BENZON SYMPOSIUM No. 52

CELLULAR RESPONSES TO DNA DAMAGE

AUGUST 22-25, 2005, COPENHAGEN, DENMARK

Organizing committee:
Jiri Bartek (Copenhagen), Jiri Lukas (Copenhagen), Jan Hoeijmakers (Rotterdam) and Arne Svejgaard (Copenhagen)

Abstracts - MONDAY, August 22, 2005

MOLECULAR AND FUNCTIONAL ANALYSIS OF THE DNA-DAMAGE RESPONSE

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Our work aims to decipher the mechanisms by which eukaryotic cells detect various forms of DNA-damage and then signal the presence of these structures to the DNA-repair, transcription and cell-cycle machineries. As many aspects of the DNA-damage response are highly conserved throughout eukaryotic evolution, we are analyzing proteins involved in these pathways both in mammalian cells and in the yeast *Saccharomyces cerevisiae*. In this seminar, I will first describe our recent data that have provided insights into the molecular co-operation between ATM and ATR in response to DNA damage caused by ionizing radiation. Second, I will describe our recent identification of the hnRNP K protein as a novel Mdm2 target and a co-activator for p53-dependent transcriptional responses to DNA damage.

MDC1 MAINTAINS GENOMIC STABILITY BY PARTICIPATING IN THE AMPLIFICATION OF ATM-DEPENDENT DNA DAMAGE SIGNALS

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MDC1 plays multiple roles in checkpoint activation and DNA repair following DNA damage. To address the physiological function of MDC1, we disrupted MDC1 gene in mice. $MDC1^{-/-}$ mice recapitulated many phenotypes of $ATM^{-/-}$ and $H2AX^{-/-}$ mice, including growth retardation, male infertility, immune defects, chromosome instability, DNA repair defects and radiation sensitivity. At the molecular level, ATM, H2AX and MDC1 form a positive feedback loop. MDC1 binds to phosphorylated H2AX through its BRCT domain and its focus localization following DNA damage depends on phosphorylated H2AX. While MDC1 is not required for initial ATM activation, MDC1, through its FHA domain, recruits ATM to the sites of DNA damage, facilitates further ATM-dependent phosphorylation of H2AX and thus amplifies the DNA damage signals. In the absence of MDC1, H2AX phosphorylation is compromised, resulting in the failure of accumulation of downstream ATM substrates (BRCA1, 53BP1, NBS1) at the sites of DNA damage. Most downstream ATM signaling events, including the phosphorylation of NBS1, SMC1, Chk1, Chk2 as well as the DNA damage checkpoints are defective in $MDC1^{-/-}$ cells. These results demonstrate the important role of MDC1, as a signal amplifier of the ATM pathway, in controlling proper DNA damage responses and maintaining genomic stability.

DNA REPLICATION CONTROL AND GENOME STABILITY

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The eukaryotic cell cycle is designed to promote and coordinate the accurate duplication and apportionment of the genome during proliferation. The large genomes of eukaryotic cells are replicated from multiple replication origins during S phase. These origins are not activated synchronously at the beginning of S phase but, instead, fire throughout S phase according to a pre-determined, cell type specific program. Only after the entire genome is completely replicated do cells proceed into mitosis.

Ensuring that each origin is efficiently activated once and only once during each S phase is crucial for maintaining the integrity of the genome. This is achieved by a two-step mechanism. Pre-RCs, which are essential for initiation, can only assemble at origins during G1 phase when cyclin dependent kinase (CDK) activity is low. Initiation is then triggered by an increase in CDK activity at the end of G1 phase which also prevents new pre-RC assembly until CDKs are inactivated in the subsequent mitosis. The prevention of pre-RC assembly by CDKs is redundant: every component of the pre-RC is, in some way, inhibited by CDKs. Mechanisms regulating pre-RC assembly will be

discussed. In particular, a novel mechanism by which Cdc6 function is inhibited by the mitotic CDK, Clb2/Cdc28, will be discussed in detail.
In human cells, CDKs including cyclin E-CDK2 can also play a positive role in pre-RC assembly in certain circumstances. We have found that phosphorylation stabilises Cdc6 by preventing its association with the Anaphase Promoting Complex/ Cyclosome (APC/C). In cells re-entering the cell cycle, Cdc6 phosphorylation is executed primarily by Cyclin E-Cdk2. This stabilisation by Cdk phosphorylation ensures that Cdc6 can accumulate prior to the licensing inhibitors geminin and cyclin A.

CELL CYCLE CHECKPOINTS AND THE INTEGRITY OF REPLICATING CHROMOSOMES

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Cells overcome intra-S DNA damage and replication impediments by coupling cell cycle checkpoints with chromosome replication, recombination and sister chromatid cohesion.
We have studied the molecular mechanisms and the genetic pathways controlling the integrity of replicating chromosomes in *S. cerevisiae*, under normal conditions and in response to replication stress or intra-S DNA damage.
We found that cells couple sister chromatid tethering with replication initiation by generating specialised cruciform joint molecules that are likely implicated in promoting sister chromatid-mediated recombination and replication processes. The same structures, however, contribute to the pathological accumulation of reversed forks and DNA breaks in the absence of a functional checkpoint. We found that these pathological transitions are influenced by the unscheduled dissociation of the replisome from stalled forks, by the lagging strand replication apparatus and by the activity of the Exo1 nuclease. Exo1 is actively implicated in counteracting fork reversal when the replisome dissociates from stalled forks and in resecting newly synthesized strands, thus promoting the formation of gapped molecules and the resolution of the sister chromatid junctions.
We also found that in *sgs1* and *top3* mutants, recombination-dependent cruciform structures accumulate at damaged forks. Their accumulation requires the Rad51 protein, is counteracted by the Srs2 DNA helicase and does not prevent fork movement. Sgs1, but not Srs2, promotes resolution of these recombination intermediates. A functional Rad53 checkpoint kinase is required for the accumulation of recombination intermediates in *sgs1* mutants. Finally, *top3* and *top3 sgs1* mutants accumulate the same structures as *sgs1* cells.
Altogether, our findings suggest that the checkpoint response protects the integrity of replication forks by stabilising the replisome–fork complexes when forks stall and that the coordinated action of RecQ helicases and Type 3 topoisomerases promote the maturation of recombination-dependent intermediates at damaged forks that originate from the replication-related sister chromatid junctions.

THE CELLULAR DNA DAMAGE RESPONSE: DIVERSE FUNCTIONS CONVERGE

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Work in our laboratory is focused on understanding the molecular transactions that govern chromosome stability and replication. The association of cancer predisposition and other pathology with mutations that affect chromosomal metabolism forms the basis of our interest in this process. In this regard, we focus on the Mre11 complex, a conserved multiprotein complex that includes Mre11, Rad50 and Nbs1 in mammals or Xrs2 in the budding yeast, *S. cerevisiae*. Congenitally acquired hypomorphic defects in *NBS1* and *MRE11* are associated with the cancer predisposition and chromosome instability syndromes, Nijmegen breakage syndrome (NBS) and the ataxia-telangiectasia like disorder (A-TLD) respectively. We have modeled these diseases in the mouse, in addition to deriving a hypomorphic *Rad50* murine mutant. The characterization of these animals, as well as yeast mutants affecting the complex will be discussed in the larger context of the Mre11 complex's influence on chromosome metabolism.

DNA DAMAGE CHECKPOINTS: MECHANISMS AND ROLE IN TUMORIGENESIS

Bartek J; Danish Cancer Society, Copenhagen, Denmark
The lecture will provide a brief outline of our most recent work on mechanisms of DNA damage responses in mammalian cells, with emphasis on the following specific issues, and their potential implications for oncogenesis and cancer therapy. First, the emerging biological differences between the ATR-Chk1-regulated and ATM-Chk2-regulated pathways in unperturbed cell cycles and in response to genotoxic insults will be discussed. This part may include presentation of our results on new roles and substrates of the Chk1 kinase, and consideration of Chk1 kinase inhibition as a potential strategy to sensitize cancer cells to DNA damaging treatment modalities. Second, our data supporting a concept of the DNA damage response machinery as an inducible barrier against progression of early stages of human tumours in vivo, and in response to various oncogenes in cell culture models, will be presented. Here, emphasis will be mainly on unpublished results on distinct patterns of DNA damage response activation seen in diverse types of human malignancies, and discussion of potential cellular and molecular basis of such differences, as well as consideration of the various cancer-associated molecular defects capable of inducing the DNA damage checkpoint machinery.

Poster No. I-1

MUTATIONS IN S. CEREVISIAE CHROMATIN ASSEMBLY FACTORS CAF-I AND RCAF CAUSE REPLICATION-DEPENDENT DNA DAMAGE WHICH IS RELIANT ON DIFFERENT CHECKPOINT PATHWAYS FOR SUPPRESSION

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The chromatin-assembly factor I (CAF-I) and replication-coupling assembly factor (RCAF) complexes function in chromatin assembly during DNA replication and repair. We have previously shown that CAF-I and RCAF play a role in the maintenance of genome stability as evidenced by increased genomic rearrangements in CAF-I and RCAF defective mutants. One of the explanations for the high genomic instability rates observed in these mutants is that they might have a checkpoint defect. The present study addresses this hypothesis by examining checkpoint proficiency of various RCAF and CAF-I mutants. We find that in contrast to the well-described checkpoint mutant *rad53sml1*, treatment of *asf1*, *cac1*, *cac2*, and *cac3* single mutants and the *asf1 cac1/2/3* double mutants with hydroxyurea (HU) did not cause increased lethality or permanent G2/M arrest, as evidenced both by HU-survival assays as well as FACS analysis. Furthermore, release of chromatin assembly mutants into 0.03% MMS resulted in a marked decrease in the rate of S-phase progression, demonstrating these mutants' intra-S checkpoint proficiency. Additional FACS analysis of S-phase progression in mutants lacking Asf1 or Cac1 as well as various checkpoint proteins indicate that in *asf1* and *cac1* mutants replication defects occur that make the mutants dependent on different checkpoint functions in order for S-phase progression to occur at normal rates. We also examined the endogenous formation of DDC2.GFP loci in our mutants and found that while both *asf1* and *cac1* mutants exibit an increase in damage foci formation, the damage caused by the *asf1* mutation is processed via the DUN1 pathway, while this is not the case in *cac1* mutants. Our data further support the hypothesis that both CAF-I and RCAF play important yet different roles in replication and suppression of DNA damage.

Poster No. I-2

ATM AND ATR PREVENT DOUBLE-STRAND BREAK ACCUMULATION DURING CHROMOSOMAL DNA REPLICATION BY PROMOTING MRE11 DEPENDENT REPAIR AND RESTART OF DAMAGED REPLICATION FORKS

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ATM and ATR play a key role in maintaining genome integrity. We have set up several assays based on Xenopus laevis cell free extracts to study ATM and ATR function[1, 2, 3]. Here, we show that ATM and ATR prevent double-strand breaks (DSBs) accumulation during unperturbed and disrupted chromosomal DNA replication. Chromosomes replicating in cell free extracts in which ATM and ATR have been inhibited by chemical inhibitors or depleted with specific polyclonal antibodies accumulate DSBs as demonstrated by TUNEL assay. DSBs accumulation is replication dependent as it is inhibited by geminin. ATM and ATR kinases addition to depleted extracts prevents DSBs accumulation. DSBs result from inefficient repair of damaged replication forks. Damaged forks are efficiently repaired and replication resumes from repaired forks when damaged chromosomes are transferred to extracts containing active ATM and ATR. In contrast, repair and restart of damaged forks is highly inefficient in extracts in which ATM and ATR activities have been inhibited. Addition of purified active ATM and ATR kinases to depleted extracts restores efficient DNA repair and DNA replication restart. We show that damaged forks retain functional MCM complex regardless of ATM and ATR status. In contrast, retention of functional Cdc45 at damaged forks requires active ATM and ATR. Repair and restart of damaged forks is Mre11/Rad50/Nbs1 dependent as it is inhibited in Mre11 depleted extracts and it is restored in Mre11 depleted extracts supplemented with recombinant Mre11/Rad50/Nbs1 complex. Using a novel FRET based technique we demonstrate that ATM and ATR promote Mre11 localization to damaged forks. This study provides direct biochemical evidences that ATR and ATM ensure genomic stability during chromosomal DNA replication by promoting DNA damage repair and fork restart. *[1]Costanzo et al. Mol Cell 2000, [2] Costanzo et al. Mol Cell 2001, [3]Costanzo et al. Mol Cell 2003*

Poster No. I-3

POLO-LIKE KINASE-1 REGULATES CHECKPOINT ADAPTATION AND RECOVERY

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It is well established that DNA damaging agents elicit a potent block in the cell division process, through the action of so-called DNA damage checkpoints. These checkpoints ensure that the damage can be repaired before new daughter cells are formed. However, little is known about the factors that determine how and when a restart of the cell cycle is permitted following a checkpoint-mediated arrest. Ideally, this restart should only occur once repair of the damage is completed (recovery), but occasionally cells may escape from a checkpoint-induced arrest (checkpoint escape). Also, if the damage cannot be repaired, cells may eventually abrogate the checkpoint (adaptation). The latter two options will lead to propagation of mutations and are thought to contribute to the formation of secondary tumors that frequently arise as a result of genotoxic anti-cancer therapies.

We have recently set out to investigate the mechanism of checkpoint recovery in human cells and found that Polo-like kinase-1 (Plk1) and the phosphatase Cdc25B play essential roles during checkpoint recovery in cells arrested in the G2 phase of the cell cycle following DNA damage. During recovery, Plk1 promotes the degradation of Wee1, a Cdk1 inhibitory kinase. In fact, the requirement for Plk1 during recovery was completely lost in Wee1-depleted cells, indicating that Wee1 is a crucial target of Plk1 in this process. Importantly, Plk1 and Cdc25B appeared to be dispensable for mitotic entry in an unperturbed cell cycle, making it possible to selectively interfere with the recovery process. In addition, we found that while Cdc20 protein levels drop in response to DNA damage, they are quickly restored to normal levels during checkpoint recovery, coincident with mitotic entry.
We are currently investigating the requirement for Plk1 during recovery in greater detail by protein replacement in RNAi-depleted cultures. In addition, we are addressing the role of Cdc20 and cyclin stability in the recovery process, i.e. what are the consequences of a failure to downregulate Cdc20 by overexpressing Cdc20 in DNA damage-arrested cells, and is recovery prevented if Cdc20 fails to be up-regulated? In parallel, we are studying if checkpoint adaptation is possible in human cells and whether Plk1 can induce adaptation. The results of these studies will be discussed.

Poster No. I-4

DNA DAMAGE-INDUCED DISSOCIATION OF CHK1 FROM CHROMATIN

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Checkpoint signalling pathways are of crucial importance for maintenance of genomic integrity. Within these pathways, the kinase Chk1 plays a central role mediating a cell cycle arrest. After DNA damage, the ATR and Rad17 complexes are recruited to single strand DNA lesions. The loading of Rad9 onto chromatin is required for the ATR-dependent phosphorylation of Chk1. Recent studies have also implicated the mediator protein Claspin in phosphorylation of Chk1. Upon phosphorylation, Chk1 is activated, phosphorylates key cell cycle regulators and thereby inhibits cell cycle progression.
When investigating the localisation of Chk1, we observed that around 20% of cellular Chk1 localises to chromatin. In response to UV damage, Chk1 dissociates from the chromatin. Chk1 was found to localise to chromatin during all phases of the cell cycle, suggesting that the observed dissociation of Chk1 from the chromatin after DNA damage is not an indirect effect of the induced cell cycle arrest. All conditions that activate Chk1 cause a dissociation of Chk1 from the chromatin. We also observed a tight correlation between Chk1 phosphorylation and chromatin dissociation, which suggests that phosphorylated Chk1 does not stably associate with chromatin. Inhibition of the activity of PI3K-like kinases ATR and ATM, as well as downregulation of Claspin by siRNA, resulted in an abrogation of the DNA damage-induced dissociation of Chk1 from chromatin. From these results we conclude that active checkpoint signalling is required for the DNA damage-induced dissociation of Chk1 from chromatin. Recent progress into understanding the functional significance and the mechanism of the DNA damage-induced dissociation of Chk1 from the chromatin will be discussed.

Poster No. I-5

DEGRADATION OF HUMAN EXONUCLEASE 1B UPON DNA SYNTHESIS INHIBITION

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In response to DNA damage, signaling pathways are triggered that either block the cell division cycle at defined transitions (G1/S and G2/M), or slow down progression through S-phase. Nucleases play important roles in DNA synthesis, recombination, repair and apoptosis. In this study we have examined the regulation of human exonuclease 1 (hEXO1b). The endogenous hEXO1b protein was only detected upon enrichment by immunoprecipitation. We found that hEXO1b was constantly expressed throughout the cell cycle. However, treatment of cells with agents that cause arrest of DNA replication led to rapid degradation of hEXO1b. This effect was fully reversed upon removal of the block. Analysis of synchronized cells showed that degradation of hEXO1b during S-phase was strictly dependent on DNA synthesis inhibition. DNA damage caused by UV-C radiation, ionizing radiation, cisplatin or the alkylating agent MNNG did not affect hEXO1b stability. We show that hEXO1b was phosphorylated in response to inhibition of DNA synthesis and that phosphorylation coincided with rapid protein degradation through ubiquitin-proteasome pathways. Our data support the evidence that control of exonuclease 1 activity may be critical for the maintenance of stalled replication forks.

Poster No. I-6

HUMAN ASF1 REGULATES THE FLOW OF S PHASE HISTONES DURING REPLICATIONAL STRESS

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Maintenance of chromosomal integrity requires tight coordination of histone biosynthesis with DNA replication. We have recently shown that extracts from human cells exposed to replication inhibitors display an increased capacity to support replication-coupled chromatin assembly (Groth et al. 2005). This enhanced activity is dependent on the histone chaperones Asf1a and Asf1b (hAsf1), which can synergize with the chromatin assembly factor CAF-1 in repair- and replication-coupled chromatin assembly (Tyler et al. 1999; Mello et al. 2002). In cytosolic extracts from

unperturbed S-phase cells, hAsf1 exists in equilibrium between an active histone-containing multi-chaperone complex and an inactive histone-free form. Interference with DNA replication results in accumulation of soluble S-phase histones and mobilization of the majority of hAsf1 into the active complex. The active complex is limiting for chromatin assembly in S-phase extracts and accumulation of such complex can thus account for the enhanced activity in cells exposed to replication inhibitors. Our data suggest that hAsf1 provides the cells with a buffering system for histone excess generated in response to stalled replication and explains how mammalian cells maintain a critical "active" histone pool available for deposition during recovery from replicational stresses. In yeast *S. cerevisiae* loss of Asf1 function confers high sensitive to replication inhibitors and agents causing single and double strand breaks (Le et al. 1997; Singer et al. 1998; Tyler et al. 1999). Furthermore, yeast Asf1 has been directly implicated in checkpoint responses via its regulated interaction with Rad53 (Emili et al. 2001; Hu et al. 2001). Nonetheless, we find that the ability of hAsf1 to buffer histone excess is checkpoint independent. We are currently aiming to isolate and characterize hAsf1 complexes from unperturbed S-phase cells and cells exposed to replication inhibitors to identify components that may integrate information about histone availability with DNA replication and histone biosynthesis.

Poster No. I-7

MOLECULAR MECHANISMS REGULATING DNA DAMAGE-ACTIVATED DIFFERENTIATION CHECKPOINT IN MUSCLE CELLS

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In proliferating cells exposed to genotoxic stress, DNA damage-induced cell cycle arrest allows the repair of damaged DNA to prevent the propagation of chromosomal abnormalities to daughter cells. In multicellular organisms cell proliferation is often directed at generating specialized cell types through differentiation. The induction of terminal differentiation requires a global re-programming of gene expression, which occurs in parallel with the withdrawal from the cell cycle. The coupling between differentiation and cell cycle arrest raises the question on how differentiation-committed progenitors respond to DNA damage. We show that DNA damage activates a differentiation checkpoint, superimposed to the cell cycle arrest, to prevent the re-programming of damaged genome toward differentiation, until the DNA lesion has been repaired. Exposure to different classes of genotoxic agents, which leads to arrest of the cell cycle at discrete boundaries – i.e. the G0/G1 and G2/M - inhibits muscle-gene expression by distinct mechanisms. While the inhibition of MyoD function by agents that induce G0/G1 arrest occurs via cAbl-mediated tyrosine phosphorylation of chromatin bound MyoD, agents that induce G2/M phase arrest prevent muscle-gene transcription by precluding MyoD binding to chromatin. Consistently, exposure to caffeine, which bypasses the G2 arrest, restores muscle transcription in the presence of G2-arresting genotoxic agents, and allow the formation of myotubes, with unrepaired DNA lesions. We are currently characterizing these myotubes enforced to differentiate despite the exposure to DNA damaging agents by analyzing the expression and re-localization of repair, muscle-specific and DNA damage-induced proteins. These findings are instrumental to investigate whether bypassing the differentiation checkpoint could generate genetically unstable differentiated cells, displaying constitutive activation of DNA-damage signaling, and will likely shed light on the chromatin re-programming toward senescence in differentiated cells.

