

Oral Presentations

**Session 1: Poly(ADP-ribose) metabolism
and base excision repair**

Poly(ADP-ribose) polymerase-2: from the structure to the function.

Gilbert de Murcia

Unité 9003 du CNRS, Ecole Supérieure de Biotechnologie de Strasbourg, Boulevard Sébastien Brant, BP 10413, 67412 Illkirch Cedex, France.

Five years ago, we isolated a cDNA encoding a 62 kDa protein sharing considerable homology with the catalytic domain of PARP-1. The assumption that we were dealing with a novel PARP enzyme was supported by the presence of a residual PARP activity in PARP-1 KO cells (Shieh et al. 1998, *J. Biol. Chem.* 273, 30069), the discovery in *A. thaliana* of a gene coding for a PARP related polypeptide of a calculated mass of 72 kDa (Doucet-Chabeau et al. 2001, *MGG*, 265, 954) and by the identification of two PARP homologues: one present at telomeres (Tankyrase 1; Smith et al. 1998, *Science*, 282, 1484) and a second present in Vault particles (VPARP; Kickhoefer et al. 1999, *J. Cell Biol.* 146, 917). The protein encoded by this new cDNA was therefore named PARP-2.

We now know that poly(ADP-ribose) polymerases (PARPs) constitute a large family of 18 proteins, encoded by different genes and displaying a conserved catalytic domain. PARP-1, the founding member and PARP-2 its closest relative are so far the sole enzymes whose catalytic activity is immediately stimulated by DNA discontinuities.

This review summarizes the data obtained from molecular and genetic approaches developed in our laboratory to better understand the structure-function relationship of PARP-2.

Supported by CNRS, Association pour la Recherche contre le Cancer, Ligue contre le Cancer, Electricité de France, and Commissariat à l'Energie Atomique

References:

- Amé et al. 1999, *J. Biol. Chem.* 274, 17860
- Amé et al. 2001, *J. Biol. Chem.* 276, 11092
- Schreiber et al. 2002. *J. Biol. Chem.* 277, 23028
- Ménissier-de Murcia et al. 2003, *EMBO J.*, 22, 2255
- Dantzer et al. 2004, *Mol. Cell. Biol.* 24, 1595
- Oliver et al. 2004, *Nucleic Acids Res.* 32, 456
- Amé et al, 2004, *BioEssays*, in press

Inhibition of PARP activity leads to reversible telomere shortening

S. Beneke(p)(1,2), A. Bürkle(1), and P.Boukamp(2)

(1) Lehrstuhl Molekulare Toxikologie, Universität Konstanz

(2) Abt. Genetik der Hautkarzinogenese, DKFZ Heidelberg

Telomeres are repetitive sequences protecting the ends of chromosomes against degradative and inappropriate repair processes. This is achieved by the specific t-loop structure, in which the 3'-overhang folds back and invades the double-stranded region, thus hiding the single-stranded stretch of DNA from recognition by the repair machinery. Important proteins involved are the telomeric repeat binding factors TRF1 and 2. Every replication round leads to a loss of 50 to 150 bp at the very 5'-end of chromosomal DNA, due to degradation of the initial RNA primer. This is counteracted in germline and stem cells, as well as most tumor cells, by telomerase, a reverse transcriptase carrying a RNA-encoded telomeric template. But evidence is accumulating that the t-loop has to be transformed to an open state to facilitate access of telomerase. This is achieved at least in part by members of the poly(ADP-ribosyl) polymerase (PARP) family, enzymes involved in genomic integrity maintenance. Tankyrases 1 and 2 and the repair PARPs 1 and 2 modify TRF1 and TRF2, respectively. In our investigation of the role of poly(ADP-ribosyl)ation at telomeres, we observed that PARP inhibition mediated by 3-aminobenzamide in hamster or HeLa cells leads to a rapid but reversible decline in telomere length in a dose-dependent fashion, as detected by quantitative fluorescence in-situ hybridization (Q-FISH). This was also evident by suppression of PARP activity in hamster cells in a dominant negative approach. We detected a telomere loss of about 300 bp per cell division, and therefore propose that the loss of poly(ADP-ribose) formation triggers an active degradation process at telomeres. Our observations may have implications for carcinogenesis as well as for the ageing process.

Mammalian base excision repair

G. Dianov (p), I. Dianova, J. Parsons

MRC Radiation and Genome Stability Unit, Harwell, Oxfordshire, OX11 0RD, UK

Base excision repair (BER) is a major pathway for the removal of simple lesions in DNA including base damage and base loss (abasic site). BER requires the coordinated action of several repair and ancillary proteins, the impairment of which can lead to genetic instability. Several current models account for molecular events during BER, considering different sequential order and protein complexes involved, however the precise molecular dynamics of BER proteins on damaged DNA is unclear. Using formaldehyde crosslinking during repair in human cell extracts, we followed BER proteins involved in repair of a substrate containing a site-specific abasic site in DNA. We find that after incision of the abasic site by AP endonuclease, poly(ADP-ribose) polymerase binds to the incised abasic site followed by a transient complex of DNA polymerase β and DNA ligase III α -XRCC1 heterodimer. After accomplishing its function, DNA polymerase β dissociates from the complex, however, DNA ligase III α -XRCC1 heterodimer remains bound to the repair intermediate until ligation is accomplished. This approach allows visualisation of real time protein dynamics on damaged DNA during BER in human cell extracts and provides a detailed view of the molecular events leading to repair.

Poly(ADP-Ribose) Metabolism in DNA Base Excision Repair

S.L. Oei (p), T. Gröbe, C. Keil, and E. Petermann

Institut für Biochemie, Freie Universität Berlin, Thielallee 63, D-14195 Berlin, Germany

Multiple mechanisms have evolved to restore the integrity of the genome following damaging of DNA. One immediate reaction to the occurrence of DNA breaks is poly(ADP-ribosyl)ation, a modification of proteins with poly(ADP-ribose), catalysed mostly by Poly (ADP-ribose) polymerase-1 (PARP-1). This post-translational reaction is functionally associated with the stimulation of DNA base excision repair (BER). BER is conducted by a multi enzyme complex including DNA polymerase beta (Pol beta), DNA ligase III (Lig III), PARP-1, and further enzymes, assembled around the scaffold protein XRCC1. Previously we have demonstrated that the decision between two BER subpathways is governed by the cellular ATP concentration and includes the regulation of Pol beta and PARP-1 activities [1]. While in single-nucleotide BER only the damaged nucleotide is substituted by Pol beta, in long-patch BER a DNA stretch of up to 8 nucleotides is exchanged, accompanied by the transient occurrence of poly(ADP-ribose) synthesized by PARP-1. Employing a reconstituted BER complex, we present evidence that Lig III and XRCC1 together are essential mediators of this regulation. Further analysis revealed that adenylation of Lig III is the crucial step in the switch between Pol beta –dependent single-nucleotide and long-patch BER. In another investigation the cDNA of the human enzyme responsible for degradation of poly(ADP-ribose), the poly(ADP-ribose) glycohydrolase (PARG), was cloned, overexpressed and purified to perform studies of protein-protein interaction [2]. An interaction between immobilized PARG and endogenous PARP-1 from HeLa cell extracts could be observed. From further analysis we suppose that PARG is yet another component of the BER machinery.

[1] Petermann, E., Ziegler, M. & Oei, S. L. (2003) ATP-dependent selection between single nucleotide and long patch base excision repair. *DNA Repair* 2, 1101-1114.

[2] Keil, C., Petermann, E. & Oei, S. L. (2004) Tannins elevate the level of poly(ADP-ribose) in HeLa cell extracts. *Arch Biochem Biophys*, 425, 115-121.

DNA-*N*-Glycosylase Deficient Mice.

Rhoderick H. Elder

Cancer Research UK Carcinogenesis Group, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Wilmslow Road, Manchester. M20 4BX. UK.

A cell's first line of defence against DNA base damage, base excision repair comprises a battery of DNA-*N*-glycosylases that release a variety of chemically modified bases from DNA. Base damage can arise through spontaneous hydrolytic deamination, interaction with endogenous reactive oxygen species and reaction with exogenous chemicals, such as those used in alkylating agent chemotherapy. In our lab, we have generated several novel strains of mice lacking specific DNA-*N*-glycosylases involved in the release of both alkylated, and oxidatively damaged bases from DNA. However, contrary to exhibiting various detrimental effects of the gene disruption, the different DNA-*N*-glycosylase deficient mouse strains have proved to be remarkably resilient to the loss of the major activities that catalyse the removal of altered bases from DNA. Indeed, with a few exceptions, there is little evidence for the accumulation of endogenously produced oxidised bases in tissues and organs of the glycosylase null mice, a finding that is highly suggestive of hitherto unknown backup mechanisms for dealing with the removal of oxidative base damage from genomic DNA. Recently, we have generated a mouse strain deleted in 8-oxoguanine-DNA-glycosylase (OGG1) and NTH1, DNA-*N*-glycosylase/lyases involved in the release of 8-oxoguanine and oxidised pyrimidines respectively. As with the single knockout strains, these mice exhibit no outward abnormalities and murine embryonic fibroblasts do not show an increased sensitivity to oxidative stress. However, incision assays using oligonucleotides containing a single oxidised pyrimidine base, indicated the presence of one or more compensatory DNA glycosylase activities in cell extracts from the null mice. While subsequent partial protein purification by ion-exchange chromatography revealed the presence of two polypeptides with DNA glycosylase/lyase activity, the activity of both polypeptides could be specifically inhibited by antibodies raised against mNEIL1 and the antibodies bound both polypeptides following western blotting. Thus, with the exception of NEIL1, we have found no evidence of other, compensatory DNA-*N*-glycosylases in either whole cell extracts, or fractionated nuclear and mitochondrial extracts. Furthermore, while the expression of NEIL1, NEIL2 and NEIL3 transcripts was detected in various organs by reverse-transcriptase PCR, no increased expression was seen in the double knockouts that might compensate for the lack of OGG1 and NTH1. Thus, the biological consequences of increased levels of DNA base damage have still to be fully addressed. In this respect, the application of RNAi techniques in cell culture systems and mouse models may provide a more rapid means of determining the true protective effect of DNA-*N*-glycosylases *in vivo*.

