerase II (RNAPII) to transcribe past blockages while DNA repair mechanisms constantly preserve the genetic codec’s indispensable integrity by repairing lesions (Garinis et al., 2008; Kamileri et al., 2012). Acknowledging the mechanistic synchronization that must be taking place, coordination among DNA repair and arrest-relief is inevitable once RNAPII encounters a lesion.

Revealing their involvement in gene expression regulation activities, specific NER factors urge upon an additional level of coordination among the administrators, that necessitates the apportionment of common factors and their functions among these pathways. In addition, the exact mechanism by which CSB is attracted on the damaged site, for recognition and repair initiation steps to ignite, is not fully understood. Designated by the notion that backtracked RNAPII allows for the damaged site to me revealed, pin-pointed for repair, several studies result in controversy regarding substantiation of TFII-S inter-playing synchronization with NER via interaction (Hill et al., 2014; Brueckner et al., 2007; Fousteri et al., 2006). Experimental approaches, have been specifically directed towards the comprehension of lesion recognition kick-off events for Transcription-Coupled Repair (TC-NER), as well as the delineation of functional complexes that grant synchronization among lesion repair and transcription reboot. Following the generation of biotin-tagged TCEA2 knock-in and conditional knock-out mice, TCEA2 associated protein complexes will be initially identified and compared upon UV, with the use of pull-down assays coupled to LC/MS/MS performed on Mouse Embryonic Fibroblasts’ (MEFs) and Mouse Primary Dermal Fibroblasts (PMDF) nuclear extracts. Streptavidin-bound bioTCEA2 ChiP assays, coupled with high-throughput sequencing (ChiP-seq) will be performed in trans-retinoic acid treated or UV irradiated MEFs NE. Within a UV induced scenario, follow-up ChIP-qPCR on the respective targets, with identified protein partners, will allow for the acknowledgment of specific interactions as the means based on which TCEA2 is recruited to rescue UV induced RNAPII arrest.

Furthermore, crossing the conditional knock-out with the newly acquired, ubiquitously expressing CPD-Photolyase, transgenic mice will allow assessment of TCEA2’s involvement in DDR and NER specifically by the determination of TCEA2 knock-out PMDFs repair kinetics. Follow up experimental procedures include the estimation of certain newly identified protein interactions’ lose that contributes to the expected low efficiency on CPD lesions repair in the KO, due to interpolations of transcription reboot and NER.

Nucleotide excision repair—Annual aDDReSS meeting - A2

Presenter: Mariangela Sabatella

In vivo function and regulation of ERCC1-XP

Mariangela Sabatella, Karen Thijssen, Wim Vermeulen, Hannes Lans
Department of Molecular Genetics, Erasmus MC, Rotterdam, The Netherlands

The ERCC1-XP structure-specific endonuclease has a critical role in Nucleotide Excision Repair, incising the damaged strand 5’ to the lesion. In addition, it is essential to unhook DNA crosslinks during Interstrand Crosslink (ICL) repair. In humans, deficiency of ERCC1-XP is associated with cancer predisposition, severe developmental defects and accelerated aging depending on which DNA repair pathways are affected. Although the function of ERCC1-XP has been studied in great detail in biochemical and cell biological experiments, it is not entirely clear how the activity of this complex in response to different kind of lesions is regulated and how its deficiency can lead to such a variety of tissue-specific symptoms.

To identify factors that specifically regulate ERCC1-XP in response to UV and ICLs, we stably expressed fluorescently tagged ERCC1-XP in different mammalian cell lines and determined its interactome by quantitative proteomics. This confirmed the known association with NER core factors XPA, XPG and TFIIH specifically after UV damage. We also identified several putative novel interactors, which are currently being investigated. Furthermore, we set up new imaging methods to monitor the recruitment of ERCC1-XP to both UV-lesions and intra- and interstrand crosslinks and to evaluate the involvement of (novel) regulatory proteins. Results so far confirm that indeed XPA and FANCD2 promote the recruitment of ERCC1-XP to DNA damage. Previously, our lab showed that ERCC1/XPF loss-of-function in C. elegans causes development defects and accelerated aging, reminiscent of human patients. To study potential tissue-specific activities of ERCC1-XP, we expressed and imaged fluorescently tagged ERCC1-XP in the C. elegans germline, hypodermis, intestine, neurons and muscles. Preliminary results show that in oocytes the complex quickly but only transiently re-localizes to damaged chromosomes upon UV irradiation. Moreover, we find that the mobility of the complex differs depending on chromatin content and cell type. By combining genetic analysis, imaging and proteomic screening, we aim to uncover the regulatory mechanisms that underlie damage and cell type specific responses of ERCC1-XP.
Characterization of a putative DNA repair nuclease, C15ORF41

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Congenital dyserythropoietic anaemia (CDA) consists of a heterogeneous group of very rare hereditary disorders characterized by distinct morphological abnormalities of the erythroid precursors in bone marrow, ineffective erythropoiesis and suboptimal reticulocyte response [1, 2]. CDA has been defined by 3 major subtypes, as classified by Heimpel & Wendt [3]: CDA-I, CDA-II and CDA-III.

Congenital dyserythropoietic anaemia type I (CDA-I) is characterized by moderate to severe macrocytic anaemia, hepatomegaly, spongy heterochromatin and inter-nuclear bridges. A vast majority (~80%) of the known cases of CDA type I disease have been found to be associated with mutations in the CDAN-1 gene [4-6]. Mutations (substitutions) identified using a complete genome study [7] in the previously uncharacterized locus, C15ORF41 suggests a possible causative gene underlying the CDA type I disease. This could address the ~20% of CDA-I cases lacking any CDAN1 mutations.

Structural analysis of the protein provided evidence of 2 N-terminal AraC/XylS-like helix-turn-helix domains followed by a PD-(D/E)XK nuclease domain, implying C15ORF41 is a nuclease. The PD-(D/E)XK superfamily of proteins exhibits sequence divergence and variable structural elements interspersed within a relatively small and evolutionary conserved core which makes it difficult to identify new members of this family. However, several diverse and previously uncharacterized proteins (including C15ORF41) have been identified using bioinformatics studies [8].

Based on these findings, we hypothesize that C15ORF41 may play a role in chromatin reassembly during replication. We present the ongoing studies to test C15ORF41 for nuclease activity and to understand the basis of pathogenesis associated with mutations in C15ORF41 and congenital dyserythropoietic anaemia type I.

Nucleotide excision repair–Annual aDDResss meeting - A2

Presenter: Angela Helfricht

SWI/SNF chromatin remodelling in Nucleotide Excision Repair

Angela Helfricht, Wim Vermeulen, Hannes Lans
Department of Molecular Genetics, Erasmus MC, Rotterdam, The Netherlands

Nucleotide Excision Repair (NER) is a highly versatile DNA repair pathway, capable of removing a wide range of helix-destabilizing DNA lesions, including those induced by solar UV-light (CPDs and 6-4PP photolesions), cigarette smoke and several commonly used chemotherapeutics. Despite our detailed knowledge on the core NER machinery, it is still largely unclear how NER operates within the complex chromatin environment as present in mammalian cells and how the dynamic remodelling of chromatin takes place during NER to regulate its efficiency. ATP-dependent chromatin remodelling factors are thought to regulate chromatin accessibility through movement of nucleosomes along the DNA or by transfer of nucleosomes from and to DNA. In mammals, different ATP-dependent chromatin remodelling complexes have been identified of which the SWI/SNF family is among the best characterized. Previously, we identified different SWI/SNF chromatin remodelling subunits that play a role in the UV-induced DNA damage response by showing that mutation of BRM/BRG1, SNF5, BAF155, PBRM1 and BAF250 sensitizes C. elegans to UV irradiation. Preliminary experiments with the respective mammalian orthologs of these remodelling proteins suggest that different SWI/SNF chromatin remodelling subunits differently affect mammalian global genome NER and transcription coupled NER. However, the precise molecular mechanism of SWI/SNF involvement in the DNA damage response remains undefined. To better understand how these important remodelling complexes act during NER, we started a systematic in-depth analysis of the function of SWI/SNF-dependent chromatin remodelling subunits during NER.
Low-dose gamma-radiation enhances DNA excision repair in mouse spleen in vivo

Youssef Ismail, Ron Mitchel, Matthew Flegal, Dmitry Klokov
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Ionizing radiation is a known carcinogen. However, carcinogenic potential of low-dose radiation is subject to continuous controversy and arguments, mostly due to differential responses of DNA damage signaling and DNA repair observed at low vs. high doses (1). We have previously shown that exposure to low-dose gamma-radiation can increase life span and the latency of cancer development in C57BL/6J mice (2). In this study, we examined whether DNA repair mechanisms can be activated by low-dose radiation and enable cells to response more efficiently to damage induced by high-dose challenging radiation. Exposure of mice to 20 or 100 mGy of gamma-radiation 24 h prior to a 2 Gy challenging dose did not result in enhanced repair of DNA double-strand breaks in splenic lymphocytes, measured as gamma-H2AX formation and loss by flow cytometry and western blot. In contrast, base (BER) and nucleotide excision repair (NER) rates, measured by the DNA excision and synthesis repair functional assay, were significantly higher in cells from low-dose irradiated compared to non-irradiated mice. In quantitative RT-PCR experiments we measured the expression levels of 84 key genes involved in various DNA repair pathways. We showed that only DNA excision repair genes were substantially modulated by low-dose radiation. Using western blot, we validated these results at the protein level for APEX2, XPD and DDB1 genes. Altogether, our data provide strong evidence that DNA excision repair is activated in response to low-dose radiation in vivo. Given our previous results showing increased latency of cancer in mice following exposure to low-dose radiation, our results suggest the role of BER and NER in increased genome stability and anti-tumor effects triggered by low-dose radiation.

DNA repair mechanisms involved in resistance to and removal of the nucleoside analogue Gemcitabine

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1. NWCR Institute, Bangor University, UK; 2. Norwegian University of Science and Technology, Norway; 3. Birmingham University, UK

Replication fork blockage interferes with cell division and contributes to genome instability. While DNA damage response mechanisms that deal with blockages caused by obstacles on the template (e.g. DNA damage or tightly bound proteins) or nucleotide pool depletion (e.g. after hydroxyurea treatment) have been more widely studied, not much is known about replication blockage caused by integration of modified nucleotides into the nascent strand during DNA replication. The cytotoxicity of the deoxycytidine analogue Gemcitabine (2',2'-difluoro-2'-deoxycytidine) is predominantly dependent on DNA integration followed by replication termination. For a cell to survive treatment with this drug, the modified nucleotide needs to be removed from the DNA to allow replication restart. In collaboration with Prof. Hans Krokan (NTNU, Norway) we have developed a method to quantify the amount of Gemcitabine in genomic DNA and have used this to show that many DNA repair factors are involved in removal of Gemcitabine from the DNA during replication.

The Mre11/Rad50/Nbs1 (MRN) protein complex is highly conserved among eukaryotes and is involved in a wide range of early responses to DNA damage, often mediated by its role in DNA end processing. Central to DNA-end processing are the Mre11 single strand endonuclease and 3'-5' exonuclease activities. While these activities have been implicated in the repair of DNA double strand breaks (DSBs), their contribution to other DNA damage responses remains largely unknown. We present data suggesting that MRN is able to remove Gemcitabine from the nascent strand during DNA replication. This function has not previously been attributed to MRN.

We have also found that some Nucleotide Excision Repair (NER) genes contribute to Gemcitabine removal and resistance. Deletion of the S. pombe homologues of XPA, XPC and ERCC1 leads to a defect in Gemcitabine removal and increased sensitivity. Surprisingly, XPF and XPG deletions are not defective in Gemcitabine removal and are not hypersensitive to this drug. We also present evidence that NER genes provide resistance to Gemcitabine in human cells. Both in S. pombe and human cells, defects in NER reduce S-phase progression in Gemcitabine-treated cells.

Finally, we found that Gemcitabine treatment is mutagenic, and that this mutagenicity is dependent on translesion synthesis (TLS). We present evidence that suggests that TLS acts on replication forks during the first round of DNA replication after Gemcitabine treatment. This suggests a novel role for TLS polymerases at replication forks that have been stalled due to problems in the nascent DNA strand, additional to its well-established role in allowing replication past DNA lesions in the template strand.

We have thus uncovered novel roles of various DNA repair mechanisms in resistance to the nucleoside analogue Gemcitabine.
Persistent DNA damage signaling triggers aberrant chromatin organization changes during mammalian development

Georgina Chatzinikolaou, Zisis Apostolou, Tamara Aid-Pavlidis, Anna Ioannidou, Theodore Kosteas, George A. Garinis
Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, Heraklion, Crete, Greece

Inborn defects in genome maintenance pathways are associated with complex developmental disorders whose causal mechanisms are poorly understood. Using an in vivo tagging approach in mice and high-throughput proteomics strategies, we show that the nucleotide excision repair (NER) structure-specific endonuclease ERCC1-XPF complex interacts with protein factors involved in chromatin architecture and organization during mammalian development. Loss of Ercc1 or exposure to various genotoxins triggers the aberrant localization of chromatin regulators, altered histone marks and the aberrant expression of selected gene targets. The response is cell-autonomous and requires functional DDR. We propose that persistent DNA damage signaling triggers chromatin changes that affect gene expression programs associated with NER developmental disorders.
Nucleotide excision repair—Annual aDDRess meeting - A2

Presenter: Andriy Khobta

Vectors with the elements of synthetic nucleic acids for quantitative assessment of specific DNA repair pathways

Nataliya Kitsera, Andriy Khobta
Institute of Toxicology, University Medical Centre Mainz, Germany

Host-cell reactivation assay (HCR) is an indicator test, which estimates DNA repair proficiency of cells based on their capacity to restore the functionality of a virus or plasmid vector after these had been inactivated by a DNA damaging agent (typically UV) [1,2]. HCR proved to be useful for screening for the abnormalities of DNA repair and for identification of DNA repair genes, particularly those associated with hereditary defects of nucleotide excision repair (NER). However, because of structural heterogeneity and random distribution of DNA modifications generated by exposure to damaging agents, it has been difficult to develop an HCR-based assay, which would unambiguously and quantitatively assign reactivation of damaged vectors to a certain DNA repair pathway.

We have overcome these limitations by purposed design of reporter vectors suited for DNA strand- and sequence-specific incorporation of short stretches of synthetic nucleic acid containing precisely defined modifications of nucleobases and/or of the sugar-phosphate backbone [3]. With the help of reporter constructs carrying a variety of structurally different modifications, we have improved the HCR technology and developed it towards a sensitive and robust detection of distinct DNA repair activities. Successful applications include differential measurement of the global genome (GG-NER) and transcription-coupled (TC-NER) subpathways of NER [4] as well as new insights into cellular base excision repair (BER), such as assessment of DNA glycosylase substrate preferences under physiological conditions [5-7] and determination of the hierarchy and functional coupling between separate enzymatic steps of BER [unpublished]. In the present and future, we consider an ever broader range of applications of vectors containing the elements of synthetic nucleic acids to the fields of DNA repair and epigenetics.

Increased oxidative metabolism in response to acute and chronic DNA damage

Lear E. Brace(1), Sarah C. Vose(2), Kristopher Stanya(1), Rose M. Gathungu(3), Vasant R. Marur(3), Alban Longchamp(1), Pedro Mejia(1), Dorathy Vargas(1), Karen Inouye(1), Roderick T. Bronson(4), Chih-Hao Lee(1), Edward Neilan(5), Bruce S. Kristal(3)

1. Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, USA; 2. Vermont Departments of Health, Environmental Health Division, Burlington, Vermont, USA; 3. Departments of Neurosurgery, Harvard Medical School, Brigham and Women’s Hospital, Boston, USA; 4. Rodent Histopathology Laboratory, Harvard Medical School, Boston, USA; 5. Genetics and Metabolism, Boston Children’s Hospital, Harvard Medical School, Boston, USA

Free radicals from endogenous oxidative energy metabolism can damage DNA, but whether DNA damage itself exerts feedback control over cellular or organismal energy metabolism to potentially limit further oxidative DNA damage is unknown. We modeled endogenous and exogenous genotoxic stress using Csa-/-/Xpa-/- (CX, a model of Cockayne Syndrome) mice, deficient in DNA repair, and wildtype cells and mice treated with genotoxic chemotherapeutics, respectively. Chronic endogenous and acute exogenous genotoxic stress were both associated with increased oxidative metabolism, in particular fatty acid oxidation (FAO), at the organismal level, manifested by increased oxygen consumption, reduced respiratory exchange ratio, progressive adipose loss and increased fatty acid oxidation in tissues ex vivo. Surprisingly, this adaptive, metabolic response to DNA damage occurred cell-autonomously, subsequent to PARP-1 activation and a transient decline in steady-state ATP levels, and required the energy-deprivation sensing kinase, AMPK. The functional relevance of increased FAO upon genotoxic stress was tested by genetic and/or pharmacological inhibition of AMPK or FAO, each of which decreased cellular viability upon genotoxic stress in vitro. On the other hand, further activation of FAO in vivo by dietary methionine restriction significantly increased lifespan of short-lived CX mice despite increased adipose loss. Together, these data support a model in which PARP-1 activation upon acute or chronic genotoxic stress imposes an energetic cost and drives an AMPK-dependent switch to energy-efficient oxidative mitochondrial metabolism.
Oxidative Damage to RPA Limits the Nucleotide Excision Repair Capacity of Human Cells

Melisa Guven, Reto Brem, Peter Macpherson, Matthew Peacock, Peter Karran
Francis Crick Institute, Clare Hall Laboratory, South Mimms, Herts, UK.

Solar ultraviolet radiation (UV) which comprises ≥ 95% UVA (320-400nm) and around 5% UVB (280-320nm), is a carcinogen. Nucleotide excision repair (NER) removes UVB-induced DNA lesions and defective NER is associated with photosensitivity and an increased risk of sunlight-induced skin cancer. Some drugs including the immunosuppressant azathioprine and the fluoroquinolone antibiotics, cause photosensitivity and increased skin cancer risk. They interact with UVA radiation to generate reactive oxygen species (ROS) that damage proteins, including those involved in NER. This protein damage results in NER inhibition. The replication protein A (RPA), a single-stranded DNA binding protein which has an essential role in numerous DNA processes including NER, is particularly susceptible to oxidation damage. We investigated the relationship between protein oxidation and NER inhibition in cultured human cells expressing different levels of RPA. We have identified different oxidised forms of RPA in NER inhibited cells. We show that RPA is limiting for NER and that damage to RPA is the main contributor to oxidation-related NER inhibition. The vulnerability of NER to inhibition by oxidation links cutaneous photosensitivity, protein damage, and increased skin cancer risk and indicates that in addition to DNA damage itself, damage to DNA repair proteins is likely to be an important factor in skin cancer risk.

Co-localization and interaction of p21CDKN1A with PCNA-interacting NER factors after DNA damage

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The cyclin-dependent kinase inhibitor p21CDK1NA is a multifunction protein involved in cell cycle arrest, transcription regulation, apoptosis, and cell motility. Another important role of p21 in the DNA damage response is mediated by the interaction with Proliferative Cell Nuclear Antigen (PCNA), an essential factor required in DNA replication and repair. However, the role in DNA repair has been complicated by the evidence that p21 undergoes proteolytic degradation after DNA damage. Several studies have demonstrated that p21 does not inhibit DNA repair and instead it might have a regulatory role in this process. In fact, previous findings demonstrated a positive role of p21 in Nucleotide Excision Repair (NER), by regulating the interaction between PCNA and the acetyl transferase p300, thereby removing the inhibition of enzyme activity. In addition, p21 was found to influence the interaction between XPG and p300, further suggesting a regulatory function mediated through PCNA binding. Since it was previously observed that p21 interacts with DNA polymerase δ (DNA pol δ) after UV damage, we have investigated whether p21 may also interact with proteins participating in NER through PCNA binding. In addition, we have assessed whether p21 accumulation at UV damage sites might influence the recruitment of PCNA partners, such as DNA pol δ, XPG, and CAF1. Accumulation of high p21 protein levels was obtained in primary human fibroblasts and in HeLa cells by treatment with the proteasome inhibitor MG132. The results have shown that after local exposure to UVC light, p21 accumulates at UV damage sites in concomitance with PCNA, DNA pol δ, and also with XPG and CAF1. In addition, p21 interacted with PCNA in a complex containing DNA pol δ, both in the absence and in the presence of proteasomal inhibition. MG132 induced persistent high levels of XPC, PCNA and p21 proteins at local DNA damage sites, together with accumulation of XPG, DNA pol δ and CAF1. In vivo live-cell imaging confirmed that p21 did not inhibit, rather limited the recruitment of PCNA partner proteins. In fact, DNA pol δ retention at DNA damage sites was observed after several hours from UV exposure in p21-null, but not in normal fibroblasts. These results suggest that p21 protein is physiologically present at DNA damage sites, and that further accumulation consequent to inhibition of its degradation did not block the recruitment of NER factors interacting with PCNA. Therefore, p21 appears to be a regulator rather than an inhibitor of PCNA partners assembling at DNA damage sites, thereby coordinating in time and space the interaction of PCNA with its multiple partners.
Oxidative stress-induced protein damage inhibits DNA repair and determines mutation risk and anticanccer drug effectiveness

Lizzy McAdam, Reto Brem, Peter Karran
The Francis Crick Institute, Clare Hall Laboratory, South Mimms, UK

Oxidative stress, the production of excessive reactive oxygen species (ROS) is a common property of tumors and can be enhanced by anticanccer drugs. Along with potentially mutagenic DNA lesions, ROS induce widespread protein damage. We show here that oxidative stress inhibits DNA repair. ROS generated by ultraviolet A (UVA) radiation or by interventions that alter metabolism to deplete antioxidants inhibit excision repair in HaCaT human keratinocytes. DNA repair inhibition is a consequence of oxidation damage to proteins. Consistent with inhibition of the nucleotide excision repair (NER) system that protects against sunlight-induced mutation and skin cancer, UVA irradiation increases their susceptibility of HaCaT cells to mutation by UVB. Oxidative stress also enhances HaCaT sensitivity to killing by cisplatin, a DNA damage-inducing anticanccer drug for which NER status determines therapeutic effectiveness. Oxidative-stress related damage to DNA repair proteins therefore has implications for both cancer risk and for the effectiveness of anticanccer therapy.
A global switch of RNA polymerase II elongation optimizes the elimination of genotoxic stress

Matthieu D. Lavigne, Katerina Ntakou, Anastasios Liakos, Dimitris Konstantopoulos, Maria Fousteri
Biomedical Sciences Research Center 'Alex. Fleming', Vari, Athens, Greece.

Monitoring and repair of DNA lesions is facilitated by elongating RNA Polymerase II (RNAPII) regularly scanning a significant proportion of the genome (transcription-coupled repair (TC-NER)). This specialized sub-pathway of nucleotide excision repair (NER), which ensures fast gene repair to decrease mutagenesis, prevents persistent stalling of transcription machinery on impeding DNA lesions that can be detrimental for the cells. Thorough and correlated high-throughput ChIP-seq and gene expression profiling analyses (RNA-seq and GRO-seq) allowed us to decipher the global modulation of RNAPII dynamics in response to DNA damage and gain insights in the functional consequences on the transcriptome and on genome stability. We define an RNAPII switch into elongation “safe mode” that is depended on the global release of the transcription machinery into a maximized number of expressed genes. This phenomenon accounts for the observed increase in stalling of RNAPII at DNA lesions for efficient repair before transcription kinetics are re-established. Our findings further extend and clarify the current and paradox mechanistic views on transcription arrest following UV stress.
POSTER SESSION A3: 'Nucleotide Excision Repair'

Poster viewing: Tuesday, April 19, 20:00 – 22:00 h

Discussion: Thursday, April 21, 16:55 – 18:25 h
Ultraviolet (UV) radiation causes skin cancer and photoaging as a result of the damage it is able to cause to a range of biomolecules. Among these it is well established as being capable of causing both direct and indirect damage to DNA. Shorter wavelength UVB (280-315nm) can be directly absorbed by DNA bases, causing cross-linked mutagenic adducts i.e. cyclobutane pyrimidine dimers (CPDs) and 6-4-photoproducts (6-4PP). By contrast, for longer wavelength (UVA; 315-400 nm) radiation photosensitiser-mediated processes are thought to be primarily responsible for genotoxic damage, which may include both pyrimidine dimers and directly induced double strand breaks, although there are conflicting reports of the latter.

Here we compare the kinetics of activation of the DNA damage response (DDR), as measured by γH2AX foci formation, in human keratinocytes by varying equitoxic doses of UVA and UVB radiation. We demonstrate that UVA elicits an earlier response of shorter duration than the equivalent dose of UVB and that this response is independent of cell cycle stage. We have also characterised the activation of downstream components such as p53 and find substantial differences in both the intensity and duration of the responses to UVA and UVB radiation. The apical kinases involved in each case have been identified by co-incubation with DDR inhibitors. We additionally show that, similar to effects previously observed for ionising radiation, UVA-exposed cells are able to induce a DDR in co-cultured unirradiated cells. Collectively our results show important differences in the damage response elicited by UVA and UVB, and shed new light on the underlying mechanisms of photo-carcinogenesis.
**Nucleotide excision repair - A3**

Presenter: Diu Nguyen

**ATRX suppresses R-loops**

*Diu Nguyen, Hsiao Voon, Caroline Scott, Douglas Higgs, Richard Gibbons*

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ATRX is a chromatin remodeling protein associated with X-linked Alpha-Thalassemia Mental Retardation syndrome and cancers that use the Alternative Lengthening of Telomere pathway. In the absence of ATRX, there is a DNA damage response associated with telomeres (Wong et al., 2010; Lovejoy et al., 2012) and the expression of certain genes are perturbed (Law et al., 2010). Previous studies (Law et al., 2010) have shown that ATRX is preferentially enriched at GC-rich tandem repeats in the genome and many targets appeared to be transcribed. The mechanism for this localisation is unknown but may be related to the potential for these GC-rich tandem repeats to adopt nucleic acid secondary structures. This study aims to understand the specific features of the repeats that signal ATRX targeting.

First, to see if ATRX targeting is tissue-specific at genome-wide scale, we performed ATRX-ChIP in three mouse tissues MEF, FL and ESCs. The results show that the vast majority of ATRX binding sites are cell-type specific. Moreover, this variability of ATRX targeting is associated with differential transcription.

Next, to test if ATRX recruitment is dependent on transcription, known ATRX targets, the human pseudo-zeta VNTR and telomere repeats of different sizes and orientations were inserted into an inducible ectopic gene in the 293T-Rex cell line by site-directed recombineering. ATRX enrichment was found to increase significantly upon transcription of the ectopic gene, and this was dependent on the orientation of the repeat such that the non-template strand was G-rich. Moreover, there was a direct correlation between the repeat size and level of ATRX bound: the longer the repeat, the higher the increase in ATRX enrichment.

To determine the signal for ATRX binding, assays were performed to look for features which reflected the distribution of ATRX. It appears that H3K9me3, which is known to recruit ATRX to pericentric heterochromatin (Eustermann et al., 2011), did not seem to play a role in ATRX binding at the GC-rich repeats of interest. R loops, a structure containing a RNA:DNA hybrid and a single-stranded DNA that forms co-transcriptionally in GC-rich regions, appear to be a strong candidate as their formation tends to be biased towards the G-rich strand (Review by Skourti-Stathaki et al., 2014). Interestingly, we found that R-loop enrichment in our experimental system correlates with ATRX recruitment, suggesting that ATRX targeting may be triggered by R-loops. Moreover, when ATRX is depleted we observed an increase in R-loop enrichment at the ectopic G-rich sequence and other endogenous ATRX targets, suggesting the role of ATRX in suppressing R-loop formation. As these structures are linked to DNA damage and breaks (Aguilera and Garcia-Muse, 2012) which may lead to gene silencing and genomic instability, this study may shed light on the cellular events that lead to alpha thalassemia and tumorigenesis upon loss of ATRX function.
In response to DNA damage, cells activate elaborate molecular networks to ensure genomic fidelity and tissue homeostasis. DNA damage response (DDR) signaling pathways coordinate these networks and determine cellular fates, in part, by modulating RNA metabolism. A fundamental step in eukaryotic RNA metabolism is nascent RNA splicing catalyzed by the spliceosome, a highly intricate and dynamic molecular machine which is subject to complex regulation by developmental and environmental cues. We have obtained evidence that the core spliceosome is also a critical DDR target [1]. Transcription-blocking DNA lesions, as those generated after UV-irradiation, cause selective chromatin displacement of late-stage spliceosomes by a two-step mechanism involving a stochastic (cis) and an DDR signaling-mediated (trans) stage.

UV-induced photolesions pose strong impediments to forward translocation of elongating RNA Polymerase II (RNAPII). RNAPII-pausing is followed by rapid displacement from DNA damage-containing areas, of splicing factors participating in mature co-transcriptional spliceosomes; an event presumably required to facilitate the removal/backtracking of RNAPII and subsequent damage access and repair by TC-NER. Spliceosome displacement, in combination with negative supercoiling behind lesion-arrested RNAPII, facilitates hybridization of intron-retaining pre-mRNA with template DNA adjacent to the transcription bubble. In non-replicating cells, the resulting structure (R-loop) activates ATM via a non-canonical, double-strand break (DSB)-independent mechanism as evidenced by absence of detectable DSB-foci and the dispersed ATM localization throughout the nucleus. Active ATM, signals to further mobilize spliceosomes, presumably by destabilizing their interaction with nascent transcripts. Unlike spliceosome-displacement from lesion-arrested RNAPII, which is a stochastic event, ATM-dependent spliceosome mobilization is an organized response, possibly to prohibit spliceosome occupancy on transcripts attached to polymerases located distal to DNA lesions and thereby, avoid additional transcriptional stress. In addition, ATM signals via its other effectors to orchestrate the DDR in part, by imposing wide-spread gene expression changes. The importance of this dual role of ATM in the UV-induced transcriptome is highlighted by the vast number of genes whose expression and splicing depend on ATM activity. Thus, ATM may coordinate the cellular response to transcription-blocking lesions by a regulatory strategy which acts at the genome-wide level to control splicing and transcription profiles, and at the gene level, to prevent further cycles of transcription that could hamper DNA repair mechanisms. This reciprocal regulation between ATM and the spliceosome highlights the importance of ATM signaling in the cellular response to transcription-blocking DNA lesions and supports a key role of the splicing machinery in this process. 

New insights into epigenome maintenance in response to DNA damage by real-time tracking of histone dynamics in human cells

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The response to DNA damage in the cell nucleus proceeds on a chromatin substrate, whose integrity is central to cell functions and identity. The coordinated maintenance of genome stability and of its organization into chromatin when challenged by genotoxic stress is thus critical. Yet, the underlying mechanisms are still largely unknown and how much the DNA damage response impacts the chromatin landscape is poorly understood. We approach these issues by investigating alterations in histone variant patterns at sites of DNA damage in human cells. By combining in vivo tracking of newly synthesized histones and localized UVC damage, we have uncovered histone deposition pathways involved in restoring chromatin structure and transcriptional activity in response to genotoxic stress. We have also set up an innovative system allowing simultaneous visualization of new and parental histone dynamics at sites of DNA damage in live cells, providing interesting insights into how the original information conveyed by chromatin can be preserved. I will present our latest findings on these topics and discuss their implications for the memory of chromatin identity in response to genotoxic stress.
Nucleotide excision repair - A3

Presenter: Erina Kakumu

Dynamics of chromatin structure regulating nucleotide excision repair

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Eukaryotic genomic DNA binds histones to be packed into a higher-order structure called ‘chromatin’. Coordinated actions of histone modifications and chromatin remodeling factors are known to regulate gene expression by altering the chromatin structure. Although one can assume that DNA repair and other nuclear functions besides transcription must be influenced by states of chromatin structure, the precise underlying mechanism still remains unclear. Since it is difficult for cells to predict where and when DNA damage occurs within the genome, this suggests the presence of specialized mechanisms regulating the chromatin structure to allow DNA repair.

Nucleotide excision repair (NER) is one of the major DNA repair pathways, which eliminates various DNA base lesions caused by UV, chemical mutagens and so on. To better understand the chromatin states at the sites of DNA damage recognition, we performed chromatin immunoprecipitation (ChIP) assays targeting XPC and DDB2, both of which are damage recognition factors initiating NER. These results indicated that certain types of acetylated histones tend to be absent in chromatin regions bound by XPC or DDB2. We also take advantage of the live cell imaging system combined with local UVC irradiation, suggesting that inhibition of histone deacetylases delays recruitment of XPC to sites of DNA damage and removal of UV-induced (6-4) photoproducts. In addition, we found that XPC co-localizes with heterochromatin protein1 (HP1) as punctate foci in mouse embryonic fibroblast cells without exogenous DNA damage. This focus formation of both XPC and HP1 disappeared when the cells were treated with inhibitors of histone deacetylases. Then, we examined mobility of XPC by using fluorescence recovery after photobleaching technique which revealed that XPC is highly mobile even in the heterochromatic foci. These results suggest that certain features of heterochromatin could be advantageous to damage recognition in NER.
Nucleotide excision repair - A3

Presenter: Arjan F. Theil

New paradigms for understanding the rare progeroid DNA repair disorder trichothiodystrophy


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The human syndrome Trichothiodystrophy (TTD) is a rare, autosomal recessive disorder, characterized by a low content of sulfur-rich proteins in both hair and nails in addition to neurodevelopmental and premature aging features. The clinical symptoms observed among TTD patients are variable in expression and severity, including: photosensitivity, ichthyosis, brittle hair and nails, intellectual impairment, decreased fertility and short stature and stretches from very mild forms of the disease, characterized by normal development with only brittle hair and scaling skin (typical TTD-features) to very severe cases, characterized by high mortality at young age combined with severe developmental and progressive neurologic defects. Approximately 50% of TTD patients are photosensitive and carry inactivating mutations in either the XPB, XPD or TTDA genes, each of which encode for subunits of the transcription factor IIH (TFIIH). TFIIH is essential for both transcription initiation and nucleotide excision repair (NER). The affected NER function by these mutations explains the photosensitivity as NER is the main DNA repair process in mammals to remove UV-induced DNA lesions. Progressive premature aging features, including neurological decline are thought to be derived by the inefficient repair of endogenously produced DNA lesions. The more developmental abnormalities are suggested to be caused by a subtle TFIIH-dependent transcription deficiency. Mutations in the TTDN1 gene are associated with the non-photosensitive form of TTD, although the function of TTDN1 remains elusive. However, the causative gene(s) for the majority of non-photosensitive TTD patients (approximately 80%) has not yet been identified.

In order to dissect the contribution of DNA lesions from affected transcription function in photosensitive TTD patients with respect to aging and developmental problems, it is important to identify the genes and their function causing the non-photosensitive form of TTD. We have selected a group of unrelated non-photosensitive patients, with unequivocal TTD-specific features and without a mutation in TTDN1 for massive parallel whole genome sequencing. Among others, we have identified a novel potential pathogenic mutation in the General Transcription Factor IIE Subunit Beta (GTF2E2/TFIIEβ). Functional defects in TFIIEβ were verified in patient cells. Currently, the effect of the identified TFIIEβ mutation on transcription is being analyzed. In transcription initiation TFIIE is directly coupled to the action of TFIIH, but in contrast to TFIIH is TFIIE not linked to a DNA repair function.
Proper cell function requires unperturbed expression of our genes. Eukaryotic gene transcription by RNA polymerase II (RNAP2) is a tightly controlled process, which is regulated at every step in the transcription cycle from initiation, promotor escape, elongation to termination. Faithful transcription of DNA is constantly threatened by genotoxic lesions from both endogenous and environmental origin. DNA injuries can stall or strongly impede the progression of elongating RNA polymerases and advancing replication forks can collide with stalled RNAP2 complexes. If unrepaired, the consequences of transcription blocking lesions can cause severe cellular dysfunction, apoptosis and senescence, finally resulting in DNA damage induced aging.

To overcome these serious implications transcription-coupled repair (TCR), has evolved to specifically remove transcription blocking DNA lesions from the transcribed strand of a gene and restore transcription. The severe symptoms associated with genetic TCR defects underscore its importance but are strikingly diverse, ranging from mild photosensitivity to severe developmental, neurological and premature aging features.

Lesion stalled RNAP2 blocks the access of repair factors to the damage and thereby impedes repair. In order to make lesions accessible for the TCR machinery, RNAP2 has to be processed: it might backtrack, bypass the lesion or be targeted for degradation.

To study the fate and dynamic behavior of endogenous RPB1 in living cells we generated a GFP-tagged knock-in of RPB1, the largest subunit of the RNAP2 complex, using the CRISPR/Cas9 mediated gene targeting. RPB1 protein levels, viability and proliferation rates of the homozygous GFP-RPB1 cells are equal to wild type MRC5 cells, indicating that GFP-RPB1 is fully functional.

Using this cell line we can determine the protein levels by fluorescence measurements and the protein mobility by fluorescence recovery after photo bleaching (FRAP), which enables us to detect RNAP2 in the different phases of the transcription cycle. Preliminary data show that upon UV damage RPB1 is highly immobilized in a dose dependent manner, most likely representing the stalling of elongation RNAP2 on transcription blocking lesions. In addition we also observed a proteasome and VCP/p97 segregase dependent loss of a mobile fraction of RPB1, most likely representing the initiation form of RNAP2. Together this suggests that UV induced DNA damage does not only affect RNAP2 that stall on bulky DNA lesions, but also non-elongating RNAP2 complexes.
Nucleotide excision repair (NER) is specialized on the removal of bulky lesions caused by the UV radiation of sunlight, chemical carcinogens, certain drugs and oxygen radicals. Although the global-genome NER (GG-NER) reaction has been fully reconstituted using naked substrates, the so far deduced mechanism is not representative for the condensed mammalian genome were chromatin modifiers are thought to guide DNA repair factors to nucleosome arrays. To test the contribution of histone methylation in the mammalian GG-NER reaction, we depleted human cells of the 332-kDa histone methyltransferase KMT2H (also known as ASH1L). This KMT2H down regulation impairs the methylation of histone H3 at position Lys4, causes hypersensitivity to UV-C light and slows down the excision of cyclobutane pyrimidine dimers (CPDs) but not (6-4) photoproducts. Instead, depletion of SETD2, a well-studied histone methyltrasferase with an established role in other DNA repair pathways, does not affect CPD excision. The depletion of KMT2H also slows down the UV-induced degradation of DDB2 and Immunofluorescence analyses (involving formaldehyde fixation of cells) demonstrate that the reduced KMT2H level results in a prolonged residence of a subset of early NER factors (XPC, DDB2, p62 and XPA) on nuclear spots of UV lesions. This abnormal retention of some NER subunits interferes with the recruitment of XPD (the DNA helicase required for damage verification) and ERCC1 (a subunit of the endonuclease that makes the first DNA incision at damaged sites). Chromatin-binding assays carried out without any formaldehyde fixation show that the depletion of KMT2H weakens the ability of XPC (the initial damage sensor in the GG-NER pathway) to form tight complexes with CPD sites. Our findings indicate that KMT2H activity coordinates the initial docking of the XPC sensor complex on histone-associated CPD sites, which in turn is essential for the efficiency of downstream DNA damage verification and incision processes.
**Nucleotide excision repair - A3**

Presenter: Franziska Wienholz

**Amplification of the UDS signal enables single cell based TC-NER measurements**

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Transcription-coupled Nucleotide Excision Repair (TC-NER) is a dedicated DNA repair system that detects DNA lesions in transcribed strands. To date, there is no quick and single cell based assay to measure the repair activity of TC-NER. In theory, it should be possible to measure the gap-filling activity of TC-NER in Global Genome Nucleotide Excision Repair (GG-NER) deficient (XPC-/-) cells using the standard Unscheduled DNA synthesis (UDS) assay. UDS is a commonly used assay, based on measuring DNA synthesis by EdU incorporation after excision of the DNA-damage containing fragment, which in turn is fluorescently labeled using Click-chemistry. However, this UDS assay is not sensitive enough to quantify TC-NER activity, as it only accounts for approximately 10% of the total DNA repair upon UV exposure. Therefore we have introduced a dedicated Tyramide based Signal Amplification (TSA) step, which is an HRP based amplification method that results in the high-density labeling of nucleophilic residue in close proximity of the EdU and leads to an EdU signal amplification of more than 50 times.

This assay enables us to measure repair in NER proficient cells in a dose-dependent manner even at physiological relevant UV-C doses (0.5-16J/m²). Importantly, using this approach we were able to specifically detect DNA repair synthesis by TC-NER using XPC-/- cells. As expected, this signal was completely dependent on transcription and CSB. In line with this, gap filling synthesis was also detected upon treatment with IlludinS, which induces transcription blocking lesions that are preferentially repaired by TC-NER.

Together, this confirms that we can specifically quantify TC-NER activity using a fluorescence based single cell assay. Interestingly, due to the improved detection of DNA repair synthesis of this assay, it was also possible to measure DNA repair induced by oxidative and alkylating damaging agents, which has not been demonstrated before. Together, this shows that this assay is a very promising and potent tool for measuring TC-NER efficiency and it enables us to identify factors that play a role in TC-NER.
Nucleotide excision repair - A3

Presenter: Gustavo Satoru Kajitani

Photoremoval of specific DNA lesions induced by ultraviolet light in the skin of DNA repair deficient mice: effects on cell proliferation and inflammation

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Ultraviolet (UV) irradiation is considered one of the most genotoxic agents present in our environment. It is capable of damaging DNA molecules, inducing mainly cyclobutane pyrimidine dimers (CPD) and pyrimidine 6-4 pyrimidone photoproducts (6-4PP). These lesions interfere in essential cellular processes, such as transcription and replication, thereby promoting several effects in the skin, such as pigmentation, inflammation, dysplasia and cancer. Photolyases are enzymes that repair these lesions in a light dependent mechanism known as “photoreactivation”, in which the enzyme breaks the covalent bond that binds the dimer. Photolyases act in a direct and specific manner for each lesion, CPD-photolyases repairing only CPDs and 6-4PP-photolyases only 6-4PPs. However, these enzymes are absent in placental mammals, in which the UV-induced lesions are repaired by the Nucleotide Excision Repair (NER) pathway. This pathway is subdivided into two recognition pathways, the Transcription Coupled Repair (TC-NER) and the Global Genome Repair (GG-NER).

In this work, we used NER deficient, XPA knockout (KO), and TC-NER deficient, CSA KO, mice. Both mice strains transgenically expressed either CPD or 6-4PP-photolyases in order to assess the in vivo effects of the photoremoval of each of these lesions after low, chronic UVB exposure. In CSA KO mice, the photolyase-mediated removal of CPDs resulted in a reduction of hyperplasia and cell proliferation, both in the basal and suprabasal layers of the epidermis. However, 6-4PP removal by photolyase was not able to alter these skin responses, which suggests that in TC-NER deficient mice, CPD is the main lesion triggering the hyperplastic process.

In the XPA KO mice, the removal of CPDs completely prevented the hyperplasia, while the 6-4PP removal only promoted partial reduction of this response. CPD removal was also able to fully prevent cell proliferation, both in the basal and suprabasal layers of the epidermis, while 6-4PP removal managed to partially prevent this effect in the basal layer, and abrogate it in the suprabasal one. These data suggest that cell proliferation is the main cause of the observed UV-induced hyperplasia, with CPD being the main lesion triggering the hyperplastic processes, both in TC-NER and NER deficient mice, with 6-4PP playing a minor role in NER deficient mice.

We also studied the effects of CPD or 6-4PP removal in XPA KO mice in the induction of the inflammatory process by in vivo imaging of ICAM-1, MPO and MMP expression after a single, high UVB dose. The removal of either type of lesion was able to prevent ICAM-1 and MPO expression 6 hours after UVB irradiation and reduce the expression of MPO after 24 hours. These results indicate that both lesions have a major role in UVB induced, skin targeted leukocyte migration and activation. The removal of neither type of lesion, however, was able to prevent MMP expression 24 hours after UV irradiation.
NER Deficient Cells Show Increased Sensitivity and Fork Stalling in Response to the Replication Inhibitor Gemcitabine

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Nucleotide excision repair (NER) is known to be a versatile repair process but it is not known to have a role in overcoming replication stress. However we have shown Xeroderma Pigmentosum (XP) A and XPG deficient human fibroblasts are shown to be very sensitive to the replication inhibitors gemcitabine and hydroxyurea (HU), whereas XPF deficient cells show moderate gemcitabine sensitivity and no HU sensitivity. Both drugs function by causing replication stress however their mechanisms of action are different. Gemcitabine is a cytosine analogue which is incorporated into DNA and acts as a chain terminator and it also inhibits ribonucleotide reductase (RNR) depleting nucleotide pools, which leads to more gemcitabine incorporation. HU is an RNR inhibitor which has no other known mechanisms of action. As there are differences in sensitivity between the NER complementation groups it is likely that this is not canonical NER and is instead a pathway which utilises only some of the genes.

DNA fibre analysis after treatment with gemcitabine suggest the increased sensitivity of XPA and XPG deficient cells is due to increased fork stalling and decreased fork speed after drug exposure, as opposed to downstream processing of these stalled forks. As a nascent DNA strand is not thought to elongate more than one base further than an incorporated gemcitabine molecule this suggests XPA, XPG and XPF may have a role in gemcitabine removal from DNA, allowing fork progression. However, their sensitivity to HU suggests XPA and XPG may also have a role mitigating RNR inhibition. Additionally XPA deficient cells show decreased fork speed under control conditions suggesting a role in normal fork progression. This could compliment Isolation of Proteins on Nascent DNA (iPOND) data (Gilljam et al., 2012) that showed XPA bound in close proximity to the replication fork.

Altogether this work provides evidence for a role of NER components in mitigating replication stress caused by gemcitabine and HU, adding to the repertoire of this already versatile pathway. Exploring this process is likely to be clinically useful as gemcitabine is used against many types of cancer and predicting resistance based on a tumour genotype could be used in the future to improve outcomes.

Chromatin remodeling is tightly linked to all DNA transacting activities including DNA repair. Nucleotide Excision Repair (NER) is a dedicated DNA repair system that removes a wide variety of DNA helix-distorting lesions, including UV-induced DNA damage. The severe clinical consequences associated with inherited NER defects, including (premature) aging and extreme cancer susceptibility underscore its biological relevance. Despite detailed insight into the core NER reaction, much less is known about its dynamic interplay with chromatin.

Chromatin structure and dynamics are regulated by ATP-dependent chromatin remodelers, histone chaperones and post translational modifications. Recent studies have shown that post translational modifications with ubiquitin play an important role in the UV induced DNA damage response. To identify proteins that are ubiquitylated upon UV-induced DNA damage, a SILAC based quantitative mass spectrometry approach in combination with isolation procedures for ubiquitylated peptides was used. In addition to known UV-induced ubiquitylated proteins like XPC, we observed a strong increase in Histone H1 ubiquitylation upon UV. H1 ubiquitylation was confirmed using HIS-tagged ubiquitin pulldown experiments and reached its maximum 1 hour after UV-irradiation. Thus far most research on the interplay of chromatin with DNA repair has focused on core histones, however these data suggest also an important role for the linker histone H1 in the UV-damage response.

Interestingly, down regulation of the histone H1 chaperone protein SET results in an enhanced survival after UV-induced DNA damage. This improved survival is not caused by enhanced nucleotide excision repair, as shown by measurements of Unscheduled DNA synthesis (UDS). Subsequently we tested whether SET knockdown might affect the DNA damage signaling. One of the main DNA damage signaling pathways involves phosphorylation of histone H2AX. Cells with a reduced expression of SET show enhanced γH2A.X signaling after UV damage. This suggests that SET may function as a more general modulator of the DNA damage response. In line with this, clonogenic survival assays showed increased survival upon SET knockdown to different genotoxic agent including IR and H2O2. Future experiments will help to uncover the function of histone H1 and its chaperone SET in the DNA damage response.
Nucleotide excision repair - A3

Presenter: Jana Slyskova

Transcription-coupled but not global genome nucleotide excision repair protects cancer cells against oxaliplatin

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Cancer sensitivity and resistance to chemotherapy is to a large extent determined by the efficiency of the DNA damage response (DDR). In this study, we have explored DDR mechanisms to oxaliplatin, a crosslinking agent routinely used as adjuvant treatment of advanced colorectal cancer. It is assumed that the combination of conventional genotoxic chemotherapy and specific inhibition of DDR mechanisms can improve the patient’s prognosis, however, the exact DDR network that protects cells against oxaliplatin lesions is currently not well understood.

To obtain a comprehensive view of the oxaliplatin-induced DDR, we conducted a negative selection screen using a lentiCRISPR/Cas9 library constructed against 40 core DNA repair genes. Besides genes involved in classical inter-strand crosslink repair, genes participating in other DNA repair pathways were identified. Surprisingly, loss of functional CSA and CSB, but not XPC, sensitized cells to oxaliplatin. In line with this, oxaliplatin lesions triggered the chromatin retention of CSB, but not of XPC. We further observed that oxaliplatin treatment blocked transcription and this was only resumed in the presence of functional transcription-coupled repair. In contrast, global DNA repair synthesis was not induced after oxaliplatin exposure. Our findings suggest the involvement of transcription-coupled but not global genome nucleotide excision repair in oxaliplatin damage recognition and removal, independently of cell type or p53 status. Additionally, our screen revealed that oxaliplatin-treated cancer cells depend on base excision repair for survival, with the highest sensitivity observed in cells lacking POLB activity.