Poster No. I-8

REGULATION OF DNA DAMAGE-INDUCED APOPTOSIS BY CAMP AND ITS IMPLICATION IN CANCER THERAPY

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cAMP exerts an antiproliferative effect on the majority of cell types. We and others have shown that activation of the cAMP signal transduction pathway in lymphocytes leads to arrest of cells in G1 through inhibition of cyclin D3 expression. More recently, we reported that cAMP also inhibits DNA replication and leads to arrest of cells in S phase in an RB- and $p21^{Cip1}$-dependent manner. In accordance with this inhibitory effect of cAMP on S-phase progression, activation of the cAMP signaling pathway rendered cells resistant to the cytotoxic effect of S phase-specific DNA damaging drugs in wt but not RB- or $p21^{Cip1}$-deficient cells.

Here we present evidence showing that cAMP also exerts a protective effect against apoptosis induced by other DNA damaging agents, such as ionizing radiation (IR), whose cytotoxic activity is regarded as being cell cycle nonspecific. Elevation of intracellular levels of cAMP prior to exposure of cells to IR substantially inhibited IR-induced apoptosis. cAMP was found to prevent IR-induced satbilization of p53 without inhibiting the IR-mediated activation of ATM, Chk1 and Chk2 proteins. These results suggest that cAMP inhibits apoptosis mediated by IR through modulation of the p53 protein stability downstream of the ATM-Chk pathway. We are currently investigating the mechanism(s) responsible for the regulatory effect of cAMP on the stability of p53. Results of this investigation will be presented.

Finally, we will present evidence showing that activation of the cAMP signaling cascade subsequent, but not prior, to exposure of cells to IR augments IR-induced apoptosis. Taken together, these results suggest that the status of intracellular cAMP plays an important role in regulation of the cytotoxic effect of IR, and indicate that cAMP, in principle, could be used as a rational target to devise therapeutic strategies for either restoring apoptosis sensitivity in cancer cells or increasing the efficacy of IR to induce apoptosis in cancer cells.

Poster No. I-9

TARGETING HUMAN CHECKPOINT KINASE CHK1 FOR CANCER TREATMENT

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Human checkpoint kinase Chk1 has been suggested as a target for cancer treatment. The present study was initiated to explore the effects of Chk1-inhibition in the presence and absence of DNA damage induced by ionizing radiation (IR). In the presence of IR, we have found that a new inhibitor of Chk1 kinase, Cep-3891, efficiently abrogates the IR-induced S and G2 checkpoints, leading to accelerated onset of mitotic nuclear fragmentation and increased cell death. The formation of nuclear fragmentation in IR-treated human cancer cells was directly visualized by time-lapse videomicroscopy of cells expressing a green fluorescent protein-tagged histone H2B protein.

In the absence of IR, inhibition of Chk1 by two distinct drugs, UCN-01 and Cep-3891, or Chk1 siRNA, causes a rapid and strong phosphorylation of ATR targets in S-phase cells. This was associated with stabilization of human phosphatase Cdc25A, increased activity of cyclin dependent kinase 2 (cdk2), increased initiation of DNA replication, massive induction of single-stranded DNA and generation of DNA strand breaks. We propose a model where Chk1 is required during normal S phase progression to avoid aberrantly increased initiation of DNA replication, thereby protecting against DNA breakage. These results may help explain why Chk1 is an essential kinase, and should be taken into account when drugs to inhibit this kinase are considered for use in cancer treatment.

Poster No. I-10

THE ROLE OF POLO-LIKE KINASE-1 IN RESTARTING THE CELL CYCLE AFTER A G2 DNA DAMAGE CHECKPOINT ARREST

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DNA damage triggers multiple checkpoint pathways to arrest cell cycle progression. Less is known about the mechanisms that allow resumption of the cell cycle once checkpoint signaling is silenced. Here we show that while in undamaged cells several redundant pathways can promote the onset of mitosis, this redundancy is lost in cells recovering from a DNA damage-induced arrest. We demonstrate that Plk1 is crucial for mitotic entry following recovery from DNA damage. However, Plk1 is no longer required in cells depleted of Wee1, and we could show that Plk1 is involved in the degradation of Wee1 at the onset of mitosis. Thus, our data show that the cell cycle machinery is reset in response to DNA damage and that cells become critically dependent on Plk1-mediated degradation of Wee1 for their recovery.

Poster No. I-11

ANALYSIS OF CHECKPOINT ACTIVATION AND DNA TURNOVER AT DOUBLE STRAND BREAKS

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DNA double-strand breaks (DSBs) are amongst the most hazardous chromosomal lesions because of the possibility of a total loss of sequence information. It is therefore of vital importance to ensure the activation of the DNA damage checkpoint and repair machineries in order to deal with such a threat.

We have analysed the response to single and multiple DSBs in the yeast *Saccharomyces cerevisiae* introduced by controlled expression of the site-specific HO-endonuclease in strains harbouring different numbers of HO recognition-sites. We found a dosage-effect on checkpoint activation that is especially pronounced in G_1 arrested cells which, in agreement with published data, do not show any detectable checkpoint activation upon introduction of a single DSB. Upon formation of at least two DSBs, however, a robust checkpoint activation was detected even in cells at the G_1 stage.

Furthermore, we analysed the formation of single-stranded DNA (ssDNA) at a DSB in a novel assay utilising quantitative real-time PCR (RT-PCR). Using this method, we detected a significant increase in ssDNA formation in strains containing multiple DSBs, proportional to the strength of checkpoint activation observed. We hypothesise that the initial activation of checkpoint proteins leads to a change in end-turnover, with enhanced rates of ssDNA formation, thus allowing for the possibility of a positive feedback loop in checkpoint activation. Experiments are currently under way to directly test this hypothesis.

Poster No. I-12

THE EFFECT OF P53 STATUS ON RADIO-SENSITIZATION OF HUMAN CANCER CELLS BY INHIBITION OF THE CHECKPOINT KINASE 1

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p53-deficient cancer cells are considered to be more therapy-resistant than cancer cells with wild-type p53, and p53 mutations have been correlated with poor prognosis. Recently, several laboratories have tried different approaches to sensitize cancer cells towards radiation or chemotherapy. One of the more promising models proposed based on these studies suggest that p53-deficient cancer cells, which lack the G1 checkpoint and therefore may rely more on the G2 checkpoint to repair DNA damage, may be selectively affected by G2 checkpoint abrogation. In this study we use two inhibitors of human Checkpoint Kinase 1 (Chk1), Cephalon-3891 and UCN-01, to abrogate the G2 checkpoint. The

aim is to investigate if cancer cells, that lack the p53 dependent G1 checkpoint, are selectively radio-sensitized by Chk1 inhibition. We decided to study the effects of Chk1 inhibition in isogenic cell line systems based on the U-2-OS sarcoma cells, where we can assay the effects of different p53 and p21 status in the cells with otherwise identical genetic background. Our preliminary results show that p53-deficient cancer cells treated with Chk1 inhibitors and exposed to radiation are more radio-sensitive and undergo cell death earlier compared to wt p53 cancer cells. Furthermore, p53-deficient cancer cells treated with radiation and Chk1 inhibitors show different patterns of cell cycle progression after the first division compared to wt p53 cancer cells. These studies suggest the potential of Chk1 inhibition as a strategy to sensitize p53-deficient cancer cells to radiation.

Poster No. I-13

MODULATION OF THE CELL CYCLE BY DNA DAMAGE RESPONSE

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In mammalian cells, cell cycle progression is governed by distinct cyclin-dependent kinases (cdks) whose activities are regulated by binding of their activating cyclin subunits and through negative regulation by inhibitor proteins such as Cip/Kip kinase inhibitor proteins. In addition to the normal cell cycle regulation, cells have developed so-called DNA damage responses which act as surveillance systems reacting to DNA insults caused by both intrinsic and extrinsic sources of genotoxic stress. Once activated, DNA damage responses disrupt the function of the cell cycle and can result in a number of outcomes including short or long term cell cycle arrest, apoptosis and necrosis. There is immense interest in the cross-talk/relationship between the DNA damage response and the cell cycle regulatory elements. Growing evidence suggests that the cell cycle regulatory proteins play roles in both pathways to ensure the maintenance of genomic integrity. Our research aims to compare and contrast the cellular responses linked to various specific DNA damaging agents in terms of cell cycle regulatory proteins (G1 cyclins and cdk inhibitor proteins) and ultimately to understand the molecular mechanisms underlying the regulation of such responses.

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Abstracts - TUESDAY, August 23, 2005

NEW INSIGHTS INTO ATM-MEDIATED RESPONSES TO DNA DAMAGE

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The intricate signaling network mobilized by DNA damage spans numerous pathways linking DNA repair, cell cycle checkpoints, and chromatin organization, and modulates many processes that control various aspects of cellular life. The prototype DNA lesion that activates this response to its fullest extent is the double strand break (DSB). The chief transducer of the DSB response is the ATM protein, a nuclear protein kinase that is rapidly activated following DSB induction and phosphorylates a multitude of substrates, each involved in a specific process. ATM is missing or non-functional in patients with the cancer predisposing, genomic instability syndrome ataxia-telangiectasia (A-T). The current list of ATM substrates is far from complete. Identification of new ATM targets is expected to elucidate novel branches of the ATM-mediated network. Recently we identified several such substrates using a variety of methods. Four of them will be discussed: the p53 inhibitor Mdmx, whose phosphorylation leads to its proteasome-mediated degradation and thus contributes to p53 activation; the COP9 signalosome complex, whose phosphorylation is involved in DNA damage-induced apoptosis; the KAP-1 protein, whose phosphorylation is required for chromatin relaxation following DNA damage; and the transcription factor Sp1, whose activity is enhanced in the course of the DNA damage response. These novel ATM effectors provide further evidence of the variety of pathways controlled by ATM in response to DNA damage.

SIGNALING TO AND FROM ATM AFTER DNA DAMAGE

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Many of the insights that we have gained into the mechanisms involved in cellular DNA damage response pathways have come from studies of human cancer susceptibility syndromes that are altered in DNA damage responses. ATM, the gene mutated in the disorder, Ataxia-telangiectasia, is a protein kinase that is a central mediator of responses to DNA double strand breaks in cells. Recent studies have elucidated the mechanism by which DNA damage activates the ATM kinase and initiates these critical cellular signaling pathways. ATM normally exists as an inactive homodimer in unperturbed cells and introduction of DNA damage induces intermolecular autophosphorylation on serine 1981 in both ATM molecules. This phosphorylation causes a dissociation of the ATM molecules and frees up the kinase to circulate around the nucleus and phosphorylate substrates that influence cell cycle progression and DNA repair processes. The SMC1 protein appears to be a particularly important target of the ATM kinase, playing critical roles in controlling DNA replication forks and DNA repair after the damage. A major role for the NBS1 and BRCA1 proteins appears to be in the recruitment of an activated ATM kinase molecule to the sites of DNA breaks so that ATM can phosphorylate SMC1. Generation of mice and cells that are unable to phosphorylate SMC1 demonstrated the importance of SMC1 phosphorylation in the DNA damage induced S-phase checkpoint, in determining rates of repair of chromosomal breaks, and in determining cell survival after DNA damage. Focusing on ATM and SMC1, the molecular controls of these pathways will be discussed along with consideration of how to potentially manipulate these pathways to benefit cancer patients.

TUMOR-ASSOCIATED REPLICATION STRESS AND THE CELLULAR RESPONSE TO ANTICANCER AGENTS

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A major function of the eukaryotic cell cycle is to insure that mitosis and cytokinesis yield two daughter cells bearing accurate and complete copies of the parental genome. DNA replication is a mechanistically intricate, energy-consuming process that is sensitive to both environmental and intracellular conditions. Accordingly, cells have evolved an elaborate surveillance network that monitors both the progress of active DNA replication forks and the fidelity of DNA strand copying during S phase. Central components of this S-phase surveillance machinery are the PI 3-kinase related kinase ATR, and its downstream substrate Chk1, which is protein serine-threonine kinase whose

activity is regulated by ATR-mediated phosphorylation at two carboxyl-terminal Ser-Gln motifs (Ser-345 and Ser-317). Studies in ATR- and Chk1-deficient mice and cultured cell lines indicate that both protein kinases are essential for cell viability, and that even a partial reduction in gene dosage is sufficient to cause increases in genetic instability and cell death. In response to replication stress, the ATR-Chk1 pathway inhibits the firing of new replication origins and maintains the integrity of stalled replication forks. In the absence of ATR-Chk1 function, stalled forks collapse, leading to irreversible S phase arrest and DNA strand breaks. Here we report that chronic replicative stress induces the poly-ubiquitination and degradation of Chk1 in human cells. This response is dependent on the phosphorylation of Chk1 at Ser-345, a known target site for the upstream regulatory kinase, ATR. The ubiquitination of Chk1 is mediated by at least two E3 ligase complexes containing Cul1 or Cul4A. Treatment of cells with the S-phase specific genotoxic agent, camptothecin (CPT), triggers Chk1 destruction, which blocks cellular recovery from drug-induced S-phase arrest and ultimately leads to cell death. Several cancer cell lines that display high-level CPT resistance exhibit profound defects in CPT-induced Chk1 degradation, and cellular resistance to this drug is reversed by depletion of Chk1 protein with small-interfering RNA duplexes. These findings indicate that ATR-dependent phosphorylation of Chk1 delivers a signal that both activates Chk1 and ultimately marks this protein for proteolytic degradation. Furthermore, replication stress-induced Chk1 destruction may play a crucial role in tumor cell killing by CPT and related drugs.

SPINOCEREBELLAR ATAXIA, OXIDATIVE STRESS AND DNA DAMAGE RESPONSE

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Overlap in the neurological phenotype has been described for a number of human syndromes including ataxia-telangiectasia (A-T),A-T-like disorder (A-TLD),ataxia oculomotor apraxia type 1 (AOA1) and ataxia oculomotor apraxia type 2 (AOA2).Since a defective response to DNA double strand breaks contributes to the phenotype in A-T and A-TLD it was of intertest to determine whether this was also the case for AOA1 ,AOA2 and other related disorders.We have previously shown that AOA1 cells are sensitive to agents that cause single strand breaks in DNA and there is also evidence that the protein defective in this syndrome is involved in DNA repair.Senataxin the product of the gene defective in AOA2 is related in its sequence to the yeast protein sen1p which is implicated in the stress response and in RNA processing.This protein has both RNA and DNA helicase activities.We have recently shown that cells from AOA2 patients are also sensitive to agents that cause singlestrands breaks in DNA.A novel form of AOA is also described that is characterized by a defective p53 response but has normal ATM kinase function.The importance of the DNA damage in neurodegeneration will be discussed.

A TWO-STEP MECHANISM OF ATM ACTIVATION

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Following DNA damage triggered by external insults or normal cell physiology, cells have designed a response that sense aberrant DNA structures and coordinate DNA repair, cell cycle arrest and apoptosis. A critical component of the response to double-strand breaks (DSBs) is the ATM protein kinase. Previous work has established that ATM exists as inactive dimers in intact cells. Activation of ATM correlates with its autophosphorylation and its dissociation into active monomers. This process involves the Mre11/Rad50/Nbs1 (MRN) complex. MRN could facilitate ATM's recruitment to DSBs, induce its dissociation and stimulate ATM's affinity for substrates. Here, we have investigated the role of damaged DNA and MRN complex in ATM activation. Using biotinylated DNA fragments incubated into Xenopus egg extracts, we have been able to separate two distinct steps in ATM activation. We demonstrate that DSBs-containing DNA can recruit ATM and induce the dissociation of ATM dimers in the absence of MRN complex. Monomeric ATM generated in the absence of MRN is not phosphorylated on S1981. MRN is required for autophosphorylation and full activation of ATM. Our experiments suggest that MRN complex plays a dual role in ATM activation. It facilitates the recruitment of ATM to damaged DNA, possibly raising the concentration of DSBs locally. DSBs containing DNA then promotes the dissociation of ATM dimers. MRN complex is dispensable for this step. Finally, inactive ATM monomers are activated by MRN complex as seen by autophosphorylation at S1981.

SEARCHING FOR A SPECIFIC CONTRIBUTION BY LOSS OF BRCA1 FUNCTION TO BREAST CANCER DEVELOPMENT*

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BRCA1 is a tumor suppressing gene, loss of function of which is associated with highly penetrant breast and/or ovarian cancer. It encodes at least 3 proteins, p220, BRCA1-IRIS, and delta 11b. p220 is known to play a role in genome integrity control at multiple levels, including double strand DNA repair and multiple instances of cell cycle checkpoint control. p220 is also a large protein with numerous protein partners, a number of which are elements engaged in DNA repair and/or checkpoint control, on one hand, and/or in the suppression or promotion cancer development, on the other. p220 also participates in a complex network of post damage signal transduction events in

which the checkpoint- associated kinases, ATM and ATR, are involved. Indeed, both BRCA1 and several of its partners are ATM and/or ATR substrates. A growing body of evidence strongly suggests that BRCA1 operates as a scaffolding/signal integrating element following DNA damage and that its role in genome integrity control is tightly associated with its tumor suppression function.
p220 is also engaged in the process that leads to the stable maintenance of the heterochromatic state of the X chromosome. In a growing series of BRCA1 -/- breast carcinoma samples, none, thus far, have revealed a clearly definable Barr Body (Xi), nor decoration of an X chromosome with Xist RNA, macrohistone H2A1.2, or histone H3 meK27-all known signatures of Xi. All such tumors contained at least 2 X chromosomes per cell. Furthermore, in immunohistochemical, gene expression array, and SNP array analyses of a number of sporadic breast cancers, a subset-i.e. basaloid breast cancer (BLC)-was also found to be composed of tumors the vast majority of which lack a definable Barr Body and any of the Xi-associated markers, noted above. In a sizeable set of non-BLC sporadic breast cancers, Xi abnormalities were exceedingly uncommon. All sporadic BLC tested proved to be BRCA1 wt by direct genotyping, and nearly all revealed BRCA1 nuclear dot immunofluorescence, a sign of intact p220 function. Both BLC and BRCA1 -/- tumors also revealed similar immunostaining phenotypes for a number of breast cancer-associated proteins (ER. PR, erbB2, certain cytokeratins), and p53 mutations are exceedingly common in both. Thus, one can argue that nearly all sporadic BLC and BRCA1 -/- tumors are phenocopies. Furthermore, given that p220 is active in both Xi heterochromatinization and in BLC suppression, it seems reasonable to hypothesize that 'misbehavior' at Xi is material to BLC development, both BRCA1 -/- and sporadic.
*This abstract was also submitted to another meeting

MAKING AND BREAKING RECOMBINATION INTERMEDIATES
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Homologous recombination (HR) plays an essential role in two critical cellular processes. In germ-line cells, recombination is required for gene rearrangements at meiosis, whereas in somatic cells it provides an important mechanism for the repair of DSBs. Using partially purified cell-free extracts and purified recombinant proteins, we show that the human repair synthesis DNA polymerase η (polη), but not polδ, preferentially binds and extends DNA synthesis from D-loop recombination intermediates. Extracts from human XP-V cells, containing a truncated polη polymerase, have severely reduced D-loop extension activity. Furthermore, we find that polη forms discrete foci on meiotic chromosomes undergoing homologous pairing and recombination. Our results suggest a novel role for polη in the promotion of DNA synthesis during HR.
The mechanism by which recombination intermediates are resolved has been a puzzle for many years. However, we recently found that RAD51C and XRCC3, form a complex that binds specifically to Holliday junctions (HJ), is associated with HJ resolvase activity, and localizes to discrete foci on meiotic chromosomes at the stage when HJs are processed and resolved. RAD51C and XRCC3 foci were not observed at early stages of meiotic recombination when RAD51 foci were present, but instead were visualised at late times when they partially co-localized with MLH1 foci that are thought to identify sites of meiotic crossovers. We conclude that RAD51C/XRCC3 complex is associated with crossover sites in vivo, providing new insight into the composition of the human HJ resolvasome.