In-vivo-Selection of Genetically Corrected Cells by MGMTP140K-Expression

U.R.Sorg (p)(1), V.Kleff,(1) G.Nagel(2), B. Opalka(1), M.Flasshove(1), B.Kaina(2), T.Moritz(1)

(1) Dept. of Internal Medicine (Cancer Research), University of Duisburg-Essen, Germany

(2) Institute of Toxikology, Division of Applied Toxicology, University of Mainz, Germany

O6-methylguanine-DNA-methyltransferase (MGMT) protects cells from the toxicity of O6-alkylating drugs such as BCNU, or temozolomide. Additionally, MGMT gene transfer into hematopoietic stem cells reduces the dose-limiting hematotoxic side effects of these antineoplastic agents. This strategy also allows stable selection of genetically modified cells, rendering MGMT an attractive candidate gene to enrich for genetically corrected cells in the context of gene therapy. This applies in particular to diseases in which the therapeutic gene does not confer a selective advantage to corrected cells. We have evaluated this strategy utilising the O6-benzylguanine (BG)-resistant MGMTP140K-mutant in combination with the EGFP reporter gene or the gene coding for the IL-3 receptor-superfamily common- β -chain, deficiency of which causes pulmonary alveolosis. Since the position of a gene within a vector (e.g. upstream/downstream of an internal ribosomal entry site; IRES) is critical for protein expression and the better upstream position will be required for the therapeutic gene, we have investigated vectors, expressing MGMTP140K downstream of IRES. In comparison to control constructs (MGMTP140K upstream of IRES), comparable MGMTP140K-activity resulting in functional protection from O6-alkylator-induced toxicity was observed in fibroblast cell lines. In addition, in a murine gene transfer/chemotherapy model efficient and stable enrichment of transgenic granulocytes (40,9% versus 71,8%, before versus 5 weeks after CTX) as well as lymphocytes (49,8% versus 77,6%, respectively) was demonstrated in the context of combined BG/TMZ chemotherapy when MGMTP140K was expressed downstream of IRES. So far MGMT activity was analysed in animals expressing MGMT upstream of the IRES with mean values of 955 versus 82 fmol/mg in spleen and 693 versus <50 fmol/mg in bone marrow cells of MGMTP140K-transduced versus control animals. However, EGFP-expression data in human CD34+ progenitor/stem cells and murine granulocytes and lymphocytes indicate substantial effects of transgene positioning in primary hematopoietic cells, therefore, this is currently investigated for MGMT.

**Session 2: DNA lesion processing
and bypass**

The genome cleaning company: synthesizers, proofreaders, sensors and trimmers

Ulrich Hübscher

Institute of Veterinary Biochemistry and Molecular Biology, University of Zürich, 8057 Zürich, Switzerland

In the last few years, numerous studies suggested a tight implication of DNA replication factors in several DNA transaction events that maintain the integrity of the genome. This is illustrated by the numerous factors that on the one hand have a well-defined function at the DNA replication fork, but on the other also play crucial roles in different DNA repair pathways such as base excision repair (BER), nucleotide excision repair (NER), double strand break repair (DSBR), and mismatch repair (MMR) pathways. Data will be presented suggesting that *synthesizers* (DNA polymerases, pols), *proofreaders* (3'→5' exonucleases), *sensors* (the checkpoint clamp complex Rad9/Rad1/Hus1, the checkpoint clamp loader complex Rad 17/RF-C2-5) and *trimmers* (flap endonuclease 1) contribute to the quality control of DNA synthesis in different DNA transactions. In particular (i) it will be shown that pols from the X family have been conserved from the radioresistant bacterium *Deinococcus radiodurans* to human and are likely involved in DSBR (Leçointe et al., 2004); (ii) novel properties of pol λ , pol μ , and TdT will be presented (Ramdan et al., 2004); (iii) the interaction of pol β , another pol X family member, with the checkpoint clamp complex Rad9/Rad1/Hus1 will be documented (Touaille et al., 2004). Finally (iv), the novel tumour suppressor flap endonuclease 1 can trim flap structures during DNA transactions. The detailed mechanism of its action appears to be conserved in evolution from Archaea to human (Friedrich-Heinecken and Hübscher, 2004).

Friedrich Heinecken, E. and Hübscher, U.: *Nucl. Acids Res.*, 32, 2520-2528, 2004

Ramadan, K., Shevelev, I.V., Maga, G. and Hübscher, U.: *J. Mol. Biol.*, in press, 2004

Shevelev, I.V., Lecointe, F, Bailone, A., Sommer, S., and Hübscher, U.: *Mol. Microbiol.*, in press, 2004

Touaille, M., El-Andaloussi, N., Frouin, I., Freire, R., Shevelev, I.V., Friedrich-Heinecken, E., Villani, G., Hottiger, M. and Hübscher, U. : *Nucl. Acids. Res.*, in press, 2004

Induction of flap endonuclease 1 (fen1) by UV-C light enhances recovery from the DNA replication block

M. Christmann (p), M.T. Tomicic-Christmann, J. Origer and B. Kaina

Institute of Toxicology, University of Mainz, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany

Mouse fibroblasts deficient in p53 are hypersensitive to the cytotoxic and genotoxic effects of ultraviolet (UV-C) light. Here we present data indicating that p53 deficient cells are unable to remove pyrimidine dimers from DNA; they also display a defect in the recovery from the UV-C induced DNA replication block. The phenotype of p53 deficient mouse fibroblasts cannot be explained by p53 dependent regulation of genes involved in NER or translesion synthesis, since RT-PCR did not reveal differential expression of these genes on basal level or upon UV-C exposure. To identify candidate genes correlated with the reported phenotypes, a microarray analysis was performed. It revealed the induction of flap endonuclease 1 (fen1). Beside microarray analysis, Fen1 induction by UV-C was also shown at the RNA level by RT-PCR and at the protein level by western blot analysis. The induction of Fen1 is dose- and time-dependent and requires p53 since it was found only in p53 wild-type but not in p53 null (p53^{-/-}) fibroblasts. Fen1 upregulation paralleled the increase in p53 protein level in replicating p53 wild-type cells. In non-replicating cells, Fen1 and p53 were not induced. Fen1 induction is due to transcriptional activation of the fen1 gene since actinomycin D completely inhibited UV-C induced upregulation of fen1 mRNA. In promoter experiments, we identified an active p53 binding site in the fen1 promoter, and in co-transfection experiments p53 was shown to stimulate the expression of a fen1 promoter-reporter construct. Transfection mediated over-expression of Fen1 in p53^{-/-} cells attenuated the UV-C light induced DNA replication block, supporting the view that Fen1 induction is involved in abolishing the genotoxic S-phase blockade. This is in line with the fact that Fen1 is required not only for BER, but also for the removal of Okazaki fragments during replication. Based on the reported data, we suggest a model for the role of Fen1 in the recovery of MEFs from the UV-C induced replication blockade.

Phenotype-Genotype Correlation of a Xeroderma Pigmentosum Variant Patient

S. Emmert (p)(1), H. Inui (2), C.S. Seitz (3), A. Gratchev (4), K. Zachmann (1), S. Goerdtt (4), C. Neumann (1), and K.H. Kraemer (2)

(1) Department of Dermatology, Goettingen University, von-Siebold-Strasse 3, 37075 Goettingen, Germany,

(2) Basic Research Laboratory, National Cancer Institute/NIH, Bldg. 37, Room 4002, Bethesda, Maryland 20892, USA

(3) Department of Dermatology, Wuerzburg University, Josef-Schneider-Strasse 2, 97080 Wuerzburg, Germany,

(4) Department of Dermatology, University Clinic Mannheim, Heidelberg University, Theodor-Kutzer-Ufer 1-3, 68135 Mannheim, Germany.

We examined the clinical, molecular, and genetic features of a newly diagnosed xeroderma pigmentosum variant (XPV) patient. XP3GO is a 33 y old Caucasian male who is not sun sensitive. Around puberty he noticed freckling in the UV-exposed areas of his skin. At age 22 he started to develop skin cancers including multiple primary melanomas, but also basal cell and squamous cell carcinomas. There is no known consanguinity of his parents. We established a XP3GO fibroblast strain and found normal DNA repair levels as reflected by normal host cell reactivation of a UV-treated luciferase gene-containing reporter plasmid. Post-UVC cell survival of XP3GO fibroblasts was normal, but was reduced in the presence of 2 mM caffeine, a hallmark of XPV cells. XPV cells are defective in polymerase ϵ . Western blot analysis revealed undetectable levels of polymerase ϵ protein. Mutational analysis of the polymerase ϵ gene showed a deletion of exon 6 in cDNA due to a homozygous deletion of an about 1.3 kb region in the genomic DNA that includes exon 6. This deletion leads to a severely truncated and potentially non-functional polymerase ϵ protein. The ensuing loss of effective translesional synthesis past UV photoproducts produces an increased tendency of XP3GO cells to incorporate incorrect bases in DNA that results in a skin cancer-prone phenotype.