Our data show that the cellular response to oxaliplatin-based chemotherapy is more complex than initially anticipated and involves the activity of several distinct DNA repair pathways. This knowledge potentially provides new insight into the mechanisms of oxaliplatin resistance and may help to develop new strategies for cancer treatment.
Nucleotide excision repair - A3

Presenter: Jan Fischer

The Role of Poly(ADP-Ribosyl)ation in the Cellular Response to BPDE-induced Genotoxicity

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Poly(ADP-ribosylation) is a complex and reversible posttranslational modification catalyzed by poly(ADP-ribose) polymerases (PARPs), which thereby orchestrate other proteins’ functions and localizations. PARP1 plays significant roles in many different cellular aspects, such as DNA repair, chromatin remodeling, gene transcription and regulation of cell death. While a role of PARP1 in base excision (BER) and DNA double strand break repair (DSBR) is firmly established, its role in nucleotide excision repair (NER) is less well understood.

Recently, we addressed the role of PARylation in NER and revealed that the central NER factor XPA is controlled by high-affinity non-covalent interaction with PAR and further, suggested a mutual regulatory mechanism between XPA and PARP1 [1]. In the current study, we used benzo[a]pyrene-7,8-dihydro-9,10-epoxide (BPDE) as a model substance to study the role of PARylation in NER in HeLa cells [2].

First, PAR quantification using isotope dilution mass spectrometry as well as NAD+ measurements using an enzymatic NAD+ cycling assay revealed that BPDE induces PAR formation in a time- and dose-dependent manner. We further observed a counteracting mechanism which led to strongly increased NAD+ levels later on. Cellular studies, such as cell proliferation, cell cycle, and clonogenic survival experiments, demonstrated a functional relevance of PARP activity in BPDE-induced genotoxic stress response. While cytotoxic impact of BPDE during the first 48 hours after treatment was not affected when PARylation activity was impaired, PARP inhibition strongly sensitized cells on longer terms. Increased levels of BPDE-induced replicative stress, altered DNA damage signaling and as a consequence cell cycle delay in PARP inhibited cells can be adduced to explain this finding. Finally, the absence of PARP activity after exposure to BPDE enhanced its genotoxic potency and strongly increased the cellular mutation load.

In contrast, to previous studies using UV-induced DNA damage to study the role of PARylation in NER [1], the role of PARylation in BPDE-induced genotoxic stress response appears to be related to its function in the resolution of replicative stress. This points to lesion-specific roles of PARylation in the repair of DNA damage related to NER.

In conclusion, this study establishes a role for PARylation in BPDE-induced genotoxic stress, molecular mechanisms of which are currently being evaluated in greater detail.

(1) Fischer et al. “Poly(ADP-ribose)-mediated interplay of XPA and PARP1 leads to reciprocal regulation of protein function.” FEBS J. doi: 10.1111/febs.12885
(2) Fischer et al. “The role of poly(ADP-ribosyl)ation in the cellular response to BPDE-induced genotoxicity.” Manuscript in preparation for publication
Nucleotide excision repair - A3

Presenter: Julie Soutourina

Novel Mediator function connecting transcription and nucleotide excision DNA repair in eukaryotes

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Maintenance of genome integrity and transcription are two key functions of the cell, allowing the transmission and expression of the genetic material. How these processes are coordinated in vivo in eukaryotes remains a key biological question that relates to serious human diseases.

Mediator is a large multisubunit complex conserved in all eukaryotes, which plays a crucial role in transcription activation. We have discovered a novel role of Mediator as a link between transcription and DNA repair via a direct contact between the Med17 Mediator subunit and Rad2/XPG DNA repair protein (Eyboulet et al. 2013 Genes & Development). Rad2/XPG endonuclease is involved in nucleotide excision DNA repair (NER) and mutations in human XPG gene give rise to severe diseases, xeroderma pigmentosum (XP) associated with Cockayne syndrome (CS). Genome-wide location analyses revealed that Rad2 is associated with Pol II-transcribed genes in the absence of exogenous genotoxic stress in yeast and that Rad2 occupancy of class II gene promoters is highly correlated with that of Mediator. Furthermore, Mediator med17 mutants that are defective in Mediator-Rad2 interaction are UV-sensitive in a global-genome repair deficient background and are epistatic with a transcription-coupled repair deficient mutant. This UV sensitivity of Mediator mutants is correlated with reduced Rad2 occupancy of class II genes. Our results strongly suggest that Mediator is involved in transcription-coupled DNA repair by facilitating Rad2 recruitment to transcribed genes (Soutourina & Werner 2014 Cell Cycle). Now we are addressing the molecular mechanisms governing the Mediator link with DNA repair machinery. We are analysing an interaction interface and investigating the functional link between Mediator and NER factors, including TFIIH, Rad1/XPF-Rad10/ERCC1, Rad26/CSB, in the absence and in the presence of UV exposure. To determine the genomic distribution of Mediator and NER factors related to DNA damages, we develop advanced functional genomics approaches. Considering the conservation of Mediator and DNA repair proteins, the molecular events governing the Mediator link to DNA repair likely exist in all eukaryotes. We are directly addressing the conservation of these mechanisms in human cells. We showed that Mediator interacts with XPG protein in unmodified HeLa cells and primary fibroblasts. The analysis of Mediator role in NER in human cell lines derived from XP/CS patients is in progress.

In conclusion, our work will contribute to new concepts of the functional interplay between transcription and DNA repair and might give insights into our understanding of human diseases like XP/CS syndromes. (Eyboulet F. et al., (2013) Genes & Dev. 27, 2549-2562; Soutourina J. & Werner M. (2014) Cell Cycle 13, 1362-1363)
Contribution of parental histone dynamics to epigenome stability after DNA damage

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DNA damage challenges not only genome stability but also the integrity of its organization into chromatin, which conveys critical epigenetic information governing gene expression and cell identity. How the chromatin landscape is modified in response to DNA damage while preserving the information that it carries is thus a key issue. We address this fundamental question by focusing on histone H3 variants, which we showed are deposited de novo in UVC-damaged chromatin regions in human cells (1, 2). The deposition of newly synthesized histones raises questions about the fate of parental histones, present in chromatin before damage infliction and carrying the original information. To tackle this issue, we have developed an innovative approach based on the SNAP-tag technology (3) and UVC laser micro-irradiation (4), allowing real-time tracking of parental and newly synthesized histone dynamics at sites of UVC damage. Thus, we have uncovered a redistribution of parental histones around damage sites and we have examined their contribution to repaired chromatin. I will present the underlying molecular mechanisms and discuss the implications of our study for understanding how epigenetic information can be maintained during the chromatin rearrangements that accompany DNA repair.

Cockayne syndrome (CS) is a rare disorder with an autosomal recessive pattern of inheritance characterized by growth failure, progressive neurological dysfunction, signs of premature aging and cutaneous photosensitivity (1). Causative mutations have been identified in CSA or CSB genes, whose products are essential for transcription-coupled repair (TC-NER), the nucleotide excision repair (NER) sub-pathway that removes UV-induced DNA damage blocking the progression of the transcription machinery on the transcribed strand of active genes. Major CS clinical symptoms are not easily ascribed to the persistence of UV-induced DNA lesions and several studies point to the involvement of CS proteins in additional processes. Until now, very little is known about the functional roles of CSA outside TC-NER. New indications came from investigations on the functional consequences of CSA mutations. By overexpression experiments, we discovered that mutations in different sites of the CSA gene differentially affect the cellular response to UV and oxidative damage, thus indicating that the roles of the CSA protein in the removal of UV-induced damage and oxidative lesions may be uncoupled (2). These findings provide a rationale to explain the wide range in type and severity of CS clinical symptoms, as well as the unusually mild phenotype of UV-sensitive syndrome in a patient carrying CSA mutations resulting in the p.trp361cys change (2). These findings, together with the scaffold properties of CSA, suggest that CSA might interact with various proteins to carry out its activity in distinct processes. To identify a comprehensive set of CSA interactors by the Recombinase-Mediated Cassette Exchange (RMCE) we have generated a panel of isogenic cell lines expressing at physiological levels the wild type or mutated forms of CSA, tagged with the Flag and HA epitopes (CSAFlag-HA). These cells have provided a useful tool to demonstrate that specific mutations impair the stability of CSA protein without altering the stability of the transcript. In addition, the presence of the dual tags allowed the purification of CSA-interacting proteins by Tandem Affinity Purification, and their subsequent identification by mass spectrometry. Through this approach, we isolated all the well-known CSA interactors acting in TC-NER as well as novel CSA molecular partners. We then evaluated how specific CSA mutations or different cellular stressing agents, including UV irradiation and oxidative inducers, affect the pattern of CSA interactions. Overall, this study identifies novel signalling events involving CSA and opens new scenarios on the molecular alterations underlying CS pathogenesis.

Proliferating cell nuclear antigen (PCNA) is an essential sliding clamp protein required for DNA replication and damage repair processes. The interdomain connecting loop (IDCL) of PCNA interacts with many of its protein partners, making PCNA a binding platform and major coordinator of replication factory-related and other processes of genome stability maintenance. Its homotrimeric ring-shaped structure mediates its DNA binding ability and the presence of PCNA can greatly increase the processivity of DNA polymerases. Recently we reported the first natural pathogenic PCNA mutant in human populations. This causes the protein alteration p.Ser228Ile, leading to a severe DNA damage sensitivity syndrome, with premature aging, neurodegeneration, growth defect and UV sensitivity, which we have termed PCNA-associated repair disorder (PARD). We showed that the PCNA-S228I mutation impairs its interaction with some of its protein partners, including Flap endonuclease I (Fen1), DNA ligase I (Lig1) and XPG. To figure out how this impairment can result in DNA damage sensitivity disorder, we are currently utilising lymphoblastoid cell lines developed from affected individuals. However, considering the polymorphisms between different individuals as well as the fact that PCNA is such an essential and constitutively expressed protein for cell viability, perhaps requiring adaptive responses to its mutation in vivo, we consider that isogenic PCNA-S228I knock-in cell lines will be of great utility in such studies.

We are developing two strategies to construct the knock-in cell lines. Initial attempts to introduce a homology-directed repair (HDR) template with Cas9 and guide RNA to directly knock-in the point mutation were unsuccessful, possibly due to haploinsufficiency or extreme sensitivity of the cells to even transiently inactivating perturbations at the PCNA locus. To overcome this we added an intronic selection marker into the HDR template to increase the efficiency and did produce heterozygous edited clones in mouse ES cells. The second strategy is to stably express exogenous PCNA-S228I by lentivirally-mediated transduction, select clones with equal expression levels of exogenous and endogenous PCNA, and then apply CRISPR/Cas9 technique to knock out the endogenous locus. This strategy avoids any complications due to transient haploinsufficiency. Generation of these reagents, and eventually of mouse models, will allow experimental investigation of the molecular and cellular defects that result in PARD.

Transcription factor IIH: at the nexus of transcription and DNA repair

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Human transcription factor IIH (TFIIH) is a ten-subunit complex known to be involved in two vital processes: the DNA repair pathway Nucleotide Excision Repair (NER) and RNA polymerase II-mediated transcription initiation (1). Mutations in TFIIH core subunits XPB, XPD or p8 can result in three different autosomal recessive syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD), characterized by mild-to-extreme photosensitivity, premature ageing, neurological defects and, in the case of XP, a 1000-fold increased susceptibility to skin cancer (2). DNA repair pathways have become a target for novel cancer therapy strategies because of their ability to mend the damage caused to DNA by chemotherapeutic drugs. Understanding the mechanisms that regulate the NER pathway, in which TFIIH plays an essential role, and the relationship between the different factors participating in NER would provide us with information that could help improve current treatments (3).

We have successfully cloned and expressed the TFIIH core subcomplex (subunits XPB, XPD, p62, p52, p44, p34 and p8), using MultiBacTM, a baculovirus expression system specifically designed for the production of eukaryotic multi-protein complexes. We are currently working on scaling-up expression, improving purification procedures, and maximizing the yield of the XPD helicase subunit. Our aim is to biochemically and structurally characterize the TFIIH complex in an effort to better understand the causes behind the different phenotypes associated to the XP, CS and TTD syndromes.

Nucleotide excision repair - A3

Presenter: Donata Orioli

Relevance of transcriptional deregulations in the puzzling genotype-phenotype relationships of mutations in the XPD gene

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Mutations in genes encoding the XPD and XPB subunits of the repair/transcription complex TFIIH are responsible for different clinical entities, including the cancer-prone xeroderma pigmentosum and the cancer-free multisystem disorder trichothiodystrophy (TTD). The engagement of TFIIH in distinct cellular processes has provided a rationale for the different clinical phenotypes, implying that mutations affecting the DNA repair activity of TFIIH are associated with XP whereas those impairing also the transcriptional activity of TFIIH are responsible for TTD (1). Thus, XP and TTD patients represent a valuable model system to elucidate the TFIIH-dependent signaling pathways relevant for developmental and neurological defects, precocious ageing and cancer prevention.

By whole transcriptome sequencing of primary dermal fibroblasts from patients mutated in XPD, we identified TFIIH-dependent transcriptional deregulations affecting specific signalling pathways in TTD cells. We identified the transcriptional impairment of genes related to the extracellular matrix (ECM) that result in structural alterations and metabolic disturbances of the ECM in TTD cell cultures, skin equivalents and skin dermis (2). These ECM alterations impair the migration and wound healing features of TTD dermal fibroblasts, which can be fully recovered by inhibiting the activity of MMP-1, the metalloproteinase involved in ECM remodelling (3). Taking into account the key role of the ECM in triggering signalling events that regulate cell behaviour and tissue homeostasis, our findings highlight the relevance of ECM anomalies in TTD pathogenesis and in the phenotypic differences between TTD and XP.

Furthermore, we have recently found a causal link between the non-photosensitive form of TTD, which is DNA repair proficient, and mutations in a general transcription factor involved, together with TFIIH, in RNA polymerase II-driven transcription. Overall, these findings highlight the relevance of transcription deregulations in the clinical outcome of TTD.

(1) Stefanini M, Botta E, Lanzafame M, Orioli D. Trichothiodystrophy: from basic mechanisms to clinical implications. DNA Repair (Amst) 2010; 9:2-10
UV-induced transcription inhibition is regulated in a UV dose specific and gene specific manner

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UV-induced DNA damages represent a challenge for correct transcription regulation when RNA polymerase II stalls at UV-induced lesions. The exact molecular mechanism of transcription inhibition after UV-induced damage induction is still largely unresolved. The probability that DNA acquires a damage lesion after UV is considered to be correlated to the UV dose. Under physiological conditions, nucleotide excision repair (NER) mostly operates at low damage levels, occasionally being interrupted by higher damage levels, for instance due to intense sunlight exposure.

Previously we noted that cells exhibit bimodal switch behavior to choose upon different repair schemes dependent on their UV-induced damage exposure. We observed that at relatively low UV doses, i.e. below 5 J/m2, recruitment of the NER machinery by the global repair (GGR) damage-sensor XPC is suppressed but not fully inactivated. To study transcription coupled repair (TCR) at relatively low and high UV doses, we used a U2OS cell line containing 200 copies of an inducible reporter gene cassette, allowing to visualize the accumulation of NER proteins at extensively transcribed chromatin at the activated reporter array compared to general transcription levels in the rest of the nucleus. We showed that the core NER factor XPA is only recruited to the highly transcribed gene array at low UV dose (below 5 J/m2) indicating that at relatively low UV-dose the core NER machinery is predominantly recruited to actively transcribed genes due to the absence of active GGR.

As a next step to determine to what extend UV-induced transcription inhibition is gene and chromatin specific, we state that UV-induced transcription inhibition is UV dose and gene length dependent. To test this notion we measured transcription stalling at relatively long versus shorter genes upon exposing the cells to low UV doses. We used single molecule mRNA FISH to determine the amount of transcripts at single molecule level in the nucleus and cytoplasm in single cells. We analyzed the 92 kb PLOD2 gene as a relatively long and 4,4 kb GAPDH as a relatively short gene. Both genes are not related to stress or UV damage responses and therefore represent an appropriate readout of general transcription inhibition after UV-induced damage. We show full transcription inhibition of PLOD2 after 1 hour UV treatment with 10J/m2 and also almost full transcription inhibition with a much lower UV dose of 3J/m2 whereas transcription of GAPDH is not effected by both 3 and 10 J/m2 UV. From our data we conclude that gene size is a major determinant of UV damage-induced transcription inhibition. Our next challenge is to determine the relationship between UV-induced transcription inhibition and the epigenetic regulatory state and chromatin composition of damaged genes.
Restructuring of a crucial binding interface causes PCNA-associated repair disorder


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Recently, and for the first time, we described a disease causing mutation of the proliferating cell nuclear antigen (PCNA), which normally interacts with multiple proteins involved in DNA replication and repair, coordinating their various activities. Individuals homozygous for the hypomorphic Ser228Ile substitution show symptoms reminiscent of DNA damage disorders, and patient derived cells show deficiencies in nucleotide excision repair processes. We show here through recombinant protein pull-downs and Surface Plasmon Resonance that S228I-PCNA has reduced affinity for a number of PCNA binding partners including Cdt1 and DNMT1, although p21 binding is less affected.

We also now report the crystal structure of the mutated PCNA, which shows that S228I alters the structure of a crucial PCNA surface, the inter-domain connecting loop (IDCL). Many PCNA Interacting proteins contain a PCNA Interacting Protein or PIP-box motif, which has been shown to insert into a pocket beneath the IDCL. Our data provide a structural explanation for the altered binding properties of this disease causing variant of PCNA. We present subtle cellular defects resulting from this structural change, which likely contribute to the clinical phenotypes of affected individuals.

Our ongoing research into these interactions, as well as further dissection of the effect of the S228I change on other PCNA-dependent cellular functions will allow us to assign altered cellular responses to specific perturbed interactions. This will increase our understanding both of this debilitating condition but also of the normal function of this key DNA repair and replication organiser.
Nucleotide excision repair - A3

Presenter: Sarah Sertic

TLS Polymerases are involved in processing of EXO1-dependent lesions after UV-induced damage

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UV light mainly damages DNA by generating CPDs and 6-4PP photoproducts, which are responsible for the pathological effects of sunlight. In a healthy organism, such DNA helix distorting lesions are removed by Nucleotide Excision Repair (NER), a multistep process. Mutations in NER genes cause the onset of severe pathologies. The principal symptom common to all diseases is the strong sensitivity to UV. A high predisposition to tumors development arises in xeroderma pigmentosum (XP) patients, while neurological dysfunctions have been observed in both XP and Cockayne syndrome patients. Upon DNA damage sensing, checkpoints are activated allowing a block or delay of cell cycle progression to ensure repair of the DNA lesions. Intriguingly, while in normal cells UV irradiation activates DNA damage checkpoints in all phases of the cell cycle NER yeast mutant strains and human fibroblasts derived from XP patients fail activate the checkpoint in G1 and G2.

Recently, we demonstrated that the checkpoint response to UV light in cells that are not actively replicating their genome requires prior processing of the UV lesions. This involves NER factors but also the Exo1 nuclease. In particular, acting on NER intermediates, Exo1 generates structures containing long tracts of ssDNA in response to UV irradiation. This role of Exo1 is only observed at a subset of problematic lesions that cannot properly repaired by canonic NER. It is these Exo1-induced structures that provide the signal for checkpoint activation both in yeast and human non-replicating cells. The essential role of Exo1 in UV-induced checkpoint activation in vivo has been recently supported by in vitro reconstitution of the activation pathway.

What are the problematic lesions that require EXO1 activity is still unknown. We hypothesized that Closely Opposing UV Lesions (COLs) on the two DNA strands could exist and may be a likely candidate. This scenario would require TLS polymerases bypass during repair synthesis step. Therefore, we are investigating Y-family polymerase recruitment at EXO1-positive local UV damage sites (LUDs). We found that Pol η is recruited at both EXO1-positive and EXO1-negative LUDs, while Pol λ and κ always co-localize with the nuclease. Using the CRISPR-Cas9 system, we generated EXO1 knock out cell lines that demonstrated a requirement for EXO1 in Pol λ and κ recruitment, consistently with our working model. Finally, when we silenced TLS polymerases we observed a hyper-activation of UV-induced DNA damage checkpoint, suggesting that EXO1 continues to process UV damaged DNA enlarging the gap and eventually producing DSBs. TLS polymerases, thus are crucial to prevent dangerous situations in non-replicating UV irradiated cells.
The rates at which lesions are removed by DNA repair can vary widely throughout the genome with important implications for genomic stability. We measured the distribution of nucleotide excision repair (NER) rates for UV induced lesions throughout the yeast genome. By plotting these repair rates in relation to all ORFs and their associated flanking sequences, we reveal that in normal cells, genomic repair rates display a distinctive pattern, suggesting that DNA repair is highly organised within the genome. We compared genome-wide DNA repair rates in WT and in RAD16 deleted cells, which are defective in the global genome-NER (GG-NER) sub-pathway, demonstrating how this alters the normal pattern of NER rates throughout the genome. By examining the genomic distribution of global genome NER factor binding in chromatin before and after UV irradiation, we reveal that GG-NER is organized and initiated from specific locations within the yeast genome. The GG-NER complex regulates the histone H3 acetylation status and chromatin structure in the vicinity of these genomic sites to promote the efficient removal of UV induced lesions. This demonstrates that chromatin remodeling during the GG-NER process is organized in the genome. Importantly, we demonstrate that deleting the histone modifier GCN5, which acetylates histone H3 in response to UV radiation, and is an accessory factor necessary for chromatin remodeling during GG-NER, significantly alters the genomic distribution of NER rates. These observations may have important implications for the effect of histone and chromatin modifiers on the distribution of genomic mutations acquired throughout the genome.
Nucleotide excision repair - A3

Deciphering the Complex of Regulation of XPC in NER

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Our genome is continuously exposed to various types of DNA damage that may result in mutations or cell death. Luckily, our cells have evolved a variety of DNA damage responses to counteract the severe consequences of DNA damage. Nucleotide Excision Repair (NER) is an important DNA repair pathway that removes a wide variety of helix-distabilizing DNA lesions, including UV-induced lesions.

XPC is a key player for damage recognition in the global genome NER (GG-NER) subpathway of NER and detects DNA helix distorting lesions. Upon UV damage, XPC and DDB2 are ubiquitylated by the E3 ligase CRL4DDB2 (DDB1-DDB2-CUL4A/B-RBX1) complex with different outcomes. XPC gains higher affinity to the damaged DNA in vitro, while DDB2 is targeted for proteosomal degradation. XPC is also modified by small ubiquitin-like modifier (SUMO) upon damage. Recently it was described that SUMOylated XPC is modified with K63-linked ubiquitin chains by the SUMO-targeted ubiquitin ligase RNF111. Using different live cell imaging approaches, we further investigated the regulatory function of RNF111-mediated ubiquitylation. We showed that RNF111-dependent XPC ubiquitylation results in the release of XPC from the damage, which is necessary for the incorporation of the downstream NER endonucleases XPG and ERCC1/XPF, thereby enabling efficient repair.

Overall, XPC is tightly regulated upon UV damage with sequential events of ubiquitylation and SUMOylation for the efficient progression of NER. Our knowledge is still limited about the exact mechanism and interplay of the E3 ligases CRL4DDB2 and RNF111 in XPC regulation as well as the involved ubiquitylation sites and the ubiquitin chains. Furthermore, there are other players such as the deubiquitylating enzyme USP7 and the ubiquitin-selective segregase VCP/p97 involved in the regulation of XPC by ubiquitin. To unravel the exact mechanism of XPC regulation by ubiquitylation, we will study the effect of each of these factors on the stability, mobility and damage accumulation kinetics of XPC. For this purpose, we generated a GFP-tagged XPC knock-in cell line using CRISPR/Cas9 mediated genome editing with a one-step PCR based approach. This will enable us to study behavior of endogenous XPC upon DNA damage in the living cell to obtain novel insights in its regulation.
POSTER SESSION B1:
'Replication Stress and Mismatch Repair'

Poster viewing: Sunday, April 17, 20:00 – 22:00 h

Discussion: Monday, April 18, 16:55 – 18:25 h
Replication stress and Mismatch repair - B1

Presenter: Angela Brieger

Stability of the DNA mismatch repair complex MutLα is affected by the phosphorylation of PMS2

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DNA mismatch repair (MMR) is a pathway that corrects misincorporation and slippage errors introduced by DNA polymerase during DNA replication. This process is highly conserved in almost all organisms ranging from bacteria to human. Human MutLα, a heterodimer consisting of MLH1 and PMS2, plays an essential role in MMR and has been shown to be additionally involved in several other important cellular mechanisms as the control of cell cycle checkpoints and apoptosis. However, the regulation of the functional diversity of MutLα is still not clear.

In order to prove modification of MutLα by phosphorylation and to enrich the amount of phosphorylated proteins, we treated transiently MutLα transfected HEK293T cells with the cell permeable phosphatase inhibitor Calyculin. Consistent with MutLα being phosphorylated, Calyculin treatment produced an up-shift smear of MLH1 and PMS2 in SDS-PAGE. Interestingly, we found that the treatment with Calyculin leads to degradation of PMS2 when the interacting domain of MLH1 is missing. In addition, we discovered that the C-terminal end of PMS2 is relevant for this phosphorylation dependent degradation and that the process is inhibited by blocking the proteasome system with Bortezomib during Calyculin treatment.

Together, we show that MutLα can be posttranslationally phosphorylated and that pharmacologically induced phosphorylation can trigger the selective proteasome dependent degradation of PMS2. Therefore, we conclude that phosphorylation of MutLα may represent a new mechanism to regulate the dimerization of MutLα acquired during eukaryotic evolution.
Replication stress and Mismatch repair - B1

Presenter: Charlie Laffeber

Molecular mechanism of daughter strand incision during DNA mismatch repair

Charlie Laffeber(1), Nicolaas Hermans(1), Yannicka Mardenborough(1), Mariela Artola-Borán(2), Herrie H.K. Winterwerp(3), Peter Friedhoff(4), Josef Jiricny(2), Roland Kanaar(1,5), Joyce H.G. Lebbink(1,5)

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The DNA mismatch repair (MMR) system is responsible for the repair of base-base mismatches and small insertion-deletion loops that occur upon DNA replication. The MMR pathway is functionally conserved from bacteria to humans. Our interest focuses on the mechanistic details that govern the precisely defined cooperation between the constitutive MMR proteins. In Escherichia coli, the initial step is the recognition of a DNA mismatch by the MutS protein, followed by recruitment of the MutL protein, recognition of the strand discrimination signal and activation of the MutH endonuclease. To unravel the precise molecular mechanisms involved in these early steps of the repair process, we quantitatively study the importance of the distance between the mismatch and the strand discrimination signal, the number of strand discrimination signals and the role of the MutS and MutL ATPase activities in determining the efficiency of daughter strand incision and removal. We observed that the location and number of hemi-methylated GATC sites did not influence the rate at which E.coli MutS, MutL and MutH introduced the first strand incision following DNA mismatch recognition. Two GATC sites flanking the mismatch were incised by the same activated MMR complex in a processive manner, resulting in a significantly increased unwinding and strand excision efficiency compared to a single GATC site. Thus, reaction steps downstream of strand incision are responsible for the previously reported dependence of MMR repair on GATC site distribution. Interestingly, the introduction of multiple nicks by the human MutLα endonuclease in a bidirectional manner also contributed to increased repair efficiency. These data support a general model for prokaryotic and eukaryotic MMR in which, despite large mechanistic differences, mismatch-activated complexes facilitate efficient strand excision and repair by creating multiple daughter strand nicks.
Transient protein conformations in the DNA mismatch recognition pathway.

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Misspaired bases in DNA are recognized by the DNA mismatch repair protein MutS. Upon mismatch detection, it signals for the repair of the error. It is broadly accepted that during mismatch recognition, MutS undergoes a series of conformational changes. The structure of MutS bound to mismatch DNA is known for many years. Here we present a novel crystal structure of DNA-free MutS. This new structure allows us to reconstruct additional steps of mismatch recognition in high detail, and shows flexibility of the MutS dimer that allows for DNA to enter the dimer for initial binding. We made point mutants to verify the relevance of this structure. The DNA-binding assays with these mutants indicate the importance of this MutS dimer flexibility for DNA binding and release.
Replication stress and Mismatch repair - B1

Presenter: Hein te Riele

An oligonucleotide-directed mutagenesis screen to rapidly identify pathogenic DNA mismatch repair gene variants associated with Lynch syndrome

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We recently developed a gene modification technique in mouse embryonic stem cells (ESCs) that allows single base pair substitution at any desired location in the genome without the need for prior generation of a DNA double-stranded break. The method uses short synthetic oligodeoxyribonucleotides (ssODN) that are complementary to an endogenous target sequence except for the centrally located nucleotide that comprises the desired modification. Gene modification occurs by annealing of the ssODN to a complementary sequence in the replication fork and its subsequent stable integration into the genome. However, DNA mismatch repair (MMR) recognizes the mismatch at the position of the mutating nucleotide and blocks the planned modification. We found that this effect of MMR can be avoided when the mutating nucleotide in the ssODN is a locked nucleic acid (LNA). LNA-modified ssODNs (LMOs) of only 25 nucleotides allow base-pair substitution at frequencies of 10\(^{-3}\) in MMR-proficient cells. In vitro experiments showed that LNA modification prevents mismatch binding by purified bacterial MutS. Consistently, also in Escherichia coli the LMO design evades MMR during \(\lambda\) Red-mediated gene editing.

LMO-directed gene modification (oligo targeting) shows very high precision, but appears less efficient than CRISPR/Cas9-assisted gene modification. Nonetheless, we show that oligo targeting can effectively be used to fulfill a specific clinical need: the functional interrogation of variants of DNA MMR genes in order to establish whether or not they are causative for the cancer predisposition Lynch syndrome (LS).

Early onset colorectal and endometrial cancer in LS is caused by inherited mutations in MSH2, MLH1, MSH6 or PMS2 MMR genes that fully destroy gene function. Missense mutations in MMR genes are also frequently seen in cancer patients, however, their functional implications are often uncertain. To help clinicians diagnosing LS and offering appropriate counseling and treatment, we developed a rapid screening procedure to examine the functional implications of these MMR gene variants of uncertain significance (VUS).

The screen takes advantage of our novel oligo targeting protocol and comprises three steps: (i) ESCs are exposed to an LMO designed to create the VUS at the endogenous MMR gene in a subset of cells; (ii) the culture is exposed to 6-thioguanine (6TG) to select for MMR-deficient cells: the appearance of colonies indicates the mutation introduced by the LMO is deleterious; (iii) 6TG-resistant colonies are sequenced to confirm the presence of the planned mutation. The screen can distinguish pathogenic MSH2, MLH1 and MSH6 variants from polymorphisms with a specificity >98%. To date, we have identified 46 deleterious variants among 135 analyzed VUS.
Replication stress and Mismatch repair - B1

Presenter: Inga Hinrichsen

Deficiency of DNA mismatch repair protein MLH1 causes improved response to KU60648

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A significant portion of colorectal cancers (CRCs) exhibit microsatellite instability (MSI), use of standard chemotherapy for these cancers is still under discussion. Therefore a study published recently by Dietlein et al. attracted our interest. The group scanned for mutations which are associated with response to DNA-PKcs (DNA PK catalytic subunit) inhibitor KU60648. In their study loss of MSH3 came up as the most significant predictor of inhibitor efficiency and they suggested determining its status in MSI CRC patients to prognosticate a success of therapy with KU60648.

The MMR deficiency which leads to MSI is either due to MLH1 promotor hypermethylation (in up to 15% of sporadic CRCs) or caused by Lynch syndrome, a hereditary cancer predisposition. All together most CRCs show a MLH1 deficiency. Therefore we investigated if the response to the inhibitor KU60648 is limited to an inactivation of MSH3 or if this is true for MLH1 deficiency as well.

We analyzed a set of 12 colon cancer cell lines in response to KU60648. We verified the expression of MMR proteins via Western blotting and determined the cell viability after 72 h treatment by MTT Assay. MLH1 deficient cell lines showed a significantly stronger response compared with MMR proficient cells. To further analyze the correlation of missing MLH1 expression and elevated sensitivity we used the cell line 293Lalpha in which the suppression of MLH1 is inducible. In addition we generated a SW480 cell line stably transfected with shMLH1. The experiments with these two sets of cell lines, consisting of a MLH1 deficient cell line and its proficient sister cell line with identical genetic background, strengthened our results.

Our data suggest that loss of MLH1 sensitizes cells to DNA-PKcs inhibitor independently of the MSH3 status. This finding simplifies the identification of tumors sensitive to this treatment since determination of MLH1 status is a standard in diagnosis of MSI CRC patients.

Replication stress and Mismatch repair - B1

Presenter: Jacob Jansen

A novel DNA repair pathway correcting translesion synthesis errors.

Jacob G Jansen, Anastasia Tsaalbi-Shtylik, Gido Snaterse, Sandrine van Hees-Stuivenberg, Mark Drost, Niels de Wind
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Recently, we have described a novel DNA repair pathway, dubbed post-translesion synthesis (TLS) repair, in which the mismatch-binding heterodimer Msh2/Msh6 mediates the excision of incorrect nucleotides incorporated by TLS polymerases opposite helix-distorting, even non-instructive, DNA photolesions (Tsaalbi-Shtylik et al., J. Cell Biology 209, 33-46 (2015)). Post-TLS repair significantly reduces the mutagenicity of these lesions while inducing Rpa/Atr/Chk1-mediated checkpoints. Furthermore, post-TLS repair-induced excision tracts can be transmitted through mitosis, inducing apoptosis during the subsequent cell cycle. To further elucidate the mechanism of post-TLS repair, we are generating isogenic mouse embryonic stem cell lines with disruptions in downstream mismatch repair genes using CRISPR/Cas9 technology. Here we show the preliminary characterization of mouse cell lines defective for Exo1 or Mlh1. We found that Exo1 plays a minor role in post-TLS repair, indicating that excision of incorrect nucleotides incorporated by TLS opposite DNA photolesions occurs independent of Exo1. In contrast to Exo1, however, Mlh1 seems to play a major role in post-TLS repair, suppressing TLS-induced misincorporations following exposure to ultraviolet light to a similar extent as Msh2/Msh6. Interestingly, in Mlh1-deficient cells activation of Rpa/Atr/Chk1-mediated checkpoints can occur independently of excision, suggesting that Msh2/Msh6, bound to incorrect nucleotides opposite DNA photolesions, can induce checkpoints independent of excision.
Replication stress and Mismatch repair - B1

Presenter: Jean-Baptiste Charbonnier

Pivotal roles of MutL homologs in mismatch repair and meiosis: insights from structural studies

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Eukaryotic MutL homologs play central roles in mismatch repair and in meiosis (Guarné & Charbonnier, 2015, Prog Biophys Mol Biol). In particular, MutLα (Mlh1-Pms1 in S. cerevisiae and MLH1-PMS2 in human) and MutLγ (Mlh1-Mlh3) are considered respectively as the main endonuclease activity in MMR and as the main resolvase activity for cross-over formation in meiosis. Dysfunctions of these factors are associated with colorectal cancers called Lynch syndrome for MutLα and sterility and chromosome translocations for MutLγ. We previously reported the first crystal structure of the C-terminal region of Mlh1-Pms1 and showed an unexpected role of the extreme C-terminus of Mlh1 in Pms1 endonuclease site (Gueneau & al, 2013, Nat Struct Mol Biol). More recently, we solved the first crystal structure and SAXS analysis of a eukaryotic MutLγ C-terminal region. This allowed us to compare the resolvase and MMR endonuclease heterodimer structure and endonuclease active sites (J Dai & al, in preparation). The MutLγ heterodimer presents a similar overall MutL fold with key differences: (i) some motifs essential for MutLα formation and stability are missing in MutLγ, (ii) the regulatory domain of MutLγ adopts a different position thus redefining the electrostatic surface close to the endonuclease site, (iii) contrary to MutLα, the C-terminal domain of MutL specifically binds Holliday junctions. Finally though genetic studies, we characterized separation of function mutants of Mlh1 and proved a central role of the conserved C-terminus of Mlh1 in both MMR and meiosis.
LNA modification of single-stranded DNA oligonucleotides allows subtle gene modification in mismatch-repair-proficient cells

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Several laboratories, including ours, have previously shown that synthetic single-stranded DNA oligonucleotides (ssODNs) can be used for subtle gene modification in eukaryotic and prokaryotic cells without the requirement for prior generation of DNA double stranded breaks. Substantial evidence has been obtained that gene modification takes place during DNA replication. In this model, the ssODN anneals to single-stranded DNA in the replication fork, where it can serve as a primer for DNA synthesis by replicative polymerases. This process thus physically incorporates the ssODN and delivers the mutation to only one of the DNA strands. However, DNA mismatch repair (MMR) suppresses the efficiency of gene modification by more than 100-fold (Ref 1,2).

Here we present a novel ssODN design that allows complete evasion of MMR irrespective of the type of substitution (Ref 3). We demonstrate that inclusion of locked nucleic acids (LNA) in the ssODN at mismatching bases only or also at directly adjacent bases allows effective oligo targeting in MMR-proficient cells. Also in MMR-proficient Escherichia coli LNA modification of the ssODN enabled effective single base-pair substitution. To explain why LMOs evade MMR, we tested if LNA modification of mismatches interferes with mismatch recognition. To this aim, we measured equilibrium binding of purified bacterial MutS to LNA-modified mismatches by fluorescence polarization. We observed that LNA modification of mismatches precluded binding of purified MutS protein. Because of its precision, simplicity and cost effectiveness, these findings make ssODN-directed gene modification particularly well suited for applications that require the evaluation of a large number of sequence variants. In particular when they can be evaluated by scoring an easily detectable phenotype. Along this line, we have used oligo targeting to identify pathogenic variants of the key MMR gene MSH2 (Ref 4).

(3) van Ravesteyn T.W, et al. (2016) LNA modification of single-stranded DNA oligonucleotides allows subtle gene modification in mismatch-repair-proficient cells. Proc Natl Acad Sci USA (accepted for publication)
Replication stress and Mismatch repair - B1

Presenter: Wietske Pieters

Development of prevention strategies for intestinal cancer in Lynch syndrome using novel mouse models

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Lynch syndrome (LS) is an autosomal dominant hereditary cancer predisposition syndrome which is mostly caused by defects in DNA mismatch repair (MMR) genes Mlh1 and Msh2. LS patients have an increased lifetime risk of developing a range of neoplasias including a non-polyposis form of colorectal cancer (HNPCC) which typically manifests at a young age.

The mouse models that have been used in the past to study LS do not correspond well to the human pathology. Therefore, our research group has recently developed a novel inducible mouse model that closely mimics the clinical situation in MSH2 deficient LS patients (Wojciechowicz et al., 2014). After administration of tamoxifen, Lgr5-CreERT2;Msh2flox/− (Msh2-Lynch) mice have been shown to carry a number of MSH2 deficient intestinal crypts that remained present over time. Since the cells of these crypts lack functional MMR, they have a high mutational burden and may -in case mutations are oncogenic- develop into intestinal tumors. Interestingly, exposure of Msh2-Lynch mice to the methylating agent temozolomide led to expansion of the MSH2 deficient cell compartment and accelerated tumor development.

Using this mouse model, this study will focus on distinct factors that may influence the pathogenesis of CRC in Msh2 defective LS patients. Since prevention is better than cure, the former will be the emphasis of the project. Our first two approaches will therefore focus on the questions whether preventive measures as defined in reducing dietary intake of methylating substances or development of a prophylactic (chemo) therapy in order to reduce the size of the MSH2 deficient cell compartment will contribute to a reduction of tumor incidence in LS patients. Another facet of this study will explore whether MSH2 deficient intestinal tumors are eligible candidates for immunotherapy, which may be used both as prophylactic therapy and as a treatment strategy. The results of this study may ultimately contribute to a reduction of tumor incidence among LS patients and improve treatment options.
B cell receptor deep sequencing shows altered somatic hypermutation patterns in patients with defects in base excision repair and mismatch repair.

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Mismatch repair (MMR) and base excision repair (BER) are involved in repairing damaged or inappropriate bases. Defects in MMR and BER are linked to different types of cancer, but also to immunodeficiencies. The latter is explained by the role of MMR and BER in the somatic hypermutation (SHM) process taking place in B-cells. The SHM process is initiated after a B-cell recognizes a pathogen via its B-cell receptor (BR). The B-cell starts proliferating and SHMs are introduced in the variable parts of the BR encoding genes creating increased BR variation. Selection of B-cells with the highest affinity results in increased antigen specificity. SHM is initiated by Activation-Induced Cytidine Deaminase (AID), which converts a cytosine (C) into a uracil creating a uracyl:guanine (U:G) mismatch. This results in a 1,000,000 fold increased frequency of somatic mutations in the BR encoding genes making this an ideal location to investigate how U:G mismatches are repaired. U:G mismatches can be resolved using BER, MMR or replication. In BER, the mismatched base is excised and replaced. In MMR multiple bases surrounding the mismatch are removed and replaced. Here, errors by polymerases such as polη occur mainly at A:T pairs, especially WA/TW motives. Finally, during replication the U can be recognized as a thymidine (T), creating a C to T transition.

We hypothesize that SHM patterns will be altered in patients with defects in MMR or BER. Therefore, we used 454 sequencing to study SHM patterns in BR transcripts (IgG and IgA) of 17 healthy controls (HC) (ages 1-47), a 3 year old MSH2-deficient patient (MMR) and a 22 year old patient UNG-deficient patient (BER). We obtained 214-5,884 unique BR sequences per sample allowing us to study 2,353-85,221 mutations.

In the MSH2 patient we found a normal percentage of SHM (3.5%) compared to five aged matched HCs (3.3-6.1%). In line with reduced repair via MMR we found reduced mutations at A:T sites (18.8% versus 41-43%) and WA/TW motives (WA: 4.1% vs 13.1-13.9%; TW 4.2% vs 8.6-9.2%). The percentage of transitions at G:C pairs was slightly increased (64.6% vs 50.5-57.7%). The UNG patient had a normal SHM frequency (7.2; 6 age matched controls 6.4-9.2) and increased numbers of transitions at G:C pairs (90.7% vs 52.1-55.1%) in line with reduced BER efficiency. Mutations at A:T pairs (28.7% vs 40.0-45.1%) and WA/TW motives were reduced (WA: 11.2% vs 13.3-15.7%; TW: 6.3 vs 8.2-10.5).

Together our data shows that analysis of the BR repertoire can identify altered patterns of SHM as found in patients with defects in MMR and BER. Currently we are testing additional patients with defined defects to confirm these findings. Next, we will use BR sequencing to screen immunodeficiency patients for potential defects in MMR and BER. In combination with whole exome sequencing this might also lead to the identification of novel genes that play a role in SHM.
Replication stress and Mismatch repair - B1

Presenter: Volodymyr Halytskiy

One of the earliest stages in cancer development: tumour-related shifts in miRNAome can seriously affect all DNA repair mechanisms

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Background: Genome instability, high mutation rate as well as high variability is typical of many cancer cells. Also, tumor growth is tightly associated with regular shifts in miRNA expression pattern. Usually, expression of miRNAs miR-18a/b, miR-19, miR-21, miR-23a/b, miR-29a, miR-155, miR-181, miR-206, miR-210, miR-221/222 and miR-375 is up-regulated in cancer cells. This investigation aims to identify in what way these shifts in miRNAome contribute to the abnormalities in DNA repair.

Methods: miRNA targets within gene transcripts were predicted in silico using TargetScan software.

Results: Transcripts of around 60% of the genes involved in DNA repair carry targets for at least one of the up-regulated miRNAs:
- miR-18a/b - UNG, TDP1, MLH3, DDB1, GTF2H5 (TTDA), ERCC1, RBBP8, FANCG (XRCC9), FANCI (KIAA1794), DCLRE1C (Artemis), ATM;
- miR-19 - SMUG1, MBD4, APLF, TDP1, RAD23B, GTF2H1, GTF2H5 (TTDA), ERCC4 (XPF), RAD51D, RBBP8, FANCC, BRCA2, FAAP20;
- miR-21 - TDP2, MSH2, RPA2, GTF2H5 (TTDA), ERCC4 (XPF), RAD51B/D, GEN1, FANCI (KIAA1794), BRIP1 (FANCJ), FANCVM, ATM;
- miR-23a/b - NEIL1, MLH3, RAD23B, DDB1, GTF2H5 (TTDA), CCNH, ERCC4 (XPF), UVSSA (KIAA1530), MRE11A, GEN1, FANCA/D2, FANCG (XRCC9), FANCI (KIAA1794), DCLRE1C (Artemis);
- miR-181 - MBD4, APLF, RAD23B, GTF2H1/2, GTF2H5 (TTDA), UVSAA (KIAA1530), MRE11A, NBN (NBS1), GEN1, FANCD2 (FANCJ), FANCVM, BTBD12 (SLX4), FAAP20, DCLRE1C (Artemis), ATM;
- miR-155 - APLF, GTF2H5 (TTDA), ERCC6 (CSB), MRE11A, GEN1, FANCD2/F, RAD51C (FANCO), PRKDC;
- miR-206 - TDG, TDP1/2, MLH3, RPA1, ERCC4 (XPF), RAD50/51/51B/54B, NBN (NBS1), GEN1, PRKDC;
- miR-221/222 - GTF2H5 (TTDA), ERCC4 (XPF), RAD50/51, MRE11A, FANCD2, ATM;
- miR-375 - OGG1, APEX1 (APE1), MSH3, MLH3, RAD51B, XRCC2, MRE11A, EME1.

Conclusions: miRNAs, hyperexpression of which is essential for abnormal proliferation and surviving of cancer cells, silence also genes encoding DNA repair enzymes as well as other DNA damage response proteins that are key elements of all DNA repair systems – BER, direct reversal of damages, repair of DNA-topoisomerase crosslinks, MER, NER, HR and NHEJ. This results in an increase of genetic instability and can facilitate the damage of anti-oncogenes and tumour suppressor genes (incl. genes of anti-onco-miRNAs). Moreover, we speculate, that some of these genes can undergo the target damage (endogenous gene knockout) as a result of mutations in single-stranded R-loop that arises during transcription or RNA-dependent DNA methylation. Afterwards, miRNA-mediated silencing of DNA repair genes may cause higher risk of oncogene mutations and, therefore, underlie the cancer progression.
Replication stress and Mismatch repair - B1

Presenter: Stephen Kearsey

DNA polymerase proofreading-domain mutations associated with cancer

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Germline mutations in the exonuclease domain of DNA polymerases δ and ε have recently been shown to be associated with some cases of familial colorectal cancer (Palles et al., 2012). In addition, somatic Pol ε mutations have been found in about 7% of sporadic colorectal and endometrial cancers (Church et al., 2013). Thus a reduction in replicative fidelity can be a predisposing factor in cancer and may also be an early event in tumour development where there is no inherited mutation. To assess the pathogenicity of human polymerase variants, we have constructed equivalent mutations in S. pombe. Strains expressing germline variants, such as pol3C462N (equivalent to human POLD1 S478N) and pol2L425V (human POLE L424V), have mutation rates similar to proofreading-defective strains (pol3D386A and pol2D276,AE278A). Thus simple loss or reduction in 3’-5’ exonuclease activity may account for the pathogenicity of these mutations. In contrast, some somatic mutations affecting POLE show an increase in mutation rate in S. pombe that is an order of magnitude higher than that seen with proofreading-defective Pol2, indicating that a defect other than exonuclease activity per se must be responsible for the phenotype. In contrast to proofreading-defective strains, some POLE variants show sensitivity to DNA damaging drugs, synthetic lethality with checkpoint mutations, an increase in spontaneous DNA damage and defects in copying ‘hard-to-replicate’ genomic regions.

Replication stress and Mismatch repair - B1

Presenter: Hans Hombauer & Cemile Ümran Ceylan

A genome-wide screen to identify mutator interactions with low-fidelity DNA polymerase mutants

Cemile Ümran Ceylan(1), Tobias Schmidt(1), Ximena Reyes(1), Kerstin Gries(1), Matthias Meurer(2), Michael Knop(2), Hans Hombauer(1)

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Replication of the eukaryotic genome requires the function of DNA polymerases Polα (Pol1), Polε (Pol2) and Polδ (Pol3). The fidelity during this process is ensured by: a) the proofreading activity of Polε and Polδ, b) the DNA polymerase nucleotide selectivity (strongly influenced by the balance and availability of the different dNTPs), and c) the DNA mismatch repair (MMR) pathway, a post-replicative mechanism that corrects errors introduced by DNA polymerases.

We have performed a genome-wide screen in S. cerevisiae to identify unrecognized genes that might affect DNA replication fidelity. For this purpose, we crossed the yeast non-essential deletion collection (~4800 strains) against three low-fidelity polymerase mutants (pol1-L868M, pol2-M644G and pol3-L612M) and a wild-type strain. Single and double mutants were tested for an increased mutator phenotype with in vivo mutation reporters (CAN1 inactivation and lys2-10A frameshift reversion assay). Analysis of the single gene deletion mutants identified about 45 genes that function in different DNA repair pathways. Interestingly, analysis of the double mutants revealed a small number of gene deletions (including exo1Δ) that when combined with pol1-L868M or pol3-L612M, but not with pol2-M644G, resulted in a strong synergistic mutator interaction. The unpredicted bias of these interactions might be in agreement with a model where Polδ is responsible for the bulk of the DNA synthesis (leading and the lagging strand); however, could be also explained by a model in which leading and lagging strand are synthesized by Polε and Polδ respectively, however, with major differences in terms of checkpoint activation and repair efficiency.

Further characterization revealed that the identified mutants not only interact with low-fidelity polymerases but also with proofreading defective polymerase mutants, or strains with reduced MMR function.