Poster No. II-1
MULTIPLE ATRIP FUNCTIONAL DOMAINS REGULATE ATR LOCALIZATION AND ACTIVITY
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The ATR kinase is activated by many forms of genotoxic stress. In contrast, ATM responds primarily to double strand breaks. We hypothesize that the ATR binding protein ATRIP provides the expanded specificity to ATR allowing ATR to be recruited to and activated by various DNA lesions. To begin to test this hypothesis we have defined ATRIP functional domains. We found that ATRIP contains three distinct domains that mediate RPA-ssDNA binding, oligomerization, and ATR binding. A C-terminal ATRIP domain contacts residues in the N-terminus of ATR. This association is essential for ATR-dependent checkpoint signaling. ATRIP has been proposed to localize ATR to sites of DNA damage through its interaction with RPA-ssDNA. Indeed, ATRIP is required for ATR accumulation in DNA-damage induced nuclear foci. Deletion of the RPA-ssDNA interaction domain of ATRIP greatly diminished accumulation of ATRIP into foci. However, the ATRIP-RPA-ssDNA interaction is not absolutely essential for ATR activation since ATR phosphorylates Chk1 in cells expressing only the mutant ATRIP that does not bind to RPA-ssDNA. We also found that ATR-ATRIP exists in multimeric complexes containing at least two ATRIP and two ATR molecules in cells. Unlike ATM, our studies do not indicate any damage-dependent monomerization of the ATR-ATRIP complex. However, ATRIP oligomerization is important for its function. Deletion of the ATRIP oligomerization domain weakened its interaction with ATR although it did not delete any specific ATR-interacting residues. The oligomerization defective ATRIP protein is unable to accumulate in bright damage-induced foci despite its ability to bind RPA-ssDNA. Substitution of the ATRIP oligomerization domain with a heterologous dimerization domain restored the dimerization, strong ATR interaction and localization to damage-induced foci. However, this ATRIP chimera was unable to support ATR-dependent Chk1 phosphorylation. Our results indicate that accumulation of the ATR-ATRIP complex into bright DNA damage-induced foci is separable from ATR-dependent Chk1

phosphorylation. We are continuing to analyze how ATRIP functions to promote ATR signaling in response to various genotoxic stresses.

Poster No. II-2

SIGNALLING IONISING RADIATION-INDUCED DNA DAMAGE BY THE CELL CYCLE CHECKPOINT KINASES ATM AND ATR

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Ataxia-telangiectasia mutated (ATM) and the ATM and Rad3 related (ATR) kinases are central for the activation of cell cycle checkpoints in the response to DNA damage. ATM is rapidly activated in response to DNA double strand breaks (DSBs), but not other forms of stress such as base damage induced by UV, and mediates phosphorylation of a number of different proteins that regulate various aspects of the DNA damage response. These observations have led to a model whereby ATM is the primary kinase for signalling DNA DSBs. Conversely, disruption of ATR kinase activity results in sensitivity to a wide variety of DNA damaging agents, such as hydroxyurea and UV. Thus, in contrast to ATM, ATR is believed to be responsible for signalling replication stress and base damage.

Despite the role of ATM in signalling ionising radiation (IR) induced DNA damage, ATR is also believed to function in signalling this type of genotoxic stress. For example, disruption of ATR function renders cells sensitive to IR, and p53 phosphorylation at late time points following administration of this type of DNA damage has been shown to be dependent on ATR. Taken together, these data suggest that ATM and ATR perform overlapping roles in response to IR. However the relationship between recruitment of these two kinases to IR-induced sites of DNA damage has not been fully explored. Here we describe experiments that aim to establish the molecular mechanisms by which ATM and ATR are recruited to sites of genotoxic stress and contribute towards the signaling of IR-induced DNA damage.

Poster No. II-3

DNA-DAMAGE INDUCES RE-LOCALIZATION OF HSMG-1

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hSMG-1 (human suppressor with morphogenic effect on genitalia) is the most recent addition to the family of phosphatidyl-inositol-3 (PI3) kinases. This family includes proteins such as Ataxia telangiectasia mutated (ATM), DNA Dependent Protein Kinase (DNA-PK), ATM and Rad3 related kinase (ATR) which are involved in stress induced signal transduction, cell cycle checkpoint control and DNA damage repair.

hSMG-1 was first described in *Caenorhabditis elegans* where it was shown to be essential for Nonsense Mediated mRNA Decay (NMD). More recently hSMG-1 has been implicated in NMD and in the DNA damage response in cells of human origin.

Here we show that hSMG-1 kinase activity overlaps with that of ATM and can be activated by DNA damage in a time, dose and metal dependant manner. In response to DNA damage we have observed that hSMG-1 localizes to discrete sites within the cytoplasm. Consistent with its putative role in response to DNA damage we have also observed an increase of hSMG-1 protein in the fraction bound to chromatin. Here we propose new cytoplasmic and nuclear functions of hSMG-1 in the cellular response to DNA damage not previously described.

Poster No. II-4

CHARACTERIZATION OF THE ATM-MEDIATED DNA DAMAGE RESPONSE IN MOUSE CEREBELLAR CELLS

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The DNA damage response is a network of signalling pathways that affects many aspects of cellular metabolism following the induction of DNA damage. Double strand breaks (DSBs), a highly cytotoxic DNA lesion, activates the nuclear protein kinase ATM which phosphorylates numerous effectors that play key roles in the damage response pathways. Loss or inactivation of ATM leads to ataxia telangiectasia (A-T), an autosomal recessive disorder characterized by cerebellar degeneration, particularly the loss of Purkinje cells, immunodeficiency, genomic instability, radiosensitivity and cancer predisposition. The reason for the cerebellar degeneration in A-T is not clear and has been ascribed to cytoplasmic functions of ATM, which may not be relevant to the DNA damage response. We set to identify and characterize the ATM-mediated damage response in mouse cerebellar cells. We find that all readouts of this response are similar to those detected in commonly used cell lines, ATM autophosphorylation (indicative of its activation), as well as phosphorylation of several of its downstream substrates. Importantly, all of these responses are detected in the nuclei of granule and Purkinje cells, suggesting that ATM in these cells is nuclear and functions similarly to its mode of function in other cell types.

Poster No. II-5

PRO-APOPTOTIC BID IS AN ATM EFFECTOR IN THE DNA DAMAGE RESPONSE

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The "BH3-only" pro-apoptotic BCL-2 family members are sentinels of intracellular damage. Here, we demonstrated that the BH3-only BID protein partially localizes to the nucleus in healthy cells, is important for apoptosis induced by DNA damage, and is phosphorylated in the nucleus following induction of double-strand breaks in DNA. We also found that BID phosphorylation is mediated by the ATM kinase, and occurs in mouse BID on two ATM consensus sites. Interestingly, *BID*$^{-/-}$ cells failed to accumulate in the S phase of the cell cycle following treatment with the topoisomerase II poison etoposide; reintroducing wild-type BID restored accumulation. In contrast, introducing a non-phosphorylatable BID mutant did not restore accumulation in the S phase, and resulted in an increase in cellular sensitivity to etoposide-induced apoptosis. These results implicate BID as an ATM effector, and raise the possibility that pro-apoptotic BID may also play a pro-survival role important for S phase arrest.

Poster No. II-6

ACTIVATION OF ATM BY DNA STRAND BREAK-INDUCING AGENTS CORRELATES CLOSELY WITH THE NUMBER OF DNA DOUBLE STRAND BREAKS

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The protein kinase ataxia telangiectasia mutated (ATM) is activated when cells are exposed to ionizing radiation (IR). It has been assumed that ATM is specifically activated by the few induced DNA double strand breaks (DSBs), although little direct evidence for this assumption has been presented. DSBs constitute only a few percent of the IR-induced DNA damage, whereas the more frequent single strand DNA breaks (SSBs) and base damage account for over 98% of the overall DNA damage. It is therefore unclear whether DSBs are the only IR-induced DNA lesions that activate ATM. To test directly whether or not DSBs are responsible for ATM activation, we exposed cells to drugs and radiation that produce different numbers of DSBs and SSBs. We determined the resulting ATM activation by measuring the amount of phosphorylated Chk2 and the numbers of SSBs and DSBs in the same cells after short incubation periods. We found a strong correlation between the number of DSBs and ATM activation but no correlation with the number of SSBs. In fact, hydrogen peroxide, which, similar to IR, induces DNA damage through hydroxyl radicals but fails to induce DSBs, did not activate ATM. In contrast, we found that calicheamicin-induced strand breaks activated ATM more efficiently than IR and that ATM activation correlated with the relative DSB induction by these agents. Our data indicate that ATM is specifically activated by IR-induced DSBs, with little or no contribution from SSBs and other types of DNA damage. These findings have implications for how ATM might recognize DSBs in cells.

Poster No. II-7

REPAIR OF DNA DOUBLE-STRAND BREAKS IN DNAPK DEFICIENT CELLS

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The cells in an organism are constantly exposed to DNA damaging agents such as ionising radiation or free radicals from the metabolism. The repair of these damages, especially DNA double-strand breaks (DSBs), is critical for the stability of the genome and cell survival. We have studied the repair of DSBs after exposure to ionising radiation and cytotoxic drugs in different cell lines by using two different protocols (a new cold protocol including high salt extraction and a standard 50°C protocol) to extract chromosomal DNA fragments for PFGE-analysis. After ≥1h repair the cold protocol was up to two times more effective in extracting DNA from DNA dependent protein kinase (DNAPK) deficient cell lines. This phenomenon was only observed in the cell lines M059J, V3, Xrs5 and Xrs6, which all lack functional DNAPK. Normal repair proficient cell lines and cells deficient in other repair proteins such as NBS1, DNA Ligase IV, BLM and XRCC3 displayed no difference in the amount of DNA extracted using the warm and cold lysis. Thus this assay allows for differential DNA extraction during DSB repair that is specific for the DNAPK level in a cell. Preliminary data indicates a cell cycle dependence and suggest that the differential extraction is an indirect result of a S-phase specific process that acts when DNAPK is absent.

Poster No. II-8

PROTECTIVE ROLE OF HSMG-1 AGAINST CELL KILLING BY TNF RECEPTOR FAMILY MEMBERS

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The tumor-necrosis factor (TNF) and the related TNF-Related Apoptosis-Inducing Ligand (TRAIL) are potent inducers of apoptosis in certain cell types, including cancer cells. This observation has raised clinical interest in the possibility that systemic or local treatment of cancer patients with TNF-α or TRAIL might induce regression of primary or metastatic tumors. However, resistance to TNF-α or TRAIL-induced cell killing limits the effectiveness of therapy with these cytokines. Our laboratory has recently cloned and characterized hSMG-1 (also termed ATX) as the newest member of the phosphoinositide 3-kinase related kinase (PIKK) family. Studies performed to date indicate

that hSMG-1 is centrally involved in the nonsense-mediated mRNA decay pathway, and shares some functional overlap with ATM in genotoxic stress responses. Here, we report that hSMG-1 kinase activity protects malignant cells from TNF-α- or TRAIL-induced apoptotic death. Small-interfering RNA (siRNA)-mediated depletion of hSMG-1, but not ATM, in several human cancer cell lines markedly increases the magnitude and accelerates the rate of apoptosis induced by TNF-α or TRAIL stimulation. In contrast, non-transformed human breast epithelial MCF10A cells were not sensitized to TNF-α/TRAIL-induced killing by depletion of hSMG-1. The increase in TNF-α-mediated cell killing observed in the hSMG-1 siRNA-treated cells was not explained by indirect effects of hSMG-1 deficiency on overall protein translation or NF-κB-dependent gene transcription. Studies designed to dissect the effects of hSMG-1 on the extrinsic and intrinsic pathways of apoptotic cell death are in progress, and the results will be presented. Our findings suggest that inhibitors of hSMG-1 kinase activity may be useful as adjunct therapy to enhance the antitumor activity of exogenously administered TRAIL in human cancer patients.

Poster No. II-10

ATM IS A T ANTIGEN KINASE LINKING DNA DAMAGE AND SV40 VIRAL REPLICATION IN PRIMATE CELLS

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We recently showed that the ataxia-telangiectasia-mutated (ATM) protein kinase phosphorylates the cyclic AMP response element-binding protein, CREB, following exposure to DNA damage stimuli. Here, we show that phospho-specific antibodies recognizing the major ATM phosphorylation site in CREB cross react with a protein of approximately 80 kDa present in extracts of irradiated, SV40-immortalized, mouse embryo fibroblasts (MEFs). Large-scale immunoprecipitation and mass spectrometry identified this species as SV40 large tumor antigen (LTag), a multifunctional oncoprotein that promotes cellular transformation and is required for SV40 minichromosome replication. The relevant IR-induced phosphorylation sites in LTag recognized by phospho-CREB antibodies were mapped. IR strongly induced the phosphorylation of these residues in an ATM-dependent manner in MEFs and HEK 293T cells, and ATM phosphorylated LTag on multiple sites *in vitro*. Infection of African green monkey CV-1 cells with SV40 resulted in the activation of ATM and phosphorylation of LTag, indicating that an ATM-LTag pathway is induced during lytic viral infection. SV40 expressing a mutant form of LTag containing a single ATM phosphorylation site mutation exhibited a severe replication defect in CV-1 cells. Our findings uncover a link between ATM and SV40 that may have implications for understanding the replication cycle of oncogenic papova viruses. We propose that ATM regulates a viral replication checkpoint that regulates the timing of SV40 replication during S phase

Poster No. II-11

DEFFICENCY IN KU-86 SENSITIZE CELLS TO CISPLATIN AND OXALIPLATIN-INDUCED APOPTOSIS BY ALTERED JNK AND P38 SIGNALLING

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Cisplatin and oxaliplatin have different toxicity profiles and are not cross-resistant. DNA-PK signaling, known for its role in DNA repair, has also been implicated in apoptotic cell signaling. Here we have examined if deficency in DNA-PK_{cs} or Ku-86 influence platinum drug cytotoxicity and if the platinum carrier ligand is of importance. Moreover, we have assessed how apoptotic signaling pathways triggered by the platinum carrier ligand in question is influenced by deficiency in either DNA-PK_{cs} or Ku-86. The cell lines K1 and Xrs-6, Ku86 proficient and deficient respectively and AA8 and V3, DNA-PKcs proficient and deficient respectively were employed. Deficiency in either Ku86 or DNA-PKcs results in increased cytotoxicity for both cisplatin and oxaliplatin. In our cell systems, cisplatin-exposure activated caspase-3 regardless of the status of DNA-PK, whereas oxaliplatin did not. Both cisplatin and oxaliplatin-induced apoptotic signaling involved stress-activated protein kinase (SAPK) signaling i.e, JNK and p38, in K1 and Xrs-6 cells. We observed a higher basal level of both JNK and p38 in Ku-86 deficient cells compared to wildtype suggesting that Ku-86 deficiency might influence apoptotic propensity through altered basal SAPK activities.

Poster No. IIIA-1

AN ACTIVE ROLE FOR NIBRIN IN THE KINETICS OF ATM ACTIVATION FOLLOWING IRRADIATION

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The Atm protein kinase is central to the double-strand break (DSB)response in mammalian cells, transducing the damage response signal to downstream effectors involved in apoptosis, cell cycle checkpoint control, and DNA repair. Atm exists as inactive dimers in the absence of DNA DSBs. Following irradiation, Atm undergoes autophosphorylation at Ser 1981 and dissociates into active monomers. While activation of Atm in the presence of DNA DSBs does not require the Mre11/Rad50/nibrin (MRN) complex, full activation is attained more rapidly in the

presence of MRN. Previously we showed that nuclear expression of Mre11/Rad50 complexed with just a C-terminal fragment of nibrin was sufficient to stimulate Atm activation at early times following irradiation. Similar effects are observed in ataxia-telangiectasia-like disorder (ATLD) cells, that have mutations in Mre11 that prevent its binding to nibrin, and in cells where Mre11 has been degraded by viral action. These studies suggest that Mre11, either alone or in conjunction with Rad50, stimulates Atm activation. To test this hypothesis, we added a synthetic nuclear localization signal sequence to Mre11 and expressed it in Nijmegen breakage syndrome (NBS) fibroblasts, where the absence of nibrin results in cytoplasmic localization of endogenous Mre11/Rad50. We found that the Mre11-NLS protein localized to the nucleus of NBS fibroblasts and complexed with Rad50, similar to wildtype Mre11. However, Atm autophosphorylation was not stimulated early after irradiation in cells expressing Mre11-NLS, nor were downstream Atm targets phosphorylated. NBS cells expressing the Mre11-NLS protein also remained radiosensitive. In contrast, the Mre11-NLS transgene complemented Atm function when expressed in ATLD fibroblasts lacking full-length Mre11, indicating that the transgene was functional. Chromatin fractionation showed the Mre11-NLS protein localized to chromatin similar to wildtype MRN complex. These results indicate that nuclear expression of Mre11/Rad50 alone is insufficient to restore normal activation kinetics to Atm early after delivery of DNA DSBs. Thus, nibrin plays a role in stimulating Atm activation beyond simply translocating the Mre11/Rad50 proteins to the nucleus.

Poster No. IIIA-3

POSSIBLE FUNCTIONS OF RAD17 IN DNA DAMAGE RESPONSE AND REPLICATION

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Clamp loader Rad17 is involved in S-phase and G2/M arrest in response to DNA damage and replication problems. It mediates both ATR- and ATM- dependent checkpoint responses. To investigate the role of mammalian Rad17 we disrupted the mouse Rad17 gene, which resulted in early embryonic lethality. In contrast, mouse Rad17 mutant ES cells were viable and produced truncated Rad17 protein. These mutant cells showed hypersensitivity to a variety of DNA damaging agents and. were defective in homologous gene replacement. Compared to wild type, more DNA double strand breaks accumulated in mutant Rad17 ES cells after UV treatment. We also found that mutant Rad17 ES cells had no obvious checkpoint defects and were able to inhibit DNA replication upon DNA damage. However they experienced problems with DNA synthesis restarting. Currently we are investigating the mechanism by which Rad17 could promote DNA damage repair and restarting of stalled replication forks. Recent results will be discussed.

Poster No. IIIA-4

TELOMERE LENGTH HOMEOSTASIS INVOLVES DNA-DAMAGE-SENSORS CHECKPOINT FACTORS

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All organisms respond to interruptions in the DNA double-helix by promptly launching the DNA-damage response (DDR). Although telomeres are DNA ends, these stuctures actually prevent activating the DDR. Surprisingly, recent findings have revealed that various proteins involved in the DDR physically associate with telomeres and play important roles in regulating normal telomeric function. Nevertheless, in mammals, generation of conclusive evidence of direct contribution of DDR factors in telomere homeostasis and an understanding of their mechanism of action is still an unattained goal.

The 9-1-1 sliding-clamp complex composed of hRad9, hRad1 and hHus1 proteins is one of the main sensors of damaged DNA. By chromatin immunoprecipitation experiments, we observed that the 9-1-1 complex stably associates *in vivo* with functional telomeres in human and mouse cells in a variety of cell types. Our analysis of Hus1-/- mouse embryo fibroblasts (MEFs) revealed that loss of Hus1 cause a significant telomere shortening. In addition, conditional knockout of Hus1 in mouse thymocytes confirmed the results of MEFs analysis demonstrating that inactivation of 9-1-1 complex leads to severe disruption of telomere length homeostasis. Importantly, Hus1 inactivation does not lead to telomere deprotection, while a functional 9-1-1 complex seem to co-operate with telomerase *in vitro*. Our data are consistent with the hypothesis that the 9-1-1 complex is involved in telomerase-dependent telomere length regulation.