Yeast Mph1 promotes sister chromatid interactions in a recombinational pathway for error-free bypass of DNA lesions

C. Rudolph, K. A. Schürer, M. Rudolph, and W. Kramer (p)

Dept. of Molecular Genetics and Preparative Molecular Biology, Institute for Microbiology and Genetics, University Göttingen, Grisebachstr. 8, D-37077 Göttingen, Germany

Yeast *mph1* mutants have a spontaneous mutator phenotype and are sensitive to MMS and 4-NQO. Genetic interaction studies strongly suggest that MPH1 is not involved in BER, NER or PRR. However, a hypostatic relation of *mph1* to homologous recombination mutants *rad51*, *rad52* and *rad55* was detected for both spontaneous mutator phenotype and DNA damage sensitivity. Analysis of heteroallelic mitotic recombination demonstrated that *mph1* mutants are not generally deficient in homologous recombination. In an assay to detect sister chromatid interactions by reversion of a 266 bp direct sequence duplication the frequency of reversions after 4-NQO treatment were reduced considerably in *mph1* mutants compared to wild type, whereas reversion frequencies after camptothecin treatment were less affected. In *mms4* mutants reversion frequencies were more reduced after camptothecin than after 4-NQO treatment. The *mms4 mph1* double mutant showed an even more pronounced reduction of reversions after camptothecin treatment. *mms4* and *mph1* are also synergistically sensitive to both 4-NQO and camptothecin. From these data we propose a pathway for information transfer from the sister chromatid for error-free bypass of polymerase blocking lesions that requires Mph1 and proteins from the homologous recombination machinery. Within this process, Mph1 may play a role in stabilization of D-loops created by Rad51 mediated strand invasion. For camptothecin induced run off ds ends, Mph1 may provide an alternative pathway for replication fork restoration via Holliday junction formation if the D-loop resulting from insertion of the ds end into the sister chromatid fails to be cleaved by Mms4/Mus81.

**Session 3: Crosslink repair and
nucleotide excision repair**

Chemical biology of mammalian nucleotide excision repair and interstrand crosslink repair

Orlando D. Schärer

Institute of Molecular Cancer Research, University of Zürich, August Forel Strasse 7, 8008 Zürich, Switzerland

We are combining chemical and biological approaches to study the molecular mechanisms underlying DNA repair. One pathway under investigation is nucleotide excision repair (NER), in which over 30 proteins cooperate to excise and replace damaged residues in DNA. We have investigated the mechanisms by which the two endonucleases involved in NER, XPG and ERCC1/XPF catalyze phosphodiester bond cleavage using synthetically modified substrates and site-directed mutagenesis of enzyme active site residues. We have established conditions that allow formation of stable complexes between the two endonucleases and substrates using appropriate enzyme or substrate modifications. Together with new tools to investigate the damage recognition step in NER, the architecture of such stable NER preincision complexes will be studied. A second pathway under investigation deals with DNA interstrand crosslinks (ICLs), which covalently link two strands of DNA and are formed by many chemotherapeutic agents. One of the limitations in the study of DNA interstrand crosslink repair in mammals has been the limited availability of defined ICL adducts. We have developed new synthetic methodology to overcome this limitation. Furthermore, we have developed a reporter assay to study ICL repair in living cells to study the roles of various genes in different ICL repair pathways.

Molecular analysis of the xeroderma pigmentosum group A protein function by site-directed mutagenesis

U. Camenisch (p) (1), R. Dip (1), H. Naegeli (1)

(1) Institute of Pharmacology and Toxicology, University of Zürich-Vetsuisse, Winterthurerstrasse 260, CH-8057 Zürich, Switzerland

Xeroderma pigmentosum group A (XPA) protein is absolutely required for both the global genome and transcription-coupled pathways of the mammalian nucleotide excision repair (NER) system. To examine the molecular function of XPA, we have subjected its putative DNA binding domain (located between amino acids 98 and 219) to site-directed mutagenesis. The mutant proteins were probed by comparing their affinity for different DNA substrates in electrophoretic mobility shift assays as well as their ability to complement human XP-A fibroblasts in a host cell reactivation assay. This experimental strategy led to the identification of several single amino acid changes that abolish binding to damaged DNA duplexes without affecting the repair complementation capacity in living cells. On the other hand, we identified an XPA double mutant that is characterized not only by the lack of binding to damaged duplexes in the mobility shift assay but also by the formation of abnormal, i.e. fast migrating complexes, with 4-way DNA junction molecules. Additionally, this double mutation confers a NER defect in the host cell reactivation assay. These results are in agreement with previous studies indicating that XPA protein is not a primary damage recognition subunit of the NER process. Instead, XPA protein appears to exert a unique function as an architectural subunit that induces or stabilizes a particular deformation of the DNA substrate, thereby tagging a transient NER intermediate for cleavage by structure-specific endonucleases.

Human replication protein A functions as a central switch in cellular DNA metabolism

Nasheuer, H.P.(p)(1,2), Klaus Weisshart, K.(2), Pestryakov, P. E.(3), and Chen, S. (2)

(1) National University of Ireland, Galway; Dept. of Biochemistry, Galway, Ireland

(2) Abt. Biochemie, IMB Jena, Beutenbergstr. 11, D-07743 Jena, Germany

(3) Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of Russian Academy of Sciences, 630090 Novosibirsk, Russia

The replication protein A (RPA) has multiple functions in the eukaryotic DNA metabolism and consists of the three subunits p70, p32 and p14. In order to determine specific functions of the subunits, we have expressed the trimeric complex as well as dimeric complexes consisting of p70 and either of the smaller two subunits and the large subunit p70 alone. The dimeric complexes and p70 were sufficient for the bidirectional unwinding of double-stranded DNA. The dimeric complexes were able to bind to single-stranded DNA (ssDNA), but their DNA binding-modes and affinities were different from that of the trimeric complex. These data suggest that the co-operation of all subunits together allows efficient binding and correct positioning of the RPA complex on ssDNA, which is necessary for RPA to support DNA repair and DNA replication processes.

To study role of RPA in cellular DNA metabolism a new monoclonal antibody RAC-1, which binds the C-terminus of p70, was established and compared with monoclonal antibody 70B, which recognises the N-terminus of p70. Using these antibodies we found that at least two immunologically distinct populations of RPA exist in human cells, called RAC-1-RPA and 70B-RPA. They differ first in their phosphorylation state, second in their cellular distribution, third in their colocalisation with BrdU during DNA replication and DNA repair, and fourth in their interactions with nuclear proteins. Our findings suggest that 70B-RPA has a function during the initiation of DNA replication whereas RAC-1-RPA plays a role in the regulation of DNA synthesis processes. RAC-1-RPA sequesters DNA polymerases delta and epsilon to nuclear structures whereas 70B-RPA preferentially binds to DNA polymerase alpha.

Interference with NER and changes in p53 conformation by soluble and particulate cadmium compounds

T. Schwerdtle (p) and A. Hartwig

TU Berlin, Institute of Food Technology and Food Chemistry, Gustav-Meyer Allee 25, 13355 Berlin, Germany

The present study aims to compare the genotoxicity of particulate CdO and soluble CdCl₂ in human lung cells as primary targets of metal induced carcinogenicity. As a model for nucleotide excision repair (NER) the removal of benzo[*a*]pyrene-induced DNA adducts was measured by a highly sensitive HPLC/fluorescence assay. Both CdCl₂ and CdO inhibited the repair of the induced adducts in a dose-dependent manner at non-cytotoxic, environmentally relevant concentrations up to about 40 and 60 %, respectively. This repair inhibition started at 10 μM CdCl₂ and 0.2 μg/cm² CdO, concentrations, where both compounds showed no induction of oxidative DNA damage after 24 h incubation.

For cadmium, proteins with zinc binding motifs like the NER zinc finger protein XPA (Asmuss *et al.* 2000, Carcinogenesis 21, 2097) are potential molecular targets. Like XPA the tumor suppressor protein p53, which plays an important role in cell cycle control and DNA repair, contains a zinc binding motif, which is responsible for its function as transcription factor. By loss of zinc from its central DNA-binding domain the folded p53 wild-type conformation turns into the unfolded, so called mutant conformation, which is no longer capable for DNA-binding. As shown by immunoprecipitation with conformation-specific monoclonal antibodies, both CdCl₂ (≥ 25 μM) and even more pronounced CdO (≥ 0.2 μg/cm²) caused an increase of mutant p53, probably resulting in a loss of DNA-binding ability.

Taken together our results indicate a general NER inhibition by cadmium compounds, with p53 being one potential molecular target. As the general population is frequently co-exposed to particulate cadmium compounds and mutagens, this aspect gains increasing significance regarding cadmium-induced carcinogenicity.