In summary, we have identified a small set of genes whose function become increasingly important when DNA polymerase fidelity, proofreading activity or MMR activity is compromised. We are currently investigating the mechanism how these mutations interfere with faithful DNA replication.
Replication stress and Mismatch repair - B1

Presenter: Daniele Novarina

Identifying genes that suppress the accumulation of mutations in ageing yeast cells

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Genome instability is a shared hallmark and a possible driving force of cancer and ageing. While it was shown that aged yeast cells exhibit higher genomic instability, it is still unknown if specific mechanisms exist to protect the (increasingly unstable) genome of old cells. We exploited the Saccharomyces cerevisiae Replicative Life Span (RLS) model to screen for genome integrity maintenance genes in young and old cells, and to explore the interplay between genomic instability and ageing.

An S. cerevisiae cell divides asymmetrically, yielding an (increasingly) ageing mother cell and a fully rejuvenated daughter cell. The Mother Enrichment Program (MEP) is an estradiol-inducible genetic system which allows daughter cell-specific inactivation of two essential genes, thus enabling the analysis of a cohort of ageing mother cells. When the MEP is active, daughter cells irreversibly arrest at the G2/M transition, while the mother cells keep dividing, resulting in a linear growth rate and the formation of microcolonies on agar medium. Occasionally, some cells become capable of evading the MEP due to acquired mutations: even in the presence of estradiol, these cells, called escapers, grow exponentially and form normal colonies on plates.

We performed a genome-wide screen for genome maintenance genes where estradiol-insensitive escaper colony formation serves as a readout for the occurrence of spontaneous mutations. The MEP system was introduced in the yeast deletion collection using Synthetic Genetic Array technology. High-density arrays of MEP colonies were then grown for one week on agar plates in the presence of estradiol to allow for accumulation of spontaneous mutations in mother cells throughout their lifespan. Subsequently, colonies were further replicated on estradiol plates to detect the occurrence of escapers. High-throughput analysis of escaper formation frequency allowed identification of deletion mutants with elevated spontaneous mutation rate, uncovering new genes involved in the maintenance of genome integrity. Direct validation by fluctuation test allows us to discriminate between age-independent mutation suppression genes, responsible for genome maintenance throughout lifespan, and age-dependent mutation suppression genes, which are important to protect genome integrity only in old cells.
**Replication stress and Mismatch repair - B1**

Presenter: Mariliis Tark-Dame

**Probing the effect of local chromatin context on site-specific mutagenesis**

Mariliis Tark-Dame(1), Damar Tri Anggoro(1), Franck Lhuissier(2), Michiel de Both(2), Maike Stam(1)

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Introducing mutations in a targeted, site-specific manner in vivo is an attractive way to generate cells or whole organisms with desirable changes in their genomic sequence. ODM (Oligonucleotide-directed mutagenesis) is a process in which a mutation of interest (base substitution, addition or deletion) is induced at a specific genomic region by an oligonucleotide that is introduced into the cells.

ODM has been studied for more than 20 years in mammalian cell systems as well as in plants. The method has been successfully used in plant breeding, for instance to create crops insensitive to specific herbicides. Although ODM is highly attractive as a tool for inducing site-specific mutations in a genome, it also has its downsides. The main problem is that the frequency at which ODM occurs is rather low, making the method time consuming and costly for non-selectable targets.

Several factors affect the frequency in which ODM occurs. Local chromatin structure is amongst those, and until recently got undeservedly little attention. In our studies the local effect of chromatin context, DNA and histone modifications, on the frequency of ODM is addressed. As a model we use a set of Arabidopsis lines expressing a reporter that allows a simple ODM-based fluorescence restoration assay. We introduce changes into the chromatin structure by (1) using drugs affecting epigenetic marks and (2) testing a variety of Arabidopsis lines deficient in chromatin genes. Our aim is to obtain systematic information on the interplay between local chromatin context and ODM efficiency that allows to predict and improve ODM efficiency for a desired genomic site.

Our first results indicate that ODM can be significantly improved by changing epigenetic marks. Whether this improvement in ODM is dependent on the local sequence and chromatin context remains to be answered.
Set2 is required for MBF activation and to suppress synthetic lethality with Wee1

Chen-Chun Pai(1), Rachel Deegan(1), Elizabeth Blaikley(1), Rob de Bruin(2), Antony Carr(3), Tim Humphrey(1)

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SETD2 is a tumour suppressor, which is frequently mutated in a range of different cancer types. SETD2-dependent trimethylation of histone H3K36 performs a number of cellular functions and has been recently shown to play a key role in maintaining genome stability through facilitating checkpoint activation and DNA double-strand break (DSB) repair. We have previously showed that Set2 is required for non-homologous end joining in DSB repair. Here, we describe a novel function for Set2 in regulating efficient DNA replication. Our microarray data suggested set2Δ cells have low levels of transcription of a cluster of DNA replication genes following DNA damage, suggesting Set2 function in replication through facilitating MBF-induced gene expression. We also found that set2Δ mutant shows synthetic growth defect with a temperature sensitive wee1 mutant. Our results identify a role for Set2 in RNR expression, promoting replication fork restart and cell viability in the absence of Wee1. Consistent with this, elevated dNTP pools via Spd1 depletion suppresses set2Δ wee1.50 synthetic lethality. Our results together support a model where Wee1 inhibition results in nucleotide pool depletion in set2Δ cells, leading to permanent replication fork arrest, resulting in replication fork collapse. These findings are being exploited to target SETD2-deficient cancer cells.
POSTER SESSION B2:
'Replication Stress and Translesion Synthesis'

Poster viewing: Monday, April 18, 20:00 – 22:00 h
Discussion: Tuesday, April 19, 16:55 – 18:25 h
Identification of CtIP as a negative regulator of ATR signalling

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The accurate regulation of the ATR (Ataxia telangiectasia and RAD3-related) kinase activation plays an important role in preventing DNA damage during DNA replication. ATR activation results in stabilization of stalled forks, regulation of DNA repair and fork restart. To better understand how activation of ATR is regulated we make use of the power of the Xenopus egg extract model system to study this. Defined DNA model structures are used to specifically activate the ATR pathway in Xenopus extracts. By isolating these structures from extracts and identifying proteins binding to those by mass spectrometry, we previously identified the MRN (MRE11-RAD50-NBS1) complex and showed a new direct role for this complex in the activation of ATR. Since our model structures resemble resected DNA, this function of MRN is independent of its role in double-strand break (DSB) resectioning. Interestingly, we identified CtIP, which acts with MRN in DSB resectioning, as a strong interactor of our ATR activating structures as well. Surprisingly, we were able to show that CtIP represses ATR signalling. We present data showing that immunodepletion of CtIP in extracts results in hyperactivation of ATR in the presence of ATR activating structures. This can be rescued by adding back the recombinant version of this protein. Similar results are obtained in human cells upon depletion of this protein, showing that this function is conserved in human cells. This is a new function of CtIP and to our knowledge no other proteins have been identified that can repress ATR signalling. Current progress on this project will be presented.
Replication stress and Translesion synthesis - B2

Presenter: Anna Herlihy

Sustained E2F-dependent transcription is required for tolerance to DNA replication stress to prevent DNA damage

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DNA replication stress can cause DNA damage and subsequent genome instability. To prevent this cells have evolved a cellular response called the DNA replication stress checkpoint response. As oncogene-induced replication stress is an early event in tumorigenesis, understanding this checkpoint may give insight into how to target this response to specifically generate replication stress-induced DNA damage in cancer cells. This could be used in combination with strategies exploiting the frequently compromised DNA repair and damage response in cancers for anti-cancer treatments.

Our lab has previously shown that the DNA replication stress checkpoint response maintains E2F-dependent cell cycle transcription through Chk1 phosphorylating and inhibiting the transcriptional repressor E2F6. However, the role and importance of transcription in the cellular response to replication stress remains largely unknown. Here we show that in mammalian cells, unlike yeast, active protein synthesis is required for an efficient checkpoint response. We find that many proteins involved in the checkpoint response are E2F targets and are unstable, so require sustained E2F-dependent transcription for correct protein dynamics during replication stress. Our work shows that many essential functions of the checkpoint response, including fork stalling, stabilisation and resolution, are compromised when sustained E2F-dependent transcription is impaired. Preventing this response increases replication stress-induced DNA damage, which can ultimately lead to genome instability. Importantly, maintaining E2F-dependent transcription during DNA replication stress in cells compromised for the checkpoint response is sufficient to prevent replication stress-induced DNA damage.

Based on these results we propose that sustained E2F-dependent transcription is a key mechanism in the cellular response to prevent DNA replication stress-induced DNA damage. Interestingly an increase in E2F-dependent transcription caused by cancer associated mutations drives proliferation, resulting in unscheduled S-phase entry, which is thought to be at the basis of oncogene-induced replication stress. We find that E2F-dependent transcription is also required during oncogene-induced replication stress to prevent replication stress-induced DNA damage and cell death. In the context of oncogene-induced replication stress the increased reliance on sustained E2F-dependent transcription creates a potentially large therapeutic window for damaging cancer cells without affecting normal cells.

This work highlights a far greater role than previously suspected for transcriptional regulation, and in particular E2F-dependent transcription, in determining the outcome of DNA replication stress. It shows that it is necessary to consider the DNA replication stress transcriptional network to fully understand and alter tumour evolution in the treatment of cancer, providing a potential new strategy for cancer treatment.
Replication stress and Translesion synthesis - B2

Presenter: Bente Benedict

DNA damage in mitogen-deprived Rb-deficient cells is suppressed by inactivation of p53

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Loss of G1/S control, an important barrier to proliferation under several growth inhibitory conditions, is a critical step in cancer development and is often caused by inactivation of the retinoblastoma pathway. The consequences of a defective G1/S checkpoint have been studied in an in vitro model system consisting of mouse embryonic fibroblasts (MEFs) devoid of the retinoblastoma genes RB1, p107 and p130 (TKO-MEFs). These TKO-MEFs were still mitogen dependent: without mitogens many TKO MEFs died, whereas surviving cells arrested in the G2 phase of the cell cycle. Suppressing apoptosis (by Bcl2 overexpression) in these cells revealed that this G2 arrest resulted from the induction of cyclin-dependent-kinase inhibitors p27kip1 and p21cip1, the latter pointing to activation of the DNA damage response. Indeed, G2-arrested cells accumulated DNA double-stranded breaks (DSBs) caused by severe replication stress. Mitogen deprived TKO-Bcl2 MEFs did enter S phase but DNA replication speed was low and origin firing decreased.

Remarkably, we showed that knockout of p53 restored origin firing and thereby suppressed DSB formation in mitogen deprived TKO-Bcl2 MEFs. Therefore, inactivation of p53 allowed these cells to proliferate mitogen independently despite sustained replication stress: these cells still suffered from low replication speed.

This can provide a new rationale for the frequent co-occurrence of p53 and pRB pathway inactivation in cancer: loss of the p53 pathway not only abrogates cell cycle arrest and apoptosis; but also suppresses the induction of DNA breakage despite sustained replication stress.
Replication stress and Translesion synthesis - B2

Presenter: Betheney Pennycook

Inappropriate E2F activity during S phase deregulates replication dynamics.

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The control of DNA replication is essential to ensure accurate duplication of the genome during the cell cycle. Deregulation of this control can cause replication stress. DNA replication stress is defined as the slowing or stalling of replication fork progression and/or DNA synthesis. This exposes single stranded DNA, and makes the fork susceptible to DNA damage. Oncogene-induced replication stress accounts for early DNA damage during tumorigenesis, and is thought to be an important driver in cancer initiation. However, the mechanisms underlying this process are poorly understood.

Oncogenes such as c-myc and Cyclin E induce E2F-dependent transcription during the G1 phase of the cell cycle to drive proliferation. This causes premature entry into S phase, which is thought to be at the basis of oncogene-induced replication stress. However, it is unknown if an increase in E2F activity during the S phase of the cell cycle affects DNA replication control.

Our work suggests that inappropriate E2F activity in S phase causes replication stress and activates the DNA replication checkpoint. This is preceded by an increase in replication fork speed, which is likely caused by the hyper-accumulation of proteins involved in replication control and chromatin dynamics in S phase. Our work suggests that deregulation of E2F-dependent transcription during S phase has the potential to induce replication stress and might therefore have an important role in oncogene-induced replication stress. We suggest a model where the increase in replication fork speed causes forks to run into trouble representing a new mechanism of replication stress.
Translesion Synthesis (TLS) polymerases role in rNMPs incorporation during DNA replication

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Ribonuclease H (RNase H) are evolutionary conserved enzymes that cleave the RNA moiety in RNA:DNA hybrid molecules. It has been found that replicative DNA polymerases can incorporate rNTPs in place of dNTPs during DNA replication with an unexpected high frequency [1,2]. The high rate of rNTPs mis-incorporation observed under normal conditions suggests physiological functions for rNMPs in newly replicated DNA. It was recently demonstrated that the incorporation of rNMPs during leading strand DNA synthesis acts as a strand discrimination signal for the Mismatch DNA repair machinery [3,4]; moreover, rNMPs embedded in chromosomal DNA can represent an imprint, positioned in S-phase, that regulates DNA transactions [5]. RNase H enzymes are crucial for the removal of these rNMPs from genomic DNA and for the maintenance of chromosome integrity. Recently, we found that impairment of RNase H activity in yeast and human cells causes rNMPs accumulation in the genome and chronic activation of the post-replication repair (PRR) system, which becomes essential for cell survival [6].

Therefore, we focus on the contribution of TLS Polymerases. In particular, Pol and Pol activity is involved into genomic-rNMPs tolerance.

We observed that in the absence of RNaseH activity, TLS polymerases not only have an important role in bypassing unrepaired-rNMPs in the genome, but they can incorporate rNMPs during DNA replication. Through the analysis of yeast mutants and XP-V human fibroblasts, we suggest that the evolutionary conserved TLS polymerase Pol eta shows elevated predisposition to incorporate rNMPs when the dNTPs pool is limited.

(2) S.A.N. McElhinny, D. Kumar, et al., Genome instability due to ribonucleotide incorporation into DNA, Nat Chem Biol. 6 (2010) 774–781. doi:10.1038/nchembio.424.
Replication stress and Translesion synthesis - B2

Presenter: Christoffel Dinant

Replication stress-related DDR foci behavior during S phase progression

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Many proteins involved in the DNA Damage Response (DDR) are found in small foci inside a eukaryote nucleus. When these foci appear in the absence of exogenous damage sources they are usually restricted to a certain cell-cycle phase (e.g. 53BP1 bodies in G1 and Rad51 foci in S/G2). The consensus is that such spontaneous foci are caused by endogenous sources of DNA damage and research is mainly focused on the relation between foci and DNA replication stress. We observed that FANCD2 and BRCA1, both proteins that form spontaneous foci in S phase, do not co-localize well with each other nor do they always form at sites of active replication. This indicates a difference in substrate for these two proteins and suggests that not all spontaneous foci in S phase might be related to replication stress directly. Spontaneous FANCD2 foci appear in early S and are genetically stable throughout S phase. In U2OS cells, the DNA covered by FANCD2 foci is replicated at a very late stage at the S/G2 border. We performed ChIP-seq on FANCD2-GFP expressing cells lacking endogenous FANCD2 to analyze the location of spontaneous FANCD2 foci in detail.

Upon replication stress conditions caused by knockdown of RPA expression, the lack of co-localization between BRCA1 and FANCD2 foci increases. Detailed multidimensional analysis of DDR foci during S phase progression show that the bulk of FANCD2 foci appear in late S phase whereas BRCA1 mainly forms foci during mid-S. Rad51foci on the other hand display different dynamics of appearance and disappearance depending on the precise S phase timing. The implications of these differences on chromatin signaling versus DNA repair and on different repair mechanisms are discussed.
Replication stress and Translesion synthesis - B2

Presenter: Lucian Moldovan

HUWE1 is a novel PCNA-interacting protein that ubiquitinates H2AX during replication stress to promote repair of broken forks

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Unrepaired DNA lesions can arrest the progression of the DNA replication machinery during S-phase, causing replication stress, mutations, and DNA breaks. HUWE1 is a HECT-type ubiquitin ligase that targets proteins involved in cell fate, survival, and differentiation. Here, we report that HUWE1 is essential for genomic stability, by promoting replication of damaged DNA. We show that HUWE1-knockout cells (obtained using CRISPR/Cas9 technology) are unable to mitigate replication stress, resulting in replication defects and DNA breakage. Moreover, we show that this novel role of HUWE1 requires its interaction with the replication factor PCNA, a master regulator of replication fork restart, at stalled replication forks. Finally, we show that HUWE1 mono-ubiquitinates the histone H2AX in vitro and in vivo, and promotes H2AX-dependent signaling at stalled replication forks, resulting in recruitment of repair proteins. Our work identifies HUWE1 as a novel regulator of the replication stress response, and describes a new mechanism for signaling and recruitment of repair proteins to stalled replication forks dependent on the replication factor PCNA.
Replication stress and Translesion synthesis - B2

Presenter: Guillaume Guilbaud

Nucleotide pool depletion induces G-Quadruplex-dependent perturbation of gene expression

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Numerous lines of evidence have linked replication stress with genetic instability (1). However, its ability to induce epigenetic changes is poorly understood. Imbalanced or depleted nucleotide pools during replication are an important cause of such stress and can arise from the expression of oncogenes uncoupling entry into S phase from up regulation of nucleotide supply (2). Replication stress induced DNA damage is not completely randomly distributed across the genome, but is instead focussed on sites that often have features that make them potentially problematic to replicate even under ideal conditions (3). In this study, we examine the effect of hydroxyurea (HU)-induced nucleotide pool depletion on the epigenetic stability of a sensitive reporter locus, BU-1, in chicken DT40 cells (4-5-6). We show that chronic treatment with low-dose HU induces stochastic instability of BU-1 expression, characterized by loss of the chromatin marks H3K4me3 and H3K9/14ac seen in the normally active locus. This instability depends significantly on the presence of a G4 motif located at 3.5kb from BU-1 promoter and can be synergy with G4-ligand treatment. This G4 is oriented to stall the leading strand of a fork heading toward the transcription start site (TSS). The presence of this G4 motif not only increases the rate at which BU-1 expression is lost, but is additionally associated with phosphorylation of H2Ax and appearance of the heterochromatic mark H3K9me3. This is consistent with the G4 acting to focus DNA damage induced by the global replication stress imposed by HU, with the damage leading to repression of the locus. Further, we show that, across the genome, chronic exposure to HU results in an altered pattern of gene expression similar to that seen in cells lacking the G4-unwinding helicases FANCJ, WRN, and BLM. Together, these observations indicate that the effects of global replication stress induced by nucleotide pool depletion can be focussed by local replication impediments caused by G quadruplex formation to induce localised epigenetic instability, a mechanism that may contribute to selectable transcriptional changes in cancer.

Replication stress and Translesion synthesis - B2

Presenter: Abdelghani Mazouzi

A Comprehensive Analysis of the Dynamic Response to Replication Stress Uncovers Targets for ATM and ATMIN

Abdelghani Mazouzi(1), Alexey Stukalov(2), André C. Müller(1), Doris Chen(1), Marc Wiedner(1), Jana Prochazkova(1), Shih-Chieh Chiang(3), Michael Schuster(1), Florian P. Breitwieser(4), Andreas Pichlmair(2), Sherif F. El-Khamisy(1), Christoph Bock(1), Robert Kralovics(1), Jacques Colinge(5), Keiryn L. Bennet(1), Joanna I Loizou(1)

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The cellular response to replication stress requires the DNA-damage responsive kinase ATM and its co-factor ATMIN, however the roles of this signaling pathway following replication stress are unclear. To identify the functions of ATM and ATMIN in response to replication stress we utilized both transcriptomics and quantitative mass spectrometry-based phosphoproteomics. We found that replication stress induced by aphidicolin triggered widespread changes in both gene expression and protein phosphorylation patterns. These changes gave rise to distinct early and late replication stress responses. Further, our analysis revealed previously unknown targets of ATM and ATMIN downstream of replication stress. We demonstrate ATMIN-dependent phosphorylation of H2AX and of CRMP2, a protein previously implicated in Alzheimer’s disease but not in the DNA damage response. Overall, our dataset provides a comprehensive resource for discovering the cellular responses to replication stress and, potentially, associated pathologies.
Replication stress and Translesion synthesis - B2

Presenter: José Yélamos

PARP-2 sustains erythropoiesis in mice by limiting replicative stress in erythroid progenitors

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Erythropoiesis is a tightly regulated process in which multipotential hematopoietic stem cells produce mature red blood cells. Here, we show that deletion of poly(ADP-ribose) polymerase-2 (PARP-2) in mice leads to chronic anemia at steady-state, despite increased EPO plasma levels, a phenomenon not observed in mice lacking PARP-1. Loss of PARP-2 causes shortened erythrocytes lifespan and impaired differentiation of erythroid progenitors. In erythroblasts, PARP-2-deficiency triggers replicative stress, as indicated by the presence of micronuclei, the accumulation of γ-H2AX in S-phase cells and constitutive CHK1 and RPA phosphorylation. Transcriptome analyses revealed the activation of the p53-dependent DNA damage response pathways in PARP-2-deficient cells, culminating in the up-regulation of cell cycle and cell death regulators, concomitant with G2/M arrest and apoptosis. Strikingly, while loss of the pro-apoptotic p53 target gene puma restored hematocrit levels in the PARP-2-deficient mice, loss of the cell cycle regulator and CDK-inhibitor p21 leads to perinatal death by exacerbating impaired fetal liver erythropoiesis in PARP-2-deficient embryos. Although the anemia displayed by PARP-2-deficient mice is compatible with life, mice die rapidly when exposed to stress-induced enhanced hemolysis. Our results pinpoint an essential role for PARP-2 in erythropoiesis by limiting replicative stress that becomes essential in the absence of p21 and in the context of enhanced hemolysis, highlighting the potential effect that might arise from the design and use of PARP inhibitors that specifically inactivate PARP proteins.
Replication stress and Translesion synthesis - B2

Presenter: Meindert Lamers

Cryo-EM structures of the E. coli replicative DNA polymerase in complex with clamp, exonuclease and DNA

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The E. coli replicative DNA polymerase is a highly efficient machine that synthesizes DNA with speeds up 1000 nt/s. During DNA replication, the polymerase needs to quickly respond to different signals in the DNA. On the lagging strand, the polymerase repositions to a newly primed site each time it reaches the end of the Okazaki fragment, every ~1000 nucleotides, 3,500 times per genome. The polymerase furthermore quickly removes misincorporated nucleotides by moving the DNA to the associated exonuclease. How the polymerase can respond to these different signals on the DNA is poorly understood. Therefore, we have determined the cryo-EM structures of the DNA polymerase in complex with sliding clamp and exonuclease in a DNA-free state, a DNA synthesis state, and a DNA editing state. The three structures reveal how the polymerase discriminates between the different signals and undergoes large conformational changes to switch in-between the different states. These structures therefore providing unique insights into the regulation of this highly efficient molecular machine.

FANC pathway and endonucleases in common fragile site stability.

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Fanconi anemia (FA) is a genetically heterogeneous disorder characterized by bone marrow failure and increased cancer predisposition. The proteins encoded by the FA genes participate in the FANC/BRCA pathway, involved in replication stress response and homologous recombination. The cells derived from FA patients exhibit chromosomal instability and hypersensitivity to chemicals that induce DNA interstrand crosslinks (ICLs). At least part of the chromosomal instability in FA cells can be ascribed to a role of this pathway during mitosis (Naim and Rosselli, 2009). FANCD2, a key component of the FANC pathway, localizes to discrete sites on mitotic chromosomes and promotes the resolution of abnormal chromatin structures that persist following replication stress. Notably, the FANCD2-targeted loci in mitosis coincide with common fragile sites (CFSs), genomic regions that become unstable under replication stress conditions and are frequently deleted or rearranged in tumors.

We have previously shown that the structure-specific endonucleases XPF-ERCC1 and MUS81-EME1 are recruited to the FANCD2-associated sites in mitosis and that these sites correspond to genomic regions completing their replication in late G2-M (Bergoglio et al., 2013; Naim et al., 2013). The mitotic processing of replication intermediates by ERCC1 and MUS81-EME1, which contributes to the cytogenetic expression of CFS on metaphase chromosomes, promotes sister chromatid separation, thus preventing chromosome segregation defects and mitotic catastrophe. Interestingly, depletion of XPF had no effect on chromosome segregation suggesting an independent role of ERCC1 in this process. XPF-ERCC1 and MUS81-EME1 participate in a supramolecular complex with the SLX4/FANCP nuclease scaffold. SLX4 localizes at CFS and is required for MUS81 and ERCC1 recruitment to these regions, promoting a targeted action of these endonucleases (Guervilly et al., 2015). We are currently investigating the functional relationships between the FANC pathway and the different endonuclease complexes in regulation of CFS and chromosomal stability.

Replication stress and Translesion synthesis - B2

Presenter: Andrea Pichler

A novel enzyme family assembling SUMO chains

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Protein modification with SUMO (sumoylation) regulates diverse cellular pathways including DNA damage response. In our recent work we demonstrated that ZNF451, a poorly characterized zinc finger protein, has SUMO E3 ligase activity and efficiently assembles SUMO2/3 chains. Such chains are best understood as stress induced degradation-tags or as signal to recruit repair-factors to DNA-lesions. Detailed biochemical analysis revealed that ZNF451 functions distinct to all known E3 ligases described for SUMO and ubiquitin conjugation: ZNF451 executes catalysis via a tandem-SIM and its interSIM region. One SIM orients the donor-SUMO, while a second SIM binds SUMO on the backside of the E2 enzyme to stabilize the catalytic intermediate. Intriguingly, this short tandem-SIM region is sufficient to extend a backside-anchored SUMO chain (E4 elongase activity, E4s are specialized E3s for chain elongation), in contrast to chain initiation, which in addition requires the zinc finger region to recruit the first acceptor SUMO (E3 ligase activity). We further showed that four human proteins share the catalytic unit and are involved in stress-induced global sumo(2,3)ylation after DNA damage and proteasome inhibition. Moreover, we identified the DNA replication helicase subunit MCM4 as first endogenous ZNF451 dependent SUMO substrate upon proteasome inhibition.

Oncogene-induced replication stress is a crucial driver of genomic instability and one of the key events contributing to the onset of cancer. Despite its proven role in cancer-initiation and development, the mechanisms underlying oncogene-induced replicative stress and the ability of cancer cells to tolerate these high levels of replication stress remain poorly understood. Insight into this will help to define the best therapeutic window in which to target pathways that enable cancer cells to cope with high levels of replication stress.

To prevent replication stress-induced DNA damage cells have evolved a cellular response named the DNA replication stress checkpoint. Previously work from our lab has shown that the DNA replication stress checkpoint maintains E2F-dependent G1/S cell cycle transcription. We now show that sustained E2F-dependent transcription is a key mechanism in the DNA replication stress checkpoint response. Our work shows that sustained E2F-dependent transcription is both required and sufficient for many essential functions of the checkpoint response, including fork stalling, stabilisation and resolution.

Importantly we find that also in the context of oncogene-induced replication stress, where increased E2F activity is thought to lie at the basis of causing replication stress, E2F-dependent transcription is required to limit DNA damage levels. The increased reliance on sustained E2F-dependent transcription creates a potentially large therapeutic window for damaging cancer cells without affecting normal cells. In line with this, our work in yeast shows that many G1/S target genes acquire essential functions after pathological deregulated G1/S transcription. These G1/S target genes, regulated by E2F in human cells, may represent therapeutic targets to selectively kill cancers with high levels of replication stress.

Oncogenes induce E2F-dependent transcription during the G1 phase of the cell cycle to drive proliferation. This causes premature entry into S phase, thought to be at the basis of oncogene-induced replication stress. Our data indicates that inappropriate E2F activity during S phase increases replication fork speed, activating the DNA replication checkpoint. These data suggest that deregulation of E2F-dependent transcription during different phases of the cell cycle has the potential to induce replication stress by different mechanisms. Linking distinct deregulation of E2F-dependent transcription to specific cancer-associated mutations will aid in the diagnosis and treatment of human cancers.

Our work highlights a far greater role than previously suspected for G1/S transcription in determining the outcome of DNA replication stress. Gaining a comprehensive understanding of the mechanisms by which E2F-dependent transcription contributes both to induction of oncogene-induce replication stress and tolerance to it will therefore provide potential new strategies for cancer treatment.
Replication stress and Translesion synthesis - B2

Presenter: Ramveer Choudhary

Maintaining Genomic Integrity at Replication Termination zones.

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Faithful transmission of genetic material is challenged by the presence of natural impediments affecting replication fork progression that jeopardize genome integrity. Transcription, which competes with DNA replication for the same template, is a common barrier to replication in both prokaryotes and higher eukaryotes. Natural impediments include rDNA, tRNA, CEN and RSZ (Replication slow zone), which represent fragile sites. Multiple mechanisms minimize the consequences of DNA replication and transcription collisions in order to prevent torsional stress accumulation that occur when replication fork encounters to transcription machinery. Defects in resolving topological problems during chromosome replication lead to reversed forks, R loop formation and recombination-induced genome rearrangements. Mec1/ATR (Ataxia telangiectasia and Rad3 related), a major sensor of the DNA damage response, is activated by the topological complexity at replication/transcription domain and determines fragile sites stability. TERs are naturally major pol II transcript pausing element and this cause polar pausing of one of the fork converging at TERs (Termination zones). Our interest is centered on the processes that coordinate replication with transcription at TERs and on the molecular pathways involved in termination of DNA replication. We focus on the interaction of Rrm3, a DNA helicase that facilitates replication fork progression across the non-histone proteins, and the Sen1 DNA/RNA helicase involved in Pol II-mediated termination. Sen1 ablation leads to R loop accumulation at the sites where transcription is disrupted and it has deleterious consequences for cell viability. Our group has shown that TOP2 and RRM3 play key roles in termination sites containing tRNA, rDNA, POL II binding sites and CENs.

Interestingly sen1 rrm3 mutants yeast cells arrest in metaphase of cell cycle with un-replicated TERs. The replication checkpoint and recombination-mediated repair pathways are essential for the Metaphase arrest rescue. Double mutant cells showed transient accumulation of R-loops during S-phase of cell cycle. Moreover, we provide evidence that condensins play a detrimental role when Rrm3 and Sen1 are ablated. Further detail insights at TERs will explore the concept of fragile site expression and genomic stability during physiological condition.
Replication stress and Translesion synthesis - B2

Presenter: Saskia Hoffmann

TRAIP is a PCNA-binding ubiquitin ligase that protects genome stability after replication stress.

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Cellular genomes are highly vulnerable to perturbations to chromosomal DNA replication. Proliferating cell nuclear antigen (PCNA), the processivity factor for DNA replication, plays a central role as a platform for recruitment of genome surveillance and DNA repair factors to replication forks, allowing cells to mitigate the threats to genome stability posed by replication stress. We identify the E3 ubiquitin ligase TRAIP as a new factor at active and stressed replication forks that directly interacts with PCNA via a conserved PCNA-interacting peptide (PIP) box motif. We show that TRAIP promotes ATR-dependent checkpoint signaling in human cells by facilitating the generation of RPA-bound single-stranded DNA regions upon replication stress in a manner that critically requires its E3 ligase activity and is potentiated by the PIP box. Consequently, loss of TRAIP function leads to enhanced chromosomal instability and decreased cell survival after replication stress. These findings establish TRAIP as a PCNA-binding ubiquitin ligase with an important role in protecting genome integrity after obstacles to DNA replication.
Replication stress activates the highly conserved MEC1/RAD53, ATR/hCHK1 kinase mediated signal transduction pathways, which protect and stabilize stalled replication forks, respectively, in budding yeast and human cells. Treatments of mec1 and rad53 mutants with hydroxyurea, a DNA synthesis inhibitor, lead to cell lethality and chromosome fragmentation. Under replication stress rad53 mutants undergo fork collapse and accumulate hemireplicated, gapped, and reversed forks. The molecular mechanisms, through which Rad53 prevents aberrant transitions at the DNA replication forks and chromosome fragility, are largely unknown. It has been recently shown that Rad53 phosphorylates nuclear pore to counteract gene gating, to release super helical torsion at S-phase transcribed chromatin and prevent aberrant transitions at forks approaching transcribed genes.

The Pif1 DNA helicases (Rrm3 and Pif1) remove bulky non-nucleosomal DNA-protein complexes and unwind problematic DNA structures ahead of the replisome, assisting fork progression across natural pausing sites. We reveal that Rrm3 and Pif1 are detrimental in rad53 mutants experiencing replication stress induced by hydroxyurea. Rrm3 and Pif1 ablations synergistically rescue cell lethality, chromosome fragmentation, replisome-fork dissociation, fork reversal, and ssDNA gaps formation at DNA replication fork branching points in checkpoint defective cells exposed to replication stress. Moreover, we found that Pif1 and Rrm3 associate with stalled DNA replication forks and are regulated through phosphorylation by Rad53. Indeed, phospho-mimicking rrm3 mutants ameliorate rad53 phenotypes following replication stress without affecting replication across pausing elements under normal conditions.

We propose a new layer of complexity in the cellular response to replication stress in which Rad53 controls the activity of the Pif1 DNA helicases at the stalled replication forks preventing aberrant DNA replication fork transitions and chromosome breakages.
Replication stress and Translesion synthesis - B2

Presenter: Stefano Giustino Manzo

Topoisomerase I depletion affects genome-wide distribution of R loops in human cells

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R loops are three-stranded structures composed of a DNA/RNA hybrid and a displaced DNA strand. These structures play an essential role in genome instability as well as physiological processes such as replication, transcriptional termination and IgG class-switch recombination. Several factors can modulate R loop stability such as RNAseH, DNA/RNA helicases and DNA topoisomerases. DNA topoisomerase I (Top1) may prevent R loop formation by removing negative supercoils, which can thermodynamically favor R loop stability. However, it remains to be shown directly whether Top1 activity may modulate R loop structures at specific genomic loci in human cells. Here, we performed DRIPc (high resolution DNA-RNA immunoprecipitation) analyses coupled to Next Generation Sequencing to obtain high-resolution strand-specific genomic maps of R loops following Top1 depletion in human HEK cells. The results showed that Top1 downregulation induces an overall increase of R loops that are mainly localized in the body of genes. Interestingly, long genes are specifically affected by Top1 depletion, with an increase in R loop that was proportional to gene length. RNA-seq revealed that these genes tend to be highly expressed. We found also a particular modulation of Top1 on ribosomal DNA, with an increase in R loops upon Top1 depletion, which specifically occurred at 5’ETS region. Surprisingly, a significant number of genomic loci showed a reduction of R loop peaks. These are mainly localized throughout the body of short genes localized at highly gene-rich regions, with the highest gene rich chromosomes showing the strongest R loop decrease. RNA-seq revealed a partial downregulation of transcripts at 3’ end for these loci. We performed also RNA Polymerase II ChIP-seq to investigate how R loop modulation by Top1 is associated with transcription activity, and we found an altered RNA polymerase II profile especially at transcription termination sites. Genes with loss and gain have different pausing regions at terminator sites suggesting different mechanism for the transcription termination process that could explain the 3’ decrease of transcript detected in genes showing R loop loss.

Our data directly show, for the first time, that Top1 can modulate R loop structures at specific loci according to the genomic context and provide new insights into Top1-dependent mechanisms of transcription regulation and genome stability.
Aneuploidy leads to increased replication stress and genomic instability

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Aneuploidy is a hallmark of cancer and underlies genetic disorders characterized by severe developmental defects, but the molecular mechanisms underlying the changes in cell physiology remain elusive. To study the effect of extra chromosomes on the cell physiology, we transferred individual chromosomes into recipient cells in order to create a series of human cell lines with defined aneuploid karyotypes. Compared to the isogenic diploid parental cell lines, all analyzed trisomic and tetrasomic cells showed increased DNA damage and elevated sensitivity to replication stress. Next-generation sequencing and SNP-array analysis revealed accumulation of chromosomal rearrangements in aneuploids, with break point junction patterns suggestive of replication defects. Strikingly, we found that aneuploid cells suffer from reduced expression of the replicative helicase MCM2-7. Overexpression of MCM7 restored wild-type levels of chromatin-bound MCM helicase in our model aneuploid cells and reduced the DNA damage. Thus, gain of chromosomes leads to changes in expression of essential replicative proteins and this in turn triggers replication stress and DNA damage. Our results provide a novel insight on how aneuploidy promotes genomic instability and thus possibly contributes to tumorigenesis.
Replication stress and Translesion synthesis - B2

Presenter: Sasa Svikovic

R-loops potentiate structured DNA-associated epigenetic instability

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The accurate propagation of histone marks during replication is proposed to be an important mechanism by which cells maintain gene expression states across cell division. In order to maintain histone marks in register with original sequence, histones must be rapidly deposited on newly synthesized DNA strands during replication. Defective replication of G-quadruplex DNA can cause perturbation of histone deposition ahead of replication fork, leading to stochastic loss of epigenetic memory, a phenomenon termed epigenetic instability (1).

Using a previously described assay for G-quadruplex induced epigenetic instability, the BU-1 locus in chicken DT40 cells (2,3), we show that trinucleotide repeats can also generate stochastic epigenetic instability during replication in a length and orientation-dependent manner. We show that, as for G-quadruplexes (4), loss of the recently discovered primase/polymerase (PrimPol) accelerates trinucleotide repeat-induced epigenetic instability for given repeat lengths, suggesting that PrimPol’s ability to reprim close to replication-blocking secondary structures limits single strand DNA exposure.

DNA secondary structures have been associated with the formation of stable transcription-associated RNA:DNA hybrids (R-loops) (5) but how the formation of one influences the other has been unclear. We show that DNA secondary structure formation in the BU-1 locus promotes R-loop formation. R-loop formation is potentiated by the absence of PrimPol both in the BU-1 locus and genome wide. However, overexpression of RNaseHI (which degrades RNA:DNA hybrids in vivo) significantly reduced extent of epigenetic instability suggesting that R-loops also stabilise the formation of DNA secondary structures.

Together, our data suggest that DNA secondary structures and R-loops potentiate each others formation and that both are enhanced by the formation of excessive single stranded DNA at a stalled replication fork, an event that can be mitigated on the leading strand with close-coupled repriming by PrimPol. Since the loss of the previously active transcriptional state of BU-1 in this context is followed by its repression, these results have potential implications for understanding the mechanism by which trinucleotide repeats induce gene silencing.

(1) Sarkies, P. et al. (2010) Mol Cell, 40, 703-713
(3) Schiavone, D., Guilbaud, G. et al. (2014) EMBO J, 33, 2507-2520
Replication stress and Translesion synthesis - B2

Presenter: Vibe Oestergaard

**Investigations of Chromosomal Fragile Sites in the Avian Cell Line DT40**

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Chromosomal Fragile Sites (CFSs) are specific regions on the chromosomes, which have a high propensity to break upon inhibition of DNA replication. Moreover CFSs are mutational hotspots in cancer genomes. Various cellular processes seem to affect CFSs including DNA replication, transcription, checkpoints and DNA repair pathways, but detailed mechanistic insight is still sparse. Orthologs of human CFSs have been found in a number of other mammalian species, but the extent of conservation of CFSs beyond the mammalian lineage is unclear. We have taken advantage of the finding that FANCD2 binds CFS in mitosis. Thus we have tagged both alleles of FANCD2 in the avian cell line DT40 and we have performed ChIPseq to identify genomic regions where FANCD2 are enriched in response to replication stress by treatment with aphidicolin (APH). FISH analyses confirm that identified sites for FANCD2 enrichment are bona fide fragile sites. Interestingly many of these regions overlap with the largest genes in the chicken genome, some of which have human orthologs in CFSs. We predict that the obtained map of CFSs in combination with the tools developed for the DT40 system will enable a systematic dissection of fragility in an isogenic genetically tractable system. To this end we have inserted a counter selectable marker at the CFS PARK2 allowing us to study the mutational processes acting at CFSs. DT40 clones carrying the counter selectable marker at the PARK2 locus display high but variable mutation rates at the locus and large deletions are the predominant mutation events.
DNA Damage Tolerance (DDT) mechanisms help dealing with unrepaired DNA lesions that block replication and challenge genome integrity. Previous in vitro studies showed that the bacterial replicase is able to re-prime downstream of a DNA lesion, leaving behind a single-stranded DNA gap. The question remains of what happens to this gap in vivo. Following the insertion of a single lesion in the chromosome of a living cell, we showed that this gap is mostly filled in by Homology Directed Gap Repair in a RecA dependent manner. When cells fail to repair this gap, or when homologous recombination is impaired, cells are still able to divide, leading to the loss of the damaged chromatid, suggesting that bacteria lack a stringent cell division checkpoint mechanism. Hence, at the expense of losing one chromatid, cell survival and proliferation are ensured.

In addition to this work recently published (Plos Genetics 2015), we will present new insights on the role of both RecF and RecBCD pathways in the repair of single strand gap.

And-1 coordinates with Claspin for efficient Chk1 activation in response to replication stress

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To maintain genomic stability, cells have evolved multiple DNA damage checkpoints to coordinate cellular response to DNA damage. Among these signaling pathways, DNA replication checkpoint is activated in response to a wide spectrum of DNA damage and DNA replication stress. The central part of replication checkpoint is the activation of two kinases ATR and Chk1, which in turn trigger multiple physiological processes. Replisome components, Claspin and Timeless-Tipin, play an essential role in the activation of Chk1 at the downstream of ATR-mediated signaling pathway in response to DNA replication stress. However, how these proteins collaborate with ATR to facilitate Chk1 phosphorylation is not fully understood in human cells. In this study, we have identified a novel ATR-mediated functional link between And-1 and Claspin, which is critical for efficient Chk1 phosphorylation after DNA damage. In response to replication stress, And-1 accumulates at DNA damage sites and downregulation of And-1 compromises the Chk1 activation. Our in vivo and in vitro experiments indicate that And-1 is involved in Chk1 activation by regulating Claspin-Chk1. We therefore conclude that And-1 is an important mediator that promotes efficient Chk1 activation by linking Claspin-Chk1 to ATR in response to replication stress.
Replication stress and Translesion synthesis - B2

Presenter: Chit Fang Cheok

**p53 maintains genomic stability by preventing interference between transcription and replication**

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The p53 tumor suppressor has various roles in maintaining genomic stability, typically acting through cell cycle arrest, senescence and apoptosis. Here, we discover a novel and critical genome maintenance function of p53 surprisingly independent of its canonical roles. p53 deficiency makes cells exceptionally sensitive to Topoisomerase II (TOP2) inhibitors, resulting in DNA damage arising spontaneously during replication. Mechanistically, we show that TOP2A-DNA complexes preferentially accumulate in isogenic p53 mutant cells or cells harboring genetic knockout of p53 gene, when TOP2A is recruited to regulate DNA topology, resulting in increased DNA damage in EdU-positive cells and widespread DNA double stranded breaks (DSBs). The pronounced DNA damage incurred in p53-deficient cells is neither due to cell cycle checkpoints deficiency nor to defects in ATM/ATR signaling. Rather, p53 appears to prevent DNA topological stress originating from transcription during S phase, and promotes normal replication fork progression, through a mechanism that is independent of G1 arrest. Consequently, replication fork progression is impaired in the absence of p53, which is reversed by transcription inhibition. Furthermore, pharmacologic inhibition of transcription 1) attenuates DSBs and γH2AX formation 2) decreases TOP2-DNA complexes, and 3) restores cell viability in p53-deficient cells. Together, our results reveal a previously unrecognized function of p53 in preventing conflicts between transcription and replication, which may underlie its role in tumor suppression. Our findings offer an alternative approach to target p53 deficiency by exploiting the differences in Topo II-mediated regulation of DNA topology during S-phase in wildtype and p53-deficient cells.
Dihydropyrimidinase interacts with FANCM and prevents cell-intrinsic DNA replication stress.

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FANCM is a multifunctional DNA-translocating motor protein that is essential for the maintenance of chromosomal stability. This large superfamily 2 helicase associates with proteins implicated in DNA damage signaling, in DNA repair, and in the recovery of blocked replication forks. FANCM has been linked to Fanconi anemia, a chromosomal instability disorder characterized by congenital abnormalities, bone marrow failure and cancer proneness. FANCM exhibits high affinity for branched DNA and can remodel DNA replication fork structures.

Here we show that FANCM interacts with a pyrimidine degradation enzyme, dihydropyrimidinase (DHP), which catalyzes the hydrolysis of 5,6-dihydrothymine and 5,6-dihydrouracil. The activity of DHP is increased in solid tumors compared to their normal counterparts, suggesting that DHP may be necessary for tumors to cope with alterations in nucleotide metabolism. Although primarily cytoplasmic, we found that a sub-fraction of DHP locates to chromatin in a FANCM-dependent manner. The knockdown of DHP in cancer cell lines induced the formation of DNA:RNA hybrids, impaired the progression of replication forks and yielded DNA lesions.

Dihydropyrimidine dehydrogenase (DPYD) is the initial rate-limiting enzyme in the pyrimidine degradation pathway that produces dihydropyrimidines. The severe cellular phenotypes associated with the depletion of DHP were rescued by the knockdown of DPYD, suggesting that the accumulation of dihydropyrimidines induces transcription-associated DNA replication stress.

In conclusion, DHP connects FANCM to nucleotide metabolism and is necessary to prevent cell-intrinsic DNA replication stress in transformed cells.
Replication stress and Translesion synthesis - B2

Presenter: Karim Ashour Garrido

Genome-wide analysis of topoisomerase removal after camptothecin treatment in the context of replication and transcription

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Camptothecin (CPT) and its derivatives (e.g. Irinotecan and Topotecan) are commonly used anticancer drugs which increase the half-life of topoisomerase I (Top1) cleavage complexes (Top1cc) during the Top1 catalytic cycle. Even in the presence of CPT Top1 will eventually remove itself by resuming its catalytic cycle. However, increasing Top1cc half-life also increases the probability of transcription and/or replication encounters with the transient Top1cc, which can lead to DNA breaks. Tyrosyl-DNA phosphodiesterase 1 (Tdp1) and Mre11 nuclease activity have been implicated in the removal of Top1 in the context of transcription and replication respectively. Also, Swi10ERCC1-Rad16XP has been shown to act as a redundant pathway with Tdp1 for the repair of Top1-mediated DNA damage. To understand the interaction among Top1 removal pathways I have performed a genome-wide study of Top1cc occupancy in the context of replication and transcription in WT, mre11, tdp1 and rad16XPF mutants using the model system Schizosaccharomyces pombe. Our data reveal new insights of the specific pathways involved in Top1cc removal in the absence and presence of CPT in proliferative and quiescent cells.
Replication stress and Translesion synthesis - B2

Presenter: Kai J. Neelsen

53BP1 nuclear bodies: a DNA repair factory or a post-repair chromatin scar?