Poster No. IIIA-5

TRANSLESION SYNTHESIS ENZYMES ARE INVOLVED IN UV-INDUCED FRAMESHIFT MUTAGENESIS IN CELL CYCLE-ARRESTED YEAST CELLS

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Mutations arising during a quiescent state may provide a selective advantage for unicellular organisms. For multicellular organisms yet stationary-phase mutations in somatic cells are detrimental in that they contribute to carcinogenesis and ageing. We study the emergence of frameshift mutants during prolonged cell cycle arrest in the eukaryotic model organism *Saccharomyces cerevisiae.* We observed that the increased frequency of stationary-phase

frameshift mutations brought about by a defective nucleotide excision repair (NER) pathway or by UV irradiation was dependent on Rev3p, the catalytic subunit of the translesion synthesis polymerase Pol zeta. Employing the same two conditions, we further examined the effect of deletions of the genes coding for Pol eta (*RAD30*) and Rev1p (*REV1*). In a NER-deficient strain background the increased mutation incidence was only moderately influenced by a lack of Pol eta but completely reduced to wild type level by a knockout of the *REV1* gene. UV-induced stationary-phase mutations were abundant in wild type and *rad30Δ* strains, but substantially reduced in a *rev1Δ* as well as in a *rev3Δ* strain. The similarity of the *rev1Δ* and the *rev3Δ* phenotype and an epistatic relationship evident from experiments with a double-deficient strain suggests a participation of Rev1p and Rev3p in the same mutagenic pathway. Therefore, we propose that the response of cell cycle-arrested cells to an excess of exo- or endogenously induced DNA damage includes a novel replication-independent cooperative function of Rev1p and Pol zeta, which is highly error-prone.

Poster No. IIIA-6

THE ROLE OF BRCA1 AND BARD1 AS MEDIATORS OF THE DNA DAMAGE RESPONSE

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BACH1 is a novel member of the DEAH helicase family that has been shown to interact with the BRCT domain of BRCA1. Previous studies suggest that BACH1 has an important role in BRCA1-dependent repair of DNA double-strand breaks and enforcement of a DNA damage checkpoint. To study the role of BACH1 in DNA repair we cloned its chicken orthologue and established a homozygous knockout of *BACH1* in the avian B-cell line DT40. In contrast to ΔBRCA1 cells, DT40 lacking BACH1 are neither defective in homologous recombination, nor are they deficient in the execution of a S-M DNA damage checkpoint. We further show that *bach1* cells are profoundly sensitive to the DNA crosslinking agent, cisplatin and that this defect can be fully corrected by expression of human BACH1. Our data suggest that BACH1 functions independently of BRCA1 in the cellular response to DNA damage and more likely functions in a pathway for the repair of DNA crosslinks.

In a complementary study we examine the role of BRCA1 and BARD1 as mediators of the DNA damage response. BRCA1 and BARD1 mutant cell lines derived from DT40 exhibit defects in both homologous DNA repair and cell cycle checkpoints that can be corrected by expression of their human orthologues. This system has enabled us to perform a systematic genetic analysis of the human BRCA1 and BARD1 genes with respect to their role in maintaining genomic stability. Here I present our data concerning the role of ubiquitylation, BRCT-dependent phospho-peptide binding and ATM/ATR-mediated phosphorylation on the function of BRCA1 and BARD1.

Together these two studies challenge the current view on the molecular function of BRCA1 and BARD1.

Poster No. IIIA-7

CELL CYCLE-DEPENDENT PHOSPHORYLATION AND FOCUS-FORMATION OF HISTONE H2AX IN X-IRRADIATED B-LYMPHOCYTE CELL LINES

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We have studied phosphorylation and focus-formation of H2AX in the human malignant B-lymphocyte cell lines Reh and U698 after ionizing radiation (IR), as well as the disappearance with time post-IR. The amount of γ-H2AX (by flow cytometry) and the number of foci (by confocal laser scanning microscopy after sorting) were higher in unirradiated cells in S and G_2, compared to those in G_1. At short times after IR (10-30 minutes), there was a dose-dependent, but not linear, increase in the amount of γ-H2AX, as well as in the number of foci, in all cell cycle phases. However, the increase at short times post-IR was most pronounced in the G_1 cells, which lead to a higher amount of γ-H2AX in G_1 compared to S at the higher doses (≥4Gy). Since G_1 cells contain less DNA than cells in S and G_2, this means that the amount of IR-induced phosphorylation of H2AX in G_1 is several-fold higher per double-strand break compared to the other phases. The IR-induced γ-H2AX foci were 50% larger (diameter) than the foci in unirradiated S and G_2 cells. The decline in γ-H2AX content with time after IR was similar in the 2 cell lines, even though only Reh cells have an intact TP53 response, become apoptotic, and arrest in G_1 after higher doses of IR. After lower doses of IR (≤1 Gy), the γ-H2AX levels returned to the levels observed in unirradiated cells within 24 hours, in good agreement with the resumption of cell proliferation under those conditions. The amounts of γ-H2AX after higher doses of IR (≥4Gy) also declined with time, but reached plateau levels well above those observed in unirradiated cells, under conditions where the Reh cells eventually became apoptotic and U698 cells were arrested in G2, respectively. The apoptotic (Reh) cells were negative for γ-H2AX, which was shown by simultaneous staining for γ-H2AX and strand breaks by the TdT procedure. Caffeine did not inhibit the phosphorylation of H2AX after irradiation, showing that caffeine-sensitive kinases are not involved in H2AX phosphorylation.

Our results suggest that there is no direct coupling between repair and TP53 status, cell cycle arrest and induction of apoptosis.

Poster No. IIIA-8

INVESTIGATION OF THE FUNCTIONAL ACTIVITIES OF APRATAXIN, A NOVEL PROTEIN INVOLVED IN SSB REPAIR

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Mutations in the gene encoding the novel protein aprataxin are causal for the neurodegenerative disorder, ataxia with ocular motor apraxia type 1 (AOA1). We have previously found that AOA1 patient cells are sensitive to the DNA damaging agents MMS and H_2O_2, but not gamma irradiation, providing evidence that aprataxin is playing a role in the cellular response to single strand breaks.

Aprataxin has three putative functional domains, FHA, HINT and zinc finger. We have previously demonstrated the function of the FHA domain in binding other DNA repair proteins such as XRCC1, PARP1 and P53. Here we have investigated the other two putative functions of aprataxin to further elucidate its role in the cellular response to DNA damage. We have used recombinant human aprataxin in *in vitro* biochemical assays to investigate its catalytic activity in nucleotide hydrolysis (HINT domain) and DNA/RNA binding activities. Using gel shift assays we have found that aprataxin binds to double stranded DNA and RNA, and this was confirmed by antibody supershifts.

In vitro nucleotide substrates of HINT proteins were used for the catalytic assays. The level of hydrolysis on these substrates by aprataxin was found to be low, but at similar levels to other proteins with a HINT domain, but the substrate specificity profile was unique. These levels of activity suggest that there maybe a more biologically relevant substrate and that aprataxin has diverged since sharing a common ancestor with other HINT domain proteins.

We have found evidence that aprataxin may directly bind DNA and recruit repair proteins, such as XRCC1 via its FHA domain. The role of the HINT domain is still unclear.

Poster No. IIIA-9

MODULATING EFFECT OF γ-H2AX ON ATM-DEPENDENT DNA DAMAGE RESPONSE

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Once double-strand breaks (DSBs) are generated within genome DNA, DSBs are immdiately recognized by sensor mechanisms and then activate ATM kinase for phosphorylation of the substrates, which leads to the cellular responses such as cell cycle checkpoints, apoptosis and DNA repair. Recently, it was reported that the C-terminus of NBS1 has an indispensable role for recruitment of ATM to DSB sites and possibly for the resulting phosphorylation of substrates through the interaction with ATM. Previously, we demonstrated that γ-H2AX is involved in the recruitment of NBS1/hMre11/hRAD50 complex to DSB sites through interaction with NBS1 at the N-terminus. Hence, γ-H2AX might function, as a sensor or initial factor, in ATM-dependent damage response. We showed here that γ-H2AX forms the complex with ATM and NBS1 in irradiated cells. When the expression of H2AX was repressed by H2AX siRNA, chromatin-bound NBS1 in irradiated cells was significantly decreased. As a result, H2AX siRNA reduced the phosphorylation of ATM substrates, which were present in γ-H2AX complex in irradiated cells. However, these phosphorylations were restored a few hours after irradiation, indicating an altenative activation pathway for ATM-dependent damage response. Taken together, our results suggest that γ-H2AX could mediate a rapid activation pathway of ATM through interaction with ATM and the substrates.

Poster No. IIIA-10

VARIATIONS IN THE DNA DAMAGE RESPONSE REGULATED BY MDC1 THROUGHOUT THE DIFFERENT STAGES OF THE CELL CYCLE

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MDC1 is an early player in the DNA damage response that quickly localizes to the sites of damage. We have identified the mouse homologue of MDC1 and have characterized the response to DNA damage of the human and mouse proteins through the different stages of the cell-cycle. In response to ionizing radiation, MDC1 and γH2AX form nuclear foci in all stages of the cell-cycle studied including on condensed mitotic chromosomes. During S-phase in non-damaged cells, MDC1 does not appear to associate with the MRN complex at sites of DNA replication, however partial colocalization between MDC1 and late S-phase PCNA foci suggests that MDC1 is able to recognize background levels of DNA damage in S-phase. The colocalization of MDC1 and γH2AX suggests that MDC1 is recruited specifically to DNA double-stranded breaks, and interaction between MDC1 and the MRN complex also appears specific to the sites of double-stranded breaks. Finally, we have found that while MDC1 recognizes sites of replication damage in early S-phase, damage in late S-phase is recognized by 53BP1. We will discuss these and other data in relation to the roles of MDC1 and γH2AX in the cell-cycle specific control of DNA damage responses.

Poster No. IIIA-11

DNA DAMAGE-INDUCED PHOSPHORYLATION OF MAMMALIAN CHECKPOINT MEDIATORS AND ITS ROLE IN THE GENOME SURVEILLANCE PROGRAM

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The checkpoint mediators MDC1/NFBD1 and 53BP1 have recently been shown to play a key role in the early response to DNA double strand breaks (DSBs). After irradiation, both proteins become rapidly phosphorylated in an ATM/ATR-dependent manner. Moreover, both MDC1/NFBD1 and 53BP1 appear to facilitate phosphorylation of other DSB regulators.

The aim of this study is to identify irradiation-induced phosphorylation sites in MDC1/NFBD1 and 53BP1 and the functional consequences of these phosphorylations. We have generated *in vivo* and *in vitro* phospho-peptide maps in U2-OS cells and this revealed a large number of phosphorylation sites. Phospho-amino acid analysis showed an even distribution between serine and threonine residues. Several kinase inhibitors were tested and caffeine and wortmannin (inhibitors of PI-3 kinase-related kinases ATM and ATR) were the most potent suppressors of MDC1/NFBD1 and/or 53BP1 phosphorylation. The CDK inhibitor roscovitin also partly reduced the phosphate incorporation indicating that cyclin-dependent kinases may participate in regulating mammalian checkpoint mediators *in vivo*. By means of Edman cycle sequencing approach, we mapped several Ser/Thr-Gln and Ser/Thr-Pro residues on MDC1/NFBD1 and we are currently generating stable cell lines where the endogenous checkpoint mediators are silenced by RNA interference (RNAi) and replaced by their phosphorylation-deficient mutants. The latest status of these functional assays, including their implication for understanding the involvement of mammalian checkpoint mediators (and their phosphorylation) in the genome surveillance program will be presented.

Poster No. IIIA-12

HUMAN NBS1 REGULATES HOMOLOGY DIRECTED DNA REPAIR THROUGH ITS N- TERMINAL CONSERVED DOMAIN

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DNA double strand breaks (DSBs), which can result from exposure to ionizing radiation (IR) or from normal DNA processing, are potentially the most serious type of damage which can be inflicted on a cellular genome. In mammalian cells, DSBs are immediately repaired via at least two pathways: non-homologous end joining and homologous recombination (HR). Defects in the HR repair process can result in genomic instability and in a predisposition for cancer. NBS1, the protein responsible for Nijmegen breakage syndrome (NBS), is reported to be an essential factor in DSB repair through the HR pathway in higher vertebrate cells (Tauchi et al. 2002). To gain insight into the mechanisms through which NBS1 functions in HR, we generated a series of NBS1 mutant cDNAs and measured their ability to regulate HR repair by using the SCneo or DR-GFP systems, which were developed by the M. Jasin lab. The HR-regulating activity of NBS1 is completely abolished by deletion of the MRE11-binding domain, and severely decreased by mutations in the FHA or BRCT domains. In contrast, mutations in the serine residues which are phosphorylated by the ATM kinase had only slight affects on HR frequency. These findings were further supported by work showing that ATM defects did not reduce the HR repair frequency of specific DSBs. These results suggest that NBS1 regulates the HR pathway through the functions of the N-terminal FHA/BRCT domain, probably through recruitment and retention of MRE11/RAD50 to DSB sites, and that this function of NBS1 is independent of ATM.

Poster No. IIIA-13

ATM-DEPENDENT DOUBLE-STRAND BREAK REPAIR AND CHECKPOINT FUNCTIONS CO-OPERATE TO MAINTAIN CHROMOSOMAL STABILITY AFTER IR

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ATM is a key protein in the signalling cascades that initiate checkpoints after ionizing radiation (IR). A-T cells are impaired in their ability to induce these checkpoints and are characterized by pronounced radiosensitivity. Recently, it has been demonstrated that ATM has an additional, direct role in the repair of a subset of DSBs which contributes to the radiosensitivity of A-T cells. This ATM-dependent repair pathway includes the nuclease Artemis, and Artemis-deficient cells show the same radiosensitivity as A-T cells. Using flow cytometry analysis, we investigated the passage of BrdU-labelled cells through the cell cycle. Compared with wild-type cells, both ATM- and Artemis-deficient cells showed a longer accumulation in the G2 phase, and the duration of the arrest correlated with dose. Investigation of the G2/M checkpoint by histone H3 analysis demonstrated that Artemis-deficient cells are capable to induce this checkpoint, whereas ATM-deficient G2 cells continued to enter mitosis. γ-H2AX foci analysis in G2 cells revealed that the repair defect of A-T and Artemis cells, initially described in non-cycling G0 cells, is also observed in G2 phase. Interestingly, chromosomal studies in metaphases from ATM- and Artemis-deficient cells showed a difference

in the amount of chromosome breaks. The break number of Artemis cells that had traversed the checkpoint was lower than in A-T cells but higher than in wild-type cells. These studies provide direct evidence that ATM-dependent DSB repair and checkpoint functions cooperate to prevent cells from entering the next cell cycle phase with an excess of DSBs.

Poster No. IIIA-14

A NOVEL FUNCTION FOR BUDDING YEAST RAD9 IN REPAIR OF IR-INDUCED DOUBLE STRAND BREAKS

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After DNA damage, numerous checkpoint and repair proteins are recruited into organised, dynamic focal assemblies that represent sites of DNA repair and damage checkpoint signalling. *Saccharomyces cerevisiae* Rad9 has been proposed to play various roles in the DNA damage response, including lesion sensing, checkpoint signalling, cell-cycle arrest and DNA repair. To investigate the role of Rad9 in these processes, we analysed the recruitment of the checkpoint proteins Rad9, Ddc1 and Ddc2 to foci and bulk chromatin after ionising radiation (IR), which causes DNA double-strand breaks (DSBs). Histone H2A serine 129 phosphorylation occurs rapidly after DNA damage, resulting in a phospho-form termed gamma-H2A (gamma-H2AX in vertebrate cells). gamma-H2A/gamma-H2AX is widely recognised as a marker of DSBs, although its roles in the cellular response to DSBs are only beginning to be understood. We show that accumulation of Rad9 in foci and bulk chromatin after IR is dependent on gamma-H2A, on the PI3K-like kinase (PIKK) Mec1 and on activation of Rad9 by PIKK-dependent hyperphosphorylation. We also present evidence that Rad9 has a direct role in facilitating efficient repair of DSBs, distinct from its role in mediating cell cycle arrest in the presence of DNA damage. Taken together, our data demonstrate the existence of a novel non-checkpoint signalling function of Rad9 mediated through its gamma-H2A dependent accumulation at sites of DNA damage and repair.

Poster No. IIIA-15

IMPAIRED ELIMINATION OF DNA DOUBLE-STRAND BREAK-CONTAINING LYMPHOCYTES IN ATAXIA TELANGIECTASIA AND NIJMEGEN BREAKAGE SYNDROME

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The repair of DNA double-strand breaks is critical for genome integrity and tumor suppression. Here we show that following treatment with the DNA-intercalating agent Actinomycin D, quiescent mature T lymphocytes accumulate unrepairable DNA double-strand breaks and die. On the contrary, we found that the mature T cells from Ataxia Telangiectasia and Nijmegen Breakage Syndrome patients are resistant to this death pathway, though the treatment produces in these cells an amount of DNA double-strand breaks comparable to normal cells. We demonstrate that the death pathway induced by Actinomycin D in quiescent mature lymphocytes: i) follows DNA damage and H2AX phosphorylation; ii) is ATM-mediated and can be suppressed with the ATM inhibitor caffeine; iii) it is dependent on a normally functioning NBS1 protein; iv) it is due to cellular apoptosis. We are presently trying to better define at a molecular level the ATM/NBS1-dependent pathway induced by Actinomycin D in T lymphocytes. In response to a different genotoxic insult, 2-Gy irradiation, 100% of quiescent mature T cells from normal donors are shown to survive, following complete resolution of the induced damage. The mature T cells from Ataxia Telangiectasia and Nijmegen Breakage Syndrome patients are also demonstrated to survive, despite 15-20% of cells maintain damaged DNA and γH2AX foci. Our findings suggest that the leukemogenic risk of Ataxia Telangiectasia and Nijmegen Breakage Syndrome patients is not only due to defective DNA repair, but also to inefficient elimination of cells containing DNA double-strand breaks. This risk might be particularly relevant in association with the increased rate of secondary V(D)J rearrangements we previously demonstrated in T cells from these patients.

BENZON SYMPOSIUM No. 52

CELLULAR RESPONSES TO DNA DAMAGE

AUGUST 22-25, 2005, COPENHAGEN, DENMARK

Organizing committee:
Jiri Bartek (Copenhagen), Jiri Lukas (Copenhagen), Jan Hoeijmakers (Rotterdam) and Arne Svejgaard (Copenhagen)

Abstracts - WEDNESDAY, August 24, 2005

53BP1: EMERGING TUMOUR SUPPRESSOR INVOLVED IN EARLY STAGES OF DNA DAMAGE SIGNALLING

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53BP1 is a DNA damage checkpoint protein that localizes rapidly to sites of DNA DSBs and contributes to activation of ATM, raising the possibility that 53BP1 is a DNA DSB sensor. To explore this, we studied the mechanism by which 53BP1 is recruited to sites of DNA DSBs. Deletion mutants identified a 120 amino acid domain within 53BP1 that is sufficient and necessary for localization of 53BP1 to sites of DNA DSBs. This domain is conserved in the 53BP1 orthologs in budding yeast (Rad9) and fission yeast (Rhp9/Crb2). We solved the three-dimensional structure of this domain of human 53BP1 by X-ray crystallography. The domain consists of two tandem tudor folds with a deep pocket at their interface formed by evolutionarily conserved residues. In vitro, the tandem tudor domain of 53BP1 bound histone H3 methylated on Lys79. The residues that form the walls of the conserved pocket were required for binding to methylated histone H3 and also for recruitment of 53BP1 to sites of DNA DSBs. Suppression of Dot1L, the enzyme that methylates histone H3 on Lys79 in vivo, also inhibited recruitment of 53BP1 to sites of DNA DSBs. Because methylation of histone H3 Lys79 was not enhanced in response to DNA damage, we propose that 53BP1 senses DNA DSBs indirectly through changes in higher order chromatin structure that expose the 53BP1-binding site. Taken together, our findings suggest that 53BP1 is a sensor of DNA DSBs that functions upstream of ATM.

MOUSE BITES DOGMA: MOUSE MODELS PROVIDE NEW INSIGHTS INTO THE MECHANISMS THAT CONTROL THE P53 STRESS RESPONSE PATHWAY

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p53 is a short-lived transcription factor that has evolved to respond to a variety of stimuli to initiate cell cycle arrest or apoptotic programs, or to induce genes that participate in some forms of DNA repair. As p53 participates in life and death decisions, it is critical that its output be stringently regulated. The importance of the p53 pathway for tumor suppression is demonstrated by mutation of the p53 gene or alterations in its negative regulators in almost all cancers. We first used homologous recombination in mouse ES cells to critically test whether highly conserved C-terminal lysine residues are essential for degradation control and for activation. The surprising data to be shown demonstrate that the C-terminal modifications hypothesized to be critical for proper p53 regulation are not essential, but rather contribute to a fine-tuning mechanism of homeostatic control *in vivo*. The second model explores the p53 negative regulators MDM2 and MDMX. The data show that MDMX's role is to control p53 functional output, not MDM2 stability as previously thought. Together, these mouse models suggest the need to revise our current concepts of how p53 is regulated by MDM2 and MDMX. One new model consistent with the *in vivo* data will be discussed.