**Session 4: Mismatch repair and
emerging new repair pathways**

Mismatch Repair Defects and Infertility in Mice Carrying the Mlh1G67R Missense Mutation

Cora Reis(p)(3), Stefan J Scherer (1), Elena Avdievich (1), Paula Cohen (2), Sunhee Baek (3), Harry Hou Jr.(1), Raju Kucherlapati (4), Winfried Edelmann (1) and Burkhard Kneitz (3)

(1) Albert-Einstein-College of Medicine, Dept. of Cell Biology, 1300 Morris Park Ave, Bronx, New York 10461, USA

(2) Albert-Einstein-College of Medicine, Dept. of Medical Genetics, 1300 Morris Park Ave, Bronx, New York 10461, USA

(3) Biocenter of the University of Wuerzburg, Dept. of Physiological Chemistry I, Am Hubland, 97074 Wuerzburg, Germany

(4) Harvard-Partners Center for Genetics and Genomics, Boston, Massachusetts

Hereditary nonpolyposis colorectal cancer is frequently caused by mutations in the DNA mismatch repair gene MLH1. To study the biological functions of Mlh1 we previously generated an Mlh1 deficient mouse line. The analysis of this mouse line showed that loss of Mlh1 function resulted in increased cancer predisposition as well as male and female sterility. To further analyze the role of Mlh1 in these processes we generated a mouse line carrying the G67R missense mutation located in the conserved ATPase domain. Mice of this line are designated Mlh1G67R. The analysis of this mouse line showed that similar to Mlh1-deficient mice both male and female Mlh1G67R homozygous mutant mice are sterile. Histological analysis of testis sections revealed that the infertility in males is caused by a meiotic defect and apoptosis during prophase I in Mlh1G67R/G67R spermatocytes. Immunolocalization analysis on chromosome spreads in Mlh1G67R/G67R mice further showed that although MLH3 accumulates on synaptonemal complex structures of Mlh1G67R/G67R spermatocytes at pachynema, the MLH1G67R mutant protein fails to associate with MLH3-positive recombination nodules. Our results further show that the inability of the mutant Mlh1G67R protein to interact with meiotic chromosomes at late pachynema is responsible for the premature desynapsis of metaphase chromosomes. Based on these results we conclude that efficient ATP processing is required for the localization of Mlh1 to meiotic chromosomes. To study the effect of the G67R mutation on mitotic mismatch repair we performed a biochemical analysis in Mlh1G67R mutant mouse embryonic fibroblasts (MEF). This analysis showed that homozygous mutant Mlh1G67R/G67R cells were MMR-deficient similar to Mlh1^{-/-} cells. Finally we also analyzed the DNA damage response in Mlh1 mutant MEF cells. In contrast to Mlh1^{-/-} MEFs which were largely resistant to exposure to cisplatin, 6-TG or MNNG, Mlh1G67R/G67R mutant MEFs remained sensitive to these agents and displayed an apoptotic response similar to wild-type cells. In summary, our data show that normal ATP processing by Mlh1 is essential for its functions during mitotic DNA mismatch repair and Mlh1 localization to meiotic chromosomes. In contrast, the normal apoptotic response of Mlh1G67R cells indicate that it is not crucial for this function and suggests that Mlh1 might act as an scaffold protein in DNA damage response.

A role of human DNA topoisomerase I in DNA repair and apoptosis.

Kent Søre, Anja Rockstroh, Peter Schache, Holger Stephan, Michael Steiner, Kristin Dreffke & Frank Grosse(p)

*Institute of Molecular Biotechnology, Biochemistry, Beutenbergstrasse 11,
D-07745 Jena, Germany*

In the past years, human DNA topoisomerase I (htopoI) has been shown to be involved in cellular responses to DNA damage. We and others have demonstrated that this damage response is involved in DNA repair as well as apoptosis (Søre *et al.* 2004 DNA Repair 3, 387-393). During the so called "htopoI damage response", stabilized covalent intermediates between htopoI and the DNA backbone (cleavage complexes) are generated. These complexes seem to trigger either DNA repair or apoptosis. The pathway, in which the htopoI damage response is incorporated, is yet unclear, but recent data imply an involvement of the "classical" DNA repair machinery. Furthermore, we could demonstrate an involvement of the tumor suppressor protein p53 in the htopoI damage response. In fact, wild type as well as mutant forms of p53 strongly enhance the htopoI damage response. Wild type p53 has been shown to interact with htopoI *in vivo* in a regulated fashion, whereas the interaction with mutant p53 was found to be unregulated. The importance of the regulation of the htopoI damage response becomes clear if one considers that covalent htopoI-DNA complexes are known to trigger recombination events. Recently, we have shown that a htopoI cleavage complex can be recognized by a second htopoI molecule, which in turn removes the first covalently bound htopoI molecule and creates a single-stranded gap (Søre *et al.* 2001, NAR. 29, 3195-3203). This "double cleavage" reaction may provide an entry site for a subsequent repair event by DNA recombination, the htopoI induced recombination repair (TIRR). We have shown that p53 stimulates both the DNA relaxation activity of htopoI as well as TIRR (Stephan *et al.* 2002, NAR. 30, 5087-5093). Since p53 has been shown to physically interact with htopoI-DNA complexes during the htopoI damage response and that these complexes can consist of two closely associated htopoI cleavage complexes this indicates a potential involvement of TIRR in the htopoI damage response. We suggest that the htopoI damage response is involved in DNA repair as well as in apoptosis. Below a not yet identified threshold DNA repair is activated and above this apoptosis is induced. Thus, the htopoI damage response may be a sensor of the level of DNA damage. We suggest that p53 and TIRR play an important role in this process. Furthermore, we hypothesize that mutant p53 may induce uncontrolled levels of htopoI topol cleavage complexes that in turn can lead to genomic instability, possibly by a deregulated TIRR.

Characterization of the Human Tyrosyl-DNA phosphodiesterase mutation causing spinocerebellar ataxia with axonal neuropathy (SCAN1)

H. Interthal (p), H. J. Chen, T. Kehl-Fie and J.J. Champoux

*Department of Microbiology, School of Medicine,
University of Washington, Seattle, WA 98195, USA*

Human tyrosyl-DNA phosphodiesterase (Tdp1) catalyzes the hydrolysis of the phosphodiester bond between a tyrosine residue and a DNA 3' phosphate. The only known example of such a linkage in eukaryotic cells occurs in the enzyme-DNA covalent complex formed when a type IB DNA topoisomerase cleaves DNA. It has been proposed that Tdp1 is involved in the repair of such complexes formed when the topoisomerase fails to religate the DNA at nicks, gaps or abasic sites, or after treatment with the anti-cancer drug camptothecin. Tdp1 is a member of the phospholipase D superfamily and the hydrolytic reaction catalyzed by Tdp1 involves the formation of an intermediate in which the cleaved substrate is covalently linked to the enzyme. Our crystal structures with substrate bound in a mimic of the transition state of the reaction provided key insights into substrate binding and the catalytic mechanism.

The importance of human Tdp1 became evident with the recent discovery that a point mutation in an active site residue of Tdp1 (H493R) is responsible for the hereditary neurological disorder spinocerebellar ataxia with axonal neuropathy (SCAN1). We found that cell lines derived from SCAN1 patients are hypersensitive to camptothecin and FACS analysis revealed that the cells arrest in S-phase before they undergo apoptosis. This data implicates Tdp1 in the repair of topoisomerase I-DNA damage in humans.

Interestingly, Tdp1 activity in cell extracts obtained from these mutant cell lines is reduced, but not completely abolished. To further elucidate the Tdp1 defect of SCAN1 cells we purified recombinant Tdp1 H493R and characterized the effect of the mutation biochemically. The enzyme is active and preliminary data revealed surprising features with potential implications concerning Tdp1 substrate specificity and the SCAN1 phenotype.

Topotecan-induced DNA double-strand breaks are repaired by homologous recombination and variably affected by caffeine

R. A. El-Awady (p)(1,2), E.Saleh (1,2), J. Dahm-Daphi (1)

(1)Universitätsklinikum Hamburg-Eppendorf, Klinik für Strahlentherapie und Radioonkologie, Martinistr. 52, 20246 Hamburg, Germany

(2) Department of Tumour Biology, National Cancer Institute, Cairo University, Cairo, Egypt

Topotecan (TPT), a topoisomerase I inhibitor, is currently used as first and second line agent for the treatment of different types of tumours. It acts by inducing DNA single- and double-strand breaks but cell kill is mainly attributed to the double-strand breaks (DSBs) . TPT induces DSBs in replicating cells only during transcription and/or replication. In the present study we investigated the contribution of the main DSB repair pathways (non-homologous end joining, NHEJ, and homologous recombination, HR) to the repair of TPT- induced DSB. We found that mouse embryonic fibroblasts (MEF) defective in the Ku 80 protein (a component of the NHEJ) and their wild type cells were equisensitive to the cytotoxic effects of TPT eliminating the possibility that NHEJ is involved in the repair of TPT-induced DSB. On the other hand, irs1SF cells defective in HR were highly sensitive to TPT indicating that HR is the main repair pathway for TPT-induced DSBs. TPT-induced DSB repair as measured by γ -H2AX foci was more efficient in the wild type than in the irs1SF cells indicating that sensitivity differences to TPT were due to differences in HR efficiency. In accord, Caffeine a known inhibitor of HR was found to sensitize wild type but not the irs1SF cells. on the contrary those cells were more resistant to TPT combined with caffeine, which was largely due to a caffeine-induced G1-arrest. For clinical cancer therapy, our results suggest that tumours with a HR defect might be in particular sensitive to the treatment with topoisomerase I inhibitors, such as topotecan.

**Session 5: Double-strand break repair:
non-homologous end joining**

Structural biology of ATP-driven conformational control in Rad50 and SMC Proteins

K.-P. Hopfner

*Institute of Biochemistry and Gene Center, University of Munich, Feodor-Lynen-Str. 25,
81377 Munich, Germany*

Structural maintenance of chromosome (SMC) and Rad50 protein dimers are suggested to form large coiled-coil rings or assemblies around DNA in sister-chromatid cohesion, chromosome condensation and DNA repair. Crystal structures of nucleotide free and ATP-bound *P. furiosus* SMC ATPase domains reveal that ATP directly engages two SMC ATPase domains by binding to opposing Walker A and signature motifs. DNA stimulates ATP-hydrolysis in the SMC protein heads, suggesting that the ATPase activity of SMC ATPase domains is allosterically regulated. Structural and mutagenesis data identify an arginine finger that is required for DNA stimulation of the ATPase activity and directly connects a putative DNA interaction site to ATP. Conservation of this arginine finger suggests that allosteric control of the ATPase activity by DNA or other factors might be widespread feature in molecular action of SMC proteins. In particular the arginine finger seems to be a different mechanistic aspect of SMC proteins and the DNA repair protein Rad50.