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The formation 53BP1 nuclear bodies in G1 cells is one of the hallmarks of replication stress. Perturbed DNA replication - either drug-induced or due to oncogene activation - often results in incomplete genome duplication before mitotic entry. The residual under-replicated DNA interferes with sister chromatid separation in mitosis and therefore necessitates specialized processing, as do segregation problem that arise due to incomplete decatenation at centromeres. A subset of these lesions is transfererred to daughter cells, where they lead to formation of large chromatin compartments (nuclear bodies) occupied by 53BP1 and 53BP1-binding proteins. Our previous work has shown that during G1 53BP1 bodies shield the inherited DNA lesions from further erosion but whether this is the only function of 53BP1 remains unknown. Evidence that these structures may indeed perform additional function(s) comes from our observation that 53BP1 bodies persist beyond G1 and are usually dissolved only in late S phase. We reason that elucidating the exact mechanism of 53BP1 nuclear body resolution might hold the key to better understand the function of these nuclear compartments. To this end, we combine Quantitative Image-Based Cytometry (QIBC), with high-resolution confocal imaging, live imaging of single cells, and genetic silencing of DNA damage and cell cycle regulators to monitor 53BP1 body dynamics over successive cell generations. Our data suggest that, independent of the cause for body formation (replication stress at fragile sites or impaired DNA decatenation at centromeres), 53BP1 body dissolution is a replication-coupled process. Our mechanistic investigations aim at deciphering whether the 53BP1 body dissolution co-incides with repair of the underlying DNA lesions or whether it reflects restoration of a 'chromatin scar', i.e. reprograming of the chromatin at the DNA lesion to its pre-damaged state. We speculate that persistent DNA damage-associated chromatin modifications might be one of hitherto poorly characterized consequences of replication stress during the previous cell cycle.
Chromatin regulates genome targeting by cisplatin

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Cisplatin treatment damages DNA and is extensively used to treat several cancers including ovarian and testicular. The cellular response to cisplatin is pleiotropic and inherently complex, making it challenging to identify drug mechanisms of cisplatin for the use of this drug as an anticancer treatment and during drug resistance, a major limitation of cisplatin use in the clinic. As a means to overcome these obstacles to provide new insights into cisplatin mechanisms of action, we have developed a cisplatin derivative that can be fluorescently labeled or tagged in cells. The chemical labeling of our cisplatin probe in cells enabled the visual detection of platinated DNA lesions for the first time. Pharmacological inhibition of histone deactylases (HDACs) promoted focal drug accumulation into visible drug foci that co-localized with the translesion synthesis (TLS) regulator RAD18. The TLS pathway normally bypasses cisplatin lesions to allow damage tolerance, a mechanism involved in resistance to cisplatin. However, we observed that cisplatin and HDACi co-treatment synergystically activated apoptosis in cancer cells. These results suggest that formation of clustered platinated DNA adducts by HDACi co-treatment blocks the “DNA damage tolerance” function of TLS. These findings have important implications in cisplatin drug resistance cells that utilize TLS. Our results suggest that HDACi treatment can resensitize cisplatin-resistant cells by creating local clusters of platinated lesions that are inhibitory towards “bypass” mechanisms afforded by TLS pathways. The ability to identify platinated lesions in cells allowed for the unbiased screening of small molecule modulators of genome targeting with platinum drugs and provided unprecedented insights into how pharmacological alterations of chromatin can sensitize cancer cells and drug resistant cells to this class of chemotherapeutic compounds.
Replication stress and Translesion synthesis - B2

Presenter: Olimpia Alessandra Buoninfante

Roles of PCNA ubiquitination and TLS polymerases κ and η in the bypass of methyl methanesulfonate-induced DNA damage

Niek Wit(1), Olimpia Alessandra Buoninfante(1), Paul C.M. van den Berk(1), Jacob G. Jansen(2), Marc A. Hogenbirk(1), Niels de Wind(2), Heinz Jacobs(1)

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Translesion synthesis (TLS) provides a highly conserved mechanism that enables DNA synthesis on a damaged template. TLS is performed by specialized DNA polymerases of which polymerase (Pol) κ is important for the cellular response to DNA damage induced by benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), ultraviolet (UV) light and the alkylating agent methyl methanesulfonate (MMS). As TLS polymerases are intrinsically error-prone, tight regulation of their activity is required. One level of control is provided by ubiquitination of the homotrimeric DNA clamp PCNA at lysine residue 164 (PCNA-Ub). We here show that Polκ can function independently of PCNA modification and that Polη can function as a backup during TLS of MMS-induced lesions. Compared to cell lines deficient for PCNA modification (Pcna(K164R)) or Polκ, double mutant cell lines display hypersensitivity to MMS but not to BPDE or UV-C. Double mutant cells also displayed delayed post-replicative TLS, accumulate higher levels of replication stress and delayed S-phase progression. Furthermore, we show that Polη and Polκ are redundant in the DNA damage bypass of MMS-induced DNA damage. Taken together, we provide evidence for PCNA-Ub-independent activation of Polκ and establish Polη as an important backup polymerase in the absence of Polκ in response to MMS-induced DNA damage.
Replication stress and Translesion synthesis - B2

Presenter: Anastasia Tsaalbi-Shtylik

Aplastic anemia caused by excessive replication stress at endogenous DNA lesions

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Replicative polymerases are arrested by DNA lesions which causes replication stress. Replication of these lesions by so-called translesion synthesis (TLS) polymerases partially suppresses this replication stress. To study the effect of replication stress caused by endogenous DNA lesions on organismal fitness we disrupted Rev1, a key regulatory TLS protein. Rev1-deficient mice are born at sub-Mendelian ratios, display growth retardation and have a moderately reduced life span. Rev1 mice displayed a severely impaired regenerative capacity of hematopoietic stem cells (HSC) and a gradual decrease of the cellularity and proliferation of bone marrow cells. These phenotypes indicate an important role of Rev1-mediated TLS in the maintenance of stem and proliferating cells. Rev1 cells displayed a defect in TLS at lipid peroxidation-induced bulky DNA lesions indicating that such lesions may be causally related to the Rev1 phenotypes. To provide evidence that these phenotypes are caused by endogenous bulky DNA lesions we additionally disrupted Xpc, essential for nucleotide excision repair of such DNA lesions. Rev1Xpc double knockout mice are hardly born and they died from aplastic anaemia at the age of 3,5 months. Rev1Xpc mice displayed a significant reduction in the number of HSC. Although the cellularity of the bone marrow initially is normal in Rev1Xpc mice, the hematopoietic system collapses between 3 and 4 months of age. This is associated with a reduction in proliferation, increased DNA damage signalling, senescence and apoptosis of bone marrow progenitors, and the accumulation of genomic breaks and telomeric damage. Furthermore, Rev1Xpc progenitor cells showed mitochondrial dysfunction and high levels of associated oxidative stress. These phenotypes were mitigated by bone marrow transplantation from Xpc littermates, supporting cell-autonomous defects as cause for the attrition of the hematopoietic compartment.

Our results indicate that replication stress at endogenous lipid peroxidation-induced DNA lesions contributes to exhaustion of stem cell pools and to depletion of proliferating cells in the organism.
Replication stress and Translesion synthesis - B2

Presenter: Bas Pilzecker

**PrimPol prevents AID/APOBEC family mediated mutagenesis**

B. Pilzecker(1), O.B. Buoninfante(1), C. Pritchard(2), O.S. Blomberg(1), I.J. Huijbers(2), P. van den Berk(1), H. Jacobs(1)

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PrimPol is a DNA damage tolerant (DDT) polymerase displaying both translesion synthesis (TLS) and (re)-priming properties. This led us to study the consequences of a PrimPol deficiency in tolerating mutagenic lesions induced by members of the APOBEC/AID family of cytosine deaminases. Interestingly, during somatic hypermutation (SHM), PrimPol counteracts the generation of C>G transversions on the leading strand. Independently, mutation analyses in human invasive breast cancer confirmed a pro-mutagenic activity of APOBEC3B and revealed a genome-wide anti-mutagenic activity of PRIMPOL as well as most Y-family TLS polymerases. PRIMPOL especially prevents APOBEC3B targeted cytosine mutations within TpC dinucleotides. As C transversions induced by APOBEC/AID family members depend on the formation of AP-sites, we propose that PrimPol reprimes preferentially downstream of AP-sites on the leading strand, to prohibit error-prone TLS and simultaneously stimulate error-free homology directed repair. These in vivo studies are the first demonstrating a critical anti-mutagenic activity of PrimPol in genome maintenance.
Dynamic PCNA-interactions in DNA repair, replication, cell cycle control and epigenetic stability

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PCNA is a vital component of DNA replication and repair pathways in all eukaryotes. Its study has been complicated by its trimeric nature, and by the fact that it is extremely highly conserved and essential for viability. We have been developing methods to investigate the dynamic interactions between PCNA and its many client proteins in real time in living human cells. These will enable us to dissect its differing contributions to various aspects of genome stability.

We use a Forster resonance energy transfer (FRET) assay to monitor the proximity of PCNA to key clients, including flap endonuclease 1, DNA methyltransferase 1 and DNA ligase 1 during the cell cycle and in response to various DNA-damaging or replication-inhibiting agents. We here show that these interactions with PCNA are modulated throughout the cell cycle and in response to replication inhibition and propose a competition model to explain these findings.

To identify novel PCNA-interacting partners we developed an in-cell screening approach using bimolecular fluorescence complementation. We identified SetD3, Maf1 and RNF7 as PCNA binding partners with potential roles in chromatin maintenance and cell cycle regulation.

To better understand how the trimeric nature of PCNA affects its function we have developed a triple-fusion PCNA which is expressed as a single polypeptide. This allows us to systematically modify individual monomer units to assess their contribution to function. Using this we show that the functions of PCNA do not require all three of its binding surfaces to be operational; it appears that a single binding surface on a PCNA trimer is sufficient for activity. Thus the widely accepted “tool belt” model for PCNA function may not be appropriate in a cellular context.
Replication stress and Translesion synthesis - B2

Presenter: Carlos FM Menck

Gap-filling of 6-4PP lesions depends on Rev3L in ultraviolet-irradiated human cells

Annabel Quinet(1), Davi Jardim Martins(1), Alexandre Teixeira Vessoni(1), Denis Biard(2), Alain Sarasin(3), Anne Stary(3), Carlos Frederico M Menck(1)

1. Institute of Biomedical Sciences, University of São Paulo, Brazil; 2. CEA, IMETI, SEPIA, Team Cellular Engineering and Human Syndromes, Fontenay-aux-Roses, France; 3. Institut de Cancérologie Gustave Roussy, Villejuif, France

Ultraviolet-induced 6-4 photoproducts (6-4PP) and cyclobutane pyrimidine dimers (CPD) can be tolerated by translesion DNA polymerases (TLS Pol) at stalled replication forks or by gap-filling. Here, we investigated the involvement of Polη, Rev1 and Rev3L (Polζ catalytic subunit) in the specific bypass of 6-4PP and CPD in repair-deficient XP-C human cells. DNA fiber assay and novel methodologies were combined for detection and quantification of single-stranded DNA (ssDNA) gaps in ongoing replication forks and post-replication repair tracts in the human genome. We demonstrated that Rev3L, but not Rev1, is required for gap-filling, while Polη and Rev1 are responsible for TLS at stalled forks. Moreover, specific photolyases were employed to show that CPD stall replication forks, while 6-4PP lesions are responsible for the generation of ssDNA gaps, which are replicated independent of S-Phase. On the other hand, both lesions block fork movement in the absence of Polη or Rev1. Altogether, the data directly show that in the human genome Polη and Rev1 bypass CPD and 6-4PP at replication forks, while 6-4PP are tolerated by a Polζ-dependent, Rev1-independent, gap-filling mechanism.

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Replication stress and Translesion synthesis - B2

Presenter: Heinz Jacobs

PrimPol prevents APOBEC/AID family mediated DNA mutagenesis

Bas Pilzecker(1), Olimpia Alessandra Buoninfante(1), Colin Pritchard(2), Olga S. Blomberg(1), Ivo J. Huijbers(2), Paul C.M. van den Berk(1), Heinz Jacobs(1)

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PrimPol is a DNA damage tolerant (DDT) polymerase displaying both translesion synthesis (TLS) and (re)-priming properties. This led us to study the consequences of a PrimPol deficiency in tolerating mutagenic lesions induced by members of the APOBEC/AID family of cytosine deaminases. Interestingly, during somatic hypermutation (SHM), PrimPol counteracts the generation of C>G transversions on the leading strand. Independently, mutation analyses in human invasive breast cancer confirmed a pro-mutagenic activity of APOBEC3B and revealed a genome-wide anti-mutagenic activity of PRIMPOL as well as most Y-family TLS polymerases. PRIMPOL especially prevents APOBEC3B targeted cytosine mutations within TpC dinucleotides. As C transversions induced by APOBEC/AID family members depend on the formation of AP-sites, we propose that PrimPol reprimers preferentially downstream of AP-sites on the leading strand, to prohibit error-prone TLS and simultaneously stimulate error-free homology directed repair. These in vivo studies are the first demonstrating a critical anti-mutagenic activity of PrimPol in genome maintenance.
Replication stress and Translesion synthesis - B2

Presenter: Ivo van Bostelen

Suppression of genome instability by Y-family TLS polymerase REV-1 in C. elegans

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Translesion synthesis (TLS) polymerases promote the replication of damaged DNA via their ability to insert nucleotides opposite otherwise replication blocking lesions. In this way they allow for completion of the cell cycle in the presence of DNA damage and thus prevent checkpoint activation, genome instability and cell death. In comparison to replicative polymerases Y-family TLS polymerases have reduced fidelity. In this study we characterize mutants that lack REV-1 - one of the key players in TLS – and study how REV-1 influences spontaneous mutagenesis in C. elegans. We show that rev-1 mutants proliferate normally and are sensitive to UV-C exposure. We find REV-1 to be involved in a mutagenic sub-pathway of TLS and loss of REV-1 alters mutational footprints and increases genome instability. In the absence of REV-1 dependent TLS unresolved replication blocks form, which proved to be substrates for polymerase theta mediated end-joining. We conclude that during unperturbed growth REV-1 protects the genome of an animal from deletions and larger genomic rearrangements but at the cost of base substitutions.
Replication stress and Translesion synthesis - B2

Presenter: Leticia Koch Lerner

The induction of DNA damage tolerance in human cells involves p53-dependent translesion synthesis by DNA polymerase eta


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Genome lesions trigger biological responses that help cells manage damaged DNA, improving cell survival. Pol eta is a translesion synthesis (TLS) polymerase that bypasses lesions that block replicative polymerases, avoiding continued stalling of replication forks, which otherwise collapse, ultimately leading to cell death. We describe the p53-dependent induction of pol eta in normal and DNA repair-deficient XP-C human cells after ultraviolet (UV) exposure. Pre-irradiated primary fibroblasts showed a protective effect on cell survival after challenging UV exposures, which was absent in p53- and Pol H-silenced cells. Viability increase was associated with improved elongation of nascent DNA, indicating the protective effect was due to more efficient lesion bypass by pol eta. p53- and pol eta-dependent protective effect was marked in cells proficient or deficient in nucleotide excision repair, suggesting that, from cell survival perspective, proper bypass of DNA damage can be as relevant as removal. These results indicate p53 controls the induction of pol eta in DNA-damaged human cells, resulting in improved TLS and enhancing cell tolerance to DNA damage, which parallels SOS responses in bacteria.
Replication stress and Translesion synthesis - B2

Presenter: Ligia Castro

Differential mRNA processing of XPV patients with mutations at splicing sites.

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Xeroderma pigmentosum variant (XP-V) patients have mutations in a translesional DNA polymerase gene, POLH (XPV). POLH mRNA (NM_006502.2) has eleven exons, with the ATG start codon at exon 2. Two novel mutated POLH alleles that affect splicing sites were recently described in Brazil: one is located at the beginning of intron 6, (c.764+1 G>A), and the second was identified at a splicing site before the last exon (c.1249-1 G>A). Cells homozygous for this mutation express higher levels of mRNA expression compared to a wild type control. Furthermore, we found that the cells with different mutations in the same gene differ in the cellular response to treatment with cisplatin. The cDNA analysis from a patient with a homozygous c.764+1 G>A mutation revealed that there are at least three different mRNA species. One skipping the entire exon 6, other that keep part of exon 6, due to the usage of a new splice donor site located in the middle of these exon, and the other skipping one or more exons between exons 3 and 7. These mRNAs species possibly lead to the formation of a protein with a reduced activity and/or stability. Residual pol eta activity is likely responsible to differences in the cellular response to cisplatin between the homozygous c.764+1 G>A, and the homozygous c.1249-1 G>A patients, as well as cells from XP-V patients with no pol eta activity.
UVA light induces DNA damage, cell cycle arrested and twice more mutation in patients have defect in polymerase pol eta

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UVA is the most abundant UV light on the surface of earth (about 95% of sunlight). It can induce direct (CPD and 6-4PPs) and indirect (8-oxoG) DNA damage, which, if not correctly repaired or processed, may lead to the development of skin tumors, because the mutagenic potential of these lesions. The cells have several mechanisms that prevent or tolerate DNA damage caused by UV, ensuring the survival and controlling the mutagenesis induced by UV. Xeroderma Pigmentosum Variant patients (XP-V) are deficient in translesion synthesis (TLS) DNA polymerase (pol eta), but have a normal Nucleotide Excision Repair (NER). This deficiency in TLS leads to increased mutagenesis when XP-V cells are exposed to UVC or UVB-light, but the effects induced by UVA on these cells is not known. The aim of this work is to evaluate the genotoxic and mutagenic effects of UVA light in XP-V cells in comparison to repair proficient cells (primary and transformed cells). The cells were also treated with caffeine, which normally increase XP-V cells sensitivity to UVA. UVA induction of damage stress (γH2AX), cell cycle arrest (propidium iodide) and apoptosis (sub-G1) were detected by flow cytometry. Mutagenesis was identified by exome sequencing of transformed cells, cloned after UVA irradiation. The results showed that UVA light induced damage in all transformed cells, nevertheless the normal and complementary cells were able to solve the damage more efficiently than XP-V cells. XP-V cells show cell cycle arrest (mainly in S-phase) and a significant induction of cell death. This phenotype where exacerbated by the use of caffeine. Concerning primary cells, the UVA light induced increase of γH2AX immediately after irradiation, but this was higher in XP-V cells. Theses cell also had stronger cell cycle arrest and significant increase in sub-G1 population. Contrary to expectations, primary fibroblasts treated with caffeine showed no increase of γH2AX after UVA light but had persistent cell cycle arrest and significant increase of cell death. The mutagenesis analysis using transformed cells showed that UVA light also induced an increase twice the variant (base substitutions) levels in XP-V cells, when compared to control cells. The XP-V cells had more variants C:G→T:A (probably due to CPDs) than G:C→T:A (related with oxidative stress). These results showed that UVA light compromises the bypass of lesions resulting in damaged DNA, cycle arrest and cell death in absence of pol eta. Mutagenesis induced by UVA light is also affected in cells deficient in pol eta. Therefore, TLS by pol eta is an important protective pathway for human cells to the genetic damaging effects of UVA component of sunlight.
Replication stress and Translesion synthesis - B2

Presenter: Ronald Wong

Investigating the Requirement of the Chromatin Remodelling Ino80 Complex for Postreplicative DNA Repair

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The postreplicative repair (PRR) pathway resolves DNA replication problems arising from the presence of lesions in the template. The pathway is activated by ubiquitylation of the replication factor proliferating cell nuclear antigen (PCNA), which initiates damage bypass either by recruitment of damage-tolerant DNA polymerases or by a template switching mechanism. It has long been known that damage bypass can operate “on the fly” at the replication fork or on single stranded DNA gaps left behind upon passage of the fork. Since chromatin rapidly restores after replication, this implies that postreplicative repair occurs in the context of chromatin. Nevertheless the exact role of chromatin in PRR pathway remains enigmatic. In this study, we have investigated the role of the chromatin remodeller Ino80 in postreplicative repair using an experimental system in budding yeast that is capable of separating damage bypass from bulk genome replication.

This system harnesses the possibility to delay postreplicative repair by inducing PCNA ubiquitylation after completion of genome replication when cells reach G2 phase of cell cycle. This does not compromise overall cellular survival and yet offers a window of time to interrogate if Ino80 is required for the PRR events. With this approach, we observed that postreplicative DNA synthesis in G2 was severely impaired in Ino80-depleted cells. We further observed a reduction in postreplicative repair-mediated cellular survival in the absence of Ino80. Surprisingly, we found that - contrary to previous reports - PCNA ubiquitylation is not directly regulated by the Ino80 complex. We therefore conclude that the Ino80 complex plays a role downstream of PCNA ubiquitylation in facilitating PRR. Taking these results into perspective, the Ino80 complex possibly facilitates postreplicative repair by making the chromatin accessible to repair synthesis.
Accurate replication of the DNA is critical for the maintenance of genome integrity and cellular survival. Cancer-associated alterations often involve key players of DNA replication and of the DNA damage-signalling cascade. Post-translational modifications play a fundamental role in coordinating replication and repair and central among them is Ubiquitylation. The E3 ligase UBR5 has been found overexpressed or mutated in several tumours. We show that downregulation of UBR5 results in S phase accumulation and slower S phase progression. This is due to a reduction in replication fork speed followed by an accumulation of single strand DNA (ssDNA). After knockdown of UBR5 we also detect increased ubiquitylated PCNA, a key regulator of DNA damage tolerance mechanisms such as DNA translesion synthesis. The effect of UBR5 knockdown is related to a mis-regulation in the pathway that controls the ubiquitylation of histone H2A (uH2A) and blocking this modification is sufficient to rescue the cells from the replication defects. Finally, we show that the presence of polymerase eta is the main cause of cell death when UBR5 is silenced, indicating a novel role of uH2A in the control of polymerase eta as a central player of the DNA damage tolerance response.
Replication stress and Translesion synthesis - B2

Presenter: Shunichi Takeda

Evidence for translesion synthesis by DNA polymerase δ in vivo

Shunichi Takeda(1), Masataka Tsuda(1), Koji Hirota(2), Julian Sale(3)

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The replicative DNA polymerase Polδ synthesizes DNA with extremely high accuracy by strictly discriminating between correct and incorrect base pairs. It is believed that this intolerance of incorrect pairing inhibits translesion synthesis (TLS) by the enzyme. However, chicken DT40 B cells deficient in the POLD3 subunit of Polδ (pold3 cells) are deficient in TLS. Moreover, only pold3 clones, but not POLζ/- (polζ) clones, generated from chicken DT40 cells are deficient in maintenance of replication fork progression along UV-damaged template strands and exhibited an altered pattern of abasic bypass in the immunoglobulin light chain gene. These observations support the idea that Polδ is capable of performing TLS. To test this hypothesis, we inactivated Polδ proofreading in pold3 cells. This significantly restores the defective TLS of pold3 mutants, enhancing dA incorporation opposite to abasic sites and changing the mutation spectrum at the CPD UV lesion. In agreement with these in vivo data, purified proofreading-deficient human Polδ performs TLS of abasic sites in vitro much more efficiently than the wild type enzyme, with over 90% of TLS events resulting in dA incorporation. These data support Polδ contributing to TLS in vivo and suggest that the mutagenesis resulting from loss of Polδ proofreading activity may in part be explained by enhanced lesion bypass.
MAPKAP Kinase 2 inhibition differentially affects the sensitivity of pancreatic cancer cells towards nucleoside analogues and cisplatin

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DNA-damaging chemotherapy still represents the mainstay of available drugs against cancer. Most chemotherapeutics exploit and further increase the enhanced replicative stress levels in cancer cells. Recently, DNA damage signaling pathways have emerged as promising drug targets to increase the efficacy of classical chemotherapy (Dobbelstein & Sørensen, 2015).

In 2007, Reinhardt et al. have demonstrated that targeted deletion of the MAP kinase-activated protein kinase 2 (MK2), a kinase currently implicated in p38 stress signaling and G2 arrest, leads to sensitization of p53 mutant cells against cisplatin, effectively inducing cell death. In 2013, and in seeming contrast, Köpper et al. reported a replication stabilizing effect of MK2 inactivation in p53 wildtype cells upon simultaneous treatment with gemcitabine, leading to improved cell survival. The two reports seem contradictory, as in one situation MK2 loss of function leads to chemosensitization and in the other situation protects the cancer cells against a chemotherapeutic drug.

Using a mutant p53 pancreatic cell line (PANC-1), we recapitulated both observations in the same biological system. MK2 inhibition sensitized PANC-1 cells towards cisplatin but made them resistant against gemcitabine. Furthermore, quantifying the progression of single DNA replication forks using DNA fiber assays, we found a striking difference in the effects of cisplatin and gemcitabine: Cisplatin only mildly reduced the average fork rate, whereas gemcitabine rapidly and potently inhibits replication progression. MK2 inhibition rescued replication in the presence of gemcitabine, but not when cells were treated with cisplatin. Importantly, cisplatin-treated cells readily underwent premature mitosis when MK2 function was blocked simultaneously, providing an explanation for the enhanced sensitivity of MK2-inhibited cells towards cisplatin.

Taken together, the MK2 kinase provides chemosensitizing as well as protective functions towards chemotherapy, depending on the nature of the drugs and the phase of the cell cycle. This multilevel activity raises the need for careful adaptation of clinically used MK2 inhibitors, but it could also provide a novel approach for cell cycle specific chemotherapy.

Reinhardt et al., p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. Cancer Cell. 2007 Feb;11(2):175-89.
POSTER SESSION B3: 'Crosslink Repair'

Poster viewing: Tuesday, April 19, 20:00 – 22:00 h

Discussion: Thursday, April 21, 16:55 – 18:25 h
Cisplatin is a powerful agent used clinically to treat a wide variety of tumors such as ovarian, testis and lung. However, the efficacy of the treatment can be reduced due to the resistance development. There are multiple suggested mechanisms for cisplatin resistance in tumors and the increase of DNA repair is proposed to be one of the most relevant. Cisplatin acts by forming DNA adducts, which include monoadducts, intra- and interstrand DNA crosslinks (ICLs). The ICLs are highly toxic lesions that can inhibit and block DNA replication and transcription, which may lead to cell death. ERCC1–XPF is a structure-specific endonuclease that is required for the repair of these lesions through the Nucleotide Excision Repair and Interstrand Crosslink Repair pathways. It has been suggested that expression of ERCC1 correlates with cisplatin drug resistance in non-small cell lung cancer (NSCLC) and other kinds of tumors and the silencing of these proteins can alter the expression levels of other. In this work, different strategies were used to obtain silenced strains for these two genes to test the effect on the cytotoxicity of cisplatin in lung cancer cells (A549) and lung fibroblasts (MRC5 and IMR90). For this purpose, RNA interference techniques have been applied, with transient and permanent silencing by short RNA sequences (siRNA) and lentiviral transduction (shRNA), respectively. We also used the CRISPR/Cas9 technique to inactivate these genes. These cells, silenced for XPF, ERCC1 or both, were analyzed in comparison with fibroblasts extracted from patients deficient in XPF protein for their sensitivity to cisplatin and other ICLs inducing agents. Interestingly, XPF silencing appears to also reduce the expression of ERCC1. In general, it has been demonstrated that silenced cells present higher sensitivity to these agents. Moreover, glutathione (GSH) inside the cells functions as a barrier to cisplatin cytotoxicity. Our results show that addition of a GSH synthesis inhibitor (BSO) to the cisplatin treatment induced a strong increase in cisplatin sensitivity. Thus, XPF-ERCC1 silencing combined with cisplatin and BSO appears to be an interesting therapeutic strategy for improving the clinical protocols against lung cancer.
Crosslink repair - B3

Presenter: Anna Gueiderikh

Nucleolar mechanisms involved in Fanconi anemia pathogenesis and FANC pathway activity

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Fanconi anemia (FA) is a rare genetic disorder featuring progressive bone marrow failure (BMF) leading to pancytopenia, developmental defects and cancer predisposition. Nineteen proteins have been involved in its pathogenesis, all grouped in a common DNA repair pathway: the FANC pathway, in which ten proteins assembled in the FANC core complex monoubiquitinate FANCD2 and FANCI that, in turn, coordinate the activity of the downstream FANC proteins, including BRCA1 and BRCA2. The whole pathway helps cells to respond to DNA interstrand crosslinks (ICLs) and replication stress. Nonetheless, three FANC core complex proteins - FANCA, FANCC and FANCG - account for 90% of the patients with FA, FANCA representing more than half of the patients. The up-regulation of p53 in hematopoietic stem and progenitor cells is considered as the main factor responsible for the BMF observed in FA and is generally attributed to the progressive accumulation of DNA damage. “Nucleolar stress”, a dysregulation in ribosomal biogenesis that leads to an alteration of the nucleolar metabolism, is another way to stabilize p53 and has been identified in the other main genetic anemias.

Following the siRNA-mediated depletion of FANCA in HeLa cells, we unexpectedly observed the appearance of morphological features of nucleolar stress. We successively validated our observation in FANCA-/- mouse cells as well as in human cells depleted in other FANC core complex proteins but we failed to observe features of nucleolar stress in absence of the FANC pathway downstream proteins FANCD2 or BRCA2. We found a slow down in rRNA synthesis in FANCA-deficient cells, which may represent the cause for this new phenotype observed upon FANC core deficiency and which links FA to a ribosome biogenesis alteration. We also found an up-regulation of the 5S ribosomal RNA in FANCA-deficient cells as well as a diminution in the level of neddylated RPL11. Both situations can lead to the MDM2 inhibition by the RPL5-RPL11-5S complex followed by the nucleolar-induced p53 stabilization. Thus, work is in progress to determine if the known p53 stabilization in FA cells is due to nucleolar stress, unrepaired DNA damage or both.

We also established that exogenous nucleolar stress inductors can activate the FANC core complex. This activation differs from the DDR activity of the pathway as it induces a monoubiquitination of FANCD2 but without inducing subnuclear foci. We also showed that the depletion of FANCA causes the unexpected recruitment of the RECQ1 helicase to the nucleolus. This DDR protein can influence nucleolar stress phenotype and ICL response in FANCA deficient cells, evidencing a real crosstalk between the two functions of the pathway. We thus showed that the nucleolar compartment alterations are crucial in FA pathology and FANC pathway activity. We plan to investigate further the crosstalk between nucleolar stress and DNA damage response in FA as well as the importance of this phenotype in patients.
Cryo-EM structure of the human FANCD2/FANCI complex reveals a novel Tower domain required for FANCD2 monoubiquitination.

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The Fanconi Anemia (FA) pathway has been implied to play a significant role in DNA interstrand crosslink repair and may be the coordinator between different DNA damage repair pathways such as homologous recombination and nuclear excision repair. Within the FA pathway, the FANCD2 and FANCI proteins are key players essential for the functionality of the pathway. Neither of the proteins possesses any known conserved domains, and they are suggested as paralogs of each other. Upon genotoxic stress, both proteins are monoubiquitinated by the FA core complex, and reside on sites of DNA damage together with other DNA repair proteins. Yet, the molecular mechanism of how the two proteins coordinate the DNA repair responses remains elusive. Here we show the first structural insight into the human FANCD2/FANCI complex by obtaining the cryo-EM structure of the complex of full-length proteins. The complex has an inner cavity, large enough to accommodate a double stranded DNA helix. We also discovered a protruding Tower domain, which we have shown to be critical for the recruitment of the complex to ICLs in vitro and in vivo, and for the monoubiquitination of FANCD2. Disease-causing mutations in this domain is observed in several FA patients. We demonstrate that the complex binds strongly to a DNA structure resembling a replication fork stalled at an ICL. Finally, our work reveals that recruitment and binding of the complex to a stalled replication fork serves as the trigger for the activating monoubiquitination event. Taken together, our results uncover the mechanism of how the FANCD2/FANCI complex activates the FA pathway, and explains the underlying molecular defect in FA patients with mutations in the Tower domain.
Crosslink repair - B3

Presenter: Hyungjin Kim

FBW7 regulates DNA interstrand cross-link repair by modulating FAAP20 degradation

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Mutations that deregulate protein degradation lead to human malignancies. The SCF ubiquitin E3 ligase complex degrades key oncogenic regulators, thereby limiting their oncogenic potential. FBW7 is a substrate recognition subunit of SCF(FBW7) and is among the most commonly mutated ubiquitin-proteasome proteins in cancer. FBW7-mutated cancer cells display increased genome instability, but the molecular mechanism of FBW7 in preserving genome integrity remains elusive. Here, we demonstrate that SCF(FBW7) regulates the stability of FAAP20, a critical component of the Fanconi anemia (FA) DNA interstrand cross-link (ICL) repair pathway. Phosphorylation of the FAAP20 degron motif by GSK3β provides a platform for recognition and polyubiquitination of FAAP20 by FBW7, and its subsequent degradation by the proteasome. Accordingly, enhanced GSK3β-FBW7 signaling disrupts the FA pathway. In cells expressing non-phosphorylatable FAAP20 mutant, the turnover of its binding partner, FANCA, is deregulated in the chromatin during DNA ICL repair, and the FA pathway is compromised. We propose that FAAP20 degradation, which is prompted by its phosphorylation, controls the dynamics of the FA core complex required for completing DNA ICL repair. Together, this study provides insights into how FBW7-mediated proteolysis regulates genome stability and how its deregulation is associated with tumorigenesis.
Somatic mosaicism, the coexistence of cells with different genetic composition within an individual, has been associated with aging and cancer in the general population (1,2). Mosaicism for chromosomal events $>500$kb affecting $\geq 10\%$ of cells can be detected using SNP array of DNA from whatever tissue. Fanconi anemia (FA) is a genetic disorder characterized by congenital defects, bone marrow (BM) failure and cancer susceptibility, caused by deficient inter-strand DNA crosslink repair. Due to the high risk for hematological and solid cancers, a strict follow-up protocol is recommended including, among other exams, periodic bone marrow testing. We have studied the prevalence of clonal mosaicism in 11,944 young controls and 129 age-matched FA patients blood DNA and whether mosaicism could be used as an early marker for cancer. Blood DNA were analyzed by SNP array (Illumina 1M or Infinium HumanCore) and copy number and copy neutral chromosomal mosaic events were detected with the MAD software and experimentally validated by microsatellite and MLPA analyses. DNA from an anal squamous cell carcinoma (SCC) sample from one FA patient was also studied. We detected 45 mosaic events in blood of 14/129 FA patients (10.8%), and validated 94.7% of them by microsatellite and/or MLPA analysis. Compared to 11,944 age-matched controls, FA subjects under 18yo had a 220X rate while young adults (18-50yo) had a 109X rate of detectable mosaicism (4.39%/0.02%, $p=2.2\times10^{-7}$ and 29.4%/0.27%, $p=4.1\times10^{-17}$). Considering events, there was a 243X increase in FA patients, with an average of 0.34 mosaic events/patient and 0.0014 events/control. The risk of developing cancer 0-10 years after DNA extraction was 5.2X increased in FA patients with mosaicism with respect to FA patients without mosaicism (85.7%/16.5%, $p=5.7\times10^{-7}$). An uniparental disomy of 6p was detected both in blood and a SCC diagnosed 10y after, suggesting an early embryonic origin of the mosaicism. In conclusion we uncovered an extreme genetic heterogeneity of the soma in FA patients associated with higher risk of hematological and solid cancer.

(2) Laurie et al., Nature Genetics (2012) 44(6):642-650
Crosslink repair - B3

Presenter: Lonnie Swift

The SNM1A repair exonuclease: cellular functions, structure and inhibition

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SNM1A (DCLRE1A) is 5'-3' DNA repair exonuclease that plays a key role in replication-coupled and transcription-coupled interstrand cross-link (ICL) repair. Following initial ICL incision, SNM1A can resect past ICLs to provide a substrate for the downstream completion of repair through translesion synthesis and homologous recombination. Using CRISPR-Cas9 genome engineering, we have created SNM1A disrupted cell lines to describe the repair defective phenotype in further detail, extending the role of SNM1A, including a role in the repair of ‘dirty-ended’ DNA double-strand breaks, induced by radiation and radiomimetic drugs. This role again involves the capacity of SNM1A to digest DNA strands containing heavily damaged bases, and the contribution of SNM1A will be described.

In an attempt to understand the mechanistic basis if SNM1A’s capacity to digest heavily damaged DNA, we recently solved its crystal structure at 2.16 Å. This revealed that SNM1A possesses a putative, wide DNA-binding groove capable of accommodating highly distorted DNA structures. Testing this prediction through biochemical analyses supports a key role for the wide groove in facilitating digestion of lesion-containing DNA, including molecules containing lesions such as ICLs. This work also confirmed that structurally, SNM1A belongs to the β-CASP (CPSF, Artemis, SNM1, PSO2) family that defines a range of DNA- and RNA-processing metallo-β-lactamases (MBL). They possess a MBL fold that contains four highly-conserved motifs characteristic of the MBL superfamily. Since key residues at the active sites of SNM1A are conserved with those on bacterial MBLs (enzymes that metabolise and provide resistance to beta-lactam antibiotics), compounds that inhibit bacterial MBLs were an attractive starting-point for a screen for inhibitors of SNM1A. High-throughput screens using a real-time fluorescence assay, produced 22 hit compounds with 4 different scaffolds that all acted as competitive inhibitors of SNM1A. These compounds are either the natural substrates of bacterial MBLs or known mimics of these substrates. These molecules provide an important starting point for ongoing medicinal chemistry efforts, which will be described, to generate inhibitors to this important class of DNA repair factor.
Crosslink repair - B3

Presenter: Nigel Jones

**Diadenosine tetraphosphate (Ap4A) is synthesised in response to ICL and inhibits the initiation of DNA replication**

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The dinucleotide diadenosine tetraphosphate (Ap4A) is synthesised in cells in response to a variety of stresses and has been previously implicated in DNA damage responses, apoptosis and signalling. Here we characterise the role of Ap4A and its newly discovered derivative ADP-ribosylated Ap4A (ADPR-Ap4A) and demonstrate for the first time that Ap4A plays an important role in cellular responses to DNA crosslinking agents. To determine whether intracellular Ap4A is increased in response to interstrand crosslinking (ICL), Chinese hamster AA8 cells, mouse embryo fibroblasts and HeLa cells were treated with a variety of DNA ICL-agents. All three cell lines show a dose-dependent increase in Ap4A after mitomycin C (MMC), with each showing a 7-9-fold increase in response to 100 nM MMC. Similar results were obtained with cisplatin and diepoxybutane treatment and increases in Ap4A were also observed after treatment with aldehydes. These data indicate that a significant increase in intracellular Ap4A occurs after moderate, sublethal ICL DNA damage and to aldehydes that may be responsible for endogenous ICLs. Several DNA repair defective mammalian cell lines, including Fanconi anaemia FANCG deficient NM3 cells, exhibit elevated (3-9-fold) Ap4A in the absence of exogenous DNA-damaging agents. Fractionation of extracts from MMC-treated cells by HPLC and subsequent specific luminometric analysis has revealed that the increase in “Ap4A” is due not simply to Ap4A itself, but also ADP-ribosylated Ap4A (ADPR-Ap4A) not present in undamaged cells. Using a powerful cell-free system of DNA replication comprising isolated nuclei and cytosolic extracts prepared from synchronised HeLa cells at different cell cycle phases, we determined the effect of Ap4A and ADPR-Ap4A on the initiation and elongation phases of DNA replication. Our data show that Ap4A, but not ADPR-Ap4A, is a potent inhibitor of the initiation of DNA replication (Marriott et al, 2015). To further investigate the role of Ap4A we have utilised a human cell line, KBM-7, in which the primary enzyme responsible for the degradation of cellular Ap4A, nudix hydrolase NUDT2, is inactivated. In comparison to the reference cell line, the NUDT2 knockout cell line KBM-7 exhibits an approximately 400-fold increase in cellular Ap4A (that includes ADPR-Ap4A) in undamaged cells. Intriguingly, this cell line exhibits extreme (and specific) resistance to all ICL-agents tested, including MMC, diepoxybutane and importantly, acetylaldehyde. Work so far suggests that Ap4A is a novel DNA damage-stimulated inhibitor of the initiation of DNA replication that acts particularly in response to ICL-agents including aldehydes. We propose that Ap4A plays an important role in DNA damage responses and the removal of ICL and it will be important to establish a better understanding of the involvement of this small molecule in these processes and, thereby, determine how it mediates resistance to interstrand crosslinks.

Marriott et al, DNA Repair 33 (2015) 90-100
DNA interstrand crosslinks (ICLs) inhibit DNA metabolism by covalently linking two strands of DNA and are formed by antitumor agents such as cisplatin and nitrogen mustards. Although ICL provide an absolute block to replication, they are processed by multiple complex repair pathways in humans. All of these processes share a polymerase step past a partially processed or unhooked ICL as a common step. We have developed synthetic methods for the generation of site-specific, structurally diverse major groove ICLs and studied the ability of various polymerases to bypass ICLs that induce different degrees of distortion in DNA. We found that two main factors influenced the efficiency of ICL bypass: the length of the dsDNA flanking the ICL and the structure of the crosslink bridging two bases. We furthermore synthesized an “singe nucleotide ICL”, the most thoroughly processed form of an unhooked ICL and showed that it can be readily bypassed by TLS and replicative polymerases. Our studies show that ICL repair may in some cases be accomplished without the help of TLS polymerases.
How does the Fanconi pathway promote unhooking of DNA interstrand crosslinks?

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DNA interstrand cross-links (ICLs) are highly toxic DNA lesions as they prevent DNA strand separation. ICL repair requires several classes of repair enzymes including translesion DNA polymerases, structure-specific endonucleases, recombinases, and Fanconi anemia (FA) proteins. Mutation in any one of the 16 currently known FA genes leads to the cancer predisposition disorder Fanconi anemia. However, it is still largely unclear how the FA proteins and the other repair factors collaborate to repair ICLs.

We study the molecular mechanism of ICL repair using a Xenopus egg extract-based system that recapitulates replication-dependent ICL repair in vitro. Previously we have shown that activation of the FA pathway by ubiquitylation of the FANCI-FANCD2 (ID) complex is important for a specific step in ICL repair, namely the incisions that unhook the lesion from one of the DNA strands. We next demonstrated that binding of this activated ID complex to the crosslink promotes the recruitment of the incision-complex, composed of the adapter protein SLX4 and the structure specific endonuclease XPF-ERCC1. Both XPF and SLX4 have recently been identified as FA genes highlighting their importance in ICL repair. Although ICL unhooking appears to be a major function of the FA pathway the biochemical details of this process are still unclear. We will present our latest findings regarding the role of XPF-ERCC1 and SLX4 in ICL repair.
The activation of the XPF-ERCC1 nuclease during interstrand crosslink repair by RPA

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The human XPF (ERCC4, FANCQ) and ERCC1 proteins form a heterodimeric structure-selective endonuclease that plays a critical role in maintaining genome stability. Mutations in the XPF gene cause several heritable disorders including Fanconi anemia (FA), a condition associated with defects in the repair of DNA interstrand cross-links (ICLs).

ICL repair is triggered when the nascent leading strand of a replication fork collides with an ICL, where XPF-ERCC1 catalyses the incisions that initiate ICL processing. Consistent with previous studies, in biochemical reconstitution reactions we found that XPF-ERCC1 incises simple model fork structures containing ICLs within the duplex DNA region (5' to the junction). However, we also observed that the presence of a nascent leading strand on these model forks, mimicking the effects of replication arrest at ICLs, completely eliminates the activity of XPF-ERCC1. Strikingly, addition of the XPF-interacting factor replication protein A (RPA) restores XPF-ERCC1 activity on such structures, and permits quantitative processing of structures that model replication forks stalled at ICLs. We found that the stimulation of XPF by RPA requires both protein-protein interactions between XPF and RPA, as well as the presence of a free, single-stranded DNA region on the lagging-strand template.

SNM1A is a 5'-3' exonuclease capable of processively digesting DNA substrates containing a wide variety of abnormal DNA structures and lesions, including ICLs. Additional reconstitution studies demonstrated that SNM1A is able to load onto model fork structures from XPF-ERCC1-RPA induced incisions and digest past the ICL. This in vitro reaction is extremely efficient, producing a near-quantitative processing and, therefore, 'unhooking' of ICL-containing fork substrates.

We postulate that during replication-coupled ICL repair, the arrest of nascent leading strands by ICLs produces a substrate that is inhibitory to XPF-ERCC1. This inhibition can be overcome through the dramatic stimulation of XPF-ERCC1 by RPA. XPF-ERCC1-RPA-induced incision enables SNM1A to digest past the ICL, which unhooks the ICL from the duplex DNA, enabling subsequent fork repair by translesion synthesis and homologous recombination to occur.
Crosslink repair - B3

Presenter: Sabine Smolorz

FANCM isoforms: essential cellular components, useless accessory or just delusions?

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These days, when it comes to FANCM only few things seem to be certain. The full name of FANCM, “Fanconi Anemia determinant M”, dates back to its first description as a protein acting early in the Fanconi Anemia (FA) pathway recruiting the FA core complex to sites of DNA damage. So far 19 proteins were given the prefix “FANC” due to their role in the pathogenesis of the name-giving genetic disease. All these proteins orchestrate crucial steps during interstrand crosslink repair, though their action is not restricted to this type of DNA damage. FANCM itself, however, was meanwhile stripped of its title as an FA gene due to the lack of a patient suffering from FA solely caused by mutated FANCM. Nevertheless, FANCM is still very interesting as it emerged as a breast cancer susceptibility gene in different independent studies. On the molecular level FANCM does a lot more than recruiting the FA core complex. It is known to have functions both within and outside the FA pathway ranging from checkpoint signaling to replication resumption by fork traversal.

Like for almost all human proteins there are (predicted) natural isoforms of FANCM. Interestingly human cells seem to harbor an alternative transcript of FANCM that encodes only the first 669 (of the normal 2048) amino acids comprising the helicase domain and lacking the C-terminal XPF-like endonuclease domain. Another variant arises from an alternatively spliced mRNA and lacks 26 amino acids encoded by exon 3 within the N-terminal part of the protein. The existence of further isoforms is also discussed.

There are three lines of thought for the relevance of those isoforms: (1) they are essential components of the cellular protein equipment and might contribute to the diverse spectrum of activities assigned to FANCM; (2) they exist within the cell in detectable amounts, but do not cover any functions, they rather are the product of constant inaccuracies of the cellular gene expression machinery; (3) they do not exist in the “normal” human cell, but rather their appearance in relevant databases can be attributed to technical artifacts.

Our poster presents the chase for FANCM isoforms in the transcriptome and proteome of human cells from different origin and under different conditions as well as important methodological considerations. In addition we answer the question to what extent the isoforms are able to complement the lack of full length FANCM and how their overexpression influences normal cellular behavior. Thereby we show which of the suggested lines of thought is most worth thinking and explain how to overcome methodological pitfalls transferable to the search for natural variants of other proteins.
Crosslink repair - B3

Presenter: Wouter Hoogenboom

Deciphering the role of SLX4/FANCP in DNA interstrand crosslink repair

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DNA interstrand crosslinks (ICLs) pose a challenge to cells as they block DNA replication and transcription by covalently linking the two strands of the DNA. Because ICL inducing agents are extremely toxic to rapidly dividing cells, these agents are widely used in cancer chemotherapy. However, ICLs also form endogenously, as byproducts of cellular metabolism. If left unrepaired, these lesions can cause chromosomal aberrations and lead to Fanconi anemia (FA), a disease characterized by bone marrow failure and a predisposition to cancer. The 19 currently identified FA pathway proteins, together with proteins from other DNA repair pathways, act together to repair ICLs but the molecular mechanism of this process is largely unknown.

To gain insight into this our lab employs a Xenopus egg extract-based system that recapitulates replication-dependent ICL repair in vitro under physiological conditions. We previously showed that FA pathway activation, characterized by the mono-ubiquitylation of the FANCI-FANCD2 complex, is required for the incisions that unhook the lesion from one of the strands. We demonstrated that FA pathway activation promotes the recruitment of SLX4(FANCP) that in turn recruits the endonuclease complex XPF(FANCQ)-ERCC1 to the ICL. SLX4 is a large scaffold protein with binding domains for various structure-specific endonucleases and functions in several genome maintenance processes. It is currently unclear how SLX4 is recruited to an ICL and whether its function in ICL repair is limited to XPF recruitment.

To study the role of SLX4 in ICL repair we deplete SLX4 from Xenopus egg extract and assess the ability of purified SLX4 mutants to rescue ICL repair, nucleolytic incisions and recruitment to the lesion. We found that the N-terminal domains (UBZ, MLR, and BTB) all function in ICL repair, whereas the C-terminal half of SLX4 is dispensable. Importantly, this shows that the interaction of SLX1 with the C-terminus of SLX4 is not required for ICL repair. We also showed that both the UBZ and the MLR domain are required for the recruitment of SLX4 to the ICL during repair. Recruitment of SLX4 likely does not involve a direct interaction with ubiquitylated FANCD2. Finally, we demonstrated that the interaction between SLX4 and XPF is essential for the ICL unhooking incisions and ICL repair. Overall, our results provide novel insights into our understanding of how SLX4 acts in ICL repair.
Compensatory mechanisms in head and neck cancer cells from Fanconi Anemia patients to bypass DNA interstrand crosslinks

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Fanconi Anemia (FA) is a rare recessively inherited chromosomal instability syndrome. Today, at least 17 FA disease genes have been identified, all working together in the FA pathway that counteracts replication fork blocking DNA lesions. Disruption of the FA pathway results in spontaneous chromosomal instability, hypersensitivity to DNA crosslinking agents, and a growth disadvantage compared to normal cells. Therefore it is striking that FA patients have a high risk of developing malignancies, mainly head and neck squamous cell carcinomas (HNSCC). This raises the question how FA deficient cancer cells deal with DNA interstrand crosslinks during transcription and DNA replication. We hypothesize that tumor cells with a defect in the FA pathway depend on compensatory mechanisms to bypass the replication fork blocking effect of DNA interstrand crosslinks and survive.

Standard treatment for HNSCC is not tolerated by patients with FA, because of the hypersensitivity of FA cells to DNA crosslinking agents. Finding new therapeutic targets to treat FA patients with HNSCC, and gaining more insight into the molecular mechanisms of DNA replication in FA deficient tumor cells are both essential for the future treatment of patients with FA. We use whole genome RNA interference screens on FA-HNSCC patient cell lines to identify compensatory mechanisms that enable FA squamous cells to deal with DNA interstrand crosslinks, and transform into malignant tumor cells.
Crosslink repair - B3

Presenter: Filippo Rosselli

FANC pathway - MiTF crosstalk: consequences for melanoma and Fanconi Anemia cells

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Proteins and pathways involved in DNA damage response (DDR), maintaining genetic stability and safeguarding DNA replication, act not only as caretakers against cancer initiation but also play a major role in sustaining cancer progression and resistance to pharmacological-based therapies. The FANC pathway is central in maintaining genetic stability under conditions of replication stress and its loss-of-function is causative of the cancer predisposition and chromosome fragility syndrome Fanconi Anemia (FA).

We demonstrate here that FANC proteins are over-expressed and over-activated in metastatic melanoma cells expressing the oncogenic microphthalmia-associated transcription factor (MiTF), which high expression is maintained in 80% of melanoma cases. We identified MiTF as a critical regulator of the expression of the mRNAs coding key proteins of the FANC pathway in melanoma cells and demonstrated that MiTF-silenced cells display the primary characteristics of FA cells, i.e., the cellular and chromosomal hypersensitivity to DNA interstrand crosslink-inducing agents. Moreover, FANC pathway also modulates melanoma cell migration. Our observations point to a central role of the FANC pathway in cellular and chromosomal resistance to DNA damage in melanoma cells. Thus, the FANC pathway appears as a promising new therapeutic target for melanoma treatment.

Inversely, we observed that FANC pathway loss-of-function is associated to increased expression of MiTF in both FA patient-derived and siRNA-downregulated cells. We demonstrated that the FANC pathway negatively regulates MiTF expression at the mRNA level and have obtained preliminary data suggesting that FANCD2 associates to the MiTF promoter, impeding the action of the NF-kB transcription factor. MiTF depletion increases MMC sensitivity in FANC pathway proficient cells, but does not modify the sensitivity of FA cells, supporting the hypothesis that MiTF acts on the DDR by regulating the expression of FANC proteins. Finally, we demonstrated that MiTF expression is induced in response to inflammatory stimuli, like TNF-a or H2O2 exposure. Thus, altered MiTF expression in FA could be involved in the pigmentation defects reported in patients as well as participate in the known pro-inflammatory status that is associated to FANC pathway deficiency.