DYNAMIC ORGANIZATION OF GENOME MAINTENANCE PROTEINS IN VITRO AND IN VIVO

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The human Rad50/Mre11/Nbs1 complex (hR/M/N) functions as an essential guardian of genome integrity by directing proper processing of DNA ends. This biological function results from its ability to tether broken DNA molecules. hR/M/N's dynamic molecular architecture consists of a globular DNA binding domain from which two, 50 nm long, coiled coils protrude. The coiled coils are flexible and their apexes can self-associate. The flexibility of the coiled coils allows their apexes to adopt an orientation favorable for interaction. However, this also allows for interaction between the two coiled coils within the same complex, which competes with and frustrates the inter-complex interaction required for DNA tethering. We showed that the dynamic architecture of hR/M/N is dramatically affected by DNA

binding. DNA binding to the hR/M/N globular domain leads to parallel orientation of the coiled coils preventing intra-complex interactions and favoring inter-complex interactions needed for DNA tethering. The hR/M/N complex thus provides an example of a biological nanomachine in which ligand binding, in this case DNA, affects the functional conformation of a domain located 50 nm away.
Homologous recombination, the exchange of DNA sequence between homologous DNA molecules, is essential for accurate genome duplication and preservation of genome integrity. Homologous recombination requires the co-ordinated action of the *RAD52* group proteins, including Rad51, Rad52 and Rad54. Upon treatment of mammalian cells with ionizing radiation, these proteins accumulate into foci at sites of DSB induction. We probed the nature of the DNA damage-induced foci in living cells with the use of photobleaching techniques. These foci are dynamic structures undergoing reversibly interact with the recombination proteins. Executing DNA transactions through dynamic multi-protein complexes, rather than stable holo-complexes, allows greater flexibility during the transaction. In case of DNA repair, for example, it allows cross talk between different DNA repair pathways and coupling to other DNA transactions, such as replication.

DNA DAMAGE CHECKPOINTS AND THEIR INTRA-NUCLEAR DYNAMICS
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Unrepaired DNA breaks severely undermine genome integrity. To protect the genome, eukaryotic cells evolved genome surveillance pathways, so called checkpoints, initiated by activation of the ATM and/or ATR protein kinases. We are interested in how ATM and ATR propagate the 'damage alert' to the downstream effectors with the emphasis on how the underlying mechanisms operate in space and time in the nucleus of a living mammalian cell. I will summarize the current concepts of the intra-nuclear protein redistribution induced by DNA double strand breaks (DSBs). I will then focus on spatio-temporal aspects of DSB recognition by the Mre11-Nbs1-Rad50 nuclease complex, and the Mdc1/NFBD1 and 53BP1 checkpoint mediators. I will provide evidence that combination of microlaser-generated DSBs with siRNA technology and real-time imaging revealed local chromatin rearrangements required for a productive assembly of downstream components of the genome surveillance pathways. I will also show that a small group of proteins such as Chk2, Chk1 and Kap1 are characterized by very short residence times at the DSB sites. This enables these proteins to act in a more 'global' fashion by a rapid dissemination from the DNA lesions (where they are phosphorylated) to undamaged nuclear compartments. Such 'messenger' function might be instrumental to rapidly adjust the pace of some key pan-nuclear processes (DNA replication, gene expression) in cells exposed to DSB-generating genotoxic insults.

CHANGES IN CHROMATIN STRUCTURE AND MOBILITY IN LIVING CELLS AT SITES OF DNA DOUBLE-STRAND BREAKS
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The repair of DNA double strand breaks (DSBs) is facilitated by the phosphorylation of H2AX which organizes DNA damage signaling and chromatin-remodeling complexes in the vicinity of the lesion . Large-scale relaxation of chromatin has been proposed to activate the DNA damage transducer ATM at a distance, and may also increase the mobility of DSB-containing chromatin domains , whereas damage-mediated condensation may facilitate faithful repair by limiting the diffusion of broken ends . Although indirect evidence supports the idea that disruption of DNA integrity induces an alteration of chromatin architecture, little is known about the physical properties of damaged chromatin. Here we use a photoactivatable version of GFP-tagged histone H2B to examine the mobility and structure of chromatin containing experimentally induced DSBs in living cells. We find that bulk chromatin containing DSBs exhibits limited mobility but undergoes an energy-dependent local expansion immediately following DNA damage. To determine the ultrastructure of irradiation induced foci (IRIF) and the chromatin changes associated with the activation of ATM, we employed a correlative fluorescence and energy-filtering transmission electron microscopy imaging method. We find that the localized expansion observed in real-time constitutes a relaxation in higher order chromatin structure, corresponding to a 30-40% reduction in the density of chromatin fibers in the vicinity of DSBs. The observed opening of chromatin occurs independently of H2AX and restricts the initial activation of ATM to the locally damaged region. We propose that decondensation of chromatin at DSBs increases the accessibility of damaged DNA to repair proteins and establishes a sub-nuclear environment which facilitates DNA damage signaling and repair.

Poster No. IIIB-0
STRUCTURAL MAINTENANCE OF CHROMOSOME, SMC-PROTEINS AND THEIR ROLE IN REPAIR OF AND RESPONSE TO DNA DAMAGE
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The SMC protein family forms hetero-dimers that constitute the cores of three different multi-protein complexes; Cohesin, Condensin and the Smc5/6 complex. All important for different aspects of chromosome maintenance and genome stability. However, we focus on their role in repair of DNA double strand breaks (DSBs). Thus, we could recently show, using ChIP on chip with high density oligonucleotide arrays that Cohesin, responsible for cohesion between sister chromatids, is recruited to extended chromosome regions surrounding a DSB induced in G2. Accumulation of Cohesin at DSBs depends on Scc2, a Cohesin chromatin-loading protein, and is necessary for repair of DSBs in G2. Evidence was also provided that DSB induction elicits cohesion establishment. Currently we investigate the regulation of damage induced formation of cohesion in relation to cohesion created during an unchallenged S-phase. The Smc5/6 complex is required for DNA repair and cell cycle progression. Though, the mechanism of action so far remains largely unknown. To get further insight into the function of the Smc5/6 complex we are performing a broad investigation on these proteins. Initial experiments clearly show that Smc5/6 and additional sub-components of the complex are indeed localized at DSBs induced by the HO endonuclease. Investigations identifying factors required for this recruitment are ongoing. Furthermore, the ability to repair irradiation-induced DSBs is strongly impaired at restrictive temperature, in cells carrying temperature sensitive alleles of Smc6 and Nse1, one of the subcomponents of the Smc5/6 complex. The importance of Condensin for DNA repair is more ambiguous. Involvement in an S-phase checkpoint has been shown but no direct role in repair of DSBs, so far. In accordance with this we have not been able to detect any localization of Condensin at DSBs.

Poster No. IIIB-1

MODULATION OF CHROMATIN DURING DNA DOUBLE-STRAND BREAK REPAIR

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DNA repair must, of course, occur within the context of chromatin. While the organization of DNA into chromatin is necessary for genome compaction, this is generally inhibitory to the ability to access and manipulate DNA. Not surprisingly, therefore, mechanisms to modulate chromatin structure exist and are dynamically involved in cellular DNA metabolism. These include the covalent modification of histone tails and ATP-dependent chromatin remodeling machineries. Recently, it has become apparent that both mechanisms are used to facilitate DNA repair and recombination. In particular, phosphorylation of histone H2A (or H2AX in higher eukaryotes) is a rapid response to the detection of double-strand breaks, and this can mediate the recruitment of chromatin remodeling activities to DNA lesions. In addition to this phosphorylation event, we have identified roles for multiple residues in the histone tails in DNA damage responses, and have characterized the enzymes that carry out the covalent modifications using budding yeast as a model system. Interestingly, these enzymes are themselves regulated in response to DNA damage by the Mec1 and Tel1 protein kinases. Moreover, we find that the histone tail residues play separate and distinct roles in mediating these DNA damage responses.

Poster No. IIIB-2

MDC1/NFBD: THE PRIMARY γ-H2AX RECOGNITION MODULE OF HIGHER EUKARYOTES

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Higher order chromatin structure is important for recognition and repair of DNA damage. The histone variant H2AX is phosphorylated in response to DNA damage and is thought to facilitate recruitment of DNA repair and cell cycle checkpoint proteins to regions of damaged chromatin. Loss of H2AX causes genome instability and is associated with DNA double strand break (DSB) repair defects and failure to efficiently activate the G2/M checkpoint. Several proteins have been proposed to interact with phosphorylated H2AX (γ-H2AX) but compelling evidence for the existence of a primary γ-H2AX recognition module in higher eukaryotes has not yet been presented.

Here we show that MDC1/NFBD1 directly and specifically interacts with the phosphorylated C-terminus of H2AX via its tandem BRCT domains. Biochemical, biophysical and structural approaches revealed the molecular details of the MDC1-γ-H2AX complex. Furthermore, we show that direct interaction between MDC1 and γ-H2AX is required for ionising radiation-induced nuclear focus formation by several DNA repair and checkpoint proteins. Moreover, we present evidence that MDC1 regulates γ-H2AX maintenance at least in part by protecting it from premature de-phosphorylation. Finally, functional aspects of the MDC1-γ-H2AX interaction in regards to the DNA damage response will be discussed.

Poster No. IIIB-3

CELLULAR RESPONSE TO MR-PROCEDURE

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Changes in gene expression in human cell lines occur after as little as 0.02Gy rays, and in peripheral blood lymphocytes alter as little as 0.2Gy. Diverse genes do also have elevated expression in vivo in mice 24 h after 0.2Gy irradiation (Amundson 2001). Magnetic field (resonance) and possible temperature rise in the tissue from MR-procedure (magnetic field and radio frequency) might alter gene-expression. After Regional Ethical Committee

approval an 11- year's old female subjected to an upper-body/head MR-procedure was sampled for blood (2.5 ml) immediately before and after the MR procedure. We used the PAXgeneTM Blood RNA System and the PAXgene Blood RNA Kit, for purification of intracellular RNA from whole blood. Further subjects have been sampled.
Microarray type used is Applied Biosystems Human Genome Survey Microarray.
The Applied Biosystems Human Genome Survey Microarray is a component of the Applied Biosystems Expression Array System. The current version of Human Genome Survey Microarray contains 31,077 probes that cover 53,977 individual transcripts and target a complete, annotated, and fully curated set of 27,868 human genes from the public and Celera databases. Finally, we grouped genes after changed expression rate after MR procedure 1. Little or no change of gene expression rate, 2. Up or down regulated > 1.5 times, 3. Up or down regulated > 3 times or larger. By analyzing genes grouped in known functional pathways we searched for gene-expression changes in several pathways as the Mitogenic-, Wnt-, Hedgehog-, TGF-b-, Survival-(PI3 Kinase-/AKT-, NF-kB-), p53-, Stress-, NFkB-, NFAT-, CREB-, Jak-Stat-, Estrogen-, Androgen-, Calcium and Protein Kinase C-, Phospholipase C-, Insulin-, LDL- and Retinoic Acid pathways. Approximately a group of 200 genes were picked out for closer analysis. A general impression is that several genes (and their close relatives) related to change in cell cycle and immune response seems to be mostly affected either up or down regulated.

Poster No. IIIB-4

GENOMIC INSTABILITY, ENDO-REDUPLICATION AND DIMINISHED IMMUNOGLOBULIN CLASS SWITCH RECOMBINATION IN B CELLS LACKING NBS1

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Mre11, Rad50, and Nbs1 form an evolutionary conserved protein complex (MRN) that has been proposed to function as a DNA damage sensor. Hypomorphic mutations in Mre11 and Nbs1 result in the human ataxia-telangiectasia like (ATLD) disorder and Nijmegen breakage syndrome (NBS) respectively. Here we compare two mouse models for Nijmegen breakage syndrome. In the first (a hypomorphic mutant), the lethality of $Nbs1^{-/-}$ mice is rescued by transgenic expression of the common human NBS allele ($Nbs1^{657\Delta5}$) from a human bacterial artificial chromosome. $Nbs1^{657\Delta5}$ mice resembled their human NBS counterparts in that they exhibited female sterility, T cell developmental defects, and chromosomal instability. However, Nbs1 hypomorphic B cells proliferate normally and do not exhibit an intrinsic defect in class-switching recombination (CSR). In the second model, Cre-loxP-mediated recombination is used to restrict Nbs1 deletion to B lymphocytes. We find that complete disruption of Nbs1 results in impaired proliferation , chromosomal endo-reduplication, and accumulation of high levels of spontaneous DNA damage. Moreover, we show that in contrast to $Nbs1^{657\Delta5}$ mice, CSR is defective in Nbs1 knockout B cells. The CSR defect is B cell intrinsic, independent of switch region transcript, and is consequence of ineffective recombination at the DNA level. Our findings reveal that Nbs1 is critical for efficient Ig CSR and for maintaining the integrity of chromosome structure and number.

Poster No. IIIB-5

INTERACTION WITH DNA STRAND BREAKS INDUCES A NOVEL CONFORMATIONAL FORM OF P53

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The p53 is a potent tumor suppressor, which becomes activated in response to stress. Ionizing radiation leads to p53 stabilization via phosphorylation of p53 and its regulator Mdm2. These phosphorylations inhibit the association between p53 and Mdm2. However, the exact molecular mechanism of p53 activation by DNA damage is not clear. We now report that upon binding DNA ends p53 dissociates to monomers *in vitro*. This is accompanied by a conformational shift, which can be recognized by a novel p53 antibody, LSP16. LSP16 recognizes a cryptic N-terminal epitope exposed in p53 monomer. Using p53 immunostaining on DNA fibers, we observed LSP16-positive p53 localized to damage sites in response to ionizing radiation. These sights also contained γ-H2AX. *In vivo*, there was early formation of nuclear foci recognized by LSP16 upon γ-irradiation. Next we addressed the question about the biological significance of LSP16-positive p53. Peptide mapping showed that LSP16 epitope coincides with the binding site of Mdm2. Since the LSP16 epitope is unstructured, whereas Mdm2 requires α-helix for binding, we reason that LSP16 and Mdm2 recognize two alternative conformations of the N-terminal site. In accordance with this, we did demonstrate a reduced Mdm2 binding to LSP16-positive p53. Since the amount of LSP16-positive p53 did not further increase after initial induction, we suggest that there is a fast exchange of p53 molecules at the sites of DNA breaks. This generates p53 molecules, which are refractory to Mdm2 degradation. This process might serve to initiate p53 accumulation, independently of phosphorylation by checkpoint kinases. Furthermore, we speculate that a direct molecular link between DNA damage and p53 activation might have evolved to ensure the coordination of DNA damage response with ongoing DNA repair. We will further discuss our idea that p53 modulates DNA repair by facilitating the recruitment of DNA repair factors to the sites of DNA strand breaks and/or by preventing error-prone repair via interaction with Rad51.

Poster No. IIIB-6

ROLE OF HUMAN RECQ5β PROTEIN IN THE MAINTENANCE OF GENOMIC STABILITY

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Rescue of arrested and collapsed DNA replication forks is essential for maintenance of genomic integrity. Here, we provide evidence for the involvement of human RECQ5β protein in the rescue of stalled DNA replication forks. RECQ5β physically interacts with proliferation cell nuclear antigen (PCNA) *in vitro* and *in vivo*. Also, RECQ5β colocalizes tightly with PCNA-containing nuclear foci in HeLa cells upon replication block by hydroxyurea. Moreover, our biochemical studies have revealed that on static forked structures, hRECQ5β promotes two different reactions – unwinding of the "lagging-strand" arm and unwinding of the "parental" duplex, with both reactions being stimulated by the human single-strand DNA binding protein RPA (hRPA). The other human RecQ homologues BLM and WRN preferentially catalyzed unwinding of the parental duplex on these structures; this preference was further enhanced by hRPA. On a mobile 3'-flap structure mimicking a stalled replication fork with a single strand gap on the leading-strand arm, all three helicases exclusively displayed an efficient fork-regression activity resulting in the displacement of the lagging-strand duplex and re-annealing of the parental strands. However, addition of RPA to the reaction completely abolished the fork-regression activity of BLM and WRN and biased the action of these helicases towards unwinding of the parental duplex arm. In contrast, addition of RPA to the hRECQ5β reaction further enhanced the fork-regression activity. Analysis of a series of RECQ5β deletion variants revealed that the region encompassing the amino acids 561-651 is essential for its fork regression activity *in vitro*. Collectively, our current evidence suggests that RECQ5β might play a role in the recovery of stalled DNA replication forks by promoting fork-regression.

Poster No. IIIB-7

PHOSPHORYLATION OF ARTEMIS FOLLOWING IRRADIATION-INDUCED DNA DAMAGE

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Artemis is a DNA repair factor required for V(D)J recombination, repair of DNA damage induced by ionizing radiation (IR) or radiomimetic drugs, and the maintenance of genome integrity. During V(D)J recombination, Artemis participates in the resolution of hairpin-sealed coding ends, a step crucial to the constitution of the gene encoding for the antigen receptor of lymphocytes. The precise role of Artemis in the repair of IR-induced DNA damage remains to be elucidated.

Here we show that Artemis is constitutively phosphorylated in cultured cells and undergoes additional phosphorylation events after irradiation. The IR-induced phosphorylation is mainly, although not solely, dependent on Ataxia-telangiectasia-mutated kinase (ATM). The physiological role of these phosphorylation events remains unknown, as *in vitro*-generated Artemis mutants, which present impaired IR-induced phosphorylation, still display an activity sufficient to complement the V(D)J recombination defect and the increased radiosensibility of Artemis-deficient cells. Thus, Artemis is an effector of DNA repair that can be phosphorylated by ATM, and possibly by DNA-PKcs and ATR depending upon the type of DNA damage.

Poster No. IIIB-8

ASCIZ REGULATES RAD51 FOCI FORMATION AND APOPTOSIS IN RESPONSE TO ALKYLATING DNA DAMAGE

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The Rad51 recombinase is essential for homologous recombination (HR) repair and redistributes within the nucleus to form single-stranded DNA (ssDNA) containing foci in response to double-strand breaks (DBSs), DNA cross-links and alkylating DNA damage. Rad51 foci formation depends on recombination mediators BRCA1, BRCA2/FANCD1, XRCC2-3 and Rad51B-D, mutation of which leads to genomic instability, DNA damage hyper-sensitivity and cancer predisposition. We have isolated a novel human protein, ASCIZ, which forms foci in response to methylating DNA damage, but not in response to DSB-inducing agents. ASCIZ foci contain Rad51 and ssDNA and may therefore represent sites of HR. Cells depleted of ASCIZ have impaired Rad51 foci formation in response to methylating agents, but not DSB-inducing ionising radiation, and show increased rates of methylation-induced apoptosis. Overexpression of an ASCIZ "core" domain uncouples Rad51 from the DNA damage response, concentrating Rad51 into constitutive foci-like structures in the absence of damage. ASCIZ foci formation is increased by inhibition of the base-excision repair (BER) pathway, and thus may compete with BER for the processing of methylation induced damage. Futhermore, ASCIZ foci formation and increased apoptosis in ASCIZ-depleted cells depends on the mismatch repair protein, MLH1. ASCIZ may function as a scaffold for Rad51 foci formation to direct incompletely repaired DNA methylation damage into the HR pathway.

Poster No. IIIB-9

OVERLAPPING ROLES OF NER AND MMR IN TARGETED HOMOLOGOUS RECOMBINATION IN MAMMALIAN CELLS

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In *Saccharomyces cerevisiae,* both MutSβ (Msh2/Msh3 heterodimer) and Rad1/Rad10 endonuclease are required to precisely remove lengthy nonhomologous 'tails' from strand invasion and single-strand annealing (SSA) recombination substrates, and show epistasis for this activity. We are particularly interested in SSA in mammalian cells, since it may be involved in the repair of complex DNA damage, such as DNA interstrand crosslinks. We previously used CHO *ERCC1* knock-out cells to show that the Xpf/Ercc1 endonuclease (corresponding to Rad1/Rad10) is required for recombination functions during gene targeting in mammalian cells. To investigate the role of MutSβ in *ERCC1*-dependent or –independent recombination pathways, we generated isogenic wild-type ($MSH3^{+}/ERCC1^{+}$), and *MSH3* deletion ($MSH3^{DEL}/ERCC1^{+}$) and compound ($MSH3^{DEL}/ERCC1^{KO}$) mutant CHO cell lines, each containing a common, hemizygous *APRT* mutation that can be repaired by homologous recombination using *APRT* gene targeting vectors. In gene targeting experiments using vectors in which the ends of homologous DNA 'arms' are blocked by long nonhomologous 'tails,' $MSH3^{DEL}$ cells show ~ 2-fold reduction in targeting frequency compared to wild-type cells, whereas the compound mutant ($MSH3^{DEL}/ERCC1^{KO}$) shows ~ 10-fold reduction. These results suggest that *MSH3* and *ERCC1* are *not* epistatic in mammalian cells, but may have overlapping functions in processing these types of recombination intermediates. Analysis of recombinant structures from these experiments also indicate that deletion of *MSH3* has distinct and unexpected effects on the distribution of recombinant structures.