ARTEMIS, a structure-specific nuclease of the NHEJ pathway

K. Schwarz (p), U. Pannicke

Institute for Clinical Transfusion Medicine and Immunogenetics and Department of Transfusion Medicine, University Hospital of Ulm, Helmholtzstraße 10, 89081 Ulm, Germany

Nonhomologous DNA end joining (NHEJ) is the major pathway for repairing double-strand DNA breaks. Such breaks arise spontaneously throughout the cell cycle. Defects in NHEJ result in marked sensitivity to ionizing radiation and ablation of lymphocytes, which rely on NHEJ to complete the antigen receptor gene cutting/rejoining process called V(D)J recombination. NHEJ is intrinsically imprecise. This imprecision is useful for the immune diversification process in lymphocytes. However, such imprecision may contribute to some of the genetic changes underlying cancer and aging.

One of the NHEJ components, the structure specific nuclease ARTEMIS contributes to imprecise joinings. It forms a complex with the 469 kDa DNA-dependent protein kinase (DNA-PK_{cs}) in the absence of DNA. The purified ARTEMIS protein alone possesses single-strand-specific 5' to 3' exonuclease activity. Upon complex formation, DNA-PK_{cs} phosphorylates ARTEMIS, and ARTEMIS acquires endonucleolytic activity on 5' and 3' overhangs, as well as hairpins. Finally, the ARTEMIS:DNA-PK_{cs} complex can open hairpins generated by the RAG complex. DNA-PK_{cs} regulates ARTEMIS by both phosphorylation and complex formation to permit enzymatic activities that are critical for the hairpin-opening step of V(D)J recombination and for the 5' and 3' overhang processing in NHEJ. Biochemical analyses and a novel *in vivo* V(D)J recombination assay, allowed the identification of separation of function mutants within the β -lactamase domain of ARTEMIS which were defective in both overhang endonucleolytic and hairpin-opening activities but not in the 5' to 3' exonuclease activity.

Loss of end-joining efficiency and precision in mutant hamster and patient cells

L. Brugmans (p)(1), D. van Heemst (1), N.S. Verkaik (1), M. van der Burg (2), J.J.M. van Dongen (2), D.C. van Gent (1)

(1) Department of Cell Biology and Genetics, ErasmusMC, University Medical Center, P.O. Box 1738, 3000 DR, Rotterdam, The Netherlands

(2) Department of Immunology, ErasmusMC, University Medical Center, P.O. Box 1738, 3000 DR, Rotterdam, The Netherlands

DNA double-strand breaks (DSBs) result in ionising radiation (IR) hypersensitivity. Therefore, DNA DSB repair after IR has been studied extensively. However, the lack of site-specificity has drawbacks. Another way of creating DSBs is the use of the restriction endonuclease I-SceI. This research has yielded interesting data but after precise repair of this DSB the site will be restored, which obscures the end-joining process.

To look in more detail at all DSB repair events we developed an assay, in which mammalian cells were transfected with a linearized plasmid. To analyse the DSB repair events, this assay is set up in such a way that we could discriminate between direct joining and microhomology directed joining by restriction enzyme digestion of the joined products.

In another assay we used the *E. coli* cut-and-paste type transposon Tn5. Interestingly, a blunt 5' phosphorylated break could efficiently be repaired without nucleotide loss in wild type Chinese hamster fibroblasts. DNA-PKCS deficiency reduced the joining efficiency without reducing the precision, whereas both efficiency and accuracy of joining were affected in Ku80 or XRCC4 mutants. These results show that these factors are required for NHEJ and that other, more error prone, repair processes cannot efficiently substitute for joining blunt ends.

We also screened cells from T-B-NK+ SCID patients for defects in the end-joining pathway. Thus far, we found seven radiosensitive patients, from which six carried mutations in the Artemis gene. Cells from one patient were affected in an unknown factor. From these cells all other known NHEJ components were tested for defects, but no abnormalities were found. However, formation of IR induced Mre11 and 53BP1 foci was disturbed in fibroblasts from this patient, we did not find any alterations in the protein expression level or in the sequence of Mre11. Currently, we are working towards identification of the missing factor.

Features of Ku-independent error-prone NHEJ

Feldmann E., Kuhfittig-Kulle S., Odersky A., Kuliczowska A., Goedecke W., and Pfeiffer P. (p)

Universität Duisburg-Essen, Fachbereich 9 Genetik; Universitätsstr.5, D-45117 Essen, Germany.

Nonhomologous DNA end joining (NHEJ) is the major pathway of double-strand break (DSB) repair in mammalian cells and involves the Ku70/80 heterodimer, the catalytic subunit of the DNA-dependent proteinase kinase (DNA-PKCS), DNA ligase IV and its essential co-factor XRCC4.

Using gamma-2HAX foci formation and disappearance as a measure for DSB repair, we show that CHO mutants deficient in Ku80 (*xrs6*), DNA-PKCS (*XR-C1*), and XRCC4 (*XR-1*) exhibit strongly reduced kinetics of DSB repair compared to wild-type CHO-K1 cells following 1Gy of X-irradiation. The delay in disappearance of gamma-H2AX foci 24h post-irradiation is strongest in *xrs6* and *XR-1* cells (both 100% remaining foci) and milder in *XR-C1* cells (26%) vs. about 14% in CHO-K1 cells.

Reduced DSB repair capacity in the NHEJ mutants is confirmed in cell-free NHEJ-assays measuring the ability of extracts prepared from these cells to rejoin different types of DSB generated by restriction endonucleases. While the K1-extract is highly efficient in rejoining all types of DSB as quantified by average total substrate turnover (40%), all three mutants display strongly decreased NHEJ efficiency (*XR-C1*: 23%; *xrs6*: 15%; *XR-1*: 7%).

Parallel to reduced efficiency, NHEJ fidelity is also strongly decreased in all three mutants which is expressed by an increase of deletions (K1: 35%; *XR-C1*: 46%; *XR-1*: 69%; *xrs6*: 78%). Interestingly, substantial fractions of these deletions display microhomologies (μhom) of four basepairs at their breakpoints (K1: 9%; *xrs6*: 9%; *XR-C1*: 16%; *XR-1*: 23%) indicating a mechanistic role for short homologies in this error-prone NHEJ-pathway.

In summary, our results indicate that Ku70/80, and XRCC4 are most important for high NHEJ efficiency and fidelity. By contrast, lack of DNA-PKCS displays the least severe phenotype. In the absence of any of these factors, secondary slower, less efficient error-prone NHEJ pathways take over which create deletions and thus may contribute to genomic instability.

Role of Yku in replicative aging in *S. cerevisiae*

B. Meier (2) and H.M. Feldmann (p)(1)

(1) *Institut für Biochemie der LMU - Genzentrum, 81377 München, Feodor-Lynen-Str. 25*

(2) *Current address: Department of Genetics, University of North Carolina, Chapel Hill, NC 27599, USA*

The Ku heterodimer is involved in at least two important cellular processes: First, it is essential for DNA double strand break repair by non-homologous end-joining, second, it is essential for telomere length maintenance and proper telomere end structure formation.

Both processes – DNA repair and telomere length control – have been described to influence replicative lifespan of eukaryotic organisms. It is well established that telomere shortening in primary somatic cells determines their replicative lifespan. However, mutations in the WRN and BLM proteins, both involved in DNA repair, induce also a premature aging phenotype. Recently, the importance of telomere length for lifespan has been shown for *C.elegans*. There, telomere elongation leads to a significant lifespan extension.

There is evidence that the Ku heterodimer might be involved in maintenance of replicative lifespan. Fibroblasts derived from Ku knock out mice display a dramatic reduction in cell-doubling capacity, and deletion of Ku86 causes an early onset of senescence in mice.

To investigate the role of yeast Ku in replicative lifespan we analysed an Yku70 deletion in genetically different *S.cerevisiae* background. All tested yku70- strains displayed a significant reduction in their replicative lifespan. Additionally, a diploid homozygous yku70- strain shows lifespan shortening. This result shows that the lifespan reduction is rather caused by YKU70 deletion and not mediated by the SIR4 pathway of replicative aging, although the Yku heterodimer directly interact with the Sir4 protein. Strikingly, overexpression of the Yku heterodimer also induces lifespan shortening and this effect seems to be mediated by titrating the Sir4 protein.

DNA damage leads to a p53 mediated induction of Cyclin A1 that activates DNA Double Strand Break Repair

Carsten Müller-Tidow (1)(p), Ping Ji (1), Anja Restle(2), Sven Diederichs (1), Jenny Potratz(1), Nicole Bäumer(1), Lisa Wiesmüller(2), Wolfgang E. Berdel(1) and Hubert Serve(1)

(1) Department of Medicine, Hematology / Oncology, Albert-Schweitzer-Str. 33, University of Münster, 48129 Münster, Germany

(2) Universitätsfrauenklinik, Prittwitzstrasse 43, D-89075 Ulm, Germany

Vertebrates express two A-type cyclins; both associate with and activate the CDK2 protein kinase. Cyclin A1 is required in the male germ line but its molecular functions are incompletely understood. We observed specific induction of Cyclin A1 expression and promoter activity after UV- and γ -irradiation which was mediated by p53. Cyclin A1^{-/-} cells showed increased radiosensitivity. To unravel a potential role of Cyclin A1 in DNA repair, we performed a yeast triple hybrid screen and identified the Ku70 DNA repair protein as a binding partner and substrate of the Cyclin A1-CDK2 complex. DNA double strand break (DSB) repair was deficient in Cyclin A1^{-/-} cells. Further experiments indicated that A-type cyclins activate DNA DSB repair by mechanisms that depend on CDK2 activity and Ku proteins. Both, cyclin A1 and cyclin A2 enhanced DSB repair by homologous recombination, but only cyclin A1 significantly activated non-homologous end-joining (NHEJ). DNA DSB repair was specific for A-type cyclins since cyclin E was ineffective. These findings establish a novel function for Cyclin A1 and CDK2 in DNA DSB repair following radiation damage.