In conclusion, we will present a corpus of both validated and yet preliminary data that strongly supports the existence of an epistatic relationship between MiTF and the FANC pathway, in which MiTF positively regulates FANC pathway expression that, in turn, shuts down MiTF expression, as a part of a recovery process. This circuitry appears to have an important role in melanoma resistance to chemotherapies and in some FA pathological traits.
The folate-driven one carbon cycle is a source of endogenous genotoxic formaldehyde

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Endogenous formaldehyde is a potent genotoxin which can cause widespread damage to cells. Mammals use a two-tier protection mechanism consisting of the enzyme alcohol dehydrogenase 5 (Adh5) and the Fanconi anaemia DNA repair pathway to prevent this chemical from causing lasting DNA damage. However we know very little about the origins of endogenous formaldehyde, though enzymatic demethylation is speculated to be one such important source. Despite its potent genotoxicity, formaldehyde is also used as a building block for the synthesis of nucleotides and other essential molecules through one-carbon metabolism. Surprisingly, we have found that this fundamental metabolic pathway also releases formaldehyde. This source of formaldehyde can inflict lethal DNA damage in human and chicken cell lines and also murine haematopoietic progenitors that are defective in two-tier protection. The realisation that endogenous formaldehyde can come from instability of certain key components of the folate cycle underscore the delicate balance between essential metabolism and the generation of a ubiquitous genotoxin.
Crosslink repair - B3

Presenter: Román González-Prieto

SUMOylation and PARylation cooperate to recruit and stabilize SLX4 at DNA damage sites

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SUMOylation plays important roles in the DNA damage response. However, whether it is important for interstrand crosslink repair remains unknown. We report that the SLX4 nuclease scaffold protein is regulated by SUMOylation. We have identified three SUMO interaction motifs (SIMs) in SLX4, mutating all of which abrogated the binding of SLX4 to SUMO-2 and covalent SLX4 SUMOylation. An SLX4 mutant lacking functional SIMs is not recruited to PML nuclear bodies nor stabilized at laser-induced DNA damage sites. Additionally, we elucidated a novel role for PARylation in the recruitment of SLX4 to sites of DNA damage. Combined, our results uncover how SLX4 is regulated by posttranslational modifications.
POSTER SESSION C1: 'Double Strand Break Repair'

Poster viewing: Sunday, April 17, 20:00 – 22:00 h

Discussion: Monday, April 18, 16:55 – 18:25 h
Double strand break repair - C1

Exploiting the DNA damage response to improve peptide receptor radionuclide therapy outcome

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Targeted cancer therapy increases treatment efficacy and reduces toxic side effects. For neuroendocrine tumors targeted therapy with lutetium-labeled somatostatin analogues (peptide receptor radionuclide therapy or PRRT) has successfully been applied in clinical trials. Similar approaches may also be feasible for many other tumor types, including tumors expressing prostate specific membrane antigens (PSMA). PRRT of metastasized neuroendocrine tumors is promising because patients survive longer compared to other treatments, although overall cure rates are still low. To achieve a better tumor response, the administrated dose cannot simply be increased because of dose limiting toxicity in bone marrow and kidneys. Therefore, to improve the current treatment outcome, we investigated the DNA damaging effects of PRRT with the purpose to enhance these effects through modulation of the DNA damage response. We aim specifically to sensitize the (replicating) tumor cells without further damage to healthy tissues using PARP inhibitors (PARPi). Although PRRT induces cytotoxic double strand breaks (DSBs), the majority of the induced lesions are single strand breaks (SSBs) that require PARP activity for repair. If these breaks are not repaired efficiently (by using PARPi), they cause replication fork arrest and DSB formation in replicating cells.

Using several molecular biological and (fluorescent) microscopy techniques, we have characterized the induced DNA damage and the DNA damage response after PRRT. Our results show that cells treated with PRRT showed accumulation of the DSB repair protein 53BP1 in nuclear foci, indicating the production and repair of DSBs. These foci were detectable for up to 3 days after treatment. By contrast, treatment with unmodified lutetium causes transient DSBs that were no longer detectable after 1 day. Interestingly, addition of PARPi led to increased and prolonged DNA damage. This was also confirmed by the increase of cells with chromosomal damage, shown by micronuclei. PRRT eradicated cells in a dose dependent manner and cell killing via apoptosis was strongly enhanced by PARPi.

In conclusion, we show that PRRT triggers the DNA damage response by producing DSBs in preclinical models. Through modulation of the DNA damage response with PARPi, we were able to enhance the DNA-damaging effects of PRRT, specifically in replicating cells. We expect that our results will eventually improve the current PRRT outcome, leading to increased patient survival rates.
Double strand break repair - C1

Presenter: Alex Zelensky

H2Ax phosphorylation dependent integration of extrachromosomal DNA in Human and and mouse cells exposed to very low ionizing radiation doses

Alex Zelensky(1), Mascha Schoonakker(1), Joost Schimmel(2), Inger Brandsma(3), Marcel Tijsterman(2), Dik van Gent(1), Jeroen Essers(1), Roland Kanaar(1)

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Extrachromosomal DNA can integrate into the genome with no sequence specificity producing an insertional mutation. This process, which is referred to as random integration (RI), requires a double stranded break in the genome. Inducing additional breaks by various means, including ionizing radiation, increases the frequency of integration. Multiple previous studies investigated this effect using doses above 0.5 Gy. Here we report that physiologically relevant doses of irradiation (10-100 mGy), well within the range produced by medical equipment, stimulate RI of the transfected and viral episomal DNA in human and mouse cells with an extremely high efficiency by up to 10 fold. Unexpectedly, given the high sensitivity of the process, we found that cells deficient in the key DNA repair pathways (NHEJ and HR) are no different from the wild type cells, but that blocking H2ax S139 phosphorylation inactivates the process. Neither of the two known γH2AX interactors – 53BP1 and MDC1 – are required for stimulation. The robust responsiveness of the RI to very low doses of induced damage allows it to be used as a straightforward yet sensitive mutagenesis assay. The complete dependence of the stimulated RI on γH2AX is more drastic than the known consequences of H2AX deficiency, while independence of 53BP1 and MDC1 demonstrates that a yet unidentified signaling process also recognizes this crucial post-translation modification.
A new case of Ataxia-Telangiectasia Like Disorder (ATLD) with typical clinical features and mild impairment of the ATM pathway

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Ataxia-Telangiectasia-Like Disorder (ATLD) is a rare genomic instability syndrome caused by bi-allelic mutations of MRE11A. It is characterized by a progressive cerebellar ataxia and typical karyotype abnormalities. These symptoms are common with Ataxia Telangiectasia, which is consistent with the key role of MRE11 in ATM activation. However, impairment of the ATM pathway after ionizing irradiation (IR) is reported to be highly variable in the different ATLD patients assessed. We report the first French ATLD patient, with two unreported MRE11A mutations. In order to confirm the deleterious effect of the mutations, transcript and protein analyses were conducted. Functional consequences on the activation of the ATM pathway were studied through the analysis of the phosphorylation of ATM and its main targets (p53, CHK2 and KAP1) after IR. The patient had typical ATLD clinical features with progressive cerebellar ataxia since early childhood. Mutations lead to highly conserved amino-acid changes or splicing defects. The level of MRE11 was dramatically reduced. Contrasting to her typical clinical features, activation of the ATM pathway after IR in the patient’s cell line was found almost normal. This report highlights the variability of ATM pathway activation defects in MRE11A mutated patients, which do not correlate with the gravity of neurologic features.
Heavy particle irradiation produces complex DNA double strand breaks (DSBs) which can arise from primary ionization events within the particle track. Using imaging with deconvolution, we previously showed that at 8 hours after exposure to Fe ions (200 keV/micrometer), gH2AX foci along the particle track were large and encompassed multiple smaller and closely localized foci (clustered gH2AX foci). Importantly, clustered gH2AX foci induced by heavy particle radiation caused prolonged checkpoint arrest compared to simple gH2AX foci after X-rays. Recently, we developed an assay to simultaneously visualize both DSB sites and a chromosome position using Immunofluorescence and fluorescence in situ hybridization. Importantly, the frequency of gH2AX foci at the chromosome boundary of chromosome 1 after carbon ion (70 keV/micrometer) irradiation was >3-fold higher than that after X-ray irradiation. This observation is consistent with the idea that particle irradiation generates DSBs at the boundaries of two chromosomes along the track. Further, we showed that resolution of gH2AX foci at chromosome boundaries is prevented by inhibition of DNA-PKcs activity, indicating that the DSB repair is NHEJ-dependent. Finally, we found that gH2AX foci at chromosome boundaries after carbon-ion irradiation contain DSBs undergoing DNA-end resection, which promotes microhomology mediated end-joining during translocation formation. Together, our study suggests that frequency of cluster DSB formation at chromosome boundaries is associated with the incidence of chromosomal translocation, supporting the notion that the spatial proximity between breaks is an important factor in translocation formation.
Double strand break repair - C1

Presenter: Burkhard Jakob

Heterochromatic DNA Repair: Establishing Fluorescence Lifetime Imaging Microscopy to Monitor Radiation-Induced Chromatin Decondensation

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The repressive environment of heterochromatin (HC) makes the processing of HC double-strand breaks (DSBs) and the maintenance of genomic stability a challenging task for the cellular repair system after a radiation insult. To address the question of heterochromatic DNA accessibility and damage recognition, we applied targeted single ion irradiation to produce strictly localised DSBs directly within constitutive murine HC compartments (chromocentres). Besides a fast damage response indicated by the recruitment of repair proteins into HC and the phosphorylation of H2AX inside the chromocentre, we showed a fast local decondensation of the HC at the sites of ion traversal, both arguing against the hypothesis of a generally limited accessibility of damage response proteins to HC. The radiation-induced decondensation is accompanied by a relocation of the induced DSBs to the adjacent euchromatin (EC), which might be a requirement for subsequent DNA repair. This is in accordance with observations after sparsely ionising irradiation, where γH2AX foci at later times post-irradiation are generally found at the EC/HC interface. Surprisingly the decondensation can also be observed in ATM deficient cells, ruling out KAP1 phosphorylation as sole mechanism. Up to now it is not clear which factors are responsible for this HC decondensation and if the decondensation itself is the driving force for the observed damage relocation.

To address these important questions we aim at identifying protein factors involved in the decompaction process. However, whereas the radiation-induced decondensation can readily be observed in fixed samples after ion irradiation, it proved difficult to reveal these changes using intensity based measurements in living cells. Therefore alternative approaches are needed and we exploit Fluorescence Lifetime Imaging Microscopy (FLIM) as a promising spatially resolving technique, not relying just on the amount of bound DNA dye. Here we screened several DNA dyes on their suitability to serve as potential chromatin compaction probes using FLIM. The most important characteristics of promising probes are a pronounced compaction dependent lifetime contrast as well as sufficient photostability to allow multiple repeated recordings. Utilising enzymatically/chemically induced changes of the chromatin structure, promising candidates were benchmarked against the known FRET-sensor pair H2B-GFP/-RFP which we expressed in the same murine cell line. Especially for the HC decondensation using HDAC inhibition, a more than 5 fold increase in sensitivity could be achieved. We present first evidence for the detection of a locally confined heterochromatin decondensation at sites of ion traversal in irradiated murine chromocentres using our FLIM approach. A confocal FLIM scanner now installed at the GSI accelerator beamline microscope will allow the sensitive readout of the chromatin status in living cells during future ion irradiation experiments.

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Sensitization of Cancer Cells to Ionizing Radiation by Targeting DNA DSB Repair

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Radiation therapy is based upon the induction of lethal DNA damage, primarily DNA double-strand breaks (DSB), in tumor cells. DNA DSBs can potentially be repaired via Non-Homologous Recombination (NHEJ) or Homologous Recombination (HR). Due to the DNA DSB repair, cancer cells could be rescued from cellular death and might resist radiation treatment. This study aims to enhance the effect of radiation treatment by suppressing the DNA DSB repair systems with hyperthermia (HT) or DNA-PKcs inhibitor NU7441.

The sensitizing effect of HT and NU7441 after radiation treatment was studied in two cervical cancer cell lines (SiHA, HELA) and two breast cancer cell lines (MCF7, T47D). Clonogenic survival analysis, DNA DSBs foci studies, levels of apoptosis, and effects on cell cycle distributions after different combinations of treatments were investigated. Specificity of HT treatment was assessed by detecting the accumulation of DSB repair proteins RAD51, MRE11 and γ-H2AX at the break ends. The effect of NU7441 on DNA-PKcs activity was measured with a DNA dependent protein kinase assay. Radiation treatment combined with HT and NU7441 was also tested in an in vivo xenograft system of human cervical cancer (SiHa) growing in nude mice.

Clonogenic analysis revealed significantly lower survival rates after combination treatments compared to RT alone in all assessed cell lines, with the lowest surviving fraction after the triple treatment (RT with both HT and NU7441). This was confirmed by induced levels of apoptosis, a 2.5 fold increase after the triple treatment (2 fold induction after double treatment, RT with either NU7441 or HT). The induction and disappearance of γ-H2AX foci revealed a delay in DNA DSB repair after the addition of HT and NU7441 to radiation. Significantly more residual foci were found 6 h post triple treatment compared to radiation alone and controls. No differences in number of foci is detected 24 h after treatment, indicating a slower DSB repair rate rather than no repair in the surviving cells after the triple treatment. Radiosensitization by HT and NU7441 is accompanied with an increased G2 arrest. Tumor growth delay analysis in vivo confirmed these findings. Growth curves showed a significant reduction in tumor growth after RT with HT and NU7441 compared to RT alone or the double treatment (RT+HT or RT+NU7441).

In conclusion, hampering DNA DSB repair by HT and NU7441 leads to an enhanced radiotoxicity. As the combination of both HT and DNA-PKcs inhibition with radiotherapy has not been investigated yet, the results of this study are both novel and promising for clinical use. The use of combined hyperthermia and DNA-PKcs inhibitor NU7441 in the clinic could contribute to a more efficient treatment of cancer patients.
The genome is a long, folded three dimensional chromatin polymer with a definite 3D structure and hierarchical organization. This organization leads to the formation of ‘Topologically Associated Domains’ or TADs that fold into distinct 3D structures iteratively throughout the nucleus. The 3D organization confines the chromosomes into distinct territories called chromosome territories. Some of the chromatin is also associated with the periphery of the nucleus or lamin (lamin associated domains/ LADs). Based on histone marks, the chromatin is also divided into a transcriptionally active compartment (euchromatin) and a transcriptionally inactive compartment (heterochromatin). This compartmentalization of the genome is important for maintaining genomic stability.

Although there is extensive knowledge of double strand break (DSB) sensing and subsequent repair, the genomic landscape of the occurrences of DSBs as a result of different conditions and in different cell types remains largely unknown because of a lack of methods which can map these breaks with a high resolution. Since it is already known that DSBs exhibit a limited mobility, the position of the break might influence its repair. Work from our lab has shown earlier that non-random global genome organization is a key factor in maintaining genomic integrity. It has also been shown that transcriptionally active chromatin uses homologous recombination to repair DSBs.

We are trying to understand the influence of 3D genome organization on DNA repair. Since it is not very apparent whether there is an inherent hierarchy in repairing different genomic sequences, i.e., which breaks are repaired sooner than others, the goal is to precisely follow the kinetics of DSB repair and map in high resolution the location of the breaks as they get repaired. This is being done by two parallel approaches: in situ break labeling, enrichment on streptavidin and next-generation sequencing (BLESS); and ChIP-Seq with γH2AX.

We are studying DSB repair kinetics at several time points after treating mouse embryonic stem cells (J1 ES cells) and human fibroblasts (IMR90) with a saturating dose of the DNA damaging agent neocarzinostatin. This approach along with ChIP-Seq data enables us to map in high resolution, break regions as they get repaired. Since the order of DNA damage repair is not known, a high resolution map of the break repair kinetics along with data-sets from ChIP-Seq will give clues as to whether the repair occurs in a gene/region specific manner or depends on the 3-D organization of the genome. Using these two approaches to look at the repair kinetics will help in understanding if the DNA damage repair happens at random or does it follow a particular order for repair. The data generated along with correlations with other datasets available (ENCOD) will also lead to a better understanding of the occurrence of translocations at certain regions.
Glioblastomas (GBM) are lethal brain tumors for which ionizing radiation (IR), in combination with temozolomide (TMZ), is the first line of therapy after surgical resection of the tumor (1). Unfortunately, not all GBMs respond to therapy, and most of them quickly acquire resistance to TMZ and recur. In order to develop more effective and rational treatments for GBM, it is crucial to understand molecular mechanisms underlying radio- and chemo-resistance. We find that protracted TMZ treatment of mice bearing orthotopic tumors (derived from GBM9 neurospheres) leads to acquired-TMZ resistance resulting in tumor recurrence. In order to understand the basis for therapy-driven TMZ resistance, we generated and functionally characterized ex-vivo cultures from the primary and recurrent tumors. We found that cell lines derived from recurrent (TMZ-treated) tumors were more resistant to TMZ in vitro compared to cell lines derived from primary (untreated) tumors. We also found that the increased resistance to TMZ was due to the augmented repair of TMZ-induced DNA double strand breaks (DSBs). TMZ induces DNA replication-associated DSBs that are repaired primarily by the homologous recombination (HR) pathway. We found that cell lines from recurrent cultures exhibited faster resolution of Rad51 foci and higher levels of sister chromatid exchanges (SCEs), implicating augmented homologous recombination (HR) in TMZ resistance. We have recently shown that CDKs 1 and 2 promote HR in S and G2 phases of the cell cycle, in part, by phosphorylating the exonuclease EXO1 (2). We hypothesized, therefore, that blocking CDKs 1 and 2 might be a viable strategy for re-sensitizing recurrent tumors to TMZ. Indeed, we found that CDK inhibitors, AZD5438 and Roscovitine, could attenuate HR in the recurrent TMZ-resistant cell lines, resulting in significant chemo-sensitization. While TMZ-induced DSBs are primarily repaired by HR, IR-induced DSBs would be mainly repaired by the non-homologous end joining (NHEJ) pathway. We, therefore, developed another approach to sensitize these tumors to both radiation and TMZ by using a dual PI3K/mTOR inhibitor, NVP-BEZ235, to block both DNA-PKcs and ATM, key enzymes in the NHEJ and HR pathways, respectively (3). We found that NVP-BEZ235 inhibited both DNA-PKcs and ATM in orthotopic tumors generated from GBM9 neurospheres, thus blocking the repair of TMZ- and IR-induced DSBs in these tumors. Hence NVP-BEZ235, administered with IR, could attenuate tumor growth, and extend survival of tumor-bearing mice. Importantly, inhibition of DSB repair was more pronounced in tumor cells compared to normal brain cells, thereby providing a larger therapeutic window (4). In sum, these studies indicate that augmented DSB repair may underlie radio- and chemo-resistance in GBM, and provide support for DNA repair inhibition as an effective strategy for improving the efficacy of GBM therapy.
Double strand break repair - C1

Presenter: Bjoern Schwer

Long Neural Genes Harbor Recurrent DNA Break Clusters in Neural Stem/Progenitor Cells

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Repair of DNA double-strand breaks (DSBs) by non-homologous end-joining is critical for neural development, and brain cells frequently contain somatic genomic variations that might involve DSB intermediates. We now use an unbiased, high-throughput approach to identify genomic regions harboring recurrent DSBs in primary neural stem/progenitor cells (NSPCs). We identify 27 recurrent DSB clusters (RDCs) and, remarkably, all occur within gene bodies. Most of these NSPC RDCs were detected only upon mild, aphidicolin-induced replication stress, providing a nucleotide-resolution view of replication-associated genomic fragile sites. The vast majority of RDCs occur in long, transcribed, and late-replicating genes. Moreover, almost 90% of identified RDC-containing genes are involved in synapse function and/or neural cell adhesion, with a substantial fraction also implicated in tumor suppression and/or mental disorders. Our characterization of NSPC RDCs reveals a basis of gene fragility and suggests potential impacts of DNA breaks on neurodevelopment and neural functions.
Double strand break repair - C1

Presenter: Carole Beck

The absence of PARP3 exacerbates centrosome amplification and reduces the survival and tumor progression of BRCA1-mutated cell lines

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Poly(ADP-ribosylation) is a post-translational modification of proteins mediated by Poly(ADP-ribose) polymerases (PARPs, 17 members). We have recently reported that PARP3 (also known as ARTD3), the third member of this family, is a critical regulator of mitotic progression. A fraction of PARP3 localizes to centrosomes and has been identified as part of a protein complex containing the telomeric PARP Tankyrase 1 and the mitotic factor NuMA. PARP3 acts as a positive regulator of the Tankyrase 1 mediated PARylation of NuMA that in turn will control spindle microtubule stabilization and promote telomere integrity (1). It has been shown previously that the silencing of Tankyrase 1 exacerbates the centrosome amplification associated with BRCA1 deficiency and reduces the survival of BRCA1-depleted cells (2). In addition, both Tankyrase 1 and BRCA1 have been implicated in the control of mitotic progression and centrosome function (3-4). According to these observations, we decided to explore the impact of the absence of PARP3 in cancer cell lines mutated or depleted for BRCA1. We describe PARP3 as a novel contributor in the regulation of centrosome duplication and stability. We demonstrate that the absence of PARP3 exacerbates centrosome dysfunction in BRCA1 mutated breast cancer cells, reduces their survival and delays tumor progression on xenografts in nude mice.

(2) McCabe, N. et al. Oncogene 2009, 28, 1465
Double strand break repair - C1

Presenter: Dipanjan Chowdhury

DNA damage induced alternative polyadenylation of DNA repair gene transcripts influences the DNA damage response

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The optimum level of DNA repair proteins is critical for an efficient DNA damage response. However, global transcription is repressed upon DNA damage potentially limiting the production DNA repair factors. Therefore the cell must rely on other mechanisms to ensure the availability of DNA repair proteins. In recent years, alternative polyadenylation has emerged as an important co-transcriptional player in gene regulation. We performed 3’RNA-seq analysis of 103 DNA repair genes and found that about 40% of them display alternative polyadenylation (APA) events. We focused on Mre11, an important factor in the DNA damage response, and found that Mre11 3’UTR length is highly regulated by DNA damage. Exposure to ionizing radiation (IR) in G1 phase of the cell induced Mre11 3’UTR switching to longer 3’UTR transcripts while IR in S phase favored shorter Mre11 3’UTR isoforms. Importantly, reporter assays reveal that Mre11 transcripts with shorter 3’UTR displayed increased protein levels compared to the longer 3’UTR variant. The IR induced APA event regulating Mre11 3’UTR length is mediated by ATM. ATM phosphorylates at least two important factors of the cleavage and polyadenylation machinery. We observe that forced expression of Mre11 long 3’UTR using CRISPR knockout of the proximal polyadenylation cleavage site resulted in ~50% decrease in cellular Mre11. From the functional standpoint these cells are significantly more sensitive to IR. Overall this phenotype is reminiscent of ataxia-telangiectasia-like disorder (ATLD), a disease caused by mutation in Mre11 and characterized by lower levels of Mre11 protein and radiosensitivity. Together, our results highlight for the first time that ATM-mediated DNA damage signaling regulates alternative polyadenylation of DNA repair genes and that this regulation is important for DNA repair.
Multiple DSBs in S. cerevisiae: the role of chromosome organization in genome maintenance.

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To ensure the fidelity of the transmission of genetic information over generations, cells have evolved complex surveillance and maintenance mechanisms. Impairment of proper DNA repair metabolism can lead to genomic instability, which enhances the rate of cancer development. Although the signaling mechanisms of repair pathways have been intensively studied, the spatio-temporal regulation of the DNA damage response (DDR) remains relatively unexplored. Notably, the influence of genome organization on DNA repair mechanisms is only partially understood.

We have developed a system able to unravel the role played by genome organization during the repair of multiple specific Double Strand Breaks (DSBs) by inducing multiple DSBs at known positions within different chromatin contexts and nuclear compartments. The controlled expression of the AsiSI restriction enzyme (RE) allows the targeting and potential cut of 37 discrete 8pb loci dispersed throughout the genome of Saccharomyces cerevisiae. The induction of AsiSI leads to the rapid accumulation of the phosphorylated form of checkpoint protein Rad53, indicating the ability of the system to trigger DDR. RE expression did not appear toxic for yeast cells, while it impaired cell growth when efficient repair was impaired. Homologous recombination activation was observed through the accumulation of rad52-GFP foci in 30% of the cells, at a level similar to what is observed upon Zeocin treatment, a genotoxic drug that induces random DSBs. Quantitative PCR determined cleavage efficiency of the 37 AsiSI sites and shows an increased presence of cuts at sites within subtelomeres, revealing an increase in the accessibility of these sites to the RE and/or a decrease in repair at these positions. Dynamics of subtelomeric sites upon RE induction was increased, inducing global changes in genome contacts as detected by Chromosome Capture Configuration (3C).

Our study of global genome stability indicates how important chromosome organization is for DSB destiny and paves the way to a better understanding of the DDR control.
Double strand break repair - C1

Presenter: Eva Vesela

Quantitative evaluation of laser-induced striation patterns in large amounts of live cells

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Laser micro-irradiation is a valuable technique which enables precise photo-manipulation of cellular ultrastructure keeping the surroundings relatively intact. This approach is very convenient for investigation of protein mobility involving fluorescence bleaching and recovery experiments (FRAP). Moreover, certain laser wavelengths can induce local DNA damage which is valuable feature for studying proteins recruited to various DNA lesions. Common micro-irradiation approaches based on laser scanning microscopes involve manual definition of region of interest (ROI) which is then exposed to a particular laser and followed over time. Unfortunately, the technique is generally limited to rather low number of cells because of excessive work demands guiding the irradiation, acquisition and analysis. Therefore, a new approach for induction of particular photo-effect and software for quantitative image analysis were developed. Newly, a standard confocal laser scanning microscope is set to enable photo-manipulation in a precisely defined pattern of collinear stripes exposing simultaneously all the cells within the acquisition area. The pattern can be generated very fast and due to known parameters the photo-effects can be followed over time in dozens of cells in parallel. The method was optimized for evaluation of laser-induced DNA damage, FRAP and for both live and fixed cells. Such a quantitative analysis of large amount of photo-manipulated cells provides versatile approach for a wide range of applications in biomedicine allowing robust statistical testing. Moreover, the method has all presumptions for full automation and application in high content/throughput screens aimed e.g. at the search for new drugs and/or factors interfering with DNA damage response. Here we present how the method was used to evaluate the contributions of different components of BRCA1-A complex to BRCA1 protein recruitment to sites of DNA damage. Subsequently, recruitment of DNA damage repair proteins was investigated, e.g. Rad51 as a common marker of homologous recombination and RPA as a marker of single stranded DNA.

Double strand break repair - C1

Presenter: Federica Evangelista

Role of the mammalian nuclear pore complex-associated TREX-2 complex in DNA repair

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The maintenance of proper genetic information is essential to ensure correct cellular functions and to avoid genomic instability that is a hallmark of cancer. Different damaging sources, endogenous or exogenous, constantly challenge the DNA, leading to different types of lesions. Double Strand Breaks (DSBs) are among the most cytotoxic, because they can lead to chromosomal translocations and tumorigenesis. Cells respond to DNA damage by initiating a signaling cascade, named the DNA Damage Response (DDR), which leads to the activation of cell cycle checkpoints to arrest the cell cycle and to allow DNA repair before cell division. The DDR is characterized by the recruitment and extensive spreading of DDR proteins around the lesions, having as main hallmark the phosphorylated histone variant H2AX (γH2Ax). Recent work have revealed an exciting role for the Nuclear Pore Complex (NPC) and its interacting proteins, in the maintenance of genome integrity (Le Maitre et al., 2012; Bukata et al., 2013; Khadaroo et al., 2009).

Here we focus on the role of the TRanscription and Export complex 2, TREX-2, mainly involved in the mRNA export and stably associated with the NPC (Umlauf et al., 2013), in the maintenance of genomic stability in mammalian cells, as it has been already suggested in budding yeast (Gallardo and Aguilera, 2000; Gonzalez-Aguilera et al., 2008; Bermejo et al., 2011; Nino et al., 2016).

We demonstrate that depletion of different subunits of the TREX-2 complex leads in persistent DNA damage compared to control cells, as measured by increased γH2AX intensity and S3BP1 foci (two main markers of DNA damage) after DNA damage induction by Neocarzinostatin (NCS).

Moreover, clonogenic survival assay of human HeLa cells showed that cells depleted for the TREX-2 complex exert increased sensitivity to genotoxic stress compared to controls. These findings and other preliminary findings that will be presented suggest a role for the mammalian TREX-2 complex in efficient DNA repair.

Efficient DSBs repair requires a complex interplay involving many actors that are not fully characterized. With our present study we add a new piece to this complex puzzle linking the NPC-associated TREX-2 complex to DSB repair in mammalian cells.
Double strand break repair - C1

Identification of novel TDP2 synthetic lethal interactions

Guido Zagnoli Vieira(1), Helen Pemberton(2), Chris Lord(2), Keith Caldecott(1)


The DNA repair enzyme Tyrosyl-DNA phosphodiesterase 2 (TDP2) has been implicated in cellular and organismal sensitivity to chemotherapeutic topoisomerase 2 (TOP2) poisons, transcriptional competency during TOP2-dependent gene expression, the maintenance of normal neurological function, and more recently in viral infection. To achieve a better understanding of the relevance of TDP2 in cancer we deleted TDP2 in the breast cancer cell lines CAL51 and MCF7 and in immortalized human RPE-1 cells, using the CRISPR/Cas9 technology. We show that TDP2 knockout cells show cellular hypersensitivity and delayed DSB repair kinetics following treatment with the TOP2 poison etoposide. In addition, we performed a high throughput siRNA screen for TDP2 synthetic lethal relationships that might have utility form a mechanistic and/or clinical cancer perspective. Some of these results will be presented and discussed.

(4) Pommier Y. et al. 2014, Tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2). DNA Repair, 19, 114-129;
Leukocyte DNA damage after reduced and conventional absorbed radiation doses using 3rd generation dual-source CT technology

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Purpose: Computed tomography (CT) scans are an important source of ionizing irradiation (IR) in medicine that can induce a variety of DNA damage in human tissues. With technological improvements CT scans at reduced absorbed doses became feasible presumably lowering genotoxic side effects.

Materials and methods: For measuring DNA damage we performed γH2AX foci microscopy in peripheral blood mononuclear cells (PBMC) after exposure to reduced and conventional absorbed radiation doses using 3rd generation dual-source CT (DSCT) technology.

Results: CT scans performed at reduced absorbed doses of 3 mGy induced significant lower levels ($p < 0.0001$) of DNA damage (0.05 focus per cell ± 0.01 [mean ± standard error of mean]) at 5 min after IR compared to conventional absorbed doses of 15 mGy (0.30 focus per cell ± 0.03). With ongoing DNA repair background γH2AX foci levels (0.05 focus per cell) were approached at 24 h after CT with both protocols.

Conclusions: Our results provide evidence that reduced absorbed doses mediated by adjusted tube current in 3rd generation DSCT induce lower levels of DNA damage in PBMC compared to conventional absorbed doses suggesting a lower genotoxic risk for state-of-the-art tube current reduced CT protocols.
Recruitment of BRCA1 to DNA damage foci by the large, nuclear, K63-linkage specific deubiquitinase (DUB) complex BRCA1A is essential for DNA repair. BRCA1A shares three subunits, including the active DUB, with BRISC, a cytoplasmic DUB complex involved in regulation of inflammation and the immune response. BRCA1A is recruited to sites of DNA damage via adaptor protein Rap80, while BRISC binds to metabolic enzyme SHMT2 in the cytosol. In light of the substantial degree of identity between BRCA1A and BRISC, it is currently not understood how the markedly different biological functions arise. We have determined the crystal structure of mammalian BRCA1A complex and gained insight into the structural basis of Rap80 recruitment. We found that adaptor proteins Rap80 and SHMT2 bind to their respective complexes in substantially different manner. Our structure explains how BRCA1A was repurposed for its function in DNA repair and suggests a structural basis for the differing biological functions of BRCA1A and BRISC.
Double strand break repair - C1

Presenter: Kim de Luca

Mapping DNA double-strand break repair in single cells with DamID

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DNA double-strand breaks (DSBs) are particularly toxic lesions because failure to resolve them can induce cell cycle arrest, apoptosis, and is associated with diseases such as cancer and neurodegeneration. Despite extensive research into the molecular pathways of DSB sensing and repair, little is known about management of breaks within different chromatin contexts. The local chromatin environment influences the incidence rate of DSBs, the activation of the DNA damage response (DDR), and the choice between DSB repair pathways. Current techniques to study DSB repair in a chromatin context (e.g. by identifying DNA-binding proteins or epigenetic modifications with ChIP-seq) are inadequate. For an appropriate genome-wide resolution, populations of cells are required, which disregards cell-to-cell variability and the (assumed) inherent stochasticity of DSBs. Moreover, direct identification of DDR components at break sites is a temporal 'snapshot' and as a result does not address repair fidelity. Importantly, the response of an individual cell to a damage insult has not been systematically addressed.

We employ two complementary methods to map and track DSB repair in single cells, based on the DNA adenine methylation identification (DamID) technique. 1) Single-cell DamID allows genome-wide profiling of contacts between DNA and DSB repair proteins. 2) The m6A-Tracer is a live microscopy tool to visualize dynamics of DSB foci over extended periods of time. We currently use the U2OS-DlvA cell system in which an endonuclease generates DSBs at defined positions. The project aims to understand which chromatin regions are more vulnerable to DSBs, gain insight into repair accuracy and efficiency, distinguish between pathways, and relate cellular fate to DNA damage. Taken together, this approach enables study of DSB repair in unprecedented spatio-temporal resolution.
Identification of new proteins involved in Double Strand Break repair at the nuclear lamina

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DNA double strand breaks (DSBs) constitute an important threat to genome stability as they correlate with chromosomal translocations and cancer. Cells repair DSBs through different pathways, which can be faithful or mutagenic, and the balance between them at a given locus must be tightly regulated to preserve genome integrity. Although, much is known about DSB repair factors, how the choice between pathways is controlled within the nuclear environment is not understood. We have previously shown that DNA breaks induced at the different compartments of the nuclear periphery (lamina and pores) are repaired by different pathways (1). Here, we aim to understand the mechanism that leads to differential pathway choice at different compartments. We have combined the DamID and BioID technologies and generated an innovative cellular system to identify factors that are specifically recruited to the nuclear lamina upon DNA damage by quantitative proteomics. To this end, the bacterial Adenine methylase (Dam) is fused to LaminB1, resulting in the methylation of Lamin-associated DNA. Subsequently we have fused BirA* to an engineered protein module that binds directly to Adenine-methylated DNA (m6A-Tracer), tethering the enzymatic activity of BirA* to the LADs. Using this cellular system, we have purified and identified all biotinylated proteins in proximity with the Lamin-associated DNA by quantitative Mass spectrometry in the absence and presence of DNA damage. This work provides novel insights on DNA repair organization in the highly compartmentalized nucleus and its contribution to genomic stability with far reaching implications for the understanding of the pathogenesis of human disease and cancer.

Phosphorylated nuclear Dicer processes double-stranded RNA in the DNA damage response

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The RNAse III-type endonuclease Dicer (DICER1) is a key component of the human RNA interference (RNAi) pathway and well known for its role in cytoplasmic microRNA (miRNA) processing. Emerging evidence suggests non-canonical functions for RNAi components in regulation of RNA metabolism, genome maintenance and chromosome stability (1). Interestingly, a novel class of non-coding RNA, termed DNA damage RNA (DDRNA) derived from DNA double-strand breaks (DSBs) was shown to control the DNA damage response (DDR) in a Dicer-dependent manner (2, 3). However, mechanistic details of DDRNA biogenesis as well as Dicer activation upon DNA damage are unknown. Here we show that the Extracellular Signal-Regulated Kinase complex (ERK) phosphorylates Dicer upon DNA damage in human cells. This ERK activity triggers nuclear localisation of phosphorylated Dicer (p-Dicer) and its recruitment to DSBs in a transcription and double-stranded RNA (dsRNA) dependent manner. Inhibition of ERK activity impairs p-Dicer recruitment to DSBs and causes accumulation of dsRNA. Furthermore, Dicer depletion causes persistence of DNA damage and delayed DDR. We conclude that DNA damage activation of ERK signalling acts as a molecular switch for p-Dicer sub-cellular redistribution that allows localized processing of nuclear dsRNA into DDRNA and promotes efficient DDR.

(2) Francia et al., 2012, Nature
(3) Wei et al., 2012, Cell
Double strand break repair - C1

Presenter: Katarina Chroma

Proteotoxic stress-associated overexpression of RNF168 ubiquitin ligase alters DNA repair pathway choice, genomic instability and responses to genotoxic treatments in subsets of human cancers

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DNA double-strand break (DSB) signaling and repair is crucial to preserve genomic integrity and maintain cellular homeostasis. During the DNA damage response (DDR), histone ubiquitination by the apical RNF168 ubiquitin ligase is a critical event, which orchestrates the recruitment of downstream effectors, e.g. BRCA1 and 53BP1. While 53BP1 licenses DSBs repair in G1 through non-homologous end joining (NHEJ), BRCA1 initiates DNA end resection and repair of S phase DSBs through homologous recombination (HR). Overexpression of RNF168 results in hyper-accumulation of 53BP1 on chromatin that promotes mutagenic NHEJ even outside of the G1 phase of the cell cycle.

Under conditions of ubiquitin starvation, mostly resulting from proteotoxic stress, the ubiquitin dependent accrual of DDR proteins at the sites of damage is impaired and the ubiquitin mediated DDR is attenuated. A common manifestation of the attenuation is disappearance of the 53BP1 and BRCA1 proteins from irradiation induced foci (IRIF).

However, we have identified several cancer cell lines that display 53BP1 recruitment to IRIFs under the conditions of proteasome inhibitor (Bortezomib or MG132) induced proteotoxic stress i.e. under substantial depletion of nuclear free ubiquitin levels. We show that central to this phenotype is an elevated level of the RNF168 ligase that enables more efficient exploitation of the residual free ubiquitin. Elevated RNF168 levels harboring cells are more resistant to combined treatment by gamma irradiation and proteasome inhibitor which implies that the RNF168 upregulation may have arisen as an adaptation to constant proteotoxic stress experienced by tumor cells. Moreover, the overabundance of RNF168 E3 ligase causes a boost in 53BP1 recruitment thus shifting the repair pathway balance towards the non-homologous end-joining (NHEJ). Importantly, this imbalance might account for increased sensitivity of particular HR proficient breast cancer lines towards PARP1 and topoisomerase inhibitors.

As tumors often display heterogeneity in RNF168 expression, upregulation of the RNF168/53BP1 pathway could provide a useful biomarker for assessment of tumor sensitivity to PARP1 and topoisomerase inhibitors.
PI3K signaling and DNA repair – point of convergence?

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PI3K is a major component of the growth factor receptor signaling and through the production of PtdIns(3,4,5)P3 it controls essential physiological processes such as cell cycle entry, cell survival, metabolism and motility. Activating PI3K mutations are found at high frequency in breast, ovary and lung cancer. Therefore, immense effort is made to understand PI3K signaling and design effective inhibitors of the pathway. Recently, combinations of PI3K inhibitors with PARP inhibitors were used in in vivo studies and the combinations resulted in a beneficial synergistic effect. The mechanism for the synergistic action is, however, not clear.

We are investigating the role of PI3K signaling in the context of breast cancer. As a model system, we are using a panel of breast cancer cell lines with defined genetic background. Our aim is to decipher the mechanistic details of PI3K inhibition on DNA repair pathways and identify the reason for synthetic lethality of combined PI3K/PARP inhibition. This approach is potentially clinically relevant for sensitizing tumors to DNA damage-inducing therapy.
The Role of DNA Repair in Resisting Treatment with Gemcitabine and other Nucleoside Analogues

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To improve cancer treatment, drug choice needs to be tailored to the genetic make-up of tumours. Nucleoside analogues (NA) are molecules that share structural similarities with nucleosides and interfere with DNA replication. Many nucleoside analogues such as Gemcitabine, a cytidine analogue, are an established part of cancer therapy. The main mechanism of action of Gemcitabine depends on its integration into DNA followed by DNA chain termination. Other effects of Gemcitabine include dNTP pool depletion and inhibition of enzymes involved in DNA replication.

The cellular mechanisms involved in resisting NA treatment are not very well understood. Previous research has shown that Mre11 is involved into the Gemcitabine removal and thus contributes to cancer resistance. Mre11 is a core enzyme part of the MRN complex. It is frequently muted in certain types of colorectal cancer. The MRN complex is involved in DNA double strand break detection and repair as well as other pathways such as topoisomerase removal. Mirin, a small molecule inhibitor, has been shown to be an inhibitor of the Mre11 exonuclease function.

We could show that mammalian cells exposed to both Mirin and Gemcitabine show a decreased rate of survival compared to cells only exposed to Gemcitabine. This indicates that the Mre11 exonuclease function is involved in Gemcitabine removal and that Mirin and other small molecules inhibitor with similar activities may be suitable for increasing the efficiency of conventional cancer chemotherapy.

A novel X-ray microirradiation system to study the kinetics of DNA double-strand break repair responses

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DNA double-strand breaks (DSBs) are among the most dangerous DNA lesions and cellular DSB repair (DSBR) responses are relevant for understanding the mechanisms of tumorigenesis, as well as for cancer diagnostics and treatment. “Localized” DNA damage - induced in a restricted area of the cell nucleus - is an important tool in DSBR research because signaling and repair responses triggered by the localized DNA damage can be studied in great detail using microscopical techniques. Induction of DSBs using focused UV laser beams is a commonly applied technique, but there is a growing concern about the poor characterization of the DNA lesions produced by these methods. This is an important issue, since various repair processes are known to be involved in repair of different DNA lesions. To overcome this drawback, we constructed a novel instrument, coined the multi-microbeam microscope (M3). M3 uses ultra-soft X-rays to locally induce DNA damage, mostly single-, and double-strand breaks. Using M3, we studied the kinetics of accumulation of various DSBR factors at X-ray-damaged chromatin. We also analyzed the influence of DNA sensitizer Hoechst on accumulation kinetics and compared accumulation upon X-ray microirradiation and UV-A laser microirradiation. Our results hint at the temporal sequence of some DSBR events and demonstrate that Hoechst does not likely influence DSBR responses. However, we find that the accumulation of some DSBR factors may depend on whether the damage is induced by a UV laser or ionizing radiation, striking a cautionary note for DSBR investigations performed with help of laser-microirradiation.

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Double strand break repair - C1

Presenter: Petr Solc

DNA damage response during mouse oocyte maturation


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Because low levels of DNA double strand breaks (DSBs) appear not to activate the ATM-mediated prophase I checkpoint in full-grown oocytes, there may exist mechanisms to protect chromosome integrity during meiotic maturation. Using live imaging we demonstrate that low levels of DSBs induced by the radiomimetic drug Neocarzinostatin (NCS) increase the incidence of chromosome fragments and lagging chromosomes but do not lead to APC/C activation and anaphase onset delay. The number of DSBs, represented by γH2AX foci, significantly decreases between prophase I and metaphase II in both control and NCS-treated oocytes. Transient treatment with NCS increases >2-fold the number of DSBs in prophase I oocytes, but less than 30% of these oocytes enter anaphase with segregation errors. MRE11, but not ATM, is essential to detect DSBs in prophase I and is involved in H2AX phosphorylation during metaphase I. Inhibiting MRE11 by mirin during meiotic maturation results in anaphase bridges and also increases the number of γH2AX foci in metaphase II. Compromised DNA integrity in mirin-treated oocytes indicates a role for MRE11 in chromosome integrity during meiotic maturation.
Repair and bypass of DNA lesions requires the coordinated action of many DNA repair enzymes. While each lesion recruits a specific set of repair factors, common repair intermediates predict an extensive overlap of downstream factors in multiple repair pathways. To comprehensively survey the dynamics of protein recruitment to specific DNA lesions, we exploit Xenopus egg extracts, a powerful model system for DNA replication and repair and combine it with high-resolution mass spectrometry. Using our approach, which we refer to as chromatin mass spectrometry (CHROMASS), we previously found that more than 90 factors co-operate in the replication and bypass of chromatin containing psoralen crosslinks. In addition to previously known factors we identified several new players, which we continue to study in order to determine their precise function in the response to perturbed DNA replication.

Here we show that CHROMASS can easily be adapted to analyze other DNA repair processes. Using restriction enzymes we monitor protein recruitment to DNA double strand breaks. Besides the canonical Non-Homologous End-Joining (NHEJ) factors, we found several additional factors, whose recruitment profiles suggest important roles in the repair of DNA double strand breaks.

Combining an array of different lesions, small-molecule inhibitors and other specific perturbations, we seek to determine globally how DNA damage affects the protein chromatin landscape. The unbiased and system-wide approach will help to define DNA repair pathways at the molecular level and might lead to the identification of novel DNA repair factors.
Double strand break repair - C1

Presenters: Raquel Cuella-Martin and Catarina Oliveira

53BP1-mediated regulation of the p53-dependent transcriptional programme

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Since its discovery in 1994, tumour suppressor p53-binding protein 1 (TP53BP1, 53BP1) has been attributed important roles in DNA double-strand break (DSB) repair and tumour suppression. 53BP1 drives repair via classical non-homologous end-joining (NHEJ), the pathway responsible for the repair of programmed DSBs during immunoglobulin class-switch and V(D)J recombination. 53BP1 action relies on its ability to prevent nucleolytic processing of DNA-ends, a property critical for DSB repair pathway choice regulation that inhibits homologous recombination (HR) and promotes NHEJ. Nevertheless, as its name infers, 53BP1 was initially discovered as a p53-interacting protein (1) and has since been attributed direct interactions with the p53’s DNA-binding domain (2). Surprisingly, the physiological relevance of these interactions has remained neglected, and 53BP1’s direct contribution to the p53 response ill-defined.

To investigate the role of 53BP1 in p53-dependent tumour suppressor signalling responses we have selectively disrupted the TP53BP1 gene in human cell lines using CRISPR/Cas9 technology. Here, we show that 53BP1-deficient cells present suboptimal p53-driven cellular responses as a direct result of incomplete p53-dependent transactivation events. Further probing this phenotype in complementation studies, we have also fine-mapped the minimal protein sequence requirements in 53BP1 for an optimal p53 response. Importantly, we report the 53BP1 tandem-BRCT domains – a domain dispensable for 53BP1 canonical roles during NHEJ and resection inhibition – mediate physical interactions with p53 that are necessary for optimal p53-dependent signalling. Furthermore, we have now identified additional essential components of the 53BP1-p53 axis and found these factors to be dispensable for 53BP1-dependent DSB repair activities. Together, our data provide the first mechanistic insights into 53BP1’s contribution to the p53-dependent transcriptional programme, and reveal this activity to be distinct and separable from its better-characterised DNA repair activities. Our study therefore defines important novel functions for 53BP1 in regulating a vital tumour suppressor pathway that may better explain its previously attributed roles in tumour suppression.

Double strand break repair - C1

Presenter: Sabrina Köcher

A functional ex vivo assay to detect the response of prostate cancer patients to PARP inhibitor olaparib


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The prostate cancer (PCa) genome commonly harbors gross structural rearrangements, including translocations and deletions of chromosomal material. These reflect deficiencies in canonical DNA double-strand break (DSB) repair pathways, caused by mutational or epigenetic loss of key regulators or effectors of the cellular DNA damage response (DDR) machinery. We aim to exploit these functional repair defects to selectively sensitize tumor cells to radiotherapy using PARPi.

PARPi have shown promising results in phase I and II clinical trials, but disappointingly they did not achieve their anticipated survival endpoints in phase III trials, raising more questions than answers. One critical concern is the selection of the right patients for this treatment.

Based on tissue microarray (TMA) data from more than 13,000 PCa patients, our collaboration partners from the UKE pathology department and Martini-Klinik have previously reported frequent alterations including overexpression of the oncoproteins ERG and BCL2 and deletions in tumor suppressor genes PTEN and CHD1. In vitro, we found that these alterations are associated with DSB repair deficiencies. For example, overexpression of ERG or BCL2 results in a switch to the PARP1-dependent end-joining process, due to suppression of the classical nonhomologous end-joining pathway. Deletion of either CHD1 or PTEN leads to homologous recombination deficiency, due to failure in opening the chromatin around the DSB or deficiency in the G2/M checkpoint, respectively. All these alterations render PCa cells sensitive to PARPi olaparib either alone or combined with ionizing radiation (IR).

To directly verify this finding in vivo, we established a functional ex vivo assay, which allows us to analyze DDR and DSB repair in fresh PCa tissue. According to this system, tumor biopsies were chopped into slices of a few hundred micrometers and kept under optimum growth conditions for up to 3 weeks. During this time, samples are treated with PARPi alone or combined with IR, incubated for repair for 1-24 h, fixed, frozen and sectioned for immunofluorescence microscopic analysis of DNA repair foci (gH2AX, 53BP1, Rad51), proliferation (EdU) and hypoxia (Pimonidazol). We have validated this method by (i) comparing cell line and Xenograft material, (ii) the timely assessment of DSB-repair kinetics, and most importantly (iii) the inhibition of DNA repair proteins to enhance unrepaired DSBs and therefore the amount of repair foci. Patient-derived tumor biopsies from PCa patients were collected, cultured, treated with IR ± PARPi, and stained and analyzed for DSB repair foci. Evaluation of this data is still in process. Indeed, the status of the above mentioned alterations will also be controlled using IHC.