Poster No. IIIB-10

INFLUENCE ON MRNA STABILITY AND TRANSLATION OF A REPORTER GENE BY UUUUUAU MOTIFS IN THE 3´UNTRANSLATED REGION OF HUMAN P53

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The tumor suppressor protein p53 plays an important role in the cellular response to stressful stimuli by inhibiting growth of abnormal or stressed cells. Stressful stimuli such as DNA damage, hypoxia or nutrient deprivation that might exert harmful effects in the cell have been shown to enhance the stability, location, and activity of p53 by post-translational modifications. However accumulating evidence indicates that p53 expression might also be regulated by post-transcriptional mechanisms which alters the stability and translation efficiency of p53 mRNA. We are interested in clarifying the mechanisms behind this post-transcriptional regulation of p53.

AU-rich elements present in 3´untranslated regions (3´UTRs) are often involved in regulation of both the stability and translation of mRNAs. The 3´UTR of human p53 mRNA contain two cytoplasmic polyadenylation elements (CPE) located just upstream of the poly A signal with the consensus sequence UUUUUAU. In addition a U-rich region is located approx. in the middle of the 3´UTR. We show that the p53 wild type 3´UTR mediate a post-transcriptional regulation of a reporter gene by repressing the mRNA steady state level in transduced MCF-7 and HaCaT cells. Point mutations in the CPE motifs abrogate the repression of the mRNA steady state level in a cell type dependent manner. The CPE motifs have a significant effect on the translation of the reporter mRNA in both cell lines and the full-length 3´UTR mediate a retention of the reporter mRNA in the nucleus. Irradiation of transduced HaCaT cells with UVC shows that the wild type 3´UTR mediates a translational activation of the reporter mRNA and mutations in the CPE motifs inhibit this activation. Still unidentified cytoplasmic and nuclear proteins present in both MCF-7 and HaCaT cells binds specifically to the CPE motifs.

Our results show that the two CPE motifs present in the 3´UTR from p53 are involved in the regulation of both the stability and the translation of a reporter mRNA in non-irradiated as well as irradiated cells dependent on the cell type examined.

Poster No. IIIB-11

AN ESSENTIAL ROLE FOR THE C-TERMINUS OF REV1 IN DNA DAMAGE TOLERANCE

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Bypass of DNA damage encountered during replication is accomplished by translesion synthesis in which specialised DNA polymerases cross a lesion directly or by recombination in which an alternative undamaged template is used. Despite the use of translesion synthesis increasing the risk of mutation, it is clear that higher eukaryotes not only possess homologues of the translesion polymerases found in lower organisms but also rely on them for tolerance of DNA damage and viability. Rev1 plays a key role in translesion synthesis and DNA damage tolerance in budding yeast. However, although it possesses deoxycytidyl transferase activity, its precise role remains unclear since ablation of this activity does not result in diminished DNA damage tolerance. However, a point mutation in the N-terminal BRCT domain, the *rev1-1* mutant, confers a null phenotype, indicating that there is a second role for Rev1 in yeast involving the BRCT domain. The mammalian protein has recently been shown to interact with each of the other translesion polymerases via its extreme C-terminus. We have examined the functional significance of this interaction and that of the deoxycytidyl transferase activity and BRCT domain by complementation analysis of the human gene in

a Rev1 mutant DT40 chicken cell line. A YFP-hRev1 fusion protein complements the DT40 mutant for sensitivity to UV light and cisplatin. In stark contrast to yeast, the equivalent of the *rev1-1* BRCT mutation also complements, as does a construct completely lacking the BRCT domain. hRev1 with mutation of key catalytic residues of the deoxycytidyl transferase domain, which abolish this activity, also complements. However, deletion of the C terminal 100 amino acids results in a construct that fails to complement. Therefore, although vertebrate Rev1 apparently plays a similar role to its yeast counterpart, it appears to do this in a completely different manner. Our data support the idea that Rev1 in vertebrates plays a regulatory or scaffolding role in lesion bypass rather than being directly involved in translesion DNA synthesis.

Poster No. IIIB-12

IDENTIFICATION OF PROTEINS INVOLVED IN HOMOLOGOUS RECOMBINATION

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Homologous recombination (HR) is enhanced by transcription in all cellular organisms. However, it has not yet been determined which proteins that are involved in transcription-induced recombination In order to clarify which proteins that are essential in catalysing homologous recombination induced by transcription, Chinese hamster cell lines with defects in genes encoding proteins believed to be involved in HR were transfected with a recombination substrate.
The recombination substrate consists of two non-functional neomycin resistance genes in front of a bi-directional Tet-inducable promoter that also controls a luciferase gene making it possible to monitor the expression over the recombination substrate. A site for the rare-cutting endonuclease I-SceI has been included in one of the non-functional neomycin resistance genes in order to allow induction of a double strand break. If a recombination has taken place, the cell will possess a functional neomycin resistance gene, which can be selected for. Preliminary results using the cell line UV5, deficient in the XPD subunit of TFIIH, indicate that XPD is indeed necessary for transcription-enhanced recombination. We will attempt to confirm this by transfection with the wild type protein, thus re-establishing the wild type, and studying the recombination frequencies with and without transcription.

Poster No. IIIB-13

THE MODULAR MISPAIR BINDING DOMAIN OF MSH3 CAN BE TRANSFERRED TO MSH6 TO CONFER MSH3-LIKE MISPAIR RECOGNITION AND REPAIR PROPERTIES ONTO MSH6

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Eukaryotic mismatch repair involves two mispair recognition complexes with different but overlapping specificities. Base-base mispairs are recognized primarily by the MSH2-MSH6 complex and large insertion/deletion mispairs are recognized by the MSH2-MSH3 complex, while repair of small insertion/deletion mispairs can be initiated by either complex. While the mechanism of mispair recognition by MSH6 can be inferred from the crystal structure of the bacterial homolog MutS, MSH3 has significant differences in key residues of the mispair binding domain (MBD) and the mechanism by which it recognizes mispairs therefore remains elusive. Here we show that in *Saccharomyces cerevisiae*, the mispair recognition specificity of MSH3 is determined by a 131-residue MBD, and that substitution of these 131 residues for the 116-residue MBD of MSH6 is sufficient to confer MSH3-like mispair recognition and repair properties upon the MSH6 protein. Mutation rates of strains in which the MBD of MSH6 has been replaced with that of MSH3 and MSH3 has been deleted are comparable to those of an msh6 deletion strain expressing MSH3. Analysis of the spectrum of Can^r mutations arising in different mutant strains confirms that the msh6/msh3 MBD-swapped allele is responsible for initiating repair of the same types of mispaired bases recognized by MSH3. Biochemical analysis of mispair binding confirms that mispair specificity is conferred primarily by the 131 residues exchanged. The WT MSH2-MSH6 complex has a high specificity for base-base mispairs and +1 insertions, while the mutant complex shows greater specificity for +2 insertions and is unable to distinguish base-base mispairs from fully paired DNA. These results suggest that differences in mismatch recognition between MSH3 and MSH6 are due primarily to differences in their mispair binding domains.

Poster No. IIIB-14

HOMOLOGOUS RECOMBINATION AND NON-HOMOLOGOUS END JOINING REPAIR PATHWAYS REGULATE FRAGILE SITE STABILITY

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Common fragile sites are specific loci that form gaps and constrictions on metaphase chromosomes exposed to replication stress which slows DNA replication. These sites have a role in chromosomal rearrangements in tumors, however, the molecular mechanism of their expression is unclear. Here we show that replication stress leads to focus formation of Rad51 and phosphorylated DNA-PKcs, key components of the homologous recombination (HR) and non-homologous end joining (NHEJ), double-strand break (DSB) repair pathways, respectively. Down-regulation of Rad51 or DNA-PKcs leads to a significant increase in fragile site expression under replication stress. Replication

stress also results in focus formation of the DSB markers, MDC1 and γH2AX. These foci co-localized with those of Rad51 and phospho-DNA-PKcs. These findings suggest that DSBs are formed at common fragile sites as a result of replication perturbation. The repair of these breaks by both HR and NHEJ pathways is essential for chromosomal stability at these sites.

Poster No. IIIB-15

THE CELL-CYCLE CHECKPOINT KINASE CHK1 IS REQUIRED FOR MAMMALIAN HOMOLOGOUS RECOMBINATION REPAIR

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The essential checkpoint kinase Chk1 is required for cell cycle delays following DNA damage or blocked DNA replication. However, it is unclear if Chk1 is involved in repair of damaged DNA. Here, we establish that Chk1 is a key regulator of genome maintenance by the homologous recombination repair (HRR) system. Abrogation of Chk1 function with siRNA or chemical antagonists inhibits HRR, leading to persistent unrepaired DNA double-strand breaks (DSBs) and cell death after replication inhibition with hydroxyurea (HU) or DNA-damage caused by camptothecin. Following HU treatment, the essential recombination repair protein RAD51 is recruited to DNA repair foci performing a vital role in correct HRR. We demonstrate that Chk1 interacts with RAD51, and RAD51 is phosphorylated on threonine 309 in a Chk1-dependent manner. Consistent with a functional interplay between Chk1 and RAD51, Chk1-depleted cells failed to form RAD51 nuclear foci after HU exposure, and cells expressing a phosphorylation-deficient mutant RAD51-T309A were hypersensitive to HU. These results highlight a crucial role for the Chk1 signaling pathway in protecting cells against lethal DNA lesions through regulation of HRR.

Poster No. IIIB-16

MULTIPLE MECHANISMS CONTROL CHROMOSOME INTEGRITY AFTER REPLICATION FORK UNCOUPLING AND RESTART AT IRREPARABLE UV-INDUCED LESIONS

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DNA replication forks pause in front of lesions on the template, eventually leading to citotoxic chromosomal rearrangements. The in vivo structure of eukaryotic damaged replication intermediates has been so far elusive. We applied a combination of electron microscopy analysis and two-dimensional gel electrophoresis to investigate replication forks in S. cerevisiae cells replicating irreparable UV-lesions. We found that replication forks encountering unprocessed UV-lesions rapidly uncouple nascent leading and lagging strand, thus generating long ssDNA regions at one side of the fork, recognizable as the leading strand. Furthermore, small ssDNA regions accumulate along both replicated duplexes and likely contribute to break formation, unless promptly repaired. The DNA damage checkpoint, translesion synthesis and homologous recombination are all required to prevent gap accumulation. Further, while the last two processes do not contribute to fork progression, Rad53 activation is essential to maintain a replication-competent fork structure, possibly coordinating replication restart and polymerase-switch mechanisms.

Poster No. IV-1

HUMAN TRF2, TRF1 AND TIN2 RAPIDLY ASSOCIATE WITH GENOMIC DOUBLE-STRAND BREAKS: AN NOVEL EARLY DNA DAMAGE RESPONSE

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Human cells use complex signaling networks to sense double-strand breaks (DSBs) in genomic DNA, then activate DNA repair, cell cycle checkpoints, and apoptosis. TRF2, TRF1 and TIN2 are involved in telomere maintenance, with TRF2 preventing telomeric ends from triggering cellular DSB responses. We now report that TRF2, TRF1 and TIN2 rapidly associate with DSBs in non-telomeric DNA.

These three telomeric proteins form transient foci that tightly colocalize with laser microbeam-induced DSBs and with DSB-associated ATM foci. The kinetics of GFP-TRF1 and YFP-TRF2 accumulation at DSBs are similar, occuring more rapidly than reported forYFP-NBS1 or γH2AX. Ionizing radiation induces transient phosphorylation of TRF2 at Thr188. We detect TRF2-Thr188P at photo-induced DSBs and at γ radiation-induced γH2AX foci. The TRF2 response is dependent on its basic domain but does not require telomerase and occurs in the absence of functional DNA-PKcs, the MRE11/Rad50/NBS1 complex or the Ku70, WRN and BLM repair proteins. Our observations suggested that TRF2 is temporally and spatially poised to affect DSB activation of ATM damage responses. To test

this, we over-expressed TRF2 in primary human fibroblasts. TRF2 over-expression results in reduced accumulation of phosphorylated ATM, H2AX and p53 following γ irradiation.
This work documents novel, non-telomeric functions for TRF2, TRF1 and TIN2. Our results suggest that TRF2, TRF1 and TIN2 may interact, either independently or as a complex, with DSB-containing chromatin, provide evidence that TRF2 can compete with or attenuate ATM responses to DSBs, and implicate these telomeric proteins in the DNA damage response.

Poster No. IV-2

A HIGH-RESOLUTION MICROSCOPIC ANALYSIS OF DNA DAMAGE REGULATORS REVEALS DISTINCT SPATIAL MODES OF INTERACTIONS WITH DNA DSBS

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In response to DNA double strand breaks (DSBs), many proteins accumulate into microscopically discernible structures, known as ionizing radiation-induced foci (IRIF). This trait of the DNA damage response is highly conserved in evolution and is widely regarded to facilitate genomic integrity in cells exposed to DNA damage. However, surprisingly little is known about how DNA repair and checkpoint signaling processes benefit from the increased local concentration of their effectors. By combining DSB-generating laser micro-irradiation, immuno-staining with target-specific antibodies and high-resolution confocal microscopy, we provide evidence for several qualitatively distinct modes of focal accumulation of proteins at the DSB sites. First, the chromatin compartment marked by γ-H2AX is occupied by checkpoint mediators Mdc1 and 53BP1 as well as Nbs1 and ATM. These interactions are equally robust throughout the interphase. Second, proteins such as Rad51, FANCD2, ATRIP and ATR are excluded from chromatin and accumulate in smaller focal structures delineated by single stranded DNA, the resection of which is restricted to S and G2 phases of the cell cycle. A third class of DSB regulators, including Chk1, Chk2, Ku and DNA-PK, do not show any focal accumulation, and as we previously showed for Chk2, the interaction of these proteins with DSBs is likely too transient to manifest itself as cytologically discernible foci. Interestingly, a small group of key DSB regulators appear to simultaneously occupy several compartments. Thus, BRCA1 and Nbs1 were found to accumulate into repair- as well as chromatin-associated foci. Furthermore, these two proteins, together with TopBP1, were found to form small repair foci in G1, independently of ssDNA formation. Together, these results help sub-classify DSB regulators according to their residence sites after DNA damage, and provide a framework to validate, predict, or even exclude, functional interactions between DSB regulators.

Poster No. IV-3

ION MICROIRRADIATION: AN EXCELLENT TOOL FOR THE ANALYSIS OF FOCI FORMATION

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Localized irradiation with UVA lasers has successfully been exploited by several groups to study the recruitment of damage signalling and repair proteins into so-called foci at DNA double-strand breaks (DSB). Unfortunately, the damage types induced by this treatment are poorly characterized and it is not known to what extent the cellular response processes elicited are comparable to those induced by ionizing radiation. The ion microbeam SNAKE offers the possibility of localized irradiation with single ions, with LET values (and thus dose applied to a cell per ion traversal) ranging from 2 keV/µm to more than 1000 keV/µm. By investigating the relationship between DSB density along the tracks and the number of repair foci forming we want to elucidate whether repair factories exist and what their detachment area is. To increase the resolution, we plan also to investigate microfoci as obtained by down-regulation of Mdc1. We also study to what extent the kinetics of recruitment of the repair and signalling factors 53BP1, Mdc1, ATM, RPA, Rad51, and Artemis is affected by damage density. This, and a comparison to the kinetics after UVA and gamma-irradiation should contribute to understanding low dose hypersensitivity and damage-type specific response. In the present set-up, we achieve an accuracy of irradiation of about 500 nm. Geometric irradiation patterns are applied by use of a scanning mode. These patterns allow to investigate the mobility of damaged regions within the nucleus after immunofluorescent detection of damaged chromatin sites. Preliminary data hint at constrained diffusion of damaged chromatin.

Poster No. IV-4

DETECTION AND PROCESSING OF DNA DAMAGE FOR HOMOLOGOUS RECOMBINATION

Rothstein R*, Barlow JH*, Burgess RC* & Lisby M†; *Department of Genetics & Development, Columbia University, New York, NY , USA; †Institute of Molecular Biology and Physiology, University of Copenhagen, Copenhagen, Denmark

We have taken a genetic and cell biological approach to dissect the cellular response to DNA damage in the yeast *Saccharomyces cerevisiae*. Using live cell imaging, we have analyzed the substrate specificity of specific DNA damage checkpoint and repair proteins. In particular, the cellular response leading to homologous recombination is

governed by at least two signals: 1) DNA ends recognized by the Mre11-Rad50-Xrs2 complex and 2) single-stranded DNA recognized by replication protein A.
The processing of aberrant DNA structures into substrates for homologous recombination depends cell cycle phase and the nature of the DNA lesion. For example, endonuclease-induced DNA breaks are protected from 5' to 3' resection by the Ku70/Ku80 complex specifically in G1. In contrast, DNA breaks induced by ionizing radiation, which are likely to contain damaged bases and sugars, are processed for homologous recombination at all phases of the cell cycle. These results may explain the preferred repair of DNA double-strand breaks by non-homologous end-joining in G1 and by homologous recombination in S and G2. Finally, we characterize cellular mechanisms that suppress the recombinogenic potential of non-damage DNA structures such as telomeres and replication forks.

Poster No. IV-5

DYNAMIC ASSEMBLY AND SPATIO-TEMPORAL KINETICS OF CHECKPOINT MEDIATORS AT THE SITES OF DNA DAMAGE

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53BP1 and Mdc1/NFBD1 checkpoint mediators are key components of the genome surveillance network activated by DNA double strand breaks (DSBs) and controlled by the ATM protein kinase. Despite rapid accumulation of checkpoint mediators in the so-called ionizing radiation-induced foci (IRIF), the kinetic aspects of their interaction with the DSB-containing chromosomal microcompartments is poorly understood. Here, we combine microlaser-assisted generation of local DSBs with real-time microscopy and siRNA technology to study the DSB-induced redistribution of 53BP1 and Mdc1/NFBD1 in the nuclei of living mammalian cells. We show that within minutes after DNA damage, both 53BP1 and Mdc1/NFBD1 become progressively, yet transiently immobilized around the DSB-flanking chromatin. Interestingly, quantitative imaging of single cells revealed that the assembly of 53BP1 at DSBs was temporally delayed compared to that of Mdc1/NFBD1. Furthermore, siRNA-mediated ablation of Mdc1/NFBD1 drastically impaired interaction of 53BP1 with DSBs. As a consequence, Mdc1/NFBD1-deficient cells never accumulated 53BP1 in the DSB areas to physiological levels, and they lost 53BP1 from these regions several hours before the completion of DNA repair. In contrast, depletion of 53BP1 had little effect on the kinetics of Mdc1/NFBD1-DSB interaction. Thus, these *in vivo* measurements identify Mdc1/NFBD1 as an important upstream determinant of 53BP1's interaction with DSBs, starting from the dynamic assembly at the DSB sites, and culminating at the checkpoint recovery. Together with our previous kinetic measurements of the Mre11-Nbs1-Rad50 complex, we integrate these data into a model of the spatial and temporal organization of the DSB sites, including the key underlying chromatin modifications. We will present this model and discuss it in light of the current concepts of DSB sensors and the molecular switches between the initial assembly and the sustained retention of DSB regulators in the vicinity of the primary chromosomal lesions.