**Session 6: Double-strand break repair and
defects in genetic diseases**

Double-strand break repair and genomic integrity in mammalian cells

Maria Jasin

*Cell Biology Program, Memorial Sloan-Kettering Cancer Center and Cornell University,
Graduate School of Medical Sciences, New York, New York 10021, USA*

Double-strand break repair in mammalian cells: Competition between homologous recombination and non-homologous end-joining through cell cycle.

Y. Saintigny

*UMR CNRS/CEA 217, Direction des Sciences du Vivant, Département de Radiobiologie et
Radiopathologie, 18 route du panorama, 92265 Fontenay-aux-Roses, CEDEX, France.*

DNA double-strand breaks (DSBs) are highly toxic lesions, which can be induced by ionizing radiation or replication arrest. When faced with DSBs, cells must coordinate not only cell cycle checkpoint and apoptosis, but also the different DNA repair systems themselves. Two major classes of processes can repair DSBs: non-homologous end-joining (NHEJ) and homologous recombination (HR). To study the relationship between NHEJ and HR for DSB repair, we used mammalian cells containing intrachromosomal HR substrate.

When maintained blocked in the G1 phase, irradiation-induced recombination is abolished but not in the G2 phase. In addition, after irradiation of an asynchronous cell population, RAD51 assembles into nuclear foci at the late S/G2 phase. Irradiation of cells maintained blocked at the G1/S transition did not induce RAD51 foci even though the cellular amount of RAD51 protein is equivalent in each cell cycle phase. These data show that the HR process itself may not occur in G1 and that the regulation does not act on the expression of RAD51 protein.

NHEJ-defective cells show an increase of RAD51 foci and DSB-induced. We used an XRCC4 mutant cell line which are sensitive to radiation when irradiated in G1 but not in G2 phase. In addition, HR stimulation in XRCC4 mutant cells occurs when cells are irradiated in the G1 but not in the G2 phase. Regardless of whether the cells are irradiated in the G1 or the G2 phase, RAD51 foci are formed in the late S/G2 phase. Taken together the data suggest that unrepaired DSBs produced in G1 in an XRCC4/p53 defective cell line can cross the S phase and be processed by HR in late S/G2 phase. To demonstrate this model, we measured DSBs by H2AX phosphorylation and by pulse field gel electrophoresis. Both techniques lead to the same conclusion: when cells defective for both NHEJ and G1 checkpoint are irradiated in G1, DSBs can cross the S phase. In contrast, in NHEJ-proficient cells, most DSBs are repaired prior to replication even in a G1 checkpoint-defective cell line. We propose a model in which NHEJ and HR act sequentially on DSBs in different cell cycle phase.

"Knockin" and "Knockout" gene targeting in the *Drosophila* germline, and the involved double-strand break (DSB) repair processes.

D.-H. Lankenau

Hinterer Rindweg 21, D-68526 Ladenburg, Germany

Compared to somatic cell development metazoan germline cells undergo a distinctive developmental program possibly involving specific mechanisms of genome protection. *Drosophila melanogaster* served as a model organism, and a new gene targeting system developed by Kent Golic's lab. served to study DSB repair linked to these germline processes. Using insertion vector gene targeting we generated knockin duplications of the nucleosome assembly protein one (Nap1) gene. No wild-type NAP1 protein could be detected in the protein extracts of the functional knock-outs. Homozygous Nap1-KO knockin flies were either embryonic lethal or poorly viable adult escapers. Three additional targeted recombination products were viable.

To gain insight into the underlying molecular DNA-repair processes we examined conversion tracts in the recombination products. In all cases but one the I-SceI endonuclease site of the donor vector was replaced by wild-type Nap1 sequence. This indicated exonuclease processing at the site of the DSB, followed by replicative repair at donor-target junctions. The targeting products are best interpreted either by the classical DSB repair model or the Break-Induced Recombination (BIR) model. Synthesis Dependent Strand Annealing (SDSA), which is another important recombinational repair pathway in the germline, does not explain the duplicated Nap1-KO products. Finally we used I-CreI-triggered Single Strand Annealing (SSA), which is a DSB repair process related to SDSA and BIR, to produce single copy Nap1 knockout flies. This system allows efficient *in vivo* analysis of SSA and intermediate repair of heteroduplex DNA in the germline. We found that 92% (n= 283) of the independent SSA recombination-tracts represented patterns of either the left (47%) or the right (45%) parental Nap1-knockin duplication. Mixed tract patterns were less frequent possibly hinting towards dissociation of the involved mismatch repair machinery during SSA repair.

DNA-repair and cell cycle checkpoint defects in Nijmegen Breakage Syndrome and Fanconi anaemia

Martin Digweed (p) (1), Ilja Demuth (1), Anna Melchers (1), Lars Stöckl (1), Lars Krüger (1), Sven Kracker (2), Yun-Gui Yang (3), Andreas Radbruch (2), Zhao-Qi Wang (3), Karl Sperling (1)

(1) Institut für Humangenetik, Charité - Universitätsmedizin Berlin, Germany

(2) Deutsches Rheuma-Forschungszentrum, Berlin, Germany

(3) International Agency for Research on Cancer, Lyon, France

The human genetic disorders, Fanconi anaemia (FA) and Nijmegen Breakage Syndrome (NBS), are characterised by chromosome instability, an increased mutation rate and a high risk for cancer of the lymphatic system. The specificity of mutagen-induced chromosome breakage, DNA-crosslinkers in FA and ionising radiation in NBS, has been interpreted as a failure of discrete aspects of the cellular response to DNA damage. However, surmounting evidence indicates an interaction between the FA and NBS pathways and their convergence on the repair of DNA double-strand breaks (DSB). In addition to their deficiency in DNA repair, cells from patients with both disorders show anomalous cell-cycle characteristics and much discussion has centred on the relative importance of DNA repair versus cell cycle control for the disease phenotypes. This assessment has been further hampered, in the case of NBS, by the realisation that the common mutation affecting over 90% of patients may in fact be hypomorphic. As in previous years, I will attempt to review the current findings on FA and NBS and, in addition, describe our own work on the generation of a constitutive FA knockout mouse and constitutive and inducible NBS knockout mice in order to examine DNA repair and cell cycle regulation on a cellular and organismal level.

A sub-pathway of non-homologous end-joining dependent upon ATM, Artemis and proteins locating to γ -H2AX foci

E. Riballo(1), M. Kühne(2), N. Rief(2), P. A. Jeggo(1), and M. Löbrich(p)(2)

(1) Genome Damage and Stability Centre, University of Sussex, East Sussex BN1 9RQ, UK (2) Fachrichtung Biophysik, Universität des Saarlandes, D-66421 Homburg/Saar, Germany

The ATM protein, which is mutated in individuals with ataxia telangiectasia (AT), is central to a network of cell cycle checkpoint responses initiated by DNA double-strand breaks (DSBs). However, the role of ATM and its substrates BRCA1, 53BP1, H2AX, and NBS1 in DSB repair is currently unclear as is the basis underlying the radiosensitivity of cells with mutations in any of these factors. We applied immunofluorescence detection of γ -H2AX nuclear foci and/or pulsed-field gel electrophoresis to quantify the repair of DSBs in confluence-arrested fibroblasts with mutations in ATM, 53BP1, H2AX or NBS1. In contrast to the pronounced repair defect that is observed in cells with mutations in the classical non-homologous end-joining (NHEJ) factors, DNA ligase IV, XRCC4 and DNA-PK, fibroblasts with defects in the ATM signalling pathway repair the majority of DSBs with normal kinetics. However, a subset of DSBs exists that is repaired by a process requiring both classical NHEJ and ATM signalling factors. This subset of induced breaks depends on the kind of agent generating the DSB and correlates with the complexity of the lesions induced. Significantly, cells deficient in Artemis show the same DSB repair defect as cells deficient in ATM signalling factors, suggesting that Artemis operates in the same subpathway of NHEJ repair. Our data suggest a model in which repair of a certain class of DSBs involves, in addition to classical NHEJ factors, components of the ATM signalling pathway. These lesions likely represent complex DSBs that may require a sophisticated ensemble of proteins for their faithful repair, including nucleases for the processing of break ends (Artemis and NBS1), factors for the retention of repair enzymes at the break site (H2AX), scaffold proteins for the coordinated assembly of the repair complex (53BP1 and BRCA1) and a factor for the regulation of this process (ATM).

Homologous Recombination and Non Homologous End Joining Cooperate at the Same DSB if Both Systems are Available.