In conclusion we here provide a novel assay which can not only detect treatment responses of individual tumors, but moreover monitor repair processes and defects directly in the tumor.
Double strand break repair - C1

Presenter: Serena Bologna

Novel ubiquitylation and deubiquitylation components controlling cellular responses to DNA damage: impacts on genome stability and links to cancer.

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Each human cell experiences up to 105 DNA lesions per day. To counteract DNA damage and maintain genome integrity, cells need to adequately respond to such genotoxic stresses. This is achieved by activation of evolutionarily conserved DNA damage response (DDR) pathways that block cell cycle progression by activating cell cycle check-points and inducing DNA repair.

The cascade of events resulting from detection of DNA damage and orchestrating its repair has been best described for DNA double strand breaks (DSBs), which represent the most toxic DNA lesions. Proteins, the constitutive elements of such cascades, are hierarchically related to each other. Post-translational modifications (PTMs) of such proteins define a code that translates into distinct outputs, such as the proper/improper repair of a DNA lesion. PTMs comprise the covalent addition of either chemical groups or small peptides to one or more amino acid residues of a target protein, resulting in structural changes or alterations in important enzymatic features. Ubiquitylation refers to the attachment of the highly conserved ubiquitin polypeptide to substrates and it is carried out by three main players: an E1-activating enzyme, an E2-conjugating enzyme and lastly an E3-ligase. Mammalian cells express 2 ubiquitin E1s, ~40 E2s and >600 E3s (1). Proteins ubiquitylation is in most cases a reversible event, being reversed by deubiquitylating enzymes (DUBs) (2). Deubiquitylation and ubiquitylation control various aspects of the DDR, including DNA repair and the dynamic state of histone ubiquitylation. Around 94 DUBs are encoded by the human genome and, although the link between DDR factors regulation and ubiquitylating/deubiquitylating enzymes seems well established, it is clear that many components of such network still remain to be explored. Based on the reasoning that ubiquitylating/deubiquitylating enzymes cover an extremely essential role in regulating DNA damage response, thus representing druggable targets for cancer therapeutic treatments, we intend to perform high-throughput screenings aiming both to highlight new roles in the DDR and other fields and to search for compounds that would target the active site of these proteins or block interaction with their substrates.

Double strand break repair - C1

Presenter: Thomas Hofmann

ATM-mediated phosphorylation targets Sirtuin 1 to damaged chromatin to modulate DNA repair

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Checkpoint kinase ATM is activated by DNA double-strand breaks (DSBs) and guides the cellular response through regulatory phosphorylation of diverse target proteins. Deacetylase Sirtuin 1 (SIRT1) participates in the DNA damage response by deacetylation p53 and inhibiting its cell-death promoting function and by removing acetylation marks from various histone tails, leading to chromatin compaction, and repression of transcription. Recent reports indicated that SIRT1 is redistributed to damaged chromatin in an ATM-dependent fashion and modulates DNA repair. However, the underlying mechanisms remained unclear. Here we report that ATM physically interacts through its C-terminal kinase domain with the catalytic domain of SIRT1. Furthermore, SIRT1 is phosphorylated by ATM at Serine 693 in vitro and at endogenous levels in response to ionizing radiation (IR). Ser693 phosphorylation stimulates SIRT1 deacetylase activity towards acetylated histones (eg. H1K26ac) and acetylated-p53. Interestingly, IR-induced Ser693 phosphorylation is critical to target SIRT1 to damaged chromatin and phospho-deficient SIRT1S693A fails to efficiently accumulate at microlaser-induced DNA damage sites. Finally, stable expression of a phospho-mimetic SIRT1 increases cell survival upon IR. We currently analyse whether ATM-mediated targeting of SIRT1 to damaged chromatin modulates DNA repair pathway choice possibly by modulating histone H1 deacetylation and subsequent 53BP1 recruitment. Our results suggest that the ATM-SIRT1 axis modulates chromatin state and DNA damage-induced cell fate by enhancing SIRT1 activity and chromatin targeting.
Double strand break repair - C1

Presenter: Thomas Clouaire

Characterising the chromatin landscape at DNA double strand breaks

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DNA Double Strands Breaks (DSBs) are the most deleterious type of DNA damage and a variety of human diseases associates with DSB repair defects. In eukaryotes, DNA is organised into chromatin, which tightly regulates its accessibility and therefore impacts all aspects of DNA metabolism, including DSB repair.

We recently developed an experimental system based on a restriction enzyme fused to the ligand-binding domain of oestrogen receptor (AsiSI-ER), to generate multiples sequence-specific DSB. This cell line (called DIvA for DSB Induced via AsiSI) allows to investigate simultaneously the DSB repair/chromatin remodeling events occurring on multiples DSBs located in different chromatin contexts, using chromatin immunoprecipitation (ChIP).

We recently performed ChIP-seq mapping of XRCC4 (involved in NHEJ) and RAD51 (involved in HR) in DIvA cells. We found that distinct AsiSI induced DSBs are not repaired equivalently, and we identified a subset of DSBs that are “HR-prone”, i.e preferentially recruit RAD51, undergo resection and rely on HR for efficient repair. On the other hand, “non HR-prone” AsiSI-induced DSBs could not recruit RAD51, and relied on XRCC4 for repair (Aymard et al., 2014). Using available ENCODE data, we also showed that “HR-prone” DSB were located in transcriptionally active, H3K36me3-rich chromatin and that Setd2, the enzyme responsible for H3K36 trimethylation, is required to channel those DSB to HR repair.

In order to fully understand the role for chromatin structure in DSB repair, we are currently performing genome-wide mapping of an extended set of chromatin modifications by ChIP-seq (>15) both before and after DSB induction. Using this unique dataset, we are currently deciphering the “histone code” that associates with each repair pathway, i.e, sets of histone modifications that accompany repair by HR and NHEJ. We are also drawing a more exhaustive picture of the chromatin landscape induced around DSBs, by determining which modifications are specially induced or decreased at specific DSBs.

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Double strand break repair - C1

Presenter: Tina Thorslund

Linker histones couple initiation and amplification of ubiquitin signaling after DNA damage

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DNA double-strand breaks (DSBs) are highly cytotoxic DNA lesions that trigger non-proteolytic ubiquitylation of adjacent chromatin areas to generate binding sites for DNA repair factors. This depends on the sequential actions of the E3 ubiquitin ligases RNF8 and RNF168, and UBC13, an E2 ubiquitin-conjugating enzyme that specifically generates K63-linked ubiquitin chains. Whereas RNF168 is known to catalyze ubiquitylation of H2A-type histones, leading to the recruitment of repair factors such as 53BP1, the critical substrates of RNF8 and K63-linked ubiquitylation has remained elusive. We have now elucidated how RNF8 and UBC13 promote recruitment of RNF168 and downstream factors to DSB sites in human cells. We establish that UBC13-dependent K63-linked ubiquitylation at DSB sites is predominantly mediated by RNF8 but not RNF168, and that H1-type linker histones, but not core histones, represent major chromatin-associated targets of this modification. The RNF168 module (UDM1) recognizing RNF8-generated ubiquitylations is a high-affinity reader of K63-ubiquitylated H1, mechanistically explaining the essential roles of RNF8 and UBC13 in recruiting RNF168 to DSBs. Consistently, reduced expression or chromatin association of linker histones impair accumulation of K63-linked ubiquitin conjugates and repair factors at DSB-flanking chromatin. Our results identify histone H1 as a key target of RNF8–UBC13 in DSB signaling and expand the concept of the histone code by showing that posttranslational modifications of linker histones can serve as important marks for recognition by factors involved in genome stability maintenance, and possibly beyond.

Temporal and spatial uncoupling of DNA Double Strand Break repair pathways within mammalian heterochromatin

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Repetitive DNA is packaged into heterochromatin structures to maintain its integrity. Here, we use CRISPR/Cas9 to induce DSBs in heterochromatin and demonstrate that in pericentric heterochromatin, DSBs arising in G1 are repaired at the core of the domain by NHEJ. In G2, end-resection occurs at the core but resected DNA ends relocate to the periphery to be repaired by HR. DSBs that fail to relocate are repaired in situ through NHEJ or SSA. Mechanistically, DSB-relocalization requires end-resection and exclusion of RAD51 from the core. We propose that the spatial disconnection between end-resection and homology search prevent the activation of mutagenic pathways and illegitimate recombination. Interestingly, we also show that DSBs in centromeric heterochromatin activate both NHEJ and HR throughout the cell cycle. Our results highlight striking differences in DNA repair between pericentric and centromeric heterochromatin and reveal that the DNA repair pathway regulates the position of the breaks within heterochromatin structures.
Double strand break repair - C1

Presenter: Velibor Savic

Spatial spreading of DSB-mediated chromatin changes in mammalian cells

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Double strand DNA breaks (DSBs) are one of the most dangerous genetic lesions the DNA can incur. They can occur through exogenous physical or chemical agents or can be induced endogenously, by accident or as a part of a biological processes. DSB induction and subsequent repair does not occur in isolation but in the context of chromatin which is extensively altered during DSB recognition and repair with modifications spreading at least 500kb linearly away from the break site. In lower eukaryotes, chromatin modifications spread both linearly along the broken DNA and also to non-broken chromatin segments located in the vicinity, creating a spherical region of altered chromatin.

We hypothesized that the spherical chromatin structure occurs around DSBs in mammalian cells as well. To test this, we created a cell based system with U2OS human osteosarcoma cell line harbouring a TetR-binding array (240 TetR binding sites) coupled with a triple I-SceI homing endonuclease and a separate LacI-binding array (256 TetR binding sites), both independently inserted into the genome. Through expression of a fusion protein containing both TetR and LacI we planned to induce association of the two arrays and with the induction of I-SceI mediated DSB, to use the chromatin associated with the LacI array as a readout for the ability of the DSB-induced chromatin changes to occur on juxtaposed unbroken DNA segments.

When transfected with the TetR-LacI fusion construct, the two arrays are indeed brought into close proximity as measured by two-coloured FISH and the association is abolished through a TetR inhibitor doxycycline, confirming interaction specificity. Moreover, 3C experiments have likewise shown dependency of interaction between arrays on the expression of the fusion construct. With this information we performed ChIP experiments looking for phosphorylated histone H2AX (γH2AX) and 53BP1 association in both cis and trans. As expected, we were able to detect both events in cis on the TetR-binding array upon DSB induction and irrespective of the presence of TetR-LacI fusion protein. Crucially, we were also able to detect both γH2AX and 53BP1 in chromatin on the juxtaposed LacI-binding array itself. This was dependent on both induced proximity and the presence of a DSB, validating our initial hypothesis.

Establishing spatial distribution of DNA damage response-mediated chromatin changes brings a new dimension to our understanding of the effect DSB signalling can have on local chromatin function in mammalian cells. In euchromatin it may lead to more widespread gene silencing, not only in cis but likewise in-trans, establishing a spherical zone of reduced RNA PolII activity, similar to earlier observations. Alternatively, a DSB occurring in heterochromatin may lead to more widespread chromatin de-compaction, spreading to undamaged loci as well. We are currently actively working towards establishing and understanding these relationships.
Double strand break repair - C1

Presenter: Wael Mansour

ATM-orchestrated functional crosstalk between 53BP1/RIF1 and BRCA1/CtIP regulates DSB repair pathway choice in cell cycle dependent manner

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Double strand breaks (DSBs) are dangerous chromosomal lesions. Failure to accurately repair DSBs can lead to gross chromosomal rearrangements or mutations at the break site, which can cause cell death, cell transformation, and tumorigenesis. Two main pathways have evolved to eliminate DSBs from the genome: non-homologous end joining (NHEJ) and homologous recombination (HR). Previously we have reported a functional hierarchy between DSB repair pathways to ensure the employment of the appropriate pathway. This hierarchy is regulated by many factors including the initial processing of the DSB ends. DSB end processing is primarily regulated in a cell cycle dependent manner by CDK1/2 which phosphorylates/activates the endonuclease CtIP. Therefore, in G1 phase, the end resection step is inhibited which renders NHEJ the main repair pathway. However, as cells enter S and G2 phase end resection is activated to commit the repair to HR. Further investigations have revealed much greater complexity in the determination of DSB repair choice. Here, we confirmed the antagonistic relationship between the DNA damage response (DDR) proteins BRCA1 and 53BP1 in regulating DSB repair pathway choice. Moreover, we found that 53BP1 at DSB ends acts as a scaffold protein to facilitate the recruitment of the end protection factors RIF1 and PTIP to the DSB site and hence committing the repair to NHEJ. Importantly, depletion of either 53BP1 or RIF1 leads to activation of end resection in G1 as evidenced by formation of BRCA1, CtIP, and RPA foci, however, G1-cells failed to recruit RAD51 to the resected ends, indicating the presence of an additional regulatory arm to prevent HR in G1. Intriguingly, our data revealed that the repair is switched to the alternative end joining (Alt-EJ) pathway in G1 cells after depletion of 53BP1 or RIF1. Indeed, the recruitment of RIF1 and PTIP are found to depend on the ATM-mediated 53BP1 phosphorylation, putting ATM in the center of end protection process. This raises a paradox because it is already known that ATM is critical for end resection process and that ATM-deficient cells are deficient in end resection. Here we demonstrated that ATM-dependent 53BP1 phosphorylation is more in G1 than that in S/G2 phase. This is explained by the different binding patterns of phosphorylated ATM (pATM) to DSB in different cell cycle phases. While more intense pATM foci are formed in G1 phase, pATM foci are less intense in S/G2, indicating that more pATM molecules are bound to DSB in G1 phase. These data was confirmed by chromatin fractionation analysis. In conclusion, we provide here a model for the regulation of DSB repair pathway choice. In G1, NHEJ is favored as pATM is more bound to DSB, which efficiently phosphorylates 53BP1 to recruit RIF1 to protect DSB ends. However, as cells enter S/G2 phase, pATM is displaced from DSB sites, which diminishes 53BP1 phosphorylation and prevents RIF1 recruitment, relieving end protection to commit the repair to HR.
Double strand break repair - C1

Presenter: Aurelie Vaurijoux

Random distribution of persistent γH2AX IRIF between daughter cells

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The DNA double-stranded breaks (DSB) are key events in the cell response to ionizing radiation that may affect, with the individual genetic and epigenetic profile, the fate of healthy tissues of people exposed. A set of post-translational modifications of histones are involved in the signaling process of initial damage, including the phosphorylation on serine 139 of histone H2AX (γH2AX) few minutes after their formation, leading to the formation of ionizing radiation-induced foci (IRIF). The disappearance of the majority of these γH2AX IRIF is generally observed within hours following exposure, and is related to the repair of DSBs. However, a certain proportion of γH2AX IRIF could remain 24 hours after damage formation. Their relation with residual DSB is widely accepted but still controversial. To investigate the dynamic of IRIF in our model, we exposed G0/G1-phase synchronized HUVECs to 1 or 5 Gy of X-rays. IRIF were studied from 10 minutes up to 7 days after exposure by monitoring γH2AX foci, their temporal association with 53BP1 protein and PML NBs, and their impact on cell proliferation. We analyzed a mean of 4 000 cells for each condition using an automated detection of nuclei and foci. The analysis of a large number of cells and foci allowed us to screen subpopulations of cells or foci through different characteristics, such as size, shape or cell cycle phase among others, and to weight their representativeness in the whole population of exposed cells. We observed persistent IRIF up to 7 days post-irradiation. More than 70% of cells exposed to 5 Gy had at least one persistent IRIF 24 hours after exposure. A significant spatial association between PML NBs and IRIF was observed from 10 minutes after exposure, and 24 hours post-irradiation around 90% of persistent IRIF was associated with PML NBs. We show that this association starts soon after irradiation (10 minutes) indicating that some IRIF may acquire this characteristic of “persistent” very early. Hence, they may correspond to DNA damage with specific initial characteristics like complexity and/or location. Moreover we demonstrated that persistent IRIF did not block definitively cell proliferation. A decrease of the IRIF frequency was measured in daughter cells and was mainly due to a random distribution of IRIF between them. This specific way of transmission suggests that the nature of IRIF could not be exactly the same before and after the first cell division. We postulate that the IRIF observed in daughter cells could correspond to an atypical chromosomal structure coming from an abnormal resolution of the anaphase leading to the asymmetric distribution of pieces of sister chromatids derived from a chromosome with a residual DSB initially induced in G0/G1-phase. This chromosomal assembly may be lethal or result in a gene dosage imbalance but also may lead to an enhanced genomic instability specifically in the daughter cells.
The SET-2/SET1 H3K4 methyltransferase is required for genome stability in the C. elegans germline

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Chromatin structure and organization play important roles in regulating the dynamics of DNA repair not only by influencing accessibility to the repair machinery and restoring chromatin organization following the repair process, but also by actively promoting DNA damage signaling and repair. We have shown that loss of SET-2, the C. elegans homolog of the highly conserved Set1 H3K4 methyltransferase, is associated with a mortal germline phenotype (Mrt), in which animals become progressively sterile (Xiao et al. 2011). This phenotype is common to several DNA damage checkpoint genes, and has been attributed to the accumulation of DNA damage in the germline over multiple generations. We have found that SET-2 and H3K4 methylation are required for genome stability in the C. elegans germline. Animals lacking SET-2 show increased sensitivity to DSB inducing agents and replicative stress, which increase transgenerationally. While the DNA damage response (DDR), including DNA damage induced signaling, cell cycle arrest and apoptosis are efficient in set-2 deficient germlines, DSBs accumulate in the germline of these animals. These results suggest that SET-2 is not directly involved in DDR, but rather in downstream events involved in the repair process itself. We propose that the progressive loss of germline immortality may result from the transgenerational accumulation of additional forms of DNA damage resulting from the error prone repair of DSBs in the absence of SET-2.

Double strand break repair - C1

Presenter: Marziyeh Tolouee Nodolaghi

Dopamine protects against DNA double strand break induced by cooling

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Introduction:
Ischemia is a condition suffered by cells and tissues when deprived of the adequate supply of nutrients and oxygen. Unsuccessful repair of DNA damage result in activation of the apoptotic program, cancer, complications arises of organ transplantation, as delayed graft function, graft rejection, and chronic graft dysfunction (caused by progressive interstitial fibrosis of the transplanted organ) are the clinical outcome of ischemia-reperfusion injury. Discovery of a compound that possesses an ability to protect against DNA damage would confer prevention and aid the management of diseases related to disorders mentioned above. Similarly, many mammalian cell types are vulnerable to prolonged and profound hypothermic storage, which is related to a burst of ROS upon rewarming. We previously demonstrated dopamine to attenuate hypothermia induced cell death. To further understand its cellular effects, we investigated the type of DNA damage. As we found substantial damage, we next investigated the effects of dopamine on DNA damage induced by hypothermia.

Methods:
Smooth muscle aortic cells (SMAC) were cultured in 6 well plates until confluency, cooled at 4°C for 24 hours with or without subsequent rewarming to 37°C. Untreated cells (37°C) served as a control. Dopamine (30 uM) was added throughout the whole procedure, or during parts of the cooling-reperfusion protocol. Single and double strand DNA breaks, DNA cross-links and oxidative damage in single cells was assessed by Comet assay. Per condition > 60 Comets were analyzed and damage was expressed as the percentage of DNA in the Comet’s tail (% TailDNA) using ImageJ software. To understand the mechanisms involed, immunofluorescence staining of 53BP1 was performed in different conditions.

Results:
Compared to control cells, the %TailDNA was substantially increased both by cooling and cooling/rewarming, as reflected by an increase in the median %TailDNA from 8.4% to 87.3% and 76.4%, respectively. Dopamine treatment throughout the protocol strongly reduced DNA damage, as evidenced by a substantial decrease in the median of %TailDNA to 49.9%. Notably, when treatment with dopamine was restricted to 30 min. prior to cooling, reduction in DNA damage was absent. Expression of 53BP1 as a DNA Damage marker in dopamine treated cells was decreased in compare to the cooled cells.

Conclusion:
Our results demonstrate that dopamine protects SMAC cells from DNA damage induced by hypothermia/rewarming by protection of cells in the hypothermic and rewarming phases. Thus, dopamine represents a pharmacologically promising compound for several clinical applications that require hypothermia and rewarming, such as the cold preservation of transplant organs.
Double strand break repair - C1

Miha Milek

**DDX54 regulates transcriptome dynamics during DNA damage response**

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Cellular response to genotoxic stress is a well-characterized network of DNA surveillance pathways. However, the contribution of post-transcriptional gene regulatory networks to DNA damage response (DDR) has not been extensively studied. Here, we present a quantitative approach based on UV crosslinking, oligo(dT) capture and mass spectrometry to systematically identify the proteins differentially bound to polyadenylated transcripts upon ionizing irradiation (IR) of human breast carcinoma cells. Interestingly, a large group of proteins (n=266) showed increased poly(A)+ RNA binding in IR-treated cells and contained many spliceosomal and ribosomal proteins. We performed functional analysis of DDX54, a candidate genotoxic stress responsive RNA helicase, by applying PAR-CLIP, RNA- and 4SU-seq, to address its widespread effects on post-transcriptional gene regulation during DDR. Our findings show that DDX54 is an immediate-to-early DDR regulator that impacts the splicing fidelity of its target IR-induced pre-mRNAs. DDX54 acts by direct protein-protein, protein-snRNA and protein-pre-mRNA interactions and is required for cell survival after exposure to ionizing radiation, highlighting its potential as a novel biomarker in DDR-related pathologies. This work indicates the relevance of many uncharacterized RBPs potentially involved in DDR.
Improved genome editing efficiency and flexibility using modified oligonucleotides with TALEN and CRISPR-Cas9 nucleases

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Genome editing has now been reported in many systems using TALEN and CRISPR-Cas9 nucleases. Precise mutations can be introduced during homology-directed repair with donor DNA carrying the wanted sequence edit, but efficiency is usually lower than for gene knock-out and optimal strategies have not been extensively investigated. Here we show that using phosphorothioate-modified oligonucleotides strongly enhances genome editing efficiency of single-stranded oligonucleotide donors in cultured cells and in animal models (Renaud et al, Cell Rep., 2016). In addition, it provides better design flexibility, allowing insertions more than 100 bp long. Despite previous reports of phosphorothioate-modified oligonucleotide toxicity, clones of edited cells are readily isolated and in rat and mouse, targeted sequence insertions are achieved at very high frequency. In particular, high rates of homozygous loxP site insertion and of point mutations were achieved at the mouse ROSA and rat CFTR loci, respectively.

Finally, when detected, imprecise KI events exhibit indels that are asymmetrically positioned, consistent with genome editing taking place by two steps of single-strand annealing.

Our results demonstrate that phosphorothioate-modified oligonucleotides will significantly facilitate the generation of rat and mouse models of human disease.
Double strand break repair - C1

Presenter: Pauline Chanut

Coordinated nuclease activities release Ku from single-ended DNA double-strand breaks

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Repair of single-ended DNA double-strand breaks (seDSBs) by homologous recombination (HR) requires the generation of a free 3’ single-strand DNA overhang by exonuclease activities in a process called DNA resection. However, it is anticipated that the highly abundant DNA end-binding protein Ku can sequester seDSBs and shield them from exonuclease activities. Despite pioneering works in yeast, it is unclear how Ku is released from seDSBs in mammalian cells to allow HR to proceed. Here, we show that Ku indeed loads on seDSBs in human cells, before being released by a process controlled by ATM-dependent phosphorylation of CtIP and involving the epistatic and coordinated action of Mre11 endo- and exonuclease activities and CtIP flap endonuclease activity. Our work also provides evidence for an unsuspected mechanism that contributes to Ku release, acting downstream of Mre11 endonuclease activity and in parallel with Mre11 exo-/CtIP flap endonuclease activities.
Double strand break repair - C1

Presenter: Paul Wijnhoven

The deubiquitylating enzyme USP15 promotes DNA repair

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Over recent years, it has become evident that enzymes that process ubiquitin precursors and reverse ubiquitylation, the so-called deubiquitylases (DUBs), have important functions in DNA double-strand break (DSB) repair and related events. We performed a systematic characterization of all human DUBs for roles in DSB-repair and DSB signaling [Nishi & Wijnhoven et al., NCB (2014)], thereby identifying several DUBs with previously unreported links to DSB repair. Phylogenetic analysis furthermore revealed functional clustering within certain DUB subgroups. These included USP11, which has reported DNA repair functions, and its paralogs USP4 and USP15, thus indicating their redundant and/or complementary roles in DNA repair and providing a basis for additional studies focused on USP4 and USP15. Accordingly, we recently established that USP4 promotes DNA-end resection and DSB repair by homologous recombination, and also found that USP4 interacts with CtIP and the MRE11-RAD50-NBS1 complex and stimulates CtIP recruitment to DNA-damage sites [Wijnhoven et al., Mol. Cell (2015)]. Here, we provide data suggesting DSB repair functions for the USP4 and USP11 paralog, USP15. We also show that USP15 seems to do this in a manner that appears to be non-redundant with the resection-function of USP4. Collectively, these observations indicate that USP15 promotes DSB repair and might, like USP4 and USP11, do this at least in part by regulating homologous recombination.
Double strand break repair - C1

Presenter: Philipp Oberdoerffer

Evidence for persistent, DNA damage-induced epigenetic dysfunction with cellular age

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DNA damage is often proposed to account for age-associated epigenetic decline and its potentially detrimental physiological consequences. However, while the repair of DNA lesions is intricately linked to changes in the surrounding chromatin environment, evidence for persisting epigenetic dysfunction in response to DNA damage is missing. In agreement with the latter, recent work from our lab found the mammalian transcriptome to be sufficiently robust to accommodate short-term exposure to DNA double-strand breaks (DSBs). Epigenetic decline due to chronic DNA damage, on the other hand, remains an intriguing albeit speculative possibility. Replication stress, a major source of continuous DNA damage in dividing cells, is a candidate driver of such epigenetic change. Here, we show that the macro-histone variant macroH2A1.2, a modulator of DSB repair by HR, accumulates at fragile genomic elements in a replication stress- and DNA damage signaling-dependent manner. Isolation of proteins on nascent DNA (iPOND) demonstrates enrichment of macroH2A1.2 specifically at stalled replication forks along with key HR repair factors. Moreover, genome-wide analyses implicate macroH2A1.2 in protecting sites of replication stress from excessive DNA damage. Strikingly, fragile genomic elements display increased macroH2A1.2 levels in late compared to early passage fibroblasts, whereas macroH2A1.2 levels remain unchanged at regions with no detectable replication stress. Altogether, our findings point to macroH2A1.2 as a site-specific, epigenetic memory for replicative age, driven by a chronic replication stress response. Potential implications for epigenetic integrity, gene expression and cell function will be discussed.

Double strand break repair - C1

Presenter: Christopher Cooper

Structural analysis of the helicase domain of DNA Polymerase Theta reveals a potential role in the Microhomology-Mediated End-Joining pathway

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Of the 15 DNA polymerases responsible for human genomic replication and repair, one of the most poorly characterized is DNA polymerase theta (Polθ/POLQ). In addition to its role in translesion DNA synthesis, Polθ has been identified as a crucial factor in microhomology-mediated end-joining (MMEJ), a potential reserve system in eukaryotic double strand break repair. In contrast to other DNA polymerases, Polθ also comprises a putative N-terminal helicase domain similar to the HEL308/HELQ DNA helicase Polθ alongside an additional large central, suggesting multiple roles in genome integrity beyond DNA synthesis. A range of cancer cell types defective in homologous recombination exhibit Polθ upregulation, with knockdown inhibiting cell survival in a subset of these. This suggests Polθ is an attractive target to potentiate synthetically lethal chemotherapeutic approaches in cancer treatment. We have crystallised the helicase domain of human Polθ in the presence and absence of bound nucleotides, and performed a characterization of its associated DNA-binding and DNA-stimulated ATPase activities. Comparisons to helicases from the Hel308 family identify several unique features in Polθ. Surprisingly, Polθ exists as a tetramer both in the crystals and in solution, confirmed by a number of techniques. We propose a model for DNA binding to the Polθ helicase domain in the context of the tetrameric Polθ arrangement, suggesting a role for the helicase domain in DNA strand annealing during MMEJ, for subsequent processing by the DNA polymerase domain.
DNA double-strand breaks (DSBs) constitute the most severe lesion threatening genomic integrity. Thus, several cellular DNA damage response (DDR) mechanisms such as cell cycle checkpoints and DSB repair pathways work in concert to handle these threats. Efficient DDR pathways are especially important for embryonic or tissue-specific stem cells as they represent a life-long source for tissue regeneration. However, compared with differentiated cells, little is known about the regulation of DDR pathways in stem cells. Here, we investigated mechanisms of DSB repair and G2 checkpoint regulation in murine embryonic stem cells (ESCs) and ESC-derived neuronal stem cells (NSCs).

In differentiated cells, the majority of ionizing radiation (IR)-induced breaks are repaired very fast via non-homologous end-joining (NHEJ) with DNA-PK being one of the core enzymes. In contrast, a sub-fraction of breaks is repaired with slow kinetics in an ATM-dependent manner which represents homologous recombination (HR) in G2 and a resection-dependent end-joining pathway in G1. Since the molecular mechanisms of DSB repair are only poorly defined in stem cells, we evaluated the contribution of NHEJ and of ATM-dependent repair in G1 and G2 phase ESCs and NSCs. Wild-type (wt) ESCs and NSCs showed similar repair kinetics and the IR-induced DSBs were almost completely repaired within 8 h post IR. Surprisingly, DNA-PK inhibition in G1 and G2 ESCs and NSCs impaired repair only during the first 2 h after IR which, in ESCs, was followed by an efficient repair to background DSB levels. This indicated that ESCs can efficiently use DNA-PK-independent alternative pathways for DSB repair. In contrast to DNA-PK inhibition, ATM inhibition did not affect fast DSB repair within the first 2 h post IR but the DSBs which are repaired with slow kinetics in wt cells remained unrepaired. This indicates that ATM is specifically involved in the slow DSB repair component, similar to the situation in differentiated cells (e.g. in mouse embryonic fibroblasts, MEFs). Moreover, we investigated the role of the alt-NHEJ pathway in ESCs, NSCs and MEFs by inhibiting PARP. Importantly, PARP inhibition did not affect DSB repair kinetics in wt ESCs, NSCs or MEFs. However, PARP inhibition induced an additional significant defect in DNA-PK-inhibited ESCs, a moderate additional defect in DNA-PK-inhibited NSCs and almost no additional defect in DNA-PK-inhibited MEFs. These data demonstrate that PARP-dependent alt-NHEJ only functions in the absence of canonical NHEJ and that its contribution in NHEJ-defective cells decreases during cell differentiation. Finally, we estimated the efficiency of the G2 checkpoint in stem cells. They displayed an insensitive G2 checkpoint, resulting in cells entering mitosis before the completion of DSB repair. However, this insensitivity was more pronounced in NSCs than in ESCs, again indicating the existence of a more stringent DDR in ESCs compared with NSCs.
Double strand break repair - C1

Presenter: Larry Bodgi & Sandrine Pereira

The nucleo-shuttling of the ATM protein as a basis for a novel theory of radiation response: resolution of the linear-quadratic model

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Purpose: For 50 years, cellular radiosensitivity has been defined in vitro as the lack of clonogenic capacity of irradiated cells and its mathematical link with dose has been described by the target theory. Among the numerous formulas provided from the target theory, the linear-quadratic (LQ) model empirically describes cell survival as a negative exponential of a second degree polynomial dose-function in which alpha*D is the linear component and beta*D2 is the quadratic one. The LQ model is extensively used in radiobiology (to describe survival curves) and in radiotherapy (the alpha/beta ratio indicates whether tissue reactions can occur early or late after the treatment). However, no biological interpretation of the LQ parameters was proposed to explain together the radiation response in a wide dose range, the radiosensitivity of some genetic syndromes caused by the mutation of cytoplasmic proteins and genetics syndromes such as Hutchinson progeria syndrome (HGPS) which is caused by mutations of nuclear envelope proteins.

The Model: In discrepancy with the actual paradigm but from a solid amount of experimental data, we hypothesized that the major forms of ATM are cytoplasmic dimers. By considering that IR induce ATM monomerization in cytoplasm and the resulting ATM monomers diffuse into nucleus to facilitate DSB recognition and repair. Such hypotheses lead to a coherent molecular interpretation of the LQ model by considering the yield of recognized but unrepaired (alpha-type) DSB and the non-recognized (beta-type) DSB. The notion of cell tolerance to unrepaired DSB was introduced by considering that not all DSB are lethal. Cell survival and DSB repair and signaling immunofluorescence data from 42 normal skin fibroblast and 18 tumor human cell lines were used to verify the validity of this biomathematical model proposed. Furthermore, this model allowed us to explain the radiosensitivity of HGPS cells: we provided evidence that the presence of progerin a mutated form of lamin A, impairs the nucleo-shuttling of ATM and reduces the activation of the ATM-dependent DNA repair and signalling pathways.

Results: Our model is validated at different levels by one of the widest spectrum of radiosensitivity particularly in HGPS cells. The fact that beta is expected to be a Lorentzian function of alpha was confirmed experimentally.

Conclusions: Our model provides a very general picture of human radiosensitivity, independently of the dose, the cell type and the genetic status.
Double strand break repair - C1

Presenter: Vincent Dion

Contracting CAG/CTG repeats using the CRISPR-Cas9 nickase

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The expansion of CAG/CTG repeats causes at least 13 different neurological and neuromuscular diseases for which there is currently no cure. Because longer repeats cause more severe phenotypes, contracting the repeat tract would remove the underlying cause of the disease and may provide a therapeutic avenue. Testing this hypothesis has been hampered by the lack of an efficient reporter assay that can detect both expansions and contractions in the same human cell population. Here we present a rapid GFP-based assay that monitors CAG/CTG repeat instability in both directions. Using this assay, we find that the CRISPR-Cas9 nickase efficiently induces contractions most likely by generating DNA gaps within the repeat tract. Remarkably, we did not detect a concomitant increase in expansions as we did when generating double-strand breaks using either a Zinc Finger Nuclease or the Cas9 nuclease. We further uncover roles for DNA damage response kinases in regulating instability at Cas9-nickase-induced DNA gaps: ATM inhibition decreased CAG/CTG repeat contractions, whereas ATR loss-of-function promoted both expansions and contractions in a MSH2- and XPA-dependent manner. Taken together, our results demonstrate that a contraction bias can be achieved in human cells, paving the way towards deliberate in vivo CAG/CTG repeat contractions.
Double strand break repair - C1

Presenter: Aline Marnef

DNA double-strand breaks at rDNA are repaired by specific mechanisms

Aline Marnef, Emmanuelle Guillou, Virginie Daburon
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Previously, our group studied the role of cohesin recruitment at DSBs (Caron et al, 2012). Cohesin is a four subunits complex displaying the evolutionarily conserved function of entrapping DNA through its ring-like structure with a major function in sister chromatid cohesion. Yet, it also well established that cohesin plays important roles in DNA repair. In this work, we study the role of cohesin in the context of the repair process at rDNA in human cells.

The rDNA locus is formed of a repeated unit (300 times) that localizes in the nucleolus. Alone, it represents 35% of the transcriptional activity of the cell and produces the major rRNA species (18S, 5.8S and 28S). One can therefore easily imagine that DSBs located in such repetitive DNA elements are particularly challenging for the cell to repair due to their inherent recombinogenicity. Our knowledge about the mechanistic aspects of rDNA repair in human is very limited, though two recent studies suggested that both C-NHEJ and HR can be used to repair damaged human rDNA (Sluis et al, 2015 and Harding et al, 2015).

To study the repair mechanisms at rDNA, we use the DIvA system which has been developed by our laboratory. It generates about 100 sequence-specific DSBs at known locations via the AsiSI endonuclease. Importantly, one of them is located in rDNA. We show that the phosphorylated serine 966 (P-S966) form of SMC1 subunit of the cohesin complex is specifically involved in the rDNA DSB repair response in the G2 phase of the cell cycle, as shown through ChIP-qPCR and immunofluorescence. Our results also demonstrate that another phosphorylated residue of SMC1, P-S957, is absent from rDNA but is only involved in DNA repair at other genomic locations. Altogether, this forms the first evidence to indicate that rDNA can indeed be repaired by a specific mechanism, different to that in non-repeated single genomic locus. We are currently investigating in more depth the role of phosphorylated S966 cohesin in the context of rDNA DSB repair, through the establishment of a stable cell line expressing a phospho-S966A mutant in cells deleted for the endogenous SMC1 gene. In parallel, we are also currently examining the interacting-proteins that we identified via the mass spectrometry analysis of the SMC1 WT or the SMC1 P966A mutant cohesin complex.

This project DIvA has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement No 647344).

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Securing the genome during antigen receptor assembly: functional interplay between RAG, XLF, PAXX, and ATM

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XRCC4 and the XRCC4-like factor XLF are two distantly related members of the same protein family and function in non-homologous end-joining (NHEJ). The repair of RAG1/2-induced DNA double-strand breaks (DSBs) during V(D)J recombination is known to be fully dependent on the NHEJ factors Ku70/80, Ligase IV, DNA-PKcs/Artemis and notably XRCC4. By contrast, XLF is dispensable for antigen receptor assembly, due to functional redundancy with components of the ATM-dependent DNA damage response (DDR) (1). We recently found that XLF function in the repair of RAG-DSBs is also masked by its surprising redundancy with the RAG complex (2). Contrary to single deficiency due to loss of the C-terminal region of RAG2 (core RAG2) or XLF that do not harbor major defects in V(D)J recombination, we found that combined deficiency of core RAG2 and XLF leads to a block in lymphocyte development at the progenitor stage when V(D)J recombination occurs. Utilizing core RAG2/XLF-deficient v-Abelson (v-abl) transformed pro-B cell lines, we were able to show that i) these cells, when arrested in G1 to induce RAG expression, accumulate RAG-dependent 53BP1/γH2AX DNA repair foci to similar levels observed in other NHEJ-deficient conditions such as in Ku80- and XRCC4-deficient cells and display severe impairment in joining RAG DSBs, ii) rare coding joints detected in these cells harbor deletions and microhomologies reminiscent of rare joints seen in NHEJ-deficient cells, and iii) in the context of p53 deficiency, these cells and primary pro-B cells accumulate genomic instability in the form of chromosomal breaks and translocations. These results are consistent with a severe end-joining defect in core RAG2/XLF-deficient cells and demonstrate that core RAG2 B cells require XLF and reciprocally XLF-deficient B cells require RAG to achieve DNA end repair and maintain genome integrity during antigen receptor assembly.

PAXX (PAralog of XRCC4 and XLF), a recently discovered member of the XRCC4 superfamily, interacts with Ku and functions in NHEJ (3). We employed CRISPR/Cas9-mediated gene editing to delete PAXX from v-abl pro-B cell lines with the aim of interrogating its function(s) during antigen receptor assembly. I will present our latest results and a model outlining the relative contribution of the RAG complex, the XRCC4, XLF and PAXX paralogs, and the chromatin-associated ATM-DDR in repairing RAG-DNA breaks.

The European Research Council under the ERC starting grant agreement number 310917 supports this work

Double strand break repair - C1

Presenter: François Aymard

DNA Double Strand Breaks clustering revealed by Capture-HiC

Aymard, F(1), Guillou, E(1), Aguirrebengoa, M(1), Bugler, B(1), Maria-Javierre, B(2), Arnoult, C(1), Clouaire, T(1), Fraser, P(2), Legube, L(1)
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Translocations, that occur when two DNA Double Strand breaks are abnormally rejoined, represent highly deleterious genome rearrangements favoring cancer apparition and progression. However, the mechanisms that drive their formation are yet poorly deciphered. One prerequisite for translocation is the juxtaposition of two linearly distant DNA Double Strand Breaks, an event that may arise if DSB coalesce together within repair center.

Although largely accepted in yeast, DSB clustering is still subjected to intense debate in higher eukaryotes. Using a cell line where multiple DNA Double Strand break are induced at specific and annotated genomic positions throughout the human genome, combined with Chromosome Conformation Capture followed by deep sequencing (Capture Hi-C) we report here fundamental principles of DSB clustering in human cells.

We found that only DSBs induced in transcriptionally active chromatin and repaired by homologous recombination in post-replicative cells exhibit the ability to cluster, in contrast to DSB that occur in intergenic or transcriptionally silent chromatin and that are repaired by Non Homologous End Joining throughout the cell cycle. Unexpectedly we found that this clustering behavior is enhanced during the G1 cell cycle phase. Moreover DSB clustering depends on the MRN complex, as well as the Formin 2 (FMN2) nuclear actin organizer, suggesting that active mechanisms may be at work to promote DSB clustering.
POSTER SESSION C2: 'Double Strand Break Repair – Homologous Recombination'

Poster viewing: Monday, April 18, 20:00 – 22:00 h

Discussion: Tuesday, April 18, 16:55 – 18:25 h
Double strand break repair: HR - C2

Presenter: Anna Chambers

The Irc5/HELLS subfamily of chromatin remodelling proteins and maintenance of genome stability

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In eukaryotes DNA repair takes place in the context of chromatin and a number of chromatin remodelling and modification events are associated with efficient repair. Several ATP-dependent chromatin remodelling complexes have been found to be mutated in cancer and the involvement of these complexes in the DNA damage response is conserved from yeast to humans.

Sequence analysis identifies the human protein HELLS (LSH/PASG/SMARCA6) and its budding yeast homolog, Irc5, as superfamily 2 helicase chromatin remodelling enzymes but their biochemical activity remains uncharacterised. HELLS is mutated or misregulated in a number of cancers, and preliminary data suggest both it and its budding yeast homologue, Irc5, are involved in the DNA damage response by an, as yet, unknown mechanism. HELLS mutant MEFs (mouse embryonic fibroblasts) have been reported to display premature onset of senescence, reduced proliferation, aberrant chromosome segregation, increased DNA content and reduced global DNA methylation and it has been proposed that HELLS permits recruitment of DNA methyltransferases in order to silence transcription.

Recently, a DNA methylation-independent role for HELLS in DNA repair has emerged. MEFs deficient in HELLS displayed decreased survival, inefficient repair and defective histone H2AX phosphorylation following exposure to ionising radiation (IR) (Burrage 2012). Given that DNA methylation does not occur in yeast, it seems probable that it is the function in the response to damage that is conserved in Irc5. Indeed, yeast lacking IRC5 (Increased Recombination Centres 5) were found to display increased levels of spontaneous Rad52 foci in a large-scale screen as well as increased spontaneous recombination between homologous chromosomes (Alvaro 2007).

We are complementing investigation of HELLS in mammalian cells with in vitro, biochemical studies and characterisation of Irc5 in budding yeast, in order to elucidate mechanistic details of how this subfamily of proteins contribute to maintenance of genome stability. Our data implicate the human HELLS protein in promoting efficient double-strand break (DSB) repair by the homologous recombination (HR) repair pathway. We find that in budding yeast, Irc5 has a role in control of recombination and early steps in the DNA damage response but that unlike the catalytic subunits of many chromatin remodellers, the Irc5 protein is not part of a constitutive multi-subunit complex.

(2) Burrage J et. al, (2012) J Cell Sci. 125 (22)5524-5534
Double strand break repair: HR - C2

Presenter: Maaike Vreeswijk

Functional analysis of BRCAness in ovarian tumors


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Introduction.
BRCA1 and BRCA2 related ovarian tumors respond very well to treatment with PARP inhibitors (PARPi) because of their deficiency in homologous recombination (HR). Moreover, a substantial number of ovarian tumors might have deficiencies in HR unrelated to germline mutations in BRCA1 or BRCA2 (i.e. BRCAness). Assessment of HR efficacy in ovarian tumors might therefore allow identification of additional cancer patients that could benefit from PARPi treatment.

Experimental procedure.
Fresh ovarian tumor samples were collected from patients undergoing surgery or ascites/pleural fluid drainage. Samples were ex vivo irradiated with ionizing radiation, fixed after 2 hours incubation at 37°C and embedded in paraffin. Consecutively, we used the ability of replicating tumor cells to form RAD51 foci as a functional read out for HR proficiency.

Results.
HR status was determined in 50 ovarian tumor samples. Almost half of the tumor samples showed impairment of HR. Apart from four patients with a germline BRCA mutation, also a subset of tumors from non-BRCA mutation carriers showed a deficient HR phenotype. Apparently, germline BRCA testing is not sufficient to identify all HR deficient tumors. Genetic and epigenetic analysis of tumor DNA is ongoing.

Conclusion.
The ex vivo analysis of RAD51 foci formation in fresh tumor tissue is a promising new tool to identify HR deficient tumors. Not only is it an important time saving strategy compared to BRCA1/BRCA2 mutation analysis, it may also identify a much broader patient population that might be eligible for the treatment with PARP inhibitors.
DNA double strand break repair has been studied extensively in two dimensional cell cultures. Much less is known about three dimensional growth conditions and heterogeneous cultures, such as tumors. Therefore, we determined optimal growth conditions for patient-derived breast tumor slices with a thickness of approximately 30 cell layers. These conditions allowed maintenance of the tumor slices without loss of viability or proliferation over a period of at least 7 days, allowing extensive analysis of DNA damage response pathways. We used this experimental set up to determine the homologous recombination (HR) proficiency (using RAD51 foci formation after ionizing radiation as a read out) of a series of approximately 150 unselected primary breast tumors. We found HR deficiency in 13% of the breast tumors, in many cases associated with triple negative breast cancer. Careful analysis of the molecular defects revealed several cases of BRCA1 promoter methylation in addition to BRCA gene mutations, showing the functional defect of this epigenetic modification in tumors. We also showed proof of principle that HR deficient tumor slices were PARP inhibitor and cisplatin sensitive. We are currently adapting the technology for use on biopsy material, for which we will present the first results comparing ex vivo responses and treatment outcome in the patient.
Double strand break repair: HR - C2

Presenter: Jordi Surralles

Tumor suppressor C53 interacts with BRCA2, regulates DSB repair and drives breast and ovarian cancer prognosis

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BRCA2 is an essential protein for DNA repair by homologous recombination and its deficiency leads to genome instability and tumor progression. By means of a yeast two-hybrid screen, we identified NF-KB-dependent tumor suppressor C53 as a new BRCA2 helical domain interacting protein. C53 inhibition leads to resistance to a variety of DNA damage agents and increased spontaneous and ionizing radiation-induced chromosome fragility. Resembling BRCA2, C53 is required for homologous recombination and its absence markedly upregulates single strand annealing. Finally, we show that low C53 expression strongly correlates with poor patient survival rates in over 3,500 breast and 300 ovarian cancer datasets. Our results therefore uncover a new player in DNA repair by homologous recombination and single strand annealing that modulates the prognosis of breast and ovarian cancer patients.
Double strand break repair: HR - C2

Presenter: Florence Larminat

Polo-like kinase 1 mediates BRCA1 phosphorylation and recruitment at DNA double-strand breaks

1. Corinne Chabalier-Taste(1), Laetitia Brichese(1), Carine Racca(2), Yvan Canitrot(3), Patrick Calsou(2), Florence Larminat(2)

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Accurate repair of DNA double-strand breaks (DSB) caused during DNA replication and by exogenous stresses is critical for the maintenance of genomic integrity. There is growing evidence that the Polo-like kinase 1 (Plk1) that plays a number of pivotal roles in cell proliferation can directly participate in regulation of DSB repair. In this study, we show that Plk1 regulates BRCA1, a key mediator protein required to efficiently repair DSB through homologous recombination (HR). Following induction of DSB, BRCA1 concentrates in distinctive large nuclear foci at damage sites where multiple DNA repair factors accumulate. First, we found that inhibition of Plk1 shortly before DNA damage sensitizes cells to ionizing radiation and reduces DSB repair by HR. Second, we provide evidence that BRCA1 foci formation induced by DSB is reduced when Plk1 is inhibited or depleted. Third, we identified BRCA1 as a novel Plk1 substrate and determined that Ser1164 is the major phosphorylation site for Plk1 in vitro. In cells, mutation of Plk1 sites on BRCA1 significantly delays BRCA1 foci formation following DSB, recapitulating the phenotype observed upon Plk1 inhibition. Our data then assign a key function to Plk1 in BRCA1 foci formation at DSB, emphasizing Plk1 importance in the HR repair of human cells.
A localized nucleolar DNA damage response facilitates recruitment of the homology-directed repair machinery independent of cell cycle stage

Marjolein van Sluis, Brian McStay
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DNA double-strand breaks (DSBs) are repaired by two main pathways: non-homologous end-joining and homologous recombination (HR). Repair pathway choice is thought to be determined by cell cycle timing and chromatin context. Nucleoli, prominent nuclear subdomains and sites of ribosome biogenesis, form around nucleolar organizer regions (NORs) that contain rDNA arrays located on human acrocentric chromosome p-arms. Actively transcribed rDNA repeats are positioned within the interior of the nucleolus, whereas sequences proximal and distal to NORs are packaged as heterochromatin located at the nucleolar periphery. NORs provide an opportunity to investigate the DSB response at highly transcribed, repetitive, and essential loci. Targeted introduction of DSBs into rDNA, but not abutting sequences, results in ATM-dependent inhibition of their transcription by RNA polymerase I. This is coupled with movement of rDNA from the nucleolar interior to anchoring points at the periphery. Reorganization renders rDNA accessible to repair factors normally excluded from nucleoli. Importantly, DSBs within rDNA recruit the HR machinery throughout the cell cycle. Additionally, unscheduled DNA synthesis, consistent with HR at damaged NORs, can be observed in G1 cells. These results suggest that HR can be templated in cis and suggest a role for chromosomal context in the maintenance of NOR genomic stability.