Poster No. IV-6

IN SITU DETECTION OF DNA REPAIR-RELATED PROTEINS AFTER EXPOSURE TO HIGH-LET RADIATION

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There is an increasing interest in using high-linear energy transfer (LET) radiation in tumor therapy, but the molecular basis of its biological effectiveness is not fully understood. The aim of this study was to improve quantitative analysis of DSBs and monitor the substructure of DNA damage, repair and signalling along high-LET particle tracks, as well as to determine the influence of radiation quality in induction and rejoining of DSBs. We investigated the rapid formation of γ-H2AX, p-ATM, MRE11 and 53BP1 foci that co-localized at the sites of radiation-induced DSBs along the particle track. After 24 h, a small number of foci was persistent, in accordance to the unrejoined DNA fragments detected by PFGE. The DSB induction and repair was strongly dependent on radiation quality and LET, N ions being up to 65% more efficient in DSB induction than gamma radiation. While gamma - induced DSBs rejoined almost completely after 24 h, after nitrogen ions irradiation up to 20% of the breaks were still unrejoined for the highest LET, indicating lesions that are more severe and more difficult to repair. We report results on how the DNA repair processes are affected by clustered DNA damage induced by radiation with LET ranging from 80 to 320 eV/nm.

Poster No. IV-7

SUBCELLULAR LOCALIZATION OF DNA MISMATCH REPAIR PROTEINS

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Repair of damage in DNA is carried out through six major repair pathways: (1) direct reversal (DR), (2) nucleotide excision repair (NER), (3) base excision repair (BER), (4) mismatch repair (MMR), (5) recombinational repair (RER), and (6) translesion synthesis (TLS). A link between DNA repair deficiency and human disease is emphasized by the

finding that Hereditary Nonpolyposis Colorectal Cancer (HNPCC) patients carry mutations in MMR genes. The MMR system corrects errors, which have escaped the DNA polymerase proofreading activity. The regulation of MMR protein function is controlled through several mechanisms including transcription and translation, protein stability, post-translational modifications, and subcellular localization. The regulation of subcellular localization of MMR proteins, although important for its function, is still poorly understood. We have shown that the regulation of MMR protein localization depends on (1) specific sequences in the MMR proteins that interact with factors known to be involved in nuclear import of proteins as well as (2) specific protein-protein interactions in the MMR complexes. We also show that (3) mutations identified in HNPCC patients encode proteins that are defective in translocation to the nucleus and that this defect likely results in MMR-deficiency in these patients. In summary, our results provide evidence that MMR protein localization plays an important role in repair of DNA damage as well as in human disease.

Poster No. IV-8

A VISUAL ASSAY FOR STUDYING HOMOLOGOUS RECOMBINATION AT A SINGLE DOUBLE STRANDED BREAK IN VIVO

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A DNA DSB is one of the most severe types of DNA damage. It triggers cell-cycle arrest and if not repaired it ultimately leads to cell death. An error-free method to repair a DSB is via homologous recombination. In homologous recombination the cell must first gain access to the two broken DNA ends, then locate a suitable homologous sequence (homology searching) that serves as a template for the repair process. A key step in the pathway is "homology search", but it is unclear how this process occurs since a) homology search have not been observed in live cells and b) the factors necessary for homology search are not easily separated from the factors necessary for the following invasion reaction.

In an assay constructed in bakers yeast three important components of the recombination process can now be visualized simultaneously in living cells by fluorescence microscopy: a DSB, a repair protein (e.g. Rad52), and a homologous DNA repair template have been tagged with three different fluorescent probes. This assay makes it possible to visualize at the single cell level, when the repair process is successfully completed.

Poster No. IV-9

RAD52 REPAIR CENTRES IN SACCHAROMYCES CEREVISIAE

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In eukaryotes, DNA double strand break (DSB) repair is essential to maintain genetic stability. The major pathway of DSB repair in the yeast *Saccharomyces cerevisiae* is homologous recombination (HR). *In vivo* DNA DSBs lead to the accumulation of repair proteins into large repair complexes where multiple lesions are repaired simultaneously. Along with other members of the Rad52 epistasis group, Rad52-YFP has been shown to relocalize from diffuse nuclear distribution to form bright foci when DNA DSBs are introduced.

We are interested in the architecture of these repair centres to understand the biological consequences; how are the centres formed and why are many lesions repaired at the same locus? We use Rad52-YFP as a molecular marker and have performed mutagenesis to isolate Rad52-YFP mutants, which failed to localize at the repair centres. We have identified a *rad52* allele, which is unable to form aggregates in S-phase as well as when DNA DSBs are introduced. The mutant strain showed a very severe phenotype; it grows slowly and is sensitive to the alcylating agent MMS, which indicates that its capability to ensure proper repair is impaired.

At present, we are using this mutant as a starting point to investigate the properties of the repair centres and are testing it genetically as well as biochemically.

BENZON SYMPOSIUM No. 52

CELLULAR RESPONSES TO DNA DAMAGE

AUGUST 22-25, 2005, COPENHAGEN, DENMARK

Organizing committee:
Jiri Bartek (Copenhagen), Jiri Lukas (Copenhagen), Jan Hoeijmakers (Rotterdam) and Arne Svejgaard (Copenhagen)

Abstracts - THURSDAY, August 25, 2005

HEREDITARY DISORDERS CONFERRED BY DEFECTIVE DNA DAMAGE RESPONSES

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Ataxia telangiectasia (A-T) was one of the first human disorders shown to be caused by a defective response to DNA damage. A-T is characterised by progressive ataxia, immunodeficiency, clinical radiosensitivity and cancer predisposition. ATM, the protein defective in A-T is non-essential and most A-T patients have null ATM mutations. Recently, we identified a mutation in Ataxia telangiectasia and Rad3 related protein (ATR) in two related patients displaying Seckel Syndrome. The ATR-Seckel patients displayed severe microcephaly and developmental delay. Unlike ATM, ATR is essential and the mutation identified in the patient was a hypomorphic mutation resulting in aberrant ATR splicing. A-T like disorder (ATLD) and Nijmegen Breakage Syndrome (NBS) are two further hereditary disorders caused by mutations in Mre11 and Nbs1, respectively. ATLD patients display mild A-T like features. Mre11 has been shown to be required for ATM phosphorylation events and ATLD cells display overlapping features with A-T cell lines. NBS, in contrast, patients display features that overlap both A-T and Seckel Syndrome, namely microcephaly, developmental delay, characteristic facial features, immunodeficiency and pronounced cancer predisposition. NBS cell lines have also been shown to display cellular features that overlap with A-T and ATR-Seckel Syndrome cell lines. These results suggest that Nbs1 functions in both the ATM and ATR signalling pathways. The further analysis of cell lines from Seckel Syndrome, NBS and ATLD patients will be discussed.

PROTECTION AND MAINTENANCE OF HUMAN CHROMOSOME ENDS

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Over the past 10 years, it has become clear that human telomeres contain a complex of at least six telomere specific proteins. Two of these proteins, TRF1 and TRF2 bind to double stranded telomeric DNA and a third, POT1, binds to TTAGGG repeats in single stranded form. These three DNA binding proteins, held together by protein interactions, give the telomeric complex exquisite specificity for the sequence and structure of telomeric DNA. This complex is referred to as shelterin because it protects chromosome ends. Shelterin is implicated in the formation of t-loops, affects the structure of the telomere terminus, and controls the synthesis of telomeric DNA by telomerase. The effect of shelterin on the structure of the telomeric DNA is thought to protect chromosome ends from DNA damage surveillance and repair pathways. We are using conditional targeting in the mouse to dissect the function of shelterin and to determine the consequences of telomere dysfunction. Progress on the DNA damage phenotypes resulting from conditional deletion of TRF2 in the presence or absence of NHEJ factors will be presented.

THE FANCONI ANEMIA/BRCA PATHWAY IN THE DNA DAMAGE RESPONSE

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Fanconi Anemia (FA) is a genetic DNA Repair disorder characterized by bone marrow failure and cancer predisposition, including early hematological malignancies and, later in life, solid tumors. A common feature of cells from FA patients is their hypersensitivity to DNA-crosslinking agents such as mitomycin C, cisplatin, and diepoxybutane. Thus far, eleven complementation groups of FA have been identified, nine of which have been cloned and implicated in a biochemical pathway involved in the repair of DNA damage. Seven of the FA proteins (A,B,C,E,F,G, and L) are subunits of a large intranuclear protein complex. This complex is a monoubiquitin ligase, and its putative catalytic member is the FANCL protein. In response to DNA damage, this FA complex monoubiquitinates the FANCD2 protein on Lysine 561. Monoubiquitinated D2 protein then translocates to chromatin where it interacts with the FANCD1 protein in the process of homologous recombination (HR) repair. The *FANCD1* gene is identical to the breast cancer susceptibility gene, *BRCA2*. At least two additional FA genes, corresponding to

subtypes I and J, remain to be identified. The I gene presumably works upstream in the FA pathway, since cells derived from FA-I patients lack monoubiquitinated FANCD2. In contrast, FA-J cells do express monoubiquitinated FANCD2, suggesting that the FANCJ protein functions downstream of the ubiquitin ligase complex. Additionally, FANCD2 is regulated by ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia-related) kinases. ATR is required for full activation of DNA damage-dependent monoubiquitination, and this activation is mediated, at least in part, by direct phosphorylation of FANCD2. There may, however, be additional ATM and ATR-specific phosphorylation events on other proteins in the FA pathway. We have recently determined that the FA pathway, and perhaps the translesion DNA synthesis pathways, are negatively regulated. Specifically, we have identified a deubiquitinating enzyme, USP1, which deubiquitinates FANCD2 and turns off the FA pathway. FANCD2 deubiquitination is a highly regulated event, and it occurs after the repair of DNA damage or at the end of the normal S phase of the cell cycle. In my talk, I will discuss many of the details of the regulatory machinery which control FANCD2 monoubiquitination and deubiquitination.

DNA REPAIR DEFICIENCIES IN HUMAN PREMATURE AGING

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Werners syndrome is the hallmark example of human premature aging where the affected individuals suffer from many of the signs and symptoms of the normal aging process at an early stage in life. Another premature aging condition is Cockayne syndrome. We are studying these diseases and their proteins, looking at biochemical and cellular features involved in DNA metabolism and genomic stability. The main goal is to determine in which pathways these proteins participate because that may tell us about central molecular pathways involved in aging. Both Werner and Cockayne (group B) syndrome proteins participate in DNA repair, and in more than one pathway. They both participate in the base excision repair (BER) pathway which removes oxidative DNA base lesions. We are trying to understand the mechanisms involved. Werner protein (WRN) has a number of protein partners that are involved in BER, in particular the long patch sub pathway. WRN also can participate in the hand-off process of BER in which its catalytic activities can be regulated. The notion of a defect in BER is also supported by examination of Werner syndrome cells. Werner siRNA knockdown cells accumulate oxidative DNA base damage

NEW ISOFORMS OF P53 AND THEIR ROLE IN THE DNA DAMAGE RESPONSE

Bourdon JC; University of Dundee, Ninewells Hospital, Surgery and Molecular Oncology, UK

The recently discovered p53-related genes, p73 and p63, express multiple splice variants and amino-terminally truncated forms initiated from an alternative promoter in intron-3. To date, no such complexity has been described for the p53 gene. In this study, we show that human p53 gene expresses multiple splice variants and contains an internal promoter. Therefore, p53 gene has a similar gene organization than p63 and p73 and can encode for at least nine p53 protein isoforms.

The conservation of the internal promoter in the p53 gene from drosophila to man suggests an essential role for the p53 isoforms and reveals an unforeseen complex regulation conserved through evolution.

p53 isoforms are expressed in multiple normal human tissues. One isoform can act in a dominant-negative manner towards wild-type p53 for its function in transcription and apoptosis, revealing a new mechanism by which p53 can be inactivated. This mechanism would not be detected by the current sequencing and immunohistological methods used to determine p53 status of human cancers.

Some p53 isoforms are differentially expressed in breast cancer where they can act to modulate the p53 activity threshold and could therefore influence the susceptibility to cancer development and response to therapy.

NUCLEOTIDE EXCISION REPAIR IN CANCER AND AGEING

Hoeijmakers JHJ, Garinis G, Andressoo J-O, Niedernhofer L[b]), van der Pluijm I, Diderich K, Lalai A, Mitchell J, Beems RB[a)], van Steeg H[a)] & van der Horst GTJ; [a]MGC, CBG, Dept. of Cell Biol. and Genetics, Erasmus University, Rotterdam, The Netherlands [a)] RIVM, Bilthoven, The Netherlands; [b])University of Pittsburgh Cancer Institute, Hillman Cancer Center, Pittsburgh, PA, USA

Mutations in the multifunctional nucleotide excision repair/transcription-coupled repair (NER/TCR) helicases XPB and XPD are associated with an extreme clinical heterogeneity, ranging from xeroderma pigmentosum (XP) characterized by skin cancer predisposition to XP combined with Cockayne syndrome (CS) and trichothiodystrophy (TTD), both featuring early neurodevelopmental abnormalities. Defects in the NER/crosslink repair endonuclease, ERCC1/XPF, cause XP or XP with multi-system dysfunction. Mouse models have provided important insights into the complex genotype-phenotype relationship. XPD^{TTD} mice, with a partial defect in both NER and TCR are only moderately cancer-prone but exhibit wide spread premature ageing symptoms. $XPD^{XP/CS}$ mutant mice are highly predisposed to cancer, with a milder ageing phenotype. Complete repair deficiency in TTD x XPA mice aggravates many premature ageing symptoms, reducing life span to ~3 weeks. Mutations in the ERCC1 gene induce a distinct set of accelerated ageing features (liver and kidney failure), which occur at 14 months, 4 months or 3 weeks depending on severity of the mutation. The correlation between repair defect severity and clinical manifestation provides strong evidence for the DNA damage theory of ageing. We propose that endogenous oxidative lesions compromise

transcription, inactivate genes, and trigger apoptosis and possibly senescence inducing ageing. Very cytotoxic interstrand cross-links may also cause cell death, senescence and features of ageing. In contrast, lesions or defects in genetic stability mechanisms causing enhanced levels of DNA damage-induced mutagenesis correlate with increased carcinogenesis. Various single and double mutant mouse models including conditional mutants have been generated and microarray analysis performed to study these processes in a controlled, systematic manner.

Poster No. V-1

DYNAMIC REGULATION OF BRCA2 FOR RECOMBINATIONAL REPAIR

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Inherited mutations in *BRCA2* are associated with a predisposition to early-onset breast cancers. The underlying basis of tumourigenesis is thought to be linked to defects in DNA double-strand break repair by homologous recombination, as indicated by the spontaneous chromosomal instability phenotype of *BRCA2*-defective cell lines. We found that the C-terminal region of BRCA2, which interacts directly with the essential recombination protein RAD51, contains a site (S3291) that is phosphorylated by cyclin-dependent kinases (CDKs). Phosphorylation of S3291 is low in S-phase when HR is active, but increases as cells progress towards mitosis. The modification of S3291 blocks C-terminal interactions between BRCA2 and RAD51. However, DNA damage overcomes cell cycle regulation by reducing S3291 phosphorylation and stimulating interactions with RAD51. These results indicate that S3291 phosphorylation provides a molecular switch that can regulate RAD51 recombination activity, providing new insight into why BRCA2 C-terminal deletions lead to radiation sensitivity and cancer predisposition

Poster No. V-2

REPAIR AT DNA REPLICATION FORKS AND A TREATMENT FOR BRCA2 TUMOURS

Helleday T[1,2], Al-Minawi A[1], Bryant HE[1], Cassel TN[1], Curtin NJ[3], Dziegielewski J[1], Flower D[1], Gottipati P[1], Kumari A[1], Kyle S[3], Lopez E[1], Radillo AL[1], Lundin C[2], Mclachlan J[1], Meuth M[1], Muftic D[2], Parker KM[1] Renglin-Lindh A[2], Saleh-Gohari N[1], Savolainen L[2], Schultz N[2], Sleeth K[1], Thomas HD[3]; [1]The Institute for Cancer Studies, University of Sheffield, Sheffield, UK. [2]Department of Genetics Microbiology and Toxicology, Stockholm University, Stockholm, Sweden. [3]Northern Institute for Cancer Research, University of Newcastle upon Tyne, UK.

DNA repair and damage response pathways are activated on encountering replication roadblocks in mammalian cells. Here, we report of the DNA structures formed at stalled replication forks, following processing with Holliday Junction resolvases *in vivo* and *in vitro*. We report of homologous recombination (HR) pathways and of a repair pathway including the poly(ADP-ribose) polymerase (PARP) involved in replication repair. We propose that, in the absence of PARP-1, spontaneous single strand breaks collapse replication forks and trigger HR for repair. We further show that BRCA2 deficient cells, as a result of their deficiency in HR, are acutely sensitive to PARP inhibitors, presumably because resultant collapsed forks are no longer repaired. Thus, PARP-1 activity is essential in HR deficient BRCA2 mutated cells. We exploit this requirement to specifically kill BRCA2 deficient tumours by PARP inhibition alone. Treatment with PARP inhibitors is likely to be highly tumour specific since only the tumours (which are $BRCA2^{-/-}$) in the $BRCA2^{+/-}$ patients are completely defective in HR. The use of an inhibitor of a DNA repair enzyme alone, in the absence of an exogenous DNA damaging agent, to selectively kill a tumour represents a new concept in cancer treatment.

Poster No. V-3

NPM AND PML INTERACT IN DNA DAMAGED CELLS

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Promyelocytic leukemia protein (PML) and nucleolar phosphoprotein nucleophosmin (NPM, B23) are both regulators of the activity and stability of tumor suppressor protein p53. We have recently reported that in response to UV damage PML and NPM interact with Hdm2, a negative regulator of p53, leading to p53 stabilization, and that these events associate with subcellular redistribution of both PML and NPM (1,2). Here we identify a novel interaction between NPM and PML and a cascade of cellular UV damage induced events leading to p53 stabilization. We show that NPM and PML interact shortly after UV damage and that they colocalize in the perinucleolar compartment of the nucleoli. Expression of PML forces NPM nucleoplasmic translocation. However, the UV induced translocation of NPM as well as p53 stabilization in *Pml-/-* murine embryo fibroblasts was indistinguishable of wild type cells. Thus, NPM is essential for p53 stabilization following UV damage, while PML is dispensable. Further studies on the function of NPM and other nucleolar proteins in the UV damage response will be discussed.

References:

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Poster No. V-4

CORRELATIONS BETWEEN CLINICAL NORMAL TISSUE RADIOSENSITIVITY AND SINGLE NUCLEOTIDE POLYMORPHISMS IN ATM, XRCC1, XRCC3, APEX, SOD2, AND TGF-B1.

Alsner J, Andreassen CN, Overgaard M & Overgaard J; Department of Experimental Clinical Oncology, Aarhus University Hospital, Aarhus, Denmark

Several observations indicate that normal tissue radiosensitivity may have a genetic basis. In this study we investigated whether 8 selected single nucleotide polymorphisms (SNPs) in 6 candidate genes affect the risk of radiation-induced subcutaneous fibrosis. The 41 patients included in this study were given post-mastectomy radiotherapy between 1978 and 1982. SNPs in *ATM*, *XRCC1*, *XRCC3*, *APEX*, *SOD2*, and *TGF-B1* were analyzed and dose-response curves were established in patients with different genotypes. The *ATM* codon 1853, *XRCC1* codon 399, *XRCC3* codon 241, *SOD2* codon 16 genotypes correlated with risk of fibrosis whereas the *APEX* codon 148 genotype did not. For *TGFB1*, the codon 10 genotype correlated with risk of fibrosis. Presumably through linkage disequilibrium, similar results were obtained for the *TGFB1* codon 25 and a promoter variant at position -509. Our data demonstrate that SNPs account for some of the variability in the occurrence of normal tissue reactions. This observation supports the assumption that clinical normal tissue radiosensitivity should be regarded as a complex trait dependent on the cumulative effect of variation in several genes. To fully address these questions, carefully designed clinical studies with an accrual of very large numbers of patients have been initiated (the ESTRO GENEPI project, Radiother. Oncol. 2003; 69:121-125).

Poster No. V-5

PARP INHIBITORS AS A TREATMENT FOR BRCA2 ASSOCIATED TUMOURS

Bryant H[1], Flowers D[1], Schultz N[2], Curtin N[3] & Helleday T [1,2]; [1]The Institute for Cancer Studies, University of Sheffield, Sheffield, UK; [2]Dept of Genetic and Cellular Toxicology, Stockholm University, Sweden; [3]Northern Institute for Cancer Research, University of Newcastle upon Tyne, Newcastle upon Tyne, UK

Germline mutations in BRCA2 predispose individuals to breast, ovarian and other cancers, in addition cells containing BRCA2 mutations exhibit large spontaneous and induced chromosomal rearrangements and have enhanced mutation rates. The main defect leading to this genomic instability and thus predisposition to cancer in BRCA2 deficient cells is suggested to be in Homologous Recombination (HR) mediated DNA repair. Poly(ADP-ribose) Polymerase (PARP-1) binds DNA breaks and plays a role in maintaining genome stability.