A. Rapp(p) and K.O. Greulich

Institute of Molecular Biotechnology Jena, Beutenbergstr. 11; 07745 Jena, Germany

After induction of DNA double strand breaks (dsb) two repair systems, the error prone "non homologous end joining" (NHEJ) and the more accurate "homologous recombination repair" (HRR) can compete for the same individual dsb site. We have tested, in the human keratinocyte cell line HaCaT, the spatial colocalisation and the temporal sequence of events. As a damaging agent, UV A (365 nm) was used, which can be applied in clearly defined doses and can lead to rare dsbs via propagation of clustered single strand breaks (ssbs). Direct dsb detection was performed by immunohistochemical labelling of gamma-H2AX, a phosphorylated histone that is assumed to form one foci per dsb. Intra- and inter pathway interactions were quantified by colocalisation, FRET imaging and by co-immuno-precipitation (Co-IP) of XRCC4, DNA-PK and Ku70 as representatives of NHEJ, Rad51 and Rad52 for HRR and gamma-H2AX, Mre11 and Rad50 as representatives of both pathways. In G2 cells, where both systems are available the temporal sequence after irradiation is gamma-H2AX < Mre11 < DNA-PK Rad 51 < XRCC4, i.e. the first two proteins involved in both pathways "label" the damaged site and initiate repair, followed by the NHEJ, which is temporally overlapping with HRR activity. By counting the total number of gamma-H2AX foci it can be calculated that in G2 phase approximately 30 % of the dsb sites have activity in both pathways, whereas 50 % show solely HRR repair. The remaining dsbs are occupied solely by NHEJ even in G2. Taking all these observation together we suggest that a cell tries to repair dsbs with a combination of both HRR and NHEJ, if available.

**Session 7: Chromatin, chromosomes, and
repair defects in genetic diseases**

Fast Protein Translocations and Chromatin Migration Observed by Live Cell Microscopy after Local DNA Damage Induced by Heavy Ions

B. Jakob(p)(1), J.H. Rudolph(1), N. Gueven(2), M. Scholz(1) and G. Taucher-Scholz(1)

(1) GSI Biophysik, Planckstr.1, 64291 Darmstadt, Germany

(2) The Queensland Institut of Medical Research, Herston QLD4029, Australia

Genetic stability is based on the effective recognition of DNA lesions leading to the activation of signal cascades and ultimately to repair or cell death. Ion beams offer the possibility to generate strictly localized DNA lesions within subnuclear regions. This inhomogeneous spatial distribution of lesions can be visualized by immunocytochemical detection or, in living cells, by GFP-protein constructs of repair related proteins as ionizing radiation induced foci (IRIF). Analysis of protein accumulations along ion trajectories revealed an unexpected clustering. Chromatin movement might be a basal component of the DNA damage sensing mechanism and define the location of lesion processing generating the observed IRIF-cluster. To study protein and chromatin dynamics during and immediately after irradiation, a remote controlled microscope device was developed at the UNILAC facility of GSI. The system enables us the acquisition of high-resolution fluorescence images of stained living cells during ion irradiation, allowing to study early radiation effects without the time lag of minutes presently conditional on limitations of access to the irradiation device.

Chromophore-labeled nucleotides incorporated during S-phase were observed during irradiation with heavy ions in mammalian cells. No major chromatin migrations could be detected during the first 5 to 10 min. Time-lapse images of GFP-coupled proteins proved accumulations within seconds at sites of ion hits indicating a very fast recognition of DNA damage.

Despite still under development, the new beamline microscope in combination with live cell microscopy done in an environmental chamber for longer term observations showed to have the potential to elucidate the question of the involvement of chromatin movement in the sensing and processing of local DNA lesions. First results obtained at the new device point to only minor chromatin migration at sites of ion traversal and no indication of large-scale distortions in the first minutes after the impact. Whereas DNA-damage recognition revealed by the formation of IRIF during irradiation showed to be a fast process, occurring possibly under diffusion controlled rates.

Stable chromosomal units determine the spatial and temporal organization of DNA replication

N. Sadoni(p)(1), M.C. Cardoso(2), E.H.K. Stelzer(3), H. Leonhardt(1,2), and D. Zink(1)

(1)University of Munich (LMU), Department Biology II, Goethestr. 31, D-80336 Munich, Germany

(2)Max Delbrück Center of Molecular Medicine, Wiltbergstr. 50, D-13125 Berlin, Germany

(3)European Molecular Biology Laboratory (EMBL), Meyerhofstr. 1, D-69117 Heidelberg, Germany

DNA replication occurs in mammalian cells at so-called replication foci occupying defined nuclear sites at specific times during S-phase. It is an unresolved problem how this specific spatio-temporal organization of replication foci is determined. Another unresolved question is, to what extent DNA is redistributed during S-phase. To investigate these problems, we visualized the replicating DNA and the replication machinery simultaneously in living HeLa cells. Time-lapse analyses revealed that DNA was not redistributed to other nuclear sites during S-phase. Furthermore, the results showed that DNA is organized into stable aggregates equivalent to replication foci. These aggregates, we refer to as sub-chromosomal foci, stably maintained their replication timing from S-phase to S-phase. During S-phase progression, the replication machinery sequentially proceeded through spatially adjacent sets of sub-chromosomal foci. Thus, the replication machinery followed during the temporal progression of S-phase specific spatial arrangements of these stable chromosomal units stably occupying defined nuclear positions. These findings imply that the specific nuclear sub-structure of chromosomes and the order of their stable sub-units determines the spatio-temporal organization of DNA replication.

Disruption of Spindle Checkpoint Gene Bub1b Leads to Embryonic Lethality, Loss of Spindle Checkpoint Functions and Aneuploidy

L. Fedorov (1), B. Zöller(1), C. Steinlein(2), E. Avdievich(3), C. Leimeister(1), R. Kucherlapati(4), M. Gessler(1), W. Edelmann(3), M. Schmid(2) and B. Kneitz(1)(p)

(1) Biocenter of the University of Wuerzburg, Dept. of Physiological Chemistry I, Am Hubland, 97074 Wuerzburg, Germany,

(2) Biocenter of the University of Wuerzburg, Dept. of Human Genetics, Am Hubland, 97074 Wuerzburg, Germany,

(3) Albert-Einstein-College of Medicine, Dept. of Cell Biology, 1300 Morris Park Ave, Bronx, New York 10461, USA,

(4) Harvard-Partners Center for Genetics and Genomics, Boston, Massachusetts

The correct segregation of chromosomes into daughter cells during cell division is monitored by a mechanism known as the mitotic spindle checkpoint (MSC). Biochemical and genetic experiments in yeast described Bub and Mad genes as key components in the control of this checkpoint. Recent work indicates that the complete loss of the highly conserved MSC leads to embryonic lethality in various organisms caused by chromosomal mis-segregation and apoptosis.

A novel member of the mouse Bub/Mad gene family has been identified recently and named Bub1b. To study a potential role of Bub1b in MSC the gene was disrupted by homologous recombination in embryonic stem cells. Heterozygous mice carrying one functional copy of the gene are fertile and healthy up to one year of age, whereas no adult homozygous mice were identified in 98 mice resulting from heterozygote intercrosses. Analysis of null mutant embryos from E 3.5 to E11.5 has shown growth retardation and abnormal development causing embryonic lethality at various time points up to E14.5. To identify the biological role of BUB1B in mitotic cell division E8.5 null mutant and wildtype embryos were incubated in the presence of spindle-depolymerising agents and metaphase spreads from these embryos were prepared and analysed. Karyotype analysis showed an increased number premature sister chromatid separation, known as a hallmark of a defect MSC, in metaphases of null mutant embryos. Using SKY analysis, chromosome counts of metaphase spreads and interphase fluorescence in situ hybridization (FISH) with chromosome specific probes we analyzed the importance of the mitotic checkpoint for chromosomal stability. We detected a significant increase in the number of aneuploid cells in null mutants relative to the controls.

In summary our results demonstrate that BUB1b deficiency leads to embryonic lethality. We further showed that BUB1b is an important component of the spindle check point pathway and that cells from BUB1b deficient embryos fail to arrest in metaphase in response to drug-induced spindle disruption. Moreover, our data indicate that the spindle checkpoint defect caused by BUB1b deficiency leads to chromosomal mis-segregation and genomic instability in mammalian cells.

Chromosomal mutagen sensitivity, DNA repair deficiency and susceptibility to breast cancer

Günter Speit

Abteilung Humangenetik, Universitätsklinikum Ulm, D-89070 Ulm, Germany

Mutagen sensitivity, i. e. enhanced response to the DNA-damaging action of mutagens/carcinogens has been described repeatedly as a potential marker of susceptibility to cancer in humans. Like other inherited cancer-prone conditions, a portion of breast cancer patients showed an elevated sensitivity to the induction of chromosome damage in cells exposed to ionizing radiation. It has therefore been suggested that women with breast cancer are deficient in the repair of radiation-induced DNA damage. We are studying the induction and repair of DNA damage in lymphocytes of women from families with familial breast cancer and mutations in the breast cancer susceptibility genes BRCA1 and BRCA2. Our results indicate a close relationship between the presence of a BRCA mutation and sensitivity for the induction of micronuclei by gamma irradiation. Enhanced sensitivity towards the cytostatic drugs bleomycin, cisplatin, cyclophosphamide and BCNU was also observed. Since some of these drugs are used in breast cancer chemotherapy, it might be that women with a BRCA1 mutation are at higher risk for the induction of mutations and secondary cancers by standard therapies. In contrast to the various DNA-damaging agents, there was no clear difference in the response to vincristine and taxol. FISH analysis revealed that the two aneuploids mainly induced centromere-positive micronuclei to a similar amount in lymphocytes with and without a BRCA1 mutation. We conclude that cells containing a heterozygous mutation in BRCA1 are more sensitive towards different kinds of DNA damage in accordance with the proposed central role of BRCA1 in maintaining genomic integrity.