Double strand break repair: HR - C2

Presenter: Alexandra Duarte

BRCA1/2-Deficient mouse mammary cancer organoids as a tool for in vivo dissection of PARP inhibitor resistance mechanisms

Alexandra Duarte(1), Ewa Gogola(1), Norman Sachs(2), Marco Barazas(1), Stefano Annunziato(1), Arno Velds(3), Julian R. De Ruiter(1), Marieke van de Ven(1), Hans Clevers(2), Piet Borst(4), Jos Jonkers(1), Sven Rottenberg(1,5)

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Defects in BRCA1 and BRCA2 result in impaired homology-directed DNA double-strand breaks (DSBs) repair and predispose to breast and ovarian cancer. PARP1 inactivation results in accumulation of DSBs during DNA replication and BRCA-deficient cells are therefore selectively hypersensitive to PARP inhibitors (PARPi). Although preclinical studies and phase I and II clinical trials have demonstrated the efficacy of PARPi treatment for BRCA-associated cancers, acquired resistance to PARPi treatment was eventually observed in both settings. Some drug resistance mechanisms have been uncovered including reversion of BRCA1 or BRCA2 inactivation or HR restoration independently of BRCA1/2 via loss of key DSB repair factors such as 53bp1 or Rev7, among others. However, many mechanisms of PARPi resistance are yet to be identified and more powerful preclinical cancer models are required to better understand and combat intrinsic and acquired resistance in cancer patients. For this purpose, we used our genetically engineered mouse model (GEMM) for BRCA1/BRCA2-associated breast cancer and established organoid models from PARPi-resistant and matched sensitive mouse mammary tumors. GEMM-derived organoid cultures can be easily isolated from resected tumors preserving important in vivo features such as cellular heterogeneity and self-renewal, thus improving the ability to model tumor biology in vitro. They can be rapidly expanded and long-term cultured in vitro and specifically preserve the phenotype of the original donor. Orthotopically transplanted organoids give rise to mammary tumors that recapitulate the epithelial morphology of the original lesions and conserve the drug response of the original tumor. Importantly, organoids can be efficiently genetically modified. Inactivation of 53bp1 in PARPi-sensitive BRCA1-deficient mouse organoids using CRISPR-Cas9 gene-editing technology resulted in strong PARPi resistance in vivo. These results demonstrate that BRCA1/2-deficient tumor-derived organoids may provide a useful platform to study mechanisms of drug resistance, and to test new therapeutic approaches to overcome or circumvent resistance.
Double strand break repair: HR - C2

Presenter: Arne Nedergaard Kousholt

Role of the BRCA1 C-terminal interaction partner BRIP1 in tumour suppression and therapy response

Arne Nedergaard Kousholt(1), Joey Riepsaame(2), Peter Bouwman(1), Henri van de Vrugt(3), Hein te Riele(2), Jos Jonkers(1)

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This project aims to elucidate the functional role of BRIP1 (FANCJ) as an important complex partner of BCRA1 in preventing breast cancer and in therapy responses. It is largely unknown how BRCA1 mediates its critical role as a tumour suppressor for human breast cancer. Recent literature have implicated that BRCA1 C-terminal complex partners are required. In this project we will develop tissue-specific knockout mice for one of the main C-terminal complex partners of BRCA1, BRIP1. We will monitor the development of breast tumours, and perform functional assays on derived cell lines. In addition, we will develop mice deficient for BRIP1 helicase activity (K52R) or capacity for BRCA1 interaction (S990A), to further investigate the functional role of BRIP1 as a tumour suppressor. Moreover, we will receive BRIP1 germline mutations identified in high-risk breast cancer families by large scale sequencing efforts such as performed by the BRIDGES consortium. BRIP1 patient variants with the highest probability of pathogenicity will be selected on the basis of bioinformatics predictions as well as other sources of information, such as frequency in cases and controls. We expect to select 30 potentially pathogenic mutations including previously identified BRIP1 breast cancer mutations. This comprehensive list of patient derived mutations will be introduced endogenously in a primary breast epithelial cell line using a novel CRISPR-CAS9 approach. To monitor the effect of these mutations on BRIP1 activity, these cell lines will be tested in cancer relevant functional assays for which BRIP1 deficient cells are known to have a phenotype. This novel functional assay approach allows us to test the cancer relevance of not only exon variants, but also promoter and intron variants. This will give a comprehensive knowledge of pathogenic BRIP1 variants, and give insight into functional important domains of BRIP1. Hits from the functional assay testing, will be further investigated using in-vitro and in vivo approaches. Importantly, this project is expected to yield novel insight into the regulation of critical genomic stability pathways, and provide important knowledge for developing improved screening and treatment opportunities for patients.
Double strand break repair: HR - C2

Presenter: Andrés Cruz-García

Topological stress release by topoisomerase II facilitates DNA end processing during DNA repair.

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Double strand DNA breaks (DSBs) are the most cytotoxic lesions on the DNA. If left unrepaired, they would become deleterious and act as potent inducers of apoptosis as well as chromosomal aberrations. Thus, faithful DSB repair is critical for cell survival and fitness. Several DSB repair pathways coexist within the cells, and the balance between them is tightly regulated. A key process for this is the so-called DNA end resection, a 5'→3' degradation of a single strand of the DNA that starts at a DSB. DNA resection is an essential step of homologous recombination and, moreover, its efficiency regulates the type of repair that will occur at the broken DNA. There are many factors known to be important for DNA resection processivity. However, the role of topoisomerases has been overlooked. Theoretically, the appearance of a break on the DNA may release topological stress, thus rendering topoisomerases irrelevant. However, by using classical techniques and also a high-resolution method to study DNA end resection in combination with topoisomerases inhibitors, depletions or knock out mutations we have found that indeed they facilitate such process. Hence, here we describe a new role of topoisomerase II as a helper for DNA end resection. We have explored the consequences on DNA repair of topoisomerase II inhibition or depletion. Finally, we have investigated what factor(s) impede the release of topological stress even when the DNA is broken.
Double strand break repair: HR - C2

Presenter: Christian Biertümpfel

Human Holliday junction resolvase GEN1 uses a chromodomain for efficient DNA recognition and cleavage

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Holliday junctions (HJs) are key DNA intermediates in homologous recombination. They link homologous DNA strands and have to be faithfully removed for proper DNA segregation and genome integrity. Here, we present the crystal structure of human HJ resolvase GEN1 complexed with DNA at 3.0 Å resolution. The GEN1 core is similar to other Rad2/XPG nucleases. However, unlike other members of the superfamily, GEN1 contains a chromodomain as an additional DNA interaction site. Chromodomains are known for their chromatin-targeting function in chromatin remodelers and histone(de)acetylases but they have not previously been found in nucleases. The GEN1 chromodomain directly contacts DNA and its truncation severely hampers GEN1’s catalytic activity. Structure-guided mutations in vitro and in vivo in yeast validated our mechanistic findings. Our study provides the missing structure in the Rad2/XPG family and insights how a well-conserved nuclease core acquires versatility in recognizing diverse substrates for DNA repair and maintenance.

Double strand break repair: HR - C2

Presenter: Céline Baldeyron

Identification and characterization of new binding partners of BRCA2

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DNA within cells is continuously exposed to many sources of DNA damaging agents, both from endogenous or exogenous sources. DNA double-strand breaks (DSB) are among the most deleterious forms of DNA lesion. The ability to accurately repair the breaks is essential for the faithful maintenance of genetic information. Deficiencies in DSB repair can lead to mutations and chromosomal rearrangements that ultimately may result in genomic instability and oncogenesis.

In mammals, two mechanisms are mainly involved in the repair of DSBs: non-homologous end-joining and homologous recombination (HR). NHEJ mediates the direct re-ligation of DNA ends by using no or little sequence homology. HR requires a homology of several hundreds of base pairs between the sequence carrying the DSB and the donor sequence of homology, preferentially located on the sister chromatid, to precisely repair the break. The critical intermediate in DSB repair through HR is the formation of joint molecule between the broken DNA and a homologous double-stranded template, which is ensured by the recombinase RAD51, a functional homolog of the bacterial RecA protein. RAD51 is recruited to DSBs by the protein mediator BRCA2.

BRCA2 is a large protein that binds RAD51 by its BRC repeats. Thus BRCA2 promotes the loading of RAD51 on single-stranded DNA, prevents the binding of RAD51 to double-stranded DNA and stimulates the strand exchange mediated by RAD51. Germline mutations of BRCA2 gene are implicated in two of genetic instability syndromes predisposing to cancer, familial breast and/or ovarian cancer and Fanconi anemia. Cells derived from patients carrying mutations in BRCA2 are characterized by genomic instability, lack of nuclear RAD51 foci induced by DNA damage and hypersensitivity to ionizing irradiation or Mitomycin C.

Due to its role as a HR mediator, the efficiency of this repair pathway will therefore depend on the efficiency by which BRCA2 is recruited to the chromatin. By performing pull-down assays followed by mass spectrometry analyses, we identified new BRCA2 partners specifically enriched under DNA damage conditions. We are validating some of these interactions and investigating their function in the HR-mediated repair of DSB.
DNA damage and replication stress activate the ATM and ATR kinases that coordinate checkpoint and DNA repair pathways. An essential step in homology-directed repair (HDR) of DNA breaks is the formation of RAD51 nucleofilaments mediated by PALB2-BRCA2, which is antagonized by anti-recombinases such as FBH1 and BLM. Notably, roles of ATM and ATR in this key stage of HDR are poorly understood. Here, we show that PALB2 is markedly phosphorylated in response to genotoxic stresses such as Ionizing Radiation and Hydroxyurea. This response is catalyzed by the ATM and ATR kinases through three N-terminal SQ-consensus phosphorylation target sites in PALB2. Importantly, phospho-deficient PALB2 mutant is unable to support proper RAD51 foci formation, a key PALB2 regulated repair event, whereas phospho-mimicking PALB2 version supports RAD51 foci formation. Further, we show that PALB2 phosphorylation counteracts the anti-recombinogenic helicases FBH1 and BLM. Accordingly, the phospho-deficient PALB2 is less active in HDR than wild-type and phospho-mimicking PALB2. Finally, the phosphorylation mutants reveal a new separation in PALB2 function, as the PALB2-dependent checkpoint response is normal in cells expressing the phospho-deficient PALB2 mutant. Collectively, our findings uncover the importance of PALB2 phosphorylation as a novel regulatory step countretracting anti-recombinase activities in response to genotoxic stress.
Double strand break repair: HR - C2

Presenter: Dejan Ristic

Biochemical and structural characterization of a mutant RAD51 associated with a novel Fanconi anaemia like disorder

Dejan Ristic(1,2), Sari E. van Rossum-Fikkert(1,2), Najim Ameziane(3), Josephine C. Dorsman(3), Roland Kanaar(1,2), Claire Wyman(1,2)

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Fanconi anaemia (FA) is a recessive genetically heterogeneous disorder featuring hypersensitivity to DNA cross-linking agents together with developmental abnormalities, bone marrow failure and a strong predisposition to cancer. Novel FA subtype, ‘FA-R’ is caused by a heterozygous mutation in RAD51, Ala293Thr. This suggests that this RAD51 mutation acts in a dominant-negative fashion. We purified and characterized RAD51 Ala293Thr to determine the molecular mechanism underlying its DNA repair defect. Compared to wild-type, RAD51 Ala293Thr is defective in several key biochemical functions. The mutant protein had impaired ATPase and DNA binding activity. Scanning force microscopy revealed distorted nucleoprotein filament formation suggesting a defect in the key DNA strand exchange step of homologous recombination. In deed as assayed by joint molecule formation, homologous recombination was severely reduced. Consistent with the apparent dominant negative phenotype, the mutant RAD51 interfered with binding of wild-type RAD51 to DNA.
Double strand break repair: HR - C2

Presenter: Giuseppe De Gregorio

Control of S. cerevisiae Exo1 by phosphorylation

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In presence of DNA damage as well as upon replication stress, cells activate a finely regulated response called DNA damage checkpoint, resulting in cell cycle arrest and activation of DNA repair mechanisms. In S. cerevisiae, the checkpoint response starts with 5' to 3' resection of damaged DNA ends, which is initiated by Sae2 and the MRX complex and continued by the redundant action of Exo1 and Dna2/Sgs1. This two-step mechanism of resection leads to the generation of long ssDNA tails that facilitate activation of Mec1 (homologue to hATR), the first checkpoint kinase in the DNA damage response. Activated Mec1, in turn, triggers a cascade of events leading to phosphorylation of the effector kinases Rad53 and Dun1.

Given the important role played by DNA nucleases in processing DNA double strand breaks or stalled replication forks, these enzymes are tightly regulated. Our laboratory has previously shown that human EXO1 is controlled through interaction with CtIP as well as by ATR-dependent phosphorylation and ubiquitylation. On the other hand, we provided evidence that yeast Exo1 is controlled through interaction with 14-3-3 proteins at stalled replication forks. Others have shown that phosphorylation at four residues modulates Exo1 activity at uncapped telomeres.

In this study, we first delineate pathways controlling Exo1 phosphorylation in response to replication stress and during the recovery phase, showing a role for the kinase Rad53 and the phosphatase Pph3. Next, we undertake a comprehensive identification of Rad53-dependent sites of phosphorylation in Exo1. Functional studies of the five major sites of phosphorylation (S393, S413, S563, S587 and S692) reveal a role in the sensitivity to HU or camptothecin, an effect that is the result of abnormal Exo1 resection activity at sites of DNA damage. Finally, we describe the effect of phosphorylation on Exo1 localization and protein stability.

The implications of uncontrolled Exo1 activity on genome stability will be discussed.
Inactivating germline mutations of two important players of the homologous recombination (HR) pathway, BRCA1 and BRCA2, predispose to breast and ovarian cancers and result in HR deficiency. This defect can be specifically targeted by the inhibition of the poly (ADP-ribose) polymerase (PARP)1, an enzyme involved in single strand break (SSB) repair. When inhibited, accumulating SSBs are eventually converted to double strand breaks and cells depend on HR for error-free repair of this damage. Despite the initial success of PARP inhibitors (PARPi) both in preclinical models and in the clinic, drug resistance remains a vexing problem. Thus far, several mechanisms of resistance have been proposed for BRCA1-deficient tumors, and most of them rely on HR restoration (e.g. genetic reversion of BRCA1/2, loss of 53BP1 or REV7). Nevertheless, these mechanisms fail to explain all resistant cases. Additionally, not much is known about the potential of BRCA2-deficient tumors to restore HR.

In this project, we aim to understand how frequently BRCA1/2-deficient tumors re-activate HR pathway to escape PARPi treatment. To study this, we generated a large cohort of matched PARPi-naïve and resistant mammary tumors using our mouse models for BRCA1/2-associated breast cancer (K14cre; BRCA1/2F/F; p53F/F). To determine HR capacity of the tumors, we developed an in situ method for the analysis of ionizing radiation induced foci (IRIF) of the HR protein RAD51.

Our study reveals that HR is frequently restored in BRCA1- but not BRCA2-deficient PARPi-resistant tumors. Moreover, RAD51 IRIF were detected in BRCA1-deficient tumors that lost 53BP1 or REV7 expression, confirming previously described mechanisms of HR restoration. Interestingly, we also identified a subset of BRCA1-deficient RAD51 IRIF positive tumors in which HR activity could not be explained by any of the already known mechanisms, suggesting a yet-to-be-identified mechanism. Finally, our study showed that PARPi resistance was induced in the absence of RAD51 foci formation in all of the BRCA2-deficient tumors, underpinning the crucial role of BRCA2 in RAD51 loading.

To further elucidate PARPi-resistance in BRCA2-deficient tumors, we decided to focus on stalled replication forks, an early lesion induced by PARPi treatment. For this purpose, we performed DNA fiber assays using tumor-derived cancer organoids. Our preliminary data suggest that some PARPi-resistant BRCA2-deficient tumors restored the capacity to protect stalled forks from degradation, suggesting a novel, HR-independent mechanism of PARPi resistance.

We are currently combining several in vitro assays (DDR reporter assays, DNA fiber assays, CRISPR/Cas9 screens) with various high-throughput approaches (RNASeq, whole-exome sequencing, phospho-proteomics) of tumor samples. We hope that with this complementary approach we will be able to dissect the different mechanisms of PARPi resistance and identify markers of poor response to this treatment.
Double strand break repair: HR - C2

Presenter: Frank Rolfs

Identification and validation of protein biomarkers for homologous recombination deficiency in breast cancer using patient-derived xenograft models

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Breast Cancer is a serious health problem and the most common cancer in women worldwide. Because of its high diversity, the right choice of therapy remains a challenge for each patient and subtype, which creates a need for the identification of characteristic biomarkers.

Triple negative breast cancers (TNBCs) show very poor prognosis, with chemotherapy as the only treatment option. Because of a BRCA1 mutation and thus deficiency in homologous recombination (HR) repair, a subgroup of these tumors is sensitive to DNA damaging drugs or inhibition of non-homologous end joining (NHEJ) via PARP inhibition. Therefore, the identification of HR deficient breast cancer via biomarkers is of importance for personalized therapy.

The RAD51 IRIF assay, a current test for HR-deficiency on freshly isolated primary biopsies is complex and based on a single marker. We would like to complement this situation and identify as well as validate additional protein biomarkers for HR deficient breast cancer using (phospho)proteomics and patient derived xenograft models of HR deficiency. Moreover, we plan to develop targeted mass spectrometry assays for validation and high-throughput analysis of the most promising biomarkers for HR deficiency. To explore the predictive potential of the candidate markers, we also plan to perform an in vivo intervention study and treat xenografted mice with the PARP inhibitor Olaparib.

The identified biomarkers will likely support patient selection for breast cancer therapy in the future.
The HR factor TONSL-MMS22L recognizes post-replicative chromatin via interaction with histone H4

Giulia Saredi(1), Hongda Huang(2), Colin M. Hammond(1), Simon Bekker-Jensen(3), Ignasi Forne(4), Constance Alabert(1), Nazaret Reveron-Gomez(1), Benjamin M. Foster(5), Lucie Mlejnkova(6), Till Bartke(5), Petr Cejka(6), Niels Mailand(3), Axel Imhof(4), Dinshaw J. Patel(2), Anja Groth(1)

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The eukaryotic genome is organized into chromatin and histone dynamics are tightly integrated with DNA metabolic processes, including DNA repair. Genome maintenance mechanisms are dependent on cell cycle stage and chromatin context, yet the underlying molecular mechanisms still remain unclear. We previously identified the TONSL-MMS22L complex as a novel homologous recombination (HR) factor (Duro et al. 2010; O’Connell et al. 2010; O’Donnell et al. 2010; Piwko et al. 2010). We showed that TONSL-MMS22L form a complex with histones, as well as histone chaperones ASF1 and MCM2, although the exact architecture of the complex remained to be dissected.

In our new structure-function work, we identify TONSL as a novel histone reader protein. We have used X-ray crystallography and peptide-pull down analysis to identify the molecular determinants for TONSL recognition of H4. These data show that TONSL specifically recognizes new histones and we propose that TONSL-MMS22L is delivered to chromatin during nucleosome assembly by piggybacking on H3-H4. Importantly, this provides a novel and direct mechanism for cells to identify genomic loci where a sister chromatid is available for post-replicative-repair in S/G2 phase of the cell cycle. We will present evidence to support this model, including functional analysis of TONSL interaction mutants.
The budding yeast STUbL ULS1 acts together with the homologous recombination pathway to mediate viability in the presence of DNA intercalating drugs.

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One of the key stages of DNA repair is the alteration of chromatin structure by the Snf2 superfamily of chromatin remodelling ATPases. Additionally, SUMO targeted ubiquitin ligases (STUbLs) which mediate the cross-talk between Ubiquitin and SUMO modification are important guardians of genome stability. To better understand these processes, we chose to study Uls1, a poorly characterised Snf2-family protein that has been proposed to function as a STUbL. Our initial results show that Uls1 forms nuclear foci dependent on the integrity of its SUMO interacting motifs (SIMs). Loss of Uls1 or inactivation of its ATPase activity results in strong sensitivity to the DNA intercalating drug acriflavine and subsequent activation of the DNA damage checkpoint. These effects are synergistically enhanced by mutation of the homologous recombination (HR) but not the non-homologous end joining (NHEJ) pathway of DNA repair. We show that Uls1 is rapidly recruited to sites of DNA damage and hypothesize that Uls1 is involved in preventing unusual DNA lesions.
Double strand break repair: HR - C2

Presenter: Isabel Soria-Bretones

**Sumoylation-dependent nuclear localization of CtIP influences DNA repair**

*Isabel Soria-Bretones, Pablo Huertas*  
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DNA is constantly challenged by many physical and chemical agents that alter its structure and compromise its integrity. The most cytotoxic forms of DNA damage are double-strand breaks (DSBs), that can cause deleterious consequences, such as genomic instability and chromosomal aberrations, if they are erroneously repaired. Thus, DSB repair is related with several human diseases, including cancer.

CtIP is a key protein in the DNA damage response (DDR) due to its role in checkpoint activation and transcription regulation; but more importantly because it promotes faithful repair of double strand breaks by licensing homologous recombination (HR) pathway. As a multifunctional protein, CtIP activities need to be strictly regulated either by interaction with other proteins or through post-translational modifications.

We are characterizing the role of sumoylation, an ubiquitin-like modification, on CtIP activity. In summary, we have found that a small fraction of CtIP is constitutively sumoylated by both SUMO1 and SUMO2 in human cells. Although sumoylation happens at several sites, we have identified a single lysine which sumoylation is absolutely essential for CtIP-mediated recombination and repair. Using single point mutations that block sumoylation at this residue in combination with mutants that are constitutively sumoylated we have studied the role of this post-translational modification in the repair of DSBs. Strikingly, we have noticed that constitutive sumoylation causes the relocation of CtIP to the nuclear pore complex, where it's functionally active. Thus, CtIP sumoylation links nuclear localization at the nuclear pore complex with homologous recombination.
Double strand break repair: HR - C2

Presenter: Jo Morris

The BRCA1:BARD1 Ubiquitin ligase activity counters chromatin barriers to DNA resection

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Optimal DNA repair suppresses genomic instability which is a driver of cancer and ageing. DNA double-strand break (DSB) repair choice is partly governed by the opposing activities of the p53 binding protein (53BP1) and the Breast Cancer 1 predisposition protein (BRCA1). However, how BRCA1 counters the inhibitor influence of 53BP1 on DNA resection and homologous recombination (HR) is not known. Here we demonstrate that the ubiquitin ligase activity of the BRCA1:BARD1 complex is a key determinant. Through mutation of a novel element within the dimeric RING domains of this complex we demonstrate the ligase activity is required for 53BP1 repositioning on damaged chromatin, the promotion of DNA resection and HR repair, and not required in the response to replication stress nor to inter-strand cross-links. We confirm H2A ubiquitylation by the ligase and show that bypass of the requirement for BRCA1:BARD1 in HR repair provided by expression of a H2A-ubiquitin fusion protein requires both DNA resection enzymes and a chromatin remodeler. Optimal localisation of the remodeler to sites of damage requires its ubiquitin-binding domains and BRCA1:BARD1. It is also required for 53BP1 repositioning. Consequently need for The remodeler in Olaparib or camptothecin resistance is also alleviated by loss of 53BP1. This work identifies the BRCA1:BARD1 ligase activity and subsequent chromatin remodelling as critical regulators of DNA repair.
Double strand break repair: HR - C2

Presenter: Juan Martinez

BRCA2 regulates DMC1-mediated recombination through the BRC repeats

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The breast cancer susceptibility protein 2 (BRCA2) plays a crucial role on DNA repair by homologous recombination. Through its BRC repeats, BRCA2 promotes the loading of RAD51 on ssDNA, prevents the binding of RAD51 to dsDNA and stimulates RAD51-mediated DNA strand exchange [1-3]. The meiosis-specific homolog of RAD51, DMC1, promotes the formation of DNA strand invasion products (joint molecules) between homologous molecules in a similar fashion to RAD51 [4]. As with RAD51, BRCA2 interacts directly with DMC1 [1, 5]; however, little is known about the functionality of this interaction.

In this work, we set out to address this question. We establish that human DMC1 interacts directly with each of the BRC repeats of BRCA2. We also unveil that with the exception of BRC4, each BRC repeat stimulates joint molecule formation by DMC1. Moreover, we found that the basis for this stimulation is an enhancement of DMC1-ssDNA complex formation by the BRC repeats. Importantly, the stimulation of joint molecule formation is also observed with the full-length BRCA2 protein. Finally, we demonstrate that BRCA2 stimulates DNA strand exchange between RPA-ssDNA complexes and dsDNA, thus identifying BRCA2 as a mediator of DMC1 recombination function.

Our results suggest specialized functions for the BRC motifs of BRCA2 in promoting DMC1 function and establish BRCA2 as a mediator of meiotic homologous recombination.

Paraspeckle protein PSF is an RNA processing factor required in DNA replication and homologous recombination repair

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Defects or errors in DNA replication are a major contributing factor in genomic instability. Failure to deal with stalled or collapsed replication forks is linked to the generation of disease-causing chromosome abnormalities. Using iPOND (isolation of proteins on nascent DNA) we identified the mRNA processing factor PSF as an essential component of the DNA replication complex required for cell proliferation. PSF is commonly found as a component of nuclear sub-bodies called paraspeckles, which are present in many different cell types and are linked with transcription. Depletion of PSF in cells caused profound replicative failure, activation of the checkpoint kinase Chk1, increased chromosome aberrations and greatly decreased cell viability. Loss of PSF in cells also correlated with increased replication stress as evidenced by greatly elevated nuclear RPA foci. The dual role of PSF in mRNA processing and replication suggests it might be required to prevent replication-transcription conflicts in proliferating cells. We will discuss how replicative failure in PSF-depleted cells correlates with a specific defect in homology-dependent repair (HDR). Together our data argue for a new and important role for PSF in the orchestration of replication, transcription and DNA repair to promote genome stability.
Double strand break repair: HR - C2

Presenter: Karine Dubrana

Nuclear organization and chromatin modulate homologous recombination efficiency and outcome

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Genome integrity is insured by conserved DNA repair mechanisms among which Homologous Recombination (HR) uses an intact homologous sequence to repair a broken chromosome. Although the molecular steps of HR have been extensively described and reconstituted in vitro, their regulation in the context of the nucleus and on more or less compacted chromatin remains largely unexplored.

To understand how the different genomic, chromatin, and subnuclear context influence homologous recombination, we developed a new assay to score spontaneous and DSB induced recombination events between alleles located at different chromosomal positions. It allowed us to measure recombination frequency and outcome in yeast strains in which the physical distances between telomeres and/or the spreading of heterochromatin in subtelomeric regions were modified through overexpression of the Sir3 or Sir3A2Q protein.

In addition, the assay allows assessment of the competition between two recombination pathways, namely gene conversion (GC) or break induced replication (BIR).

Using these tools, we first observed that loci at different locations in the genome recombine with different efficiency and further demonstrated that reducing the physical distance between homologous sequences favors recombination, demonstrating that homology search is a key limiting step for recombination efficiency. In addition we showed that BIR is the prominent repair pathway at subtelomeric DSB in WT cells. Increasing heterochromatin level at subtelomeric DSB favors recombination increasing both GC and BIR events. Our molecular analysis further revealed that heterochromatin fine-tunes DSB resection, limiting the loss of the homologous sequence required to perform strand invasion. On the opposite, heterochromatinization of the recombination donor site disfavors both GC and BIR suggesting that it impairs an early step of the recombination process possibly strand invasion or priming DNA synthesis.

We will discuss how this chromatin-dependent control could impact on the evolution of subtelomeric sequences and help to minimize the risks associated with repair events at chromosome ends.
Double strand break repair: HR - C2

Presenter: Marco Barazas

Systematic in vivo evaluation of putative resistance factors to PARP inhibition in Brca1-deficient cells using CRISPR/Cas9-mediated gene editing.

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Approximately 20% of triple-negative breast cancer contain alterations in the hereditary breast cancer gene Brca1, either through mutation or promoter methylation. BRCA1-deficient cells are unable to repair DNA double-strand breaks (DSB) through the error-free DNA-repair pathway of homologous recombination (HR). This characteristic can be exploited by the chemical inhibition of PARP1. Despite an initial good response of BRCA1-deficient tumors to systemic treatment with the PARP inhibitor olaparib, acquired resistance is a major clinical problem. It was previously shown that loss of additional DNA-damage response factors, such as 53BP1 or REV7, can restore HR-activity and induce PARP-inhibitor resistance in BRCA1-deficient cells in vitro and in vivo. Recently, additional downstream effector proteins of 53BP1 have been described, however the in vivo penetrance for these factors remains largely unexplored.

In this study, we demonstrate the power and versatility of CRISPR/Cas9-mediated gene editing to systematically evaluate the role of putative resistance factors on treatment response in vivo. We make use of a brief ex vivo step in which putative resistance factors are efficiently knocked out in tumor-derived organoids. This is followed by orthotopic re-transplantation in mice. We show that the intermediate culture step and subsequent manipulation with a non-targeting gRNA has no effect on the response to continuous olaparib treatment for 56 consecutive days (median survival of 67 days). In contrast, knock out of 53BP1 or REV7 induces inherent resistance to treatment with PARP inhibitors (median survival of 8 and 41 days, respectively; P <0.001). Through this approach we also provide the first in vivo evidence for RIF1 as a driver of resistance to PARP inhibition (median survival of 25 days; P <0.001). Profiling of resistant tumors confirmed specific enrichment for non-functional allelic variants and a complete protein knock out. These findings establish a robust in vivo platform for the functional interrogation of putative resistance factors in BRCA1-deficient tumors.

The isogenic tumor panel that we established will be further characterized for cross-resistance to other DNA-damaging agents, including topotecan and cisplatin. In addition, we are currently extending our work by utilizing CRISPRa to study the effect of gene activation in driving therapy resistance. Preliminary data shows that CRISPRa mediated overexpression of Abcb1b induces resistance to olaparib in vitro, whereas cells remain sensitive to AZD2461 – a PARP inhibitor that is a poor substrate for Abcb1b. These findings are currently being validated in our in vivo model. Together, we believe these approaches open up new avenues to unravel both loss-of-function and gain-of-function resistance mechanisms and treatment vulnerabilities in an in vivo setting.

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Double strand break repair: HR - C2

Presenter: Michael Chang

Multiple Rad52-mediated homology-directed repair mechanisms are required to prevent telomere attrition-induced senescence in Saccharomyces cerevisiae

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Most human somatic cells express insufficient levels of telomerase, resulting in telomere shortening and eventual senescence, both of which are hallmarks of ageing. Homology-directed repair (HDR) is important for maintaining proper telomere function in yeast and mammals. In Saccharomyces cerevisiae, Rad52 is required for almost all HDR mechanisms and telomerase-null cells senesce faster in the absence of Rad52. However, it has been unclear how Rad52 delays senescence. In this study, we made use of rad52 separation-of-function mutants to find that multiple Rad52-mediated HDR mechanisms are required to delay senescence, including both Rad51-dependent and Rad51-independent break-induced replication, as well as sister chromatid recombination. In addition, we show that misregulation of histone 3 lysine 56 acetylation, which is known to be defective in sister chromatid recombination, also causes accelerated senescence. We propose a model where Rad52 is needed to repair telomere attrition-induced DNA replication stress at both subtelomeric and telomeric regions.
Double strand break repair: HR - C2

Presenter: Malgorzata Grosbart

Investigating structural dynamics of a nanomachine: How does BRCA2 organize RAD51 for efficient DNA repair?

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BRCA2-RAD51 complex is a central component of DNA double strand break repair. Although extensively studied, the structure and the exact functions within homologous recombination remain enigmatic. BRCA2 has challenged researchers for years- only parts of the protein were crystalized and only lower resolution reconstructions from electron microscopy are available thus far, rendering structural studies extremely difficult. Sequence analysis predicts frequent occurrence of intrinsically disordered regions in BRCA2, which can explain the difficulties in imaging the protein. We have purified human BRCA2 and employed Scanning Force Microscopy to investigate its structure and corresponding functional features. We discovered that BRCA2 exists in vitro as a heterogeneous, likely dynamic array of forms, with visible preference for dimers and pentamers, with a prominent, V shaped protrusions extending from the complex core. Single molecule tracking in live cells confirmed the oligomeric arrangement of BRCA2 in vivo. After complex formation with RAD51, BRCA2’s essential partner in DNA repair, BRCA2 complexes undergo a dramatic rearrangement to form stiff, filamentous structures in vitro. We are interested in the molecular interactions responsible for, and functional importance of these structural transitions and dynamic (re)arrangements within BRCA2 complex. Therefore, we created truncated versions of BRCA2, missing DNA-binding and/or RAD51-binding domains. We intend to investigate the structural dynamics of these BRCA2 deletion construct and the impact of its different domains on the disordered-to-ordered transition that occurs upon binding RAD51. We employ Scanning Force Microscopy for nanometer resolution imaging of proteins and state-of-the art, hybrid Total Internal Reflection-SFM microscopy for identifying individual components of multiprotein complexes.
Double strand break repair: HR - C2

Presenter: Martijn Luijsterburg

The RNF168 Ubiquitin Ligase Orchestrates PALB2-Mediated Homologous Recombination

Dimitris Typas (1), Martijn S. Luijsterburg (1), Wouter W. Wiegant (1), Marie-Christine Caron (2), Leon H. Mullenders (1), Jean-Yves Masson (2), Haico van Attikum (1)

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The PALB2 tumor suppressor protein is an essential mediator of DNA double-strand break (DSB) repair by homologous recombination (HR). PALB2 interacts with and is recruited to DSBs by BRCA1 and subsequently promotes the assembly of the core HR proteins BRCA2 and RAD51. In this study, we show that the ubiquitin ligase RNF168 promotes HR by targeting PALB2 to repair sites, in a manner that is different from its role in regulating RAP80/BRCA1-dependent signaling of DSBs. PALB2 directly associates with RNF168 through its C-terminal WD40 domain and this region in PALB2 is required for its recruitment to HR sites. Moreover, RNF168’s ability to target PALB2 to HR sites requires its catalytic activity as well as its ability to associate with nucleosomes. In particular, a mutant of RNF168 (R57D) that is unable to ubiquitylate H2A on K13/K15 failed to support PALB2 recruitment, suggesting an involvement of RNF168-mediated H2A ubiquitylation in this process. Consistently, we found that over-expression of H2AK13R/K15R, but not H2AWT, significantly decreases PALB2 recruitment to nuclease-induced DSBs. Finally, we show that PALB2 is able to associate with K63-linked ubiquitin chains in vitro, but only in the presence of recombinant RNF168 protein. Together, these findings suggest a piggyback ride mechanism for PALB2 recruitment, in which PALB2 associates with RNF168 and utilizes RNF168’s ability to drive its own recruitment by binding and amplifying ubiquitin conjugates at sites of DNA damage. Our findings reveal an RNF168-dependent mechanism that facilitates the assembly of functional HR complexes and contributes to efficient execution of PALB2-dependent DNA repair by HR.
Double strand break repair: HR - C2

Presenter: Marcin Nowotny

Structural studies of Holliday junction resolvases RuvC and Slx1-Slx4.

Marcin Nowotny(1), Vineet Gaur(1), Karolina Gorecka(1), Haley D Wyatt(2), Stephen C West(2)

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Holliday junctions (HJ) are four-way DNA structures which are intermediates in homologous recombination. HJs are dangerous for genome stability and need to be removed. One of the pathways to achieve this is through the action of specialized nucleases termed resolvases.

RuvC is the canonical bacterial HJ resolvase. We solved its first crystal structure in complex with the DNA substrate (1). In the structure RuvC homodimer binds the HJ which adopts a so-called tetrahedral conformation observed for the first time. The arms of the DNA are fully paired around the exchange point and the two active sites of RuvC dimer are located symmetrically at scissile phosphates 1 nt from the HJ exchange point. All four arms form interactions with the protein explaining the strict specificity of RuvC for four-way junctions. Two alpha-helices form the protein dimer interface. Their N-termini also stabilize the sharply bent strands of the HJs. The arrangement of the Holliday junction in the complex is very different from the previously determined structures of phage resolvases Endo I and Endo VII, demonstrating that multiple modes of HJ recognition are possible.

HJ resolution in eukaryotes relies on the action of GEN1 or SLX1-SLX4-MUS81-EME1 complex. SLX1 is a promiscuous nuclease which cleaves various DNA structures. It introduces a nick in the HJ and this nicked DNA is a substrate for MUS81-EME1. In addition to HJ resolution SLX1 is also involved in telomere maintenance and interstrand cross-link repair. It is only active when it associates with the SLX4 platform protein. We solved the first crystal structure of fungal Slx1 and its complex with the interacting domain from Slx4 (Conserved C-terminal Domain – CCD) (2). Slx1 forms an oblong molecule with N-terminal GIY-YIG nuclease domain and C-terminal RING finger zinc-binding domain. In the crystal Slx1 forms a tight homodimer in which the access to the active site and DNA-binding residues is restricted, which explains why Slx1 alone is inactive. Homodimerization and CCD binding use the same interface, so the two events are mutually exclusive. Upon CCD binding the active site is exposed, which likely leads to the activation of the enzyme. This suggests that the activity of Slx1 is regulated by autoinhibitory homodimerization.

(1) Gorecka K et al. Nucleic Acid Res, 2013
Double strand break repair: HR - C2

Presenter: Nathalie van den Tempel

Localized induction of homologous recombination deficiency by hyperthermia

Nathalie van den Tempel, Hanny Odijk, Alex Zelensky, Roland Kanaar
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Modern day anti-cancer research is focused on the development of precision and targeted therapies against various tumor types. An example of a targeted therapy based on tumor-associated DNA repair deficiency, is the use of PARP-1 inhibitors on patients that have ovarian cancer lacking the BRCA1 or BRCA2 protein and harbor a defect in homologous recombination. Our laboratory has shown that this BRCA2-deficient phenotype can be temporarily induced on demand by applying mild hyperthermia to tumor cells, increasing cell temperature to 41-42.5 °C. We have demonstrated that degradation of the BRCA2 protein is not only limited to tumor cell lines, but also occurs in tumors directly derived from patients. In the clinic, hyperthermia is used to increase the temperature of the tumor tissue by employing an exogenous source, and it is available to treat a broad spectrum of tumor types. Our findings therefore might have consequences for the possibility of expansion of PARP-1 inhibitor use. Moreover, BRCA2 degradation can now be used as a guideline for the current employment of hyperthermia as a sensitizer for radiotherapy. Therefore, we have analyzed the effects of hyperthermia treatment temperature and time (thermal dose) by heating four cell lines to clinically relevant temperatures between 40-44 °C for up to 4 hours. We determined BRCA2 protein levels after hyperthermia and linked that to their clonogenic capacity after irradiation (0-6 Gy). We demonstrate that to achieve optimal radiosensitizing effects in vitro, cells should be subjected to 41-43 °C for 30 minutes to 1 hour. Besides translating the finding that hyperthermia inhibits homologous recombination, we are trying to elucidate the mechanism by which hyperthermia induces BRCA2 degradation. With this knowledge, we hope to find new ways to enhance or lengthen the temporary DNA repair deficiency effect induced by hyperthermia and find possible biomarkers that predict the response to hyperthermia. Our data demonstrates that hyperthermia has a large therapeutic potential by temporarily inhibiting homologous recombination, which is of particular interest because hyperthermia’s broad employment possibilities in cancer therapy.
Double strand break repair: HR - C2

Presenter: Sylvie Noordermeer

Ubiquitylation and E3 ligase interplay regulating homologous recombination

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The regulation of both homologous recombination (HR) and non-homologous end joining is highly dependent on protein ubiquitylation. We recently showed the importance of PALB2 ubiquitylation in G1, resulting in the disruption of its interaction with BRCA1 and thereby inhibiting HR in this cell cycle stage. In this case, the cell cycle regulation is mediated via G1-specific inactivation of the deubiquitylase USP11. Restoring the interaction of PALB2 and BRCA2 combined with the activation of end-resection is sufficient to induce HR in G1 (1).

The process of HR is regulated by ubiquitylation on additional levels via the activity of several E3 ubiquitin ligases (e.g. RNF8, RNF168, RNF169, BRCA1). Although RNF8 and RNF168 are indispensable for BRCA1 recruitment to double strand breaks (DSBs), downstream RAD51 recruitment and HR is not dependent on the function of these two ubiquitin ligases. However, these two E3 ligases may become essential for RAD51 focus formation when BRCA1 and 53BP1 are simultaneously depleted (2).

To study the interplay between different E3 ligases during HR, we generated a panel of CRISPR-CAS9 mediated knock-out RPE1 cell lines of E3 ligases as well as the E2 conjugating enzyme UBC13. We used this panel to study the recruitment of HR factors and HR outcome.

(2) Nakada et al, Cancer Res, 2012
Double strand break repair: HR - C2

Presenter: Owen Wells

**Missense mutation in NSE3 leads to a novel chromosomal disorder with perturbed homologous recombination**

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Whilst the primary function of the SMC5/6 complex remains elusive, it has been implicated in DNA repair, maintaining accurate chromosomal segregation and DNA replication processes. The SMC5/6 proteins and its non-SMC sub-units, NSMCE 1, 2, 3 and 4, are required for cell viability and are essential in budding yeast. Furthermore, the SMC5/6 complex has been shown to supress cancer and ageing in mice. Recently, a mutation in the SMC5 gene was demonstrated to have a role in brain metastasis development, and compound heterozygous frame-shift mutations in NSMCE2 have been linked to primordial dwarfism and insulin resistance.

Here, we describe patients that contain homozygous missense mutations within the NSMCE3 gene, resulting in early death (median age of mortality = 12-14 months) caused by rapidly progressive pulmonary failure following viral pneumonia. NSMCE3 mutated patient cell lines show a rise in chromosomal rearrangement and micronuclei formation that are common features in chromosomal breakage syndromes. Cellular and biochemical assays show that these mutations lead to NSMCE3 instability, resulting in the destabilization of the SMC5/6 complex. Patient fibroblasts are hypersensitive to IR, in S and G2 phase of the cell cycle, and this is due to perturbed homologous recombination.
Double strand break repair: HR - C2

Presenter: Przemek Krawczyk

Distinct genetic control of homologous recombination repair of Cas9-induced double-strand breaks, nicks, and paired nicks

Lianne E.M. Vriend(1,2), Rohit Prakash(2), Chun-Chin Chen(2,3), Fabio Vanoli(2), Francesca Cavallo(2), Yu Zhang(2), Maria Jasin(2), Przemek M. Krawczyk(1,2)

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DNA double-strand breaks (DSBs) are known to be powerful inducers of homologous recombination (HR), but single-strand breaks (nicks) have also been shown to trigger HR. Both DSB- and nick-induced HR (nickHR) are exploited in advanced genome-engineering approaches based on the bacterial RNA-guided nuclease Cas9. However, the mechanisms of nickHR are largely unexplored. Here we applied Cas9 nickases to study nickHR in mammalian cells. We find that nickHR is unaffected by inhibition of major damage signaling kinases and that it is not suppressed by nonhomologous end-joining (NHEJ) components, arguing that nick processing does not require a DSB intermediate to trigger HR. Relative to a single nick, nicking both strands enhances HR, consistent with a DSB intermediate, even when nicks are induced up to 1kb apart. Accordingly, HR and NHEJ compete for repair of these paired nicks, but, surprisingly, only when 5' overhangs or blunt ends can be generated. Our study advances the understanding of molecular mechanisms driving nick and paired-nick repair in mammalian cells and clarify phenomena associated with Cas9-mediated genome editing.
Double strand break repair: HR - C2

Presenter: Priscilla Cooper

Role of XPG in Homologous Recombination through Direct Interactions with BRCA1 and BRCA2

Kelly S. Trego(1), Weixing Zhao(2), Torsten Groesser(1), Ann C. Parpys(1), Miaw-Sheue Tsai(1), Claudia Wiese(3), Judith Campisi(4), Patrick Sung(2), Priscilla K. Cooper(1)

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XPG is a structure-specific endonuclease required for nucleotide excision repair (NER). XPG enzymatic defects result in cancer-prone xeroderma pigmentosum (XP), whereas truncating mutations cause the severe progeroid neurodevelopmental disorder Cockayne syndrome (CS), characterized by defective transcription-coupled repair (TCR). An XPG knockout mouse model substantially recapitulates the CS patient phenotype (Barnhoorn et al., 2014), but how loss of XPG results in this devastating disease is poorly understood. An XPG role early in TCR is suggested by interactions with RNA polymerase II and CSB (Sarker et al., 2005). XPG also plays a key non-enzymatic role in base excision repair (BER) of oxidative DNA lesions. Accordingly, XP-G/CS patient cells are hypersensitive to oxidizing agents and ionizing radiation as well as UV.

Unrepaired DNA lesions, repair intermediates, and stalled transcription can impede replication fork progression and result in replication stress, DNA double-strand breaks (DSBs), and genomic instability. We have now identified XPG as a novel partner of BRCA1 and BRCA2 in maintaining genomic stability through homologous recombinational repair (HRR) (Trego et al., 2016). Loss of XPG reduces cell proliferation, elevates the DNA damage response, increases phosphoRPA32, and causes DSBs in the absence of extrinsic damage, resulting in greatly elevated chromatid breaks and micronuclei formation. XPG knockout cells have impaired recovery from camptothecin-induced replicative stress, and DNA fiber analysis reveals inability to restart hydroxyurea-stalled replication forks. During S-phase, XPG forms foci that co-localize with gammaH2AX and 53BP1 foci as DSB markers. It interacts with RAD51, BRCA2, and PALB2 in a higher-order HRR complex in human cells, and insect cell co-expression studies reveal direct protein-protein interactions with each of these. Strikingly, XPG depletion results in reduced chromatin loading of all three, decreased gene conversion, and sensitivity to PARP inhibition. These results suggest a mediator role for XPG in loading RAD51 onto RPA-coated DNA, and in vitro studies to test this hypothesis are underway. Suggesting that XPG also has an upstream role in HRR, XPG interacts directly with BRCA1, and its depletion results in significantly increased chromatin binding and foci formation by BRCA1 that is accompanied by persistent BRCA1 hyper-phosphorylation.

These unexpected findings establish XPG as an HRR protein with important roles in genome stability and predict that it both plays a role with BRCA2 in assembly of the presynaptic filament and participates in regulation of BRCA1 activity. A role for XPG in HRR and recovery of stalled replication forks, in addition to its participation in TC-NER and BER, suggests how XPG defects beyond loss of its enzymatic function in NER cause severe clinical consequences including cancer and accelerated aging.
Double strand break repair: HR - C2

Presenter: Rohit Prakash

Deciphering the Roles of RAD51 Paralogs in Human Mammary Epithelial Cells

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Like BRCA1 and BRCA2, RAD51 paralogs have been recently identified as highly penetrant breast and ovarian cancer genes. RAD51 paralogs exists in two distinct biochemical complexes, BCDX2 (RAD51B, RAD51C, RAD51D and XRCC2) and CX3 (RAD51C and XRCC3). RAD51 paralog-deficient rodent cells exhibit chromosomal instability and increased sensitivity to genotoxic agents. These phenotypic defects are likely attributable primarily to the involvement of the RAD51 paralogs in DNA double-strand break (DSB) repair by homologous recombination (HR) but they may also result from impaired protection of stalled replication forks. Mouse knockouts of RAD51 paralogs (Rad51B, Rad51C, Rad51D or Xrcc2) lead to embryonic lethality, implying a critical function in early development. How RAD51 paralogs function in normal human cells, however, has not been well studied.

In order to investigate the requirement for the RAD51 paralogs in human cells, we have used genome-editing techniques to generate mutations in all five paralog genes. For this, we used the non-transformed human mammary epithelial cell line MCF10A, given the breast cancer association of RAD51 paralog gene mutations. We were unable to recover biallelic frame shift mutations in any of the RAD51 paralogs genes, suggesting that they are cell lethal or lead to a severe growth disadvantage. To overcome this, we implemented a pre-emptive complementation strategy by introducing a floxed cDNA for each RAD51 paralog at the AAVS1 “safe harbor” locus prior to mutagenesis of the endogenous gene. Expression of Cre recombinase in these conditional, biallelically mutated clones results in spontaneous DNA damage, reduction in HR, and eventual cessation of cell growth. Thus, loss of RAD51 paralogs in nontransformed human cells compromises genome integrity, eventually leading to cell lethality. Tumor-associated RAD51 paralog mutations are being investigated for their ability to complement the survival and DNA repair defects seen with RAD51 paralog deficiency. Moreover, these conditional RAD51 paralog cell lines provide an ideal tool with which to understand factors that allow cell survival in a nontransformed-cell setting.
Functional characterization of human BRCA2 variants


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In 5-10% of the high breast cancer risk families a sequence variant is identified for which it is uncertain if BRCA1/BRCA2 protein function is impaired. Since the cancer risk associated with these variants of uncertain significance (VUS) is unknown, they represent a challenge for genetic counselling and clinical management of the families. Most VUS are so rare that insufficient data on family history and patient pathology exist to make clinically meaningful inferences on their associated cancer risks. Functional assays that assess the impact of variants on protein function are required to bridge this gap.

We optimized a previously developed mouse embryonic stem cell (mES) based model system that allows functionally testing of all types of BRCA2 variants, including variants that may affect RNA splicing. The test is based on the ability of an introduced human BRCA2 (hBRCA2) gene to rescue the lethality of Brca2 deficiency in mES cells. VUS generated in the hBRCA2 gene that fail to rescue the lethality conferred by mBrca2 deficiency are considered to be deleterious for protein function. The impact of non-lethal variants is tested in a series of biological assays, based on the role of BRCA2 in homologous recombination (HR). These assays include the analysis of double strand break repair and the sensitivity towards DNA damaging agents such as cisplatin and PARP-inhibitors.