Inhibitors of PARP are non-toxic but can enhance the cytotoxicity of cytostatic drugs. Here, we show that BRCA2 deficient cells, as a result of their defect in homologous recombination (HR), are acutely sensitive to PARP-1 inhibitors without co-treatment with any DNA damaging agent. Furthermore, we show that a PARP-1 inhibitor alone is sufficient to kill BRCA2 deficient tumors, but not BRCA2 proficient tumors in xenografts, suggesting that PARP-1 inhibitors work as specific treatment for BRCA2 deficient tumors.

We demonstrate that in normal cells lesions are formed following PARP-1 inhibition that are repaired by HR and are therefore non-toxic to cells. However, BRCA2 deficient cells fail to repair the lesions, which could account for the sensitivity of these cells. We will further discuss the mechanism behind this treatment and the clinical developments that have taken place.

Poster No. V-6

RESPONSE TO 5-FLUOROURACIL (5-FU)-INDUCED DNA DAMAGE: H2AX PHOSPHORYLATION AND APOPTOSIS IN HCT116 PARENTAL AND 5-FU-RESISTANT COLON CANCER CELL LINES

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5-FU is one of the standard drugs used in chemotherapeutic regimens for metastatic colorectal cancer worldwide, despite the fact that resistance to 5-FU is a major obstacle to successful therapy. 5-FU is a DNA-damaging and apoptosis-inducing chemotherapeutic agent. Major areas of focus for investigations of the molecular mechanisms underlying resistance to such agents are alterations in DNA repair- and apoptosis-regulatory pathways. We have previously generated two 5-FU-resistant derivatives, ContinB and ContinD cell lines, from the parental HCT116 colon cancer cell line. These treatment derivatives have been extensively characterized for specific phenotypes, genotypes and altered gene expression that may be associated with the development of resistance (*De Angelis PM et al. Int J Oncol 24: 1279-88, 2004; De Angelis, PM et al. Mol Cancer 3: 11, 2004*). All cell lines had a wild-type *TP53* genotype. Compared to parental HCT116 cells, ContinB and ContinD cells were characterized by reduced growth inhibition and reduced apoptosis in response to short-term 5-FU exposure, and by up-regulation of apoptosis-inhibiting and DNA repair genes, and down-regulation of apoptosis-promoting genes in one or both treatment derivatives. DNA damage in cells exposed to 5-FU takes the form of double- and single-DNA strand breaks and this leads to a cell cycle arrest in S phase. Phosphorylation of the H2AX histone protein at serine 139 is triggered by the induction of double-strand breaks (DSBs). Phosphorylated H2AX (γH2AX) is thus regarded as an indicator of DSBs. The aims of the present study were: a) to determine whether exposure to 5-FU actually induced H2AX phosphorylation in these cell lines; b) to assess actual levels of 5-FU-induced H2AX phosphorylation; and c) to determine whether 5-FU-induced H2AX phosphorylation was associated with (eventual) apoptosis, i.e., were γH2AX-

positive cells destined for apoptosis or were they the cells that were capable of repair? We used an antibody against γH2AX and multiparameter flow cytometry to assess levels of this protein in response to 5-FU exposure. We also assessed levels of 5-FU-induced apoptosis in the same cells using flow cytometric DNA fragmentation and other apoptosis-related assays. Differential γH2AX levels, as well as differential levels of apoptosis, were demonstrated among the three HCT116 cell lines in response to 5-FU treatment. Results will be presented and discussed in the context of relevance to demonstrated 5-FU resistance phenotypes for these cell lines.

Poster No. V-7

GENETIC VARIATION IN THE REACTIVE OXYGEN SPECIES (ROS) SIGNALLING, DNA REPAIR AND ANTIOXIDANT ENZYMES (AOE) IN RELATION TO ADVERSE SIDE EFFECTS OF RADIATION TREATMENT

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Radiotherapy (RT) exerts its antineoplastic effect either by directly attacking cellular macromolecules or indirectly by generating reactive oxygen species (ROS) and subsequent by-products leading to the induction of antioxidant enzymes (AOE) and DNA-damage repair pathways. ROS in turn signal to the cell cycle and apoptosis. Therefore, the genes selected for genotyping in this study are all involved in regulating these pathways. We selected approximately 200 genes in which 1030 SNPs were genotyped. Of these 12 genes were involved in the cell cycle, 11 in apoptosis, 21 in DNA-repair and another 163 in regulating the redox-level of the cell. The selection of SNPs in our candidate genes was based on the following inclusion criteria: SNPs probable to influence function or expression levels (cSNPs and 5'-3' SNPs) and/or with known frequency in a Caucasian population. A total of 1496 patients were treated with radiation therapy, from1975 to 1986, receiving 4.3 Gyx10 (2 treatments per week) instead of 2.5Gyx20 (4 treatments per week) for 5 weeks administered to the breast wall and/or regional lymph nodes. Circumstantial evidence for strong adverse side effects of the high dose treatment (4.3Gyx10) accumulated over the following years, and in 1996 all patients still alive (289) were systematically evaluated and categorized in 4 groups according to the extent of damage. A total number of 243 patients took part in the evaluation and blood samples from 164 patients were obtained. 97 of these patients were genotyped with the described above high-throughput strategy. Some of the SNPs, in GSTP1, T1 and P1 as well as XRCC1 and APEX1 were genotyped by low throughput method in the total set of 164 patients. Of the total 1030SNPs, 725 (~70%) SNPs were successfully genotyped in a single attempt. Only 87 of the 725 successfully genotyped SNPs were singletons – this constitutes ~12% in this sample set. An additional 40 SNPS had a frequency below 1% and 38 SNPs were not in Hardy-Weinberg equilibrium (~5%). The distribution of the genotypes in the different damage groups were studied by parametric (ANOVA) and non-parametric (QMIS) methods and the observed associations will be presented. By combining the information on the adverse side effects with the patients' genotype profile will hopefully lead to markers that can be used to design successful therapy strategies in the clinics.

Poster No. V-8

INVOLVEMENT OF THE ATM 6903INSA MUTATION IN BREAST CANCER SUSCEPTIBILITY

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The ATM kinase is essential for the maintenance of genomic integrity. Loss of both *ATM* alleles results in ataxia-telangiectasia (A-T), a neuro-immunologic disorder exhibiting chromosomal instability, radiosensitivity and increased risk of cancer. Individuals heterozygous for A-T mutations have been reported to have an increased risk for breast cancer.

In this study we examined the prevalence of 6903insA, originally identified in two Finnish A-T families displaying accumulation of breast cancer, in a large cohort of both familial and unselected breast cancer cases. *ATM* 6903insA was observed in 9/1665 of the studied patients but not in 1107 healthy controls (p=0.014). A significantly elevated frequency was observed in the patients with positive family history 4/541 (p=0.012), whereas for the unselected cases the difference did not quite reach statistical significance 5/1124 (p=0.062). Interestingly, the 6903insA allele showed a geographical clustering to the Tampere region and a common founder for all identified carriers was confirmed by haplotype analysis. Regionally the 6903insA allele contributes to a significant portion of the studied familial breast cancer cases (2.4%, 4/168, p=0.004) compared to geographically matched controls (0/508).

Functional analysis of the 6903insA germline defect demonstrates that even though 6903insA mRNA is not subjected to nonsense mediated decay, it does not produce any stable protein product. Consequently, in heterozygous individuals the total amount of endogenous ATM is reduced to half. This seems sufficient for normal function of the ATM checkpoint signaling pathway, but not to ensure normal level of cell survival after IR-induced damage; lymphoblast cell lines of 6903insA carriers proved highly radiosensitive similar to A-T patients. As the tumor of 6903insA carrier showed no evidence for the loss of the wild-type allele, our results suggest that *ATM* haploinsufficiency is sufficient to promote tumorigenesis.

Poster No. V-9

DEFECTIVE DNA DAMAGE CHECKPOINT PARHWAYS IN PRIMARY PROSTATIC EPITHELIAL CELLS

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Prostate cancer is the most common noncutaneous malingnancy in men and is multifocal by its nature. These factors suggest for a defect in the DNA damage and checkpoint control pathways of the prostate. Detailed studies on the cellular responses of primary cultures of human prostatic epithelial cells (HPEC) to different types of DNA-damage (γ-IR and UVC) were carried out. We identified two defects in IR responses of the HPEC cells, one involving suppression of p53 pathway, the other diminished cdk2Tyr15 phosphorylation resulting in defects in the intra-S checkpoint. Both of these defects will be discussed in detail. In contrast to other cell types, wild-type p53 protein is not stabilized in HPECs in response to ionizing radiation (γ-IR). In the absence of p53 stabilization, transcriptional targets of p53 such as p21 and Hdm2 are not induced, and prostatic epithelial cells failed to undergo cell cycle arrest or apoptosis. As comparison we treated HPECs with UVC-radiation, and measured cellular responses, p53 accumulation and its activity. The results showed that HPEC responded to UVC by undergoing delayed apoptosis. In response to UVC, the levels of p53 increased, but p21 was not induced and p53 lacked transcriptional activity as measured by p53-reporter assays. Furthermore, the activity of p53 was not essential for UVC-induced apoptosis since HPV/E6 transfected HPECs also underwent apoptosis following UVC-treatment. Our results show that HPEC harbor defects both in p53 activation and in the intra-S phase checkpoint pathways in response to DNA-damage. It is plausible that the apparent lack of DNA damage checkpoints after natural stress signals are related to the high incidence of cancer in the prostate.

Poster No. V-10

MODULATION OF WERNER SYNDROME PROTEIN FUNCTION BY A SINGLE MUTATION IN THE CONSERVED RQC DOMAIN

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Mutations in the human *RecQ3* gene result in a loss of Werner protein (WRN) expression and manifest as a rare premature aging disorder, Werner syndrome. Cellular and biochemical studies support the role of WRN, which has both 3′→5′ DNA helicase and 3′→5′ exonuclease activities, in DNA replication, DNA repair, recombination, and telomere maintenance. The conserved RecQ C-terminal (RQC) domain of WRN was previously determined to be the major site of interaction for DNA and other proteins. Thus, we have performed site-directed mutagenesis in the WRN RQC domain to determine which amino acids might be playing a critical role in the WRN protein function. Electrophoretic gel mobility shift assay (EMSA) results demonstrated that when WRN contained a mutation at K1016, WRN binding to fork and bubble DNA substrates was dramatically decreased. The importance of DNA interactions mediated by K1016 was confirmed in studies of WRN helicase variants, which showed that the K1016A mutation markedly reduced WRN helicase activities on fork, D-loop, and Holliday junction substrates. These results suggest that the strength of WRN-DNA interaction modulates the helicase activity of WRN. Moreover, the K1016A mutation significantly reduced the ability of WRN RQC to stimulate FEN-1 incision activities, suggesting that the stimulation of FEN-1 by WRN requires proper DNA interaction by the WRN RQC domain. Our results emphasize the importance of proper WRN-DNA interaction not only for its proper helicase function but also for affecting catalytic activities of other WRN-interacting proteins. In addition to unraveling the mechanism of WRN-DNA interaction, the existence of numerous WRN interacting proteins suggest a role of WRN in a complex network of DNA metabolism. To address which pathway of DNA metabolism WRN might participate in most prominently, the magnitude of the protein-protein interaction between WRN and its binding partners were measured *in vitro* using the dot-far Western blot analysis. The results indicate that WRN binds with differential affinities to RPA, Ku, p53, Exo1, TRF2, FEN-1, VCP, DNA polymerase beta, PKA, Bloom, Topoisomerase 1, PARP1 and PCNA.

Poster No. V-11

OXIDATIVE DNA DAMAGE PRODUCED BY THIOGUANINE BASES AND UVA LIGHT AS A RISK FACTOR FOR SKIN CANCER IN THERAPEUTICALLY IMMUNOSUPRESSED PATIENTS

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The formation of mutagenic DNA lesions by reactive oxygen species (ROS) is an inexorable consequence of oxidative metabolism. Oxidative stress and an increased level of oxidative DNA damage is linked to human diseases, including cancer. Thus, clinical treatments that induce chronic oxidative stress may carry a significant risk for therapy-related cancer. Here we report that immunosuppression by azathioprine may be one such treatment. In the body, azathioprine is converted to 6-mercaptopurine (6-MP). This is metabolized to 6-thioguanine nucleotides, which are DNA precursors. High levels of DNA substitution by 6-TG cause cell death in a process that depends on the cell's mismatch

repair (MMR) system. Lower levels of DNA 6-TG, similar to those found in cells from patients treated with azathioprine or 6-MP, are tolerated, however.
UVA light of wavelengths between 320nm and 400nm comprises approximately 95% of the solar energy reaching the earth's surface. Unlike the normal DNA bases, 6-TG absorbs strongly in the UVA region (maximally at 342nm) and can undergo photochemical reactions. We demonstrate that biologically relevant doses of UVA light generate ROS in cells containing DNA 6-TG and that the treatment is both toxic and mutagenic. DNA 6-TG is itself a target of oxidation by ROS and guanine sulfonate is identified as the major DNA photoproduct in UVA treated 6-TG DNA. This novel photoproduct blocks replication *in vitro* but can be bypassed by mutagenic Y-family polymerases. Azathioprine-treated patients display an abnormal cutaneous photosensitivity to UVA consistent with the formation of analogous DNA photoproducts in their skin cells. These photochemical reactions of DNA 6-TG have implications for the development of skin cancer in immunosuppressed patients and in particular for the huge incidence of squamous cell carcinoma in long-term survivors of organ transplantation.

Poster No. V-13

FUNCTIONAL AND PHYSICAL INTERACTION BETWEEN WERNER SYNDROME PROTEIN AND MISMATCH REPAIR PROTEINS

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Werner syndrome is a rare autosomal recessive disorder characterized by premature aging and increased incidence of cancer. The gene defective, *WRN*, encodes a protein (WRN) which has 3'-5' exonuclease and ATP-dependent 3'-5' DNA helicase activities. WRN has been reported to play a role in DNA replication, repair and recombination pathways. In addition to its role in the post-replicative mismatch repair (MMR), the yeast Msh2/Msh6 heterodimer prevents recombination between moderately divergent sequences during repair of DNA double-strand breaks by single-strand annealing. The yeast WRN homologue SGS1 is also required for heteroduplex rejection during single-strand annealing, suggesting that this reaction occurs through unwinding heteroduplex recombination intermediates. The studies in yeast encouraged us to explore the possibility that the MMR proteins co-operate with WRN in suppression of aberrant recombination events. We have observed that MSH2/MSH6 and MSH2/MSH3 heterodimers, but not MLH1/PMS2, significantly stimulated WRN-mediated unwinding of synthetic 3'-flap structures that mimic recombination intermediates formed during the single-strand annealing pathway. In addition to the functional interactions, by using GST pull-down assays we found that WRN interacts with three heterodimers of the MMR machinery, MutLα, MutSα and MutSβ *in vitro.* Furthermore*,* the coimmunoprecipitation assays showed that PMS2/MLH1 (MutLα) interacts with WRN in the cell. Interestingly, we also observed the colocalisation between WRN and MLH1 after exposure to γ-irradiation. In conclusion, our results suggest that WRN and MLH1 may act together in recombination events to prevent abnormal chromosomal rearrangements.

Poster No. V-14

COMPARISON OF APOPTOTIC SIGNALING IN RESPONSE TO LOW-AND HIGH LET RADIATION IN A PAIR OF NON SMALL LUNG CARCINOMA CELL LINES

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Non-small cell lung carcinoma (NSCLC) tumours are difficult to cure as they often present resistance to both chemo- and radiotherapy. Among these tumours, an increased tumour response have been observed when patients are irradiated with high linear energy transfer (high-LET) radiation such as accelerated nitrogen or carbon ions or protons. However, the cellular signaling processes, including apoptotic signaling activated in response to such accelerated ions, have only partly been revealed. Here we have compared apoptotic signaling after low-and high-LET radiation (γ-IR and accelerated nitrogen ions respectively) in a pair of NSCLC cell lines. We report that high-LET accelerated nitrogen ions activates apoptotic signaling in a low-LET γ-IR resistant NSCLC cell line. This apoptotic response resulted from activation of stress-activated protein kinases, mitochondrial signalling and caspase-3 activation. Besides, the activation of c-Abl and ATM in response to low-and high LET radiation in NSCLC cells have also been examined and further discussed.

Poster No. V-15

MITOCHONDRIAL REPAIR OF OXIDATIVE DAMAGE AND CHANGES WITH AGING

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Reactive oxygen species (ROS) are formed in all living organisms as a by-product of normal metabolism (endogenous sources) and as a consequence of exposure to environmental compounds (exogenous sources). Endogenous ROS are largely formed during oxidative phosphorylation in the mitochondria, and therefore, mitochondrial DNA (mtDNA) is

at particularly high risk of ROS-induced damage. Mitochondria are essential for cell viability, and oxidative damage to mtDNA has been implicated as a causative factor in a wide variety of degenerative diseases, and in cancer and aging. Changes in mitochondrial function with age have been observed in several organisms and accumulation of DNA lesions in mtDNA with age may be an underlying cause for numerous age-associated diseases. We have used a number of different approaches to explore the mechanisms of DNA damage processing of the mtDNA, and we have been able to demonstrate that mammalian mitochondria efficiently remove e.g. 7,8-dihydro-8-oxoguanine (8-oxoG) and 7,8-dihydro-8-oxoadenine (8-oxoA) from their genome. Furthermore, we have demonstrated that cells from patients suffering from the segmental premature aging syndrome, Cockayne Syndrome, are deficient in mitochondrial repair of these lesions.

Poster No. V-16

COOPERATION OF THE COCKAYNE SYNDROME GROUP B PROTEIN AND PARP-1 IN THE RESPONSE TO OXIDATIVE STRESS

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Cockayne Syndrome (CS) is a rare genetic disorder characterized as a segmental premature aging syndrome. The CS group B (CSB) protein has previously been implicated in transcription-coupled repair, transcriptional elongation, and restoration of RNA synthesis after DNA damage. Recently, evidence has also accumulated for a role of CSB in base excision repair of oxidative DNA lesions. In our search to understand the molecular function of CSB in this process, we have identified a physical and functional interaction between CSB and poly(ADP-ribose) polymerase 1 (PARP-1). PARP-1 is a nuclear enzyme that protects the integrity of the genome by responding to oxidative DNA damage and facilitating DNA repair. After oxidative stress, PARP-1 binds to single strand DNA breaks, which activate the catalytic ability of PARP-1 to add polymers of ADP-ribose to various proteins. We identify CSB as a new substrate of PARP-1, and we find that poly(ADP-ribosyl)ation of CSB inhibits its DNA dependent ATPase activity. Furthermore, we have shown that CSB relocates to sites of active PARP-1 and thus DNA damage in cells after oxidative stress. We also find that CSB deficient cell lines are hypersensitive to the PARP inhibitors 3-AB and DPQ. Our results implicate CSB in the PARP-1 poly(ADP-ribosyl)ation response after oxidative stress and thus suggest a novel role of CSB in the cellular response to oxidative damage.

Poster No. V-17

MUTATION ANALYSIS OF THE TOPBP1 GENE IN FINNISH FAMILIES WITH HEREDITARY SUSCEPTIBILITY TO BREAST AND/OR OVARIAN CANCER

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The TOPBP1 protein was originally identified through its interaction with DNA topoisomerase IIβ and is required for DNA damage and replication checkpoint control. TOPBP1 displays sequence homology as well as functional similarities with BRCA1, and the two proteins have been suggested to function partly in the same cellular processes. Based on its biological significance, we reasoned that *TOPBP1* is a plausible susceptibility gene in familial breast and/or ovarian cancer. To investigate this possibility, index cases from 125 Finnish cancer families were screened for germline alterations. The analysis revealed altogether 21 sequence variants, ten of which occurred in exon and eleven in intron regions. Three novel alterations affecting protein-encoding regions were detected, two of which were classified as neutral polymorphisms. However, one of the novel exonic variants, leading to a non-synonymous change, was observed at elevated frequency in the studied cancer family material compared to healthy controls (p=0,002), and was therefore considered to be a possible disease-related alteration. To evaluate the possible functional consequences of this amino acid substitution, TOPBP1 expression and checkpoint activation studies are being performed in lymphoblastoid cell lines from heterozygous and wild-type individuals. This is to our knowledge the first study reporting the mutation screening of *TOPBP1* in a familial cancer material.