To validate the assay we used a series of known pathogenic and neutral variants in BRCA2 for which the clinical significance had already been established on the basis of clinical data (n=30). Whereas, all neutral variants were able to complement the cell lethal phenotype of endogenous mBrca2 deficiency, mES clones containing a pathogenic variant did not survive after mBrca2 expression was obliterated thus being completely in line with clinical data. To assess the effect of neutral variants on BRCA2 protein function we tested the ability of these cells to repair a restriction-enzyme generated double stranded break by HR. We observed HR levels ranging from 55% to 110% compared with cells expressing WT hBRCA2.

Subsequently, we tested the functionality of several clinically relevant BRCA2 VUS (n=24). Although most variants were able to form clones after removal of the conditional mBrca2 allele, we observed a strong reduction in HR efficiency in a subset of them. In addition we observed a wide range of IC50 values when measuring cell survival after cisplatin treatment. Strikingly, a strong correlation appears to exist between HR levels and the cisplatin IC50 values.

The hBRCA2 mES cell assay enables efficient generation and functional characterization of hBRCA2 VUS. Once we can translate functional impact into cancer risk estimates, the assay can be used to classify BRCA2 VUS, even in the absence of sufficient family data providing carriers with the necessary information to make deliberate decisions.
FANCD2 maintains fork stability in BRCA1/2-deficient tumors allowing for alternative end-joining repair

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The BRCA1 and BRCA2 (BRCA1/2) proteins are essential for genomic integrity. BRCA1/2 proteins function in homologous recombination (HR)-mediated DNA repair and also act together with Fanconi anemia (FA) proteins to maintain genomic integrity through replication fork stabilization. As a result, loss of BRCA1/2 proteins results in both DNA repair deficiency and replicative stress, which lead to genomic instability and enhanced sensitivity to DNA damaging agents, such as cisplatin or inhibitors of poly-(ADP-ribose) polymerase (PARP). Recent studies have shown that BRCA1/2-deficient tumors upregulate the POLθ-mediated alternative end-joining (alt-EJ) repair pathway as a survival mechanism. Whether other mechanisms have evolved to maintain genomic integrity upon loss of BRCA1/2 proteins is currently unknown. Here we show that BRCA1/2-deficient tumors demonstrate a compensatory increase in FANCD2 expression levels, FANCD2 monoubiquitination, and FANCD2 foci formation. Loss of BRCA1/2 also enhances FANCD2 binding to chromatin and localization to stalled replication forks. FANCD2 is required for fork protection and restart in BRCA1/2-deficient tumors. Moreover, we found that FANCD2 is also required for both POLθ and CtIP recruitment at sites of damage and for efficient alt-EJ repair. Consistent with these results, loss of FANCD2 in BRCA1/2-deficient tumors enhances cell death in vitro and in vivo. Finally, in BRCA1/2-deficient cells, FANCD2 overexpression confers resistance to PARP inhibitors through replication fork protection. All together, our results reveal a previously unidentified synthetic lethal relationship between the FANCD2 and BRCA1/2, and identify FANCD2 as a central player orchestrating DNA repair pathway choice at the replication fork.
Double strand break repair: HR - C2

Presenter: Sandeep Burma

DNA end resection by EXO1 is regulated by an interplay of phosphorylation and ubiquitination events

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Resection of DNA double-strand breaks (DSBs) to generate tracts of ssDNA is a pivotal step at which the choice is made between non-homologous end joining (NHEJ) and homologous recombination (HR). We have previously established that the 5’ to 3’ exonuclease EXO1 plays a major role in DNA end resection and repair pathway choice in human cells (1-3). In addition, we find that EXO1 is phosphorylated and regulated by ATM/ATR in a DNA damage-dependent manner and by S/G2 CDKs in a cell cycle-dependent manner. Specifically, we find that CDKs 1 and 2 bind to EXO1 in S/G2 phases and phosphorylate it at four C-terminal S/TP sites. These modifications promote DNA end resection and HR, in part, by augmenting the localization of EXO1 at DSBs via interactions with BRCA1. We have established that activation of EXO1 by CDKs is an important mechanism by which the cell exercises accurate repair pathway choice (4). However, once activated by DNA damage, EXO1 must also be restrained in order to prevent over-resection. In this context, we find that EXO1 is rapidly degraded by the ubiquitin-proteasome system soon after its activation by DSBs. DNA damage-induced EXO1 degradation is attenuated by inhibition of ATR and augmented by inhibition of protein phosphatases, indicating that phosphorylation of EXO1 not only promotes its activation, but also targets it for subsequent degradation. In accord with these results, EXO1 with mutated ATM/ATR phosphorylation sites is relatively stable after DNA damage. We find that EXO1 is ubiquitinated upon DNA damage in a phosphorylation-dependent manner, and that this can be blocked by MLN4924, a potent inhibitor of the Cullin-RING ubiquitin ligase family. Expression of dominant negative Cullin1 (DNCul1) reduces EXO1 ubiquitination and degradation, thereby implicating the Cul1-containing SCF E3 ligases in EXO1 destabilization. Importantly, blocking EXO1 degradation increases DNA end resection, but compromises DSB repair and chromosome integrity. Taken together, these results indicate that DNA end resection by EXO1 is regulated by an interplay of phosphorylation and ubiquitination events that initially activate EXO1, but eventually target this protein for degradation in order to prevent hyper-resection and preserve genomic integrity.

(3) Exo1 plays a major role in DNA end resection in humans and influences DNA repair and damage signaling decisions. Tomimatsu et al. DNA Repair 11:441-448 (2012).
Double strand break repair: HR - C2

Presenter: Michal Zimmermann

Exploring the cellular response to PARP inhibitors with CRISPR/Cas9 screens

Michal Zimmermann(1), Olga Murina(2), Traver Hart(3), Michael Aregger(3), Megha Chandrashekhar(3), Zachary Steinhart(4), Amélie Fradet-Turcotte(1), Stéphane Angers(4), Jason Moffat(3), Andrew Jackson(2), Daniel Durocher(1)

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Inhibitors of poly(ADP)-ribose polymerase 1 (PARPi) are the first cancer therapeutics developed on the principle of synthetic lethality to be approved for clinical use (1). While the toxicity of PARPi to cells deficient in homology-directed repair (HDR) was discovered over a decade ago (2,3), much remains to be learned about the exact nature of PARPi-induced DNA damage and the accompanying cellular response under physiological and pathological conditions. Such information may help the clinical development of PARPi and may also unravel novel insights into HDR regulation and interactions of HDR with other DNA damage repair pathways.

To shed light on the response to PARPi we implemented a CRISPR/Cas9-based genomic screening platform (4) and searched for genes whose inactivation hyper-sensitized human cells to PARP inhibition. As expected, many of the known HDR genes were identified in our screens. Importantly, we also identified factors that were not previously known to promote resistance to PARPi. We are currently characterizing the mechanism of action of these newly identified genes. At my poster, I will discuss the screening pipeline as well as the initial validation and characterization of our newly identified PARPi-sensitizing mutations.

Double strand break repair: HR - C2

Presenter: Peter Bouwman

Functional analysis of BRCA1 variants and domains to improve genetic counselling and cancer treatment strategies

Peter Bouwman(1), Hanneke van der Gulden(1), Ingrid van der Heijden(1), Maaike Vreeswijk(2), Jos Jonkers(1)
1. Division of Molecular Pathology and Cancer Genomics Centre, The Netherlands Cancer Institute, Amsterdam, The Netherlands; 2. Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands

Mutations in BRCA1 strongly predispose to hereditary breast and ovarian cancer and sequence analysis of BRCA1 is routinely conducted in patients with a family history of breast-ovarian cancer. Besides mutations that abolish protein function and are known to increase cancer risk, a large number of sequence variants of uncertain significance (VUS) have been identified for which the associated cancer risk is unknown.

We developed a mouse embryonic stem (ES) cell based complementation assay for the functional classification of BRCA1 missense VUS and used it to analyze the functional effects of over 100 BRCA1 variants (Bouwman et al., 2013 and unpublished data). The results of this assay aid genetic counseling, as mutations that lead to a functional defect are likely to be associated with disease. Therefore we will further improve our assay system using synthetic gene blocks and additional controls and extend our analysis to all over 200 currently identified Dutch/Belgian BRCA1 missense VUS. These BRCA1 variants will be tested for their ability to complement cisplatin and PARP inhibitor sensitivity of mouse BRCA1 deficient ES cells. As the role of BRCA1 in DNA repair via homologous recombination (HR) is important for tumor suppression, the effects of deleterious mutations on HR will be measured using an established reporter assay. We will combine the results of our functional analyses with clinical and genetic data to calculate the likelihood that a given variant is pathogenic.

Because BRCA1 deficiency renders tumours hypersensitive to platinum compounds and PARP inhibition, our data will also be important for the design of optimal treatment strategies for cancer patients carrying BRCA1 mutations. To evaluate this further, we will use human breast cancer cell lines and patient-derived xenograft models. In addition, we use genetically engineered mouse models to analyze the effects of deleterious mutations in specific BRCA1 domains on the response of tumor cells to targeted therapy. For this purpose, we have already generated mouse breast cancer models mimicking the three most common pathogenic BRCA1 founder mutations: C61G (Drost et al., 2011), 185delAG (185stop) and 5382insC (5382stop). While all three mutations predispose to mammary tumors, Brca1-C61G and Brca1-185stop tumors respond significantly worse to HRD-targeting therapy than Brca1-5382stop tumors. These tumors express a BRCA1 protein with a defective RING domain (C61G) or completely lacking the RING domain (185stop). We show that in the absence of a (functional) RING domain, BRCA1 can still mediate therapy resistance. As these results are confirmed in human BRCA1-185delAG breast cancer cells, expression of RING-mutant or RING-less BRCA1 may serve as a marker to predict poor response to DSB-inducing therapy in human cancer patients.
POSTER SESSION C3: 'Double Strand Break Repair – Non-Homologous End Joining'

Poster viewing: Tuesday, April 19, 20:00 – 22:00 h

Discussion: Thursday, April 21, 16:55 – 18:25 h
Double strand break repair: NHEJ - C3

Presenter: Robin van Schendel

**Mechanism of DNA damage-induced deletion mutagenesis by polymerase Theta**

Robin van Schendel, Jane van Heteren, Richard Welten, Marcel Tijsterman
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For almost a century, genotoxic agents have been used to induce mutations in the genome of model organisms to establish genotype-phenotype relationships. While inaccurate replication across damaged bases can explain the formation of single nucleotide variants, it remained unknown how DNA damage induces more severe genomic alterations. Here, we demonstrate for two of the most widely used mutagens, i.e. ethyl methanesulfonate (EMS) and photo-activated trimethylpsoralen (UV-TMP), that deletion mutagenesis is the result of alternative, polymerase Theta (PolQ)-mediated end joining of double strand breaks (DSBs). This discovery allowed us to survey many thousands of available C. elegans deletion alleles. Careful examination of ~10,000 deletion breakpoints to their cognate junctions reveals a distinct order of events in which the terminal nucleotide of nascent strands blocked at sites of DNA damage can engage in one of more cycles of primer extension using a more downstream located break end as a template. Resolution is accomplished when 3’overhangs have matching ends. Our study provides a step-wise and versatile model for the in vivo mechanism of POLQ action, which explains the molecular nature of mutagen-induced deletion alleles.
DNA polymerases with distinct mechanisms for maintaining chromosome stability

Richard D. Wood
The University of Texas MD Anderson Cancer Center, USA

Mammalian DNA polymerases ζ and θ are both critical for maintaining genome stability, preventing genomic DNA breaks by different mechanisms. Inactivation of the Rev3l gene encoding the pol ζ catalytic subunit causes a high frequency of chromosomal breaks, followed by lethality in mouse embryos and in primary cells. Pol ζ is required to sustain proliferation of rapidly growing cells with intact DNA damage checkpoints. We find that when pol ζ is deleted from actively growing primary mouse cells in culture, growth ceases within a few divisions, and chromosome breaks and abnormalities rapidly accumulate. Further, our experiments show that physical wound healing is defective in mice deleting Rev3l in the basal epithelium. The REV3L protein also serves as a protein scaffold via multiple conserved structural domains (1). To investigate the role of REV3L DNA polymerase activity in vivo, a Rev3l knock-in mouse was constructed with mutations of conserved catalytic aspartate residues in conserved motif I of the polymerase domain. The homozygous mutant lead to lethality during embryogenesis. Primary fibroblasts from mutant embryos showed growth defects, elevated DNA double-strand breaks and DNA damage sensitivity as with Rev3l-null fibroblasts. These severe consequences were not rescued by concomitant deletion of mouse pol η. These results validate the approach of targeting the DNA polymerase activity of pol ζ to sensitize tumors to DNA damaging agents (2).

Mammalian pol θ (POLQ), on the other hand, defends against double-strand break-induced chromosome instability via a process of “alternative” end joining of breaks. Polq-null murine cells are selectively hypersensitive to DNA damaging agents that cause direct double-strand breaks, including ionizing radiation. Restoration of damage resistance requires the DNA polymerase activity of POLQ. A DNA break end joining assay in cells monitored repair of DNA ends with long 3’ single-stranded overhangs. End joining events retaining overhang were dependent on POLQ, and independent of Ku70. During immunoglobulin class switch joining at double strand breaks in antibody genes, some joins contain insertions with homology to regional sequences. These insertions are entirely POLQ-dependent. Biochemical experiments with purified human POLQ protein reveal the mechanism generating the insertions during DNA end joining (3). This relies on the unique ability of POLQ to extend DNA from minimally paired primers, facilitated by a tight grasp on the primer-terminus (4).

Control of alternative end joining by the chromatin remodeler p400 ATPase

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Repair of DNA double-strand breaks occurs in a chromatin context that needs to be modified and remodeled to allow adequate access to the different DNA repair machineries. Of particular importance for the maintenance of genetic stability is the tight control of error-prone pathways, such as the alternative End Joining pathway. In this work we show that the chromatin remodeler p400 ATPase is a brake to the use of alternative End Joining. Using specific genomic reporter substrates we observe that p400 depletion increases the frequency of alternative End Joining events, and induces the generation of large deletions following repair of double-strand breaks. The increase of alternative End Joining events was in large part under the dependence of DNA resection mediated by CtIP. Moreover, p400 depletion leads to the recruitment of poly(ADP) ribose polymerase (PARP) and DNA ligase 3 at DNA double-strand breaks, driving to increased sensitivity to PARP inhibitors. Together these results show that p400, acts as a brake to prevent alternative End Joining-dependent genetic instability and could be a potential target for therapy in association with PARP inhibitors and/or used to orientate therapy according to p400 status.
Double strand break repair: NHEJ - C3

Presenter: Alexander Ishchenko

PARP1 and PARP2 catalyze poly(ADP-ribosyl)ation of DNA strand break termini

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Poly(ADP-ribose) polymerases (PARPs) use nicotinamide adenine dinucleotide (NAD+) to catalyze the synthesis of a long branched poly(ADP-ribose) polymer (PAR) attached to the acceptor amino acid residues of nuclear proteins. PARP1 is activated up to 500-fold when bound to DNA strand breaks. In vivo, when DNA is in the chromatin form, PARPs work on single- and double-strand DNA breaks (SSB and DSB) by recruiting and assembling DNA repair factors. The phenomenon of NAD+-dependent PARylation was discovered more than 50 years ago, but it is still unclear how this post-translational modification governs a multitude of cellular processes including DNA repair, transcription, chromatin dynamics and cell death. Here, we investigated interactions of PARP enzymes with the intermediates of DNA excision repair. Our results revealed that both mammalian PARP1 and PARP2 can covalently modify DNA oligonucleotide duplexes by addition of multiple poly(ADP-ribose) units to DNA strand break extremities. PARP1 and PARP2 preferentially react with the recessed and nicked DNA duplexes, respectively. Based on the biochemical and mass spectrometric data, we propose that PARPs can utilise DNA termini, as an alternative of 2'-hydroxyl of ADP-ribose, to catalyse PAR chain elongation either via 2',1''-O-glycosidic ribose-ribose bond, or via phosphodiester bond formation between C1' of ADP-ribose and phosphate of a terminal nucleotide in DNA duplex. Therefore, DNA can be regarded as a substrate for PARP1 and PARP2, which catalyze a post-replicational modification of DNA. The covalent DNA PARylation is a reversible process since PARG removes the PAR polymer from DNA with high efficiency and restores native DNA structure. Finally, this newly discovered type of post-replicational modification of DNA mediated by PARPs provides an heuristic insight into molecular mechanisms involved in DNA repair, transcription and chromatin dynamics in eukaryotic cells.
Roles of a new phosphorylated form of Ku70 identified in resistant leukemic cells: DNA repair and apoptosis

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The Ku-dependent canonical nonhomologous end-joining (c-NHEJ) DNA repair system is fundamental for genome maintenance and B-cell lineage. We have identified two novel phosphorylation sites (S27, S33) of Ku70 (p-Ku70) in malignant B cells from patients suffering from a progressive form of chronic lymphocytic leukemia (CLL) resistant to chemotherapy. These patients (~15%) have been classified as resistant (R-CLL), due to their malignant cell resistance to apoptosis induced in vitro by genotoxic stress (drugs and/or irradiation). Previous work showed that resistance to apoptosis was linked to an overactivation of the NHEJ pathway, leading to a fast but error-prone DNA repair creating sub-lethal DNA deletions, as well as telomere dysfunction. Using in vitro cancer cell lines as model, we have identified pS2056-DNAPKcs and pS1981-ATM as responsible kinases for Ku70 phosphorylation, although not exclusively.

We constructed specific vectors allowing the expression of either exogenous Ku70 wt- (S27, S33, Ku70wt), mutated Ku70 - (A27, A33; Ku70Ala) or phosphomimetic Ku70- protein (E27, E33; Ku70Glu); endogenous Ku70wt expression was silenced using shRNA encoded on the same vector. We studied the role of p-Ku70 after stress induction by γ-irradiation and showed that phosphorylation of Ku70, compared to unphosphorylatable Ku70Ala, resulted in faster but error-prone DNA repair, established by comet assay, γ-H2AX kinetics and the appearance of dicentric chromosomes resulting from non- or miss-repaired DSBs. Furthermore, we demonstrated that Ku70Ala expression lead to an altered S/G2- cell cycle check-point, characterized by a longer S phase, a prolonged G2 arrest and a higher percentage of hyperploid cells compared to Ku70wt expressing cells.

These data unveil a role of p-Ku70 in fast but inaccurate DNA repair; a new paradigm linked to both the deregulation of c-NHEJ and the resistance of malignant cells.

Besides its fundamental role in NHEJ, Ku70 was reported to have a regulatory function in intrinsic apoptosis, via its interaction with the proapoptotic BH3-only molecule Bax. We find that Ku70Glu and p-Ku70wt strongly interact with Bax, whether Ku70Ala shows weaker to no interaction with Bax in several cell lines. Considering our data R-CLL cell resistance to apoptosis with a constitutive overexpression of p-Ku70, we hypothesize a role of p-Ku70 in apoptosis regulation.
Double strand break repair: NHEJ - C3

Presenter: Arthur Abello

Dynamic pattern of the repair proteins Ku70/Ku80 during programmed genome rearrangements in Paramecium tetraurelia

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During its sexual cycle, the ciliate Paramecium tetraurelia undergoes massive programmed genome rearrangements. They consist, among others, in excision of 45,000 precisely delimited sequences, called IES (Internal Eliminated Sequences) (1). A domesticated PiggyBac transposase, PiggyMac (Pgm), introduces double-strand DNA cleavage at IES ends. Highly precise repair of double-strand breaks (DSB) is handled by the Ligase IV-dependent classical Non Homologous End Joining pathway (NHEJ) (2). One of the actors of this pathway is the heterodimer Ku70/Ku80. In P. tetraurelia, the KU70 gene is present in two paralogous copies and KU80 in three. RNA-seq experiments show that only one gene for each subunit is specifically expressed during programmed genome rearrangements, namely KU70a and KU80c (3). RNA interference against KU80c showed a development-specific phenotype with complete inhibition of DNA cleavage. Furthermore, a Co-IP experiment in a heterologous system showed that both Ku70/Ku80 interact with Pgm. These results provide evidence that Ku is an essential partner of Pgm for DSB introduction (3). Ongoing experiments aim at deciphering the molecular mechanism involved in the coupling between DNA cleavage and repair, by studying the interaction between Ku and Pgm sub-domains in vitro and in vivo.

Immunofluorescence experiments using an antibody directed against the endogenous Ku70 a protein showed a nuclear localization during programmed genome rearrangements. The pattern of this localization is dynamic: Ku rushes into the early developing nuclei and forms foci, before its localization becomes more diffuse after genome rearrangements have been completed. Even though Pgm is found in the nuclei at the same time as Ku, both proteins do not completely co-localize, raising the question of the functional role of Ku foci. These structures could be the nuclear compartments where DNA cleavage takes place, where DNA repair is carried out or be post-repair structures. RNAi experiments, combined with Ku70 immunofluorescence localization, showed that every time we disrupt DSB introduction or their repair, the nuclear pattern of Ku changes from dotted to diffuse. Our results suggest that Ku foci are transient post-repair structures, and thus, markers of a correctly rearranged genome.

Double strand break repair: NHEJ - C3

Presenter: Eva Brinkman

Quantitative analysis of NHEJ fidelity and kinetics in single genomic loci

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How quickly is a double-strand break (DSB) in the human genome repaired, and how does the interplay between various repair pathways affect the kinetics of repair? So far the kinetics of re-joining of broken DNA ends has primarily been studied in bulk, after exposure of cells to very high doses of damaging agents. Repair rates have also been indirectly inferred from studying the formation and clearance of “repair protein foci” by microscopy. Yet, the kinetics of the actual re-joining of two DNA ends after induction of a DSB at a single defined genomic location are not known. In addition, we lack accurate estimates of the fidelity of such a discrete repair event.

We developed a strategy to directly measure the kinetics and fidelity of NHEJ in single loci in human cells. We use a cell line with a tightly inducible Cas9 that enables the timed generation of a DSB at a locus of choice. We then use a combination of (i) our recently developed TIDE method, (ii) a novel assay to detect DSBs at single loci, and (iii) high throughput sequencing to quantitate intact, broken and repaired DNA over time. The resulting data are fed into a mathematical model that estimates key parameters of repair kinetics and fidelity.

We focused on one locus in the human LBR gene. Cas9 induced DSBs at this locus did not result in a detectable cell cycle arrest. Application of the above-mentioned strategy revealed that the half-life of DSB repair is ~6 hours, indicating that repair is rather slow in this locus. Moreover, perfect repair of the DNA seems to be almost absent, meaning that the repair is exclusively error-prone resulting in small indels at the site of the break. Interestingly, in the LBR locus either a deletion of 7 bp or an insertion of 1 bp is generated. Time series reveal that these different types of repair have different kinetics, whereby the +1 insertion accumulates more rapidly than the -7 deletion. The ratio between the +1 and -7 can be changed by a DNAPK inhibitor or by additional IR damage elsewhere in the genome. This suggests that the classical and alternative NHEJ pathways are both active at the same time to repair the DSB at one particular location in a stochastic way.

Our strategy to determine the kinetics and fidelity of DSB repair at single loci – and to infer contributions from different repair pathways – should be generally applicable. This offers new possibilities to study the effects of local sequence and chromatin context on DSB repair and pathway choice. We expect that these quantitative analyses will contribute to a better understanding of DSB repair.
Antigen receptor diversity is generated via the process of V(D)J recombination which involves the somatic recombination of different V, D, and J genes. Three different mechanisms contribute to diversity: (A) The random rearrangement of different V, (D), and J genes during V(D)J recombination in precursor B and T cells in bone marrow and thymus, respectively; (B) The pairing of two chains to create a unique immunoglobulin or T-cell receptor (TR) molecule; and; (C) The diversification of the junctional regions between the genes. The latter process includes deletions or addition of non-templated nucleotides by terminal deoxynucleotidyl transferase (TdT), which significantly increases diversity in the antigen receptor repertoires.

RAG-induced double strand breaks (DSBs) are repaired via classical NHEJ, but family X DNA polymerases also engage in the restoration of genomic integrity at the break sites. Of the four family X polymerase members, TdT, pol lambda, and pol mu participate in NHEJ and V(D)J recombination. TdT and pol lambda are predominantly expressed during immunoglobulin heavy chain (IGH) rearrangement, whereas pol mu expression increases during immunoglobulin light chain (IGL) rearrangement. Both pol lambda and pol mu are involved in end processing.

The clinical spectrum of V(D)J recombination defects ranges from mild to severe immunodeficiency. XLF-deficient patients exhibit radiosensitivity, microcephaly, growth retardation, and a variable degree of immunodeficiency. We studied V(D)J recombination in XLF-deficient patients via T- and B-cell repertoire analysis using next generation sequencing. The TR beta and IGH repertoire showed a diverse pattern. We observed a striking reduction in the number of N-nucleotides in TR delta (7.5 vs 12.4 nt) and IGH rearrangements (4.6 vs 12.1 nt) of XLF-deficient patients and a significantly shorter CDR3 length. 50-60% of the junctions in XLF-deficient patients completely lacked N-nucleotides, which we only observed in 15-40% of the junctions in controls. TR delta and IGH rearrangements take place during developmental stages with the highest level of TdT expression. During the developmental stages with low TdT expression which coincide with IGL, IGK, and TR beta rearrangement, less N-nucleotides are added, but we still observed a significant reduction in N nucleotides in IGL (1.7 vs 3.0 nt), and IGK (1.4 vs 2.6 nt) rearrangements compared to controls. We therefore hypothesize that XLF is required for the recruitment, positioning, or activity of TdT during V(D)J recombination. Given the high degree of homology with other family X polymerases, we also postulate that pol lambda and pol mu require XLF to fully execute their functions. We observed recruitment of TdT in XLF-deficient cells to DSBs and are currently conducting superresolution microscopy experiments as well as in-depth biochemical and molecular assays to further analyse the interaction of family X polymerases and XLF.
Double strand break repair: NHEJ - C3

Presenter: Gemael Cedrick Taty-Taty

Role of chromatin remodeling enzymes in the repair of DNA double strand breaks and genetic instability

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Repair of DNA double-strand breaks (DSB) occurs in a chromatin context that needs to be modified and remodeled to allow adequate access to the different DNA repair machineries. Two main pathways are used to repair DSBs in mammalian cells, classical non homologous end joining (NHEJ) and Homologous Recombination (HR). Of particular importance for the maintenance of genetic stability is the tight control of error-prone pathways, such as the alternative End Joining (AltEJ) pathway. We previously showed that the chromatin remodeler p400 ATPase participates in HR by facilitating the action of Rad51, a major actor of the HR pathway. Here we show that p400 depletion increases the frequency of AltEJ events, as evidenced using reporter substrates and by the generation of large deletions following repair of DSBs, a characteristic of Alt-NHEJ. By ChIP experiments, we revealed that p400 depletion leads to the recruitment at DSBs of PARP1 and DNA ligase 3, two actors of the AltEJ pathway. Together these results show that p400, by facilitating late steps of homologous recombination, acts as a brake to prevent the generation of deletions by AltEJ. As such, it functions as a guardian of the genome preventing the generation of genetic instability. In addition, we observed the selective killing of cells engaged in AltEJ by the use of PARP inhibitors as a consequence of its specific recruitment to DSB in p400 depleted cells.
Double strand break repair: NHEJ - C3

Presenter: Hanna IJspeert

Dual role of NHEJ in DSB repair and V(D)J recombination

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Non-homologous end joining (NHEJ) is one of the major pathways repairing DNA double strand breaks (DSBs) and is involved in recombination of V, D and J genes of the antigen receptor loci in B and T-cells. V(D)J recombination starts with DNA cleavage by RAG proteins resulting in the formation of hairpinned coding ends, which are opened by Artemis upon activation by DNA-PKcs. Subsequently, nucleotides are deleted due to exonuclease activity and non-templated (N) nucleotides are inserted by TdT, which contributes tremendously to the diversity of the antigen receptors. Finally, the DNA ends are ligated by a complex of LIG4, XRCC4 and XLF.

Defects in NHEJ factors result in a clinical spectrum characterized by ionizing radiation (IR) sensitivity, immunodeficiency, neurological abnormalities and growth failure. We aimed to study the dual role of the NHEJ factors in DSB repair and V(D)J recombination in order to link the result to the clinical presentation of the patients.

We used primary fibroblasts derived from 12 NHEJ deficient patients to determine the IR sensitivity using a clonogenic survival assay. Peripheral blood samples were used for immunophenotyping and analysis of the B-cell receptor repertoire. The DNA-PKcs and 6 Artemis patients were sensitive to IR, but the 2 XLF,XRCC4 and 2 LIG4 deficient patients displayed a higher sensitivity. This implies that at least Artemis is not involved in repair of all the breaks, while DSB repair is dependent on proper functioning of XLF, XRCC4 and LIG4.

Artemis and DNA-PKcs deficiency result in a severe immunodeficiency with strongly reduced or absent peripheral B and T cells. Neurological abnormalities are absent in Artemis deficient patients, but can be present in DNA-PKcs deficient patients. Patients with LIG4 and XLF deficiency also present with immunodeficiency, though less severe than Artemis and DNA-PKcs patients, but they do all show neurological abnormalities and growth delay. Surprisingly, XRCC4 patients are not immunodeficient; they present with primordial dwarfism and neurological abnormalities. B-cell receptor analysis showed that Artemis and DNA-PKcs patients have increased number of P-nucleotides (6.7 and 3.0 vs 0.2 in controls), as a result of asymmetric hairpin opening. Although XLF, LIG4 and XRCC4 belong to the same complex, the junction characteristics were different. All patients had reduced number of N-nucleotides, but the XLF patients had only 4.6 N-nucleotides vs 12.1 in controls which was significantly lower than the 7.9nt in XRCC4 and LIG4 patients. This suggests that the whole ligation complex is important for N-nucleotides insertions by TdT, however XLF seems to play a dominant role.

These data show that defects in hairpin opening have a severe impact on V(D)J recombination, while DNA repair is more moderately affected. While in contrast mutations in the ligation complex result in a milder immunodeficiency, but more severe neurological complications and growth failure.
Presenter: Hind Ghezraoui

**Chromosomal translocations in human cells are generated by canonical nonhomologous end-joining.**

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Breakpoint junctions of the chromosomal translocations that occur in human cancers display hallmarks of Nonhomologous End-Joining (NHEJ). In mouse cells, translocations are suppressed by canonical NHEJ (c-NHEJ) components, which include DNA ligase IV (LIG4), and instead arise from alternative NHEJ (alt-NHEJ). Here we used designer nucleases (ZFNs, TALENs, and CRISPR/Cas9) to introduce DSBs on two chromosomes to study translocation joining mechanisms in human cells. Remarkably, translocations were altered in cells deficient for LIG4 or its interacting protein XRCC4. Translocation junctions had significantly longer deletions and more microhomology, indicative of alt-NHEJ. Thus, unlike mouse cells, translocations in human cells are generated by c-NHEJ. Human cancer translocations induced by paired Cas9 nicks also showed a dependence on c-NHEJ, despite having distinct joining characteristics. These results demonstrate an unexpected and striking species-specific difference for common genomic rearrangements associated with tumorigenesis.
Double strand break repair: NHEJ - C3

Presenter: Haser Sutcu

Implication of DNA double-strand break repair in differentiation and viability of muscle stem cells

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Adult skeletal muscle stem (satellite) cells are responsible for the homeostasis and regeneration of the muscle throughout life. Quiescent satellite cells are located between basal lamina and sarcolemma of the muscle fiber. Upon injury, satellite cells become activated, proliferate (called then myoblasts), undergo myogenic differentiation and fuse into multinucleated muscle fibers. Satellite cells also self-renew, and thus maintain the stem cell pool, which is vital for future tissue homeostasis and regeneration.

The maintenance of genome integrity plays a key role in the regeneration potential of stem cells. DNA double strand breaks (DSBs) are extremely dangerous DNA lesions, which are generated by endogenous processes such as respiration and DNA replication, and exogenous treatments such as irradiation. DNA damage repair maintains genome integrity, and its impairment may affect cell or organism survival, or promote deleterious and cancer-initiating mutations. Moreover, in adult stem cells impaired DSB repair may deplete the stem cell pool, induce anticipated differentiation, affect the entire progeny, and thereby impair tissue regeneration.

In the lab, it has been shown that satellite cells repair radiation-induced DSBs more efficiently and accurately than their committed progeny, but the underlying mechanism is still unclear. Hereby, we analyse the consequences of impaired DSB repair in satellite cells on muscle differentiation. Satellite cells isolated from Tg:Pax7-nGFP mice (Pax7 is a specific marker of stemness in myogenic cells) differentiate in culture. To impair DSB repair satellite cells are treated with either inhibitors of key DNA repair factors (DNAPK, ATM) or chromatin modifiers (HDAC, PARP1), or both. Differentiation is then evaluated with myogenic markers (Pax7, MyoD, Myogenin) or structural marker (Myosin HC). Both satellite cells proliferation and differentiation are essential for myogenesis.

Our preliminary data suggest distinct and very diverse proliferation and/or differentiation alterations in satellite cells treated with different DSB repair inhibitors or chromatin modifiers. Specifically we could suggest that DNAPK may have a role in myogenesis additional to its essential function in NHEJ. In the mean-time we are also studying the impact of PARP (Poly-ADP ribose polymerase) 1 & 2 on myogenesis as these proteins are both known as chromatin modifiers as well as their known interactions in DSB repair and in other DNA damage responses.

We aim to understand the contribution of specific DSB repair factors in maintaining the proliferation and differentiation potential of muscle stem cells. We also aim to establish whether satellite cell proliferation and differentiation can be modulated by delay or temporary alteration of DSB repair. These experiments also aim to limit the loss of engraftment potential of satellite cells in culture, which represent a dramatic limit to the use of these cells in therapy. (Vahidi Ferdousi et al, 2014, Stem Cell Research,13: 492-507)
Double strand break repair: NHEJ - C3

Presenter: Juliette Kamp

RNA splicing factors UAF-1 and PNN-1 modify Non-Homologous End Joining efficacy in vivo

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Multiple pathways exist to repair double strand breaks (DSBs), and most of the components of these pathways are conserved across species. The DSB repair pathway choice depends on the context and type of break and is highly regulated. The error-prone Non-Homologous End Joining (NHEJ) pathway is the major DSB repair route in somatic animal tissues.

To identify and characterise genetic factors involved in NHEJ and its regulation, we used the nematode Caenorhabditis elegans as a model. Using an unbiased forward genetic screen in nematodes carrying a transgenic NHEJ reporter, we were able to identify seven bona fide NHEJ mutants. Three of these mutants showed mutations in the known NHEJ factors cku-70 and cku-80. The other four mutants carried mutations in genes of the THO ribonucleoprotein complex (thoc-2, thoc-5 and thoc-7) and in pnn-1. Both the THO complex and PNN play a role in RNA processing and are conserved in humans. We found that deficiency of PNN and the THO complex also leads to sensitivity to ionizing radiation in somatic tissues, but not in the germline, which is similar to the response of animals defective in cku-70 and cku-80. This supports the role of the THO complex and PNN in NHEJ.

Transcriptome analysis by RNA sequencing did not show significant changes in the expression and splicing of known NHEJ factors. However, depletion of uaf-1, which is downregulated in THO complex mutants, mimics the NHEJ defect observed in THO mutants. uaf-1 encodes the large subunit of the conserved splicing factor U2AF.

Since uaf-1 and pnn-1 both code for splicing factors, we propose a role for splicing in the regulation of NHEJ. Further delineation of the altered transcriptome and DNA damage responses in these splicing mutants will help to understand the relation between splicing and NHEJ.
Dynamics of RIF1 SUMOylation is regulated by E3 Ubiquitin Ligase PIAS4 in Response to DNA Double strand breaks.

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RIF1 has been identified as a suppressor of BRCA1, a protein that facilitates the break resection. In response to DNA double strand breaks, ATM phosphorylated 53BP1 recruits RIF1 which in turn inhibits the 5’–3’ end resection. PIAS1 and PIAS4 E3 ubiquitin ligases have been shown to play key roles in the regulation 53BP1 and BRCA1 to promote NHEJ and HR functions respectively. Recently, many novel functions of RIF1 have been reported by different laboratories, but role of RIF1 post-translational modification has yet to be known. In this study we have identified RIF1 as a SUMOylated protein. RIF1 SUMOylation is cell cycle regulated and increased in G1 cells in response to DNA double strand breaks (DSBs). We have identified C-terminal region of RIF1 to be SUMOylated. Depletion of PIAS4 E3 ubiquitin ligase significantly affects RIF1 SUMOylation. Cell biology studies demonstrate that DNA damage induced RIF1 foci colocalise with SUMO isoforms. RIF1 and SUMO protein colocalisation was reduced in PIAS4 depleted cells. Moreover, in this study we have also compared RIF1 SUMOylation with two key regulators of DDR signaling proteins 53BP1 and BRCA1. Overall our data suggesting SUMOylation dependent regulation of RIF1 function in response to DNA double strand breaks.

Double strand break repair: NHEJ - C3

Presenter: Susan Lees-Miller

**APLF (aprataxin and polynucleotide kinase/phosphatase like factor) is intrinsically flexible and stabilizes the core non-homologous end joining (NHEJ) complex.**


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Repair of DNA double-strand breaks (DSBs) by non-homologous end joining (NHEJ) in human cells is initiated by the binding of the Ku heterodimer to a DSB, followed by recruitment of the core NHEJ factors, including Aprataxin and Polynucleotide kinase/phosphatase-Like Factor (APLF), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4-like factor (XLF) and XRCC4 (X4)-DNA ligase IV (L4). How these factors act together to tether, process and ligate DSB ends remains enigmatic. Here, small angle X-ray scattering (SAXS) and mutational analyses show that APLF is largely an intrinsically disordered protein that binds Ku, Ku/DNA-PKcs and X4-L4 within an extended NHEJ core complex. X4 and XLF assemble with Ku heterodimers linked to DNA-PKcs via flexible Ku80 C-terminal regions in a complex stabilized through APLF interactions with Ku, Ku/DNA-PKcs, and X4-L4. The defined, flexible six-protein machine informs NHEJ efficiency, observed biochemistry, evolutionary complexities, and in vivo regulation for non-sequential assembly of the NHEJ complex.
Double strand break repair: NHEJ - C3

Presenter: Martijn Luijsterburg

PARP1 Links CHD2-Mediated Chromatin Expansion and H3.3 Deposition to DNA Repair by Non-Homologous End-Joining


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The response to DNA double-strand breaks (DSB) requires alterations in chromatin structure to promote the assembly of repair complexes on broken chromosomes. Non-homologous end-joining (NHEJ) is the dominant DSB repair pathway in human cells, but our understanding of how it operates in chromatin is limited. Here, we define a mechanism that plays a crucial role in regulating NHEJ in chromatin. This mechanism is initiated by DNA damage-associated poly(ADP-ribose) polymerase 1 (PARP1), which recruits the chromatin remodeler CHD2 through a novel PAR-binding domain. CHD2 in turn triggers rapid chromatin expansion and the deposition of histone variant H3.3 at sites of DNA damage. Importantly, we find that PARP1, CHD2 and H3.3 regulate the assembly of NHEJ complexes at broken chromosomes to promote efficient DNA repair. Together these findings reveal a PARP1-dependent process that couples ATP-dependent chromatin remodeling with histone variant deposition at DSBs to facilitate NHEJ and safeguard genomic stability.
Double strand break repair: NHEJ - C3

Presenter: Pierre Caron

The HECT E3 ubiquitin ligase WWP2 regulates DNA repair by Ku70/80-dependent non-homologous end-joining

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DNA damage is considered one of the main threads to genome stability. DNA double-strand breaks (DSB) are among the most deleterious lesions that can lead to genome rearrangements and mutations. The dominant pathway for the repair of DSBs in human cells is non-homologous end-joining (NHEJ). NHEJ is initiated by binding of the Ku70/80 complex to DSBs. The DSB-bound Ku complex then recruits and activates DNA-PKcs kinase, which orchestrates downstream repair processes. This includes the recruitment of the scaffold protein XRCC4 that binds DNA Ligase 4, the enzyme responsible for sealing the break. Recent work showed that the ubiquitylation of some NHEJ factors is key to their eviction from damaged chromatin upon completion of DNA repair. However, precisely how ubiquitylation drives DSB repair via NHEJ remains unclear. A genetic screen in C. elegans, identified the E3 ubiquitin ligase WWP2 as an enzyme that protects cells against ionising radiation-induced DSBs. In line with these findings, we observed that depletion of WWP2 in human cells increases radiosensitivity. Using EJ5 reporter and random plasmid integration assays, we found that depletion of WWP2 reduces the efficiency of DSB repair by NHEJ. Quantitative mass spectrometry and co-immunoprecipitation experiments revealed that WWP2 interacts with several core NHEJ proteins, including KU70/80, DNA-PKcs and XRCC4. Finally, using micro-irradiation and chromatin fractionation assays, we demonstrate that WWP2 accumulates at sites of DNA damage and that loss of this E3 ligase substantially reduces the accumulation of Ku80, DNA-PKcs, phospho-DNA-PKcs and XRCC4 at DNA breaks. Together, this suggests a key role for WWP2 in regulating DSB repair via NHEJ. We are currently identifying the underlying mechanism.
Double strand break repair: NHEJ - C3

Presenters: Pascale Bertrand & Angela Moussa

Lamin B1 controls double-strand breaks repair through its interaction with 53BP1

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Double strand break (DSB) is a very harmful lesion, which can lead to death, senescence or genome instability. Several studies suggest a link between nuclear envelope protein and DNA damage response. Integrity and plasticity of the nuclear envelope is ensured by the nuclear lamins and their associated proteins. Here, we show that lamin B1, a major component of nuclear envelope, interacts directly with 53BP1 protein. This protein plays a pivotal role in the choice of DSB repair pathway, by favoring non-homologous end-joining (NHEJ), a prominent DSB repair in mammalian cells. This interaction is dissociated upon exposure to ionizing radiation (IR) allowing then 53BP1 to be recruited at the DSB. Consistently, overexpression of lamin B1 impedes the recruitment of 53BP1 to the DSB resulting in NHEJ defect and increased sensitivity to DSB inducer agents such as IR or etoposide. Finally, we also show that lamin B1 interacts with the region encompassing the Tudor and UDR domains, which are essential for the interaction of 53BP1 with damaged chromatin. Taken together, these data identify a new pathway of DSB repair regulation through modulation of the recruitment of 53BP1 upon DNA injury, revealing a novel link between the nuclear envelope, DSB repair and genome stability.
Inactivation of Ku70 rescues the lifespan of XLF/DNA-PKcs double deficient mice

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In our cells, DNA double-strand breaks (DSBs) are generated by environmental factors and physiologically, for instance, during the lymphocyte development. DSBs are recognized and repaired by distinct DNA repair pathways, including classical Non-Homologous End Joining (C-NHEJ), where the Ku70/Ku80 heterodimer (Ku) is recruited to the DSB site and forms the DNA-PK protein kinase holoenzyme with the catalytic subunit DNA-PKcs. Then XRCC4/DNA Ligase4 ligates the DNA ends together. XLF is a C-NHEJ factor that directly interact with Ku and XRCC4. Deficiency of Ku70, Ku80, DNA-PKcs or XLF result in live mice that possess different levels of DNA repair defects, while deficiency of XRCC4 or Ligase4 leads to embryonic lethality. Recently, we found that combined inactivation of XLF and DNA-PKcs results in perinatal lethality in mice and increased genomic instability in cells. Deletion of Ku70 completely rescues the lifespan of XLF/DNA-PKcs double deficient mice. Mice with combined deficiency for Ku70, XLF and DNA-PKcs have body size and levels of genomic instability indistinguishable from that of Ku70-deficient littermates. Among other possibilities, our finding is consistent with a model where combined function of XLF and DNA-PKcs is required for C-NHEJ and that Ku70/Ku80 heterodimer blocks alternative DNA repair pathways causing, for instance, neuronal apoptosis.

Double strand break repair: NHEJ - C3

Presenter: Yuka Nakazawa

A Screening of Cockayne syndrome like patients identified a new class of disease associated with mutations in the XRCC4 gene


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We screened patients with clinical features overlapping with Cockayne syndrome (CS), a genetic disorder associated with a deficiency in transcription-coupled nucleotide excision repair (TC-NER). CS patients display developmental abnormalities and photodermatosis as well as microcephaly. From ~200 CS like patients, we identified ~50 cases with CS like clinical manifestations but retaining normal TC-NER activity. In these cases, we performed exome sequencing and identified pathogenic mutations in several cases. One patient, CSL16NG, carried a compound heterozygous mutations in the XRCC4 gene, the product of which plays an essential role in DNA non-homologous end-joining (NHEJ), the major DNA double strand break (DSB) repair pathway. Deficiencies in several NHEJ genes, encoding DNA ligase IV (LIG4), XLF/Cernunnos, Artemis, and DNA-PKcs have been identified previously; however, patients with mutations in XRCC4 had not yet been reported until 2014/2015. In CSL16NG fibroblasts, expression of the XRCC4/LIG4 protein complex was severely compromised, the cells showed dramatically defective DSB rejoining, and a plasmid assay revealed that nearly all junctions were repaired by a microhomology-dependent mechanism, with or without deletions. Most NHEJ-deficient patients display immunodeficiency because of a malfunction in V(DJ) recombination. In contrast, CSL16NG had a normal immune response and normal V(DJ) activity with increased fidelity of rejoining assessed by a recombination assay, although the pattern of class switch recombination junctions in the patient lymphocytes were aberrant. Based on these findings, we propose a new class of disease, XRCC4 syndrome, in which patients display a severe defect in DSB repair but retain efficient V(DJ) recombination capacity and normal immune functions. Our findings reveal a cellular and clinical manifestation distinct to that caused by diminished LIG4, and a novel clinical consequence of NHEJ deficiency.
Identification of novel factors involved in ubiquitination events at uncapped telomeres

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Telomeres are specialized chromatin structures that protect natural chromosome ends from being recognized as damaged DNA. Proliferation in the absence of telomerase causes telomeres to shorten progressively with every cell division, ultimately leading to critically short and deprotected chromosome ends. These deprotected ends are processed by DNA repair factors, resulting in chromosome end-to-end fusions. In checkpoint-deficient cells, these fusions can lead to genomic instability, which is a major hallmark of cancer.

The response to telomere deprotection is accompanied by posttranslational modifications, including ubiquitination, which marks proteins for degradation or regulates their activity and transport to specific locations within the cell. Our aim is to understand the role of ubiquitin in the telomere damage response, by identification and functional characterization of factors of the ubiquitin pathway that play an important role at deprotected telomeres. We previously identified the E3 ubiquitin-ligase RNF8 as a major player in the telomere damage response, by using the well-characterized TRF2ts system, which relies on the temperature-sensitive inactivation of TRF2, a component of the shelterin complex. We have shown that RNF8 affects processing and end-joining of deprotected telomeres by facilitating DNA repair at telomeres via induction of ubiquitination of histone H2A and γ-H2AX and by promoting accumulation of 53BP1 and p-ATM at uncapped telomeres.

Currently, we focus on other players of the ubiquitin system, such as E2 ubiquitin-conjugating enzymes and deubiquitinating enzymes. Knockdown of several of these enzymes resulted in rescue from telomere-induced genomic instability. We are studying these factors from different angles by various genetic, cell-biological and biochemical assays to obtain more insight on the function of these proteins at deprotected telomeres. Results so far show reductions in telomere fusions, retention of the telomeric single-stranded G-overhang and disturbed kinetics of DNA damage response factor accumulation.

Our findings underscore the importance and complex role of ubiquitination in telomere damage responses. The associated molecular mechanisms are being investigated further to increase our understanding of the cellular responses to telomere deprotection, which affect cancer development and aging.
Double strand break repair: NHEJ - C3

Presenter: Chantal Stoepker

Loss of ZBTB24, a novel non-homologous end-joining protein, impairs class-switch recombination in ICF syndrome

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The autosomal recessive immunodeficiency, centromeric instability and facial anomalies (ICF) syndrome is a genetically heterogeneous disorder. Despite recent successes in the identification of the underlying gene defects, it is currently unclear how mutations in any of the four known ICF genes cause a primary immunodeficiency. Here we demonstrate that loss of ZBTB24 in B cells from ICF2 patients impairs non-homologous end-joining (NHEJ) during immunoglobulin class-switch recombination and consequently impairs immunoglobulin production and subtype balance. Mechanistically, we found that ZBTB24 associates with poly(ADP-ribose) polymerase 1 (PARP1) and stimulates auto-poly(ADP-riboseyl)ation of this enzyme. The zinc finger in ZBTB24 binds PARP1-associated poly(ADP-ribose) chains and mediates the PARP1-dependent recruitment of ZBTB24 to DNA breaks. Moreover, by binding to poly(ADP-ribose) chains ZBTB24 protects these moieties from degradation by poly(ADP-ribose) glycohydrolase (PARG). This enhances the poly(ADP-ribose)-dependent interaction between PARP1 and the LIG4/XRCC4 NHEJ complex and promotes NHEJ by facilitating the assembly of this repair complex at DNA breaks. Thus, we uncover ZBTB24 as a regulator of PARP1-dependent NHEJ and class-switch recombination, providing a molecular basis for the immunodeficiency in ICF syndrome.
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