In contrast to the results with lymphocytes, lymphoblastoid cell lines (LCL) with a heterozygous BRCA1 mutation do not exhibit mutagen sensitivity. Although some of these cell lines seem to be deficient in the fidelity of DNA double-strand break repair and show altered expression of BRCA1, they are not sensitive towards the induction of chromosome damage by gamma-irradiation. Therefore, the use of LCL to study the mechanisms underlying mutagen sensitivity seems to be limited.

DNA damage and repair in breast cancer

J Blasiak(p)(1), M. Arabski(1), R. Krupa(1), K. Wozniak(1), J. Rykala(2), A. Kolacinska(2), Z. Morawiec(2), J. Drzewoski(3), M. Zadrozny(4)

(1)Department of Molecular Genetics, University of Lodz, ul. Banacha 12/16, 90-237 Lodz, Poland; (2)N. Copernicus Hospital, Lodz, Poland; (3)Department of Clinical Pharmacology, Medical University of Lodz, Lodz, Poland; (4)Polish Mother's Memorial Hospital, Lodz, Poland

Impaired DNA repair may fuel up malignant transformation of breast cells due to the accumulation of spontaneous mutations in target genes and increasing susceptibility to exogenous carcinogens. Moreover, the effectiveness of DNA repair may contribute to failure of chemotherapy and resistance of breast cancer cells to drugs and radiation. The breast cancer susceptibility genes BRCA1 and BRCA2 are involved in DNA repair. To evaluate further the role of DNA repair in breast cancer we determined: 1) the kinetics of removal of DNA damage induced by hydrogen peroxide and the anticancer drug doxorubicin, and 2) the level of basal, oxidative and alkylative DNA damage before and during/after chemotherapy in the peripheral blood lymphocytes of breast cancer patients and healthy individuals. The level of DNA damage and the kinetics of DNA repair were evaluated by alkaline single cell gel electrophoresis (comet assay). Oxidative and alkylative DNA damage were assayed with the use of DNA repair enzymes endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (Fpg), recognizing oxidized DNA bases and 3-methyladenine-DNA glycosylase II (AlkA) recognizing alkylated bases. We observed slower kinetics of DNA repair after treatment with hydrogen peroxide and doxorubicin in lymphocytes of breast cancer patients compared to control individuals. The level of basal, oxidative and alkylative DNA damage was higher in breast cancer patients than in the control and the difference was more pronounced when patients after chemotherapy were engaged, but usually the level of DNA damage in these patients was too high to be measured with our system. Our results indicate that peripheral blood lymphocytes of breast cancer patients have more damaged DNA and display decreased DNA repair efficacy. Therefore, these features can be considered as risk markers for breast cancer, but the question whether they are the cause or a consequence of the illness remains open. Nevertheless, our results suggest that research on the mutagen sensitivity and efficacy of DNA repair could impact the development of new diagnostic and screening strategies as well as indicate new targets to prevent and cure cancer. Moreover, the comet assay may be applied to evaluate the suitability of a particular mode of chemotherapy to a particular cancer patient.

**Session 8: DNA repair, cancer therapy,
and apoptosis**

Cell death pathways and cancer therapy

Klaus-Michael Debatin

University Children's Hospital, Prittwitzstr. 43, 89075 Ulm, Germany

Deregulation in apoptosis signalling pathways may contribute to therapy resistance and apoptosis regulating molecules represent novel targets for future therapeutic approaches. Activation of apoptosis pathways include triggering of death ligand/receptor interaction such as the CD95 or TRAIL system, "activation" of mitochondrial apoptogenic function with subsequent release of apoptosis inducing molecules such as cytochrome c and Smac Diablo, activation of the cellular stress pathway and ultimately, activation of caspases as effectors of the cell death machinery. Although, *in vitro*, the contribution of apoptosis signalling to cell death e.g. by antileukemia agents has been described in many different experimental settings, most *in vivo* studies in leukemia have failed to directly correlate e.g. the deregulated expression of apoptosis inducing regulating molecules to treatment response or outcome. In search for molecular parameters of apoptosis sensitivity we identified downregulation of caspase-8 expression as a novel mechanism of drug resistance in a variety of human tumors. Treatment with demethylating agents such as 5-Aza-2-Deoxycidin reversed hypermethylation of the caspase-8 gene, resulting in re-expression of caspase-8 and sensitisation for TRAIL or drug-reduced apoptosis. Also, treatment with Interferon γ in neuroectodermal tumor cells lead to re-expression of caspase-8 through a Stat1/IRF-1 dependent pathway. Further strategies to increase apoptosis sensitivity in constitutively resistant tumor cells may include modulation of thresholds for caspase activation. We found that Smac gene transfer or cell permeable Smac peptides sensitized various tumor cell lines *in vitro* including leukemia cells and malignant glioma cells *in vivo* for apoptosis induced by TRAIL or cytotoxic drugs. Complete eradication of established glioma xenografts in nude mice was established by combination of locally delivered Smac peptides and TRAIL without acute or delayed neurotoxicity. In addition to the development of novel strategies to overcome intrinsic apoptosis resistance, we have analysed activation of apoptosis pathways during antileukemia treatment *in vivo*. Interestingly, in ALL and AML, significant activation of apoptosis pathways and apoptosis induction was predominantly found in CD34+ leukemic cells suggesting that induction of apoptosis is confined to the elimination of the putative leukomogenic stem cells. Further detailed analysis of caspase-3 activation in cytochrome c release in individual leukemic cells from patients undergoing chemotherapy *in vivo*, deficient cytochrome c release was identified as the most important parameter for treatment response *in vivo*. Taken together, molecular insights into the apoptosis regulation will direct our understanding of therapy response in conventional treatment strategies, may help to device molecule based rational treatment approaches and provides novel targets for future therapeutic intervention.

The antitumorogenic agent Resveratrol has an inhibitory effect on homology-directed double-strand break repair

S.A. Gatz (p) (1), S. Fulda (1), K.-M. Debatin (1), L. Wiesmüller (2)

(1) Universitätsklinik für Kinder- und Jugendmedizin, Prittwitzstr. 43, 89075 Ulm

(2) Universitätsfrauenklinik, Prittwitzstr. 43, 89075 Ulm

Resveratrol - a phytoalexin with a polyphenol structure - is synthesized in a variety of plant species in response to injury, UV-irradiation and fungal attack. A wide range of biological/therapeutic properties has been propagated for this substance including cell cycle effects, apoptosis modulation, inhibition of cell growth, hormonal activity and more recently activation of SIRT-1 - the human SIR2 analogue, an histone deacetylase (HDAC) type III.

Due to its antitumorogenic effect we investigated if there is a direct effect of RV on homologous recombination. For this purpose we employed our EGFP and I-Sce I meganuclease based test system and different cell types with extra- and intrachromosomal recombination substrates (Akyüz et al. 2002, Mol Cell Biol 22: 6306-6317).

In contrast to a recent study in which a recombination stimulatory effect of RV has been described for SPD8 Chinese hamster cells (Matsuoka et al. 2004, Mutat Res 547: 101-107), we noticed a downregulation of homology-directed double-strand break (DSB) repair by RV in human HCT 116 colon carcinoma cells and K562 leukemia cells. The inhibitory effect in HCT cells was separable from the well-described influence of RV on apoptosis and cell growth by use of the p21^{-/-} and bax^{-/-} HCT116 derivatives. Interestingly the effect was also independent of the recombination surveillance factor p53.

Using siRNA mediated knockdown and patient cell lines with mutations in genes encoding alternative recombination regulatory proteins, such as ATM, BRCA1, FANCD 1 and SIRT-1, are currently examined for a possible involvement in the inhibitory effect of RV on homology-directed DSB repair.

Determination of the exact mechanism how RV affects homologous recombination may have implications on tumor therapy.

Towards the mechanisms governing Rad51-dependent chemo-resistance of tumour cells

T. Jarutat, W. Henning, T. Kremer, B. Oidtmann, S. Haase, and H.-W. Stürzbecher (p)

Institute of Pathology, University Clinic Schleswig-Holstein, Germany

Homologous recombination (HR) of DNA is one of the driving forces of genetic variety and evolution, but on the other hand, the same mechanism guarantees maintenance of genomic stability by participation in the repair of complex forms of DNA damage, such as double strand breaks, interstrand crosslinks, or DNA adducts in close proximity on opposite strands. Cells with mutations in HR genes exhibit high levels of genetic instability and sensitivity to cross-linking agents and ionising radiation. Conversely, hyperactivity of the HR pathway also could contribute to genetic instability by causing inappropriate recombination, resulting in translocations, deletions, duplications, or loss of heterozygosity. There is an increasing number of reports about elevated protein levels of Rad51, the key factor of homologous recombination, in tumours and potential functional association with tumour suppressor proteins such as p53, BRCA1 and BRCA2, all suggesting that Rad51 up-regulation confers an advantage to tumour cells. Increased Rad51 protein levels could lead to uncontrolled recombination, genome instability and increased resistance of tumours to radio- and chemotherapy.

We present that high-level expression of Rad51 contributes significantly to the resistance to a variety of chemotherapeutic agents inducing either apoptosis or necrotic cell death like cisplatin, actinomycin D, doxorubicin, staurosporine, calicheamicin γ 1, methotrexate, 5-FU and taxol. Surprisingly, this palette of drugs not only contains DNA damaging agents which will directly or indirectly cause DNA double strand breaks but also drugs that are not known to cause DNA damage, like the kinase inhibitor staurosporine. These observations argue for a unique mechanism for cytotoxic drug resistance induced by Rad51 in human cancers. Detailed analysis revealed that apoptosis inducing drugs cause rapid degradation of Rad51 within four hours of treatment in a variety of cell lines (UIRad51, UiLacZ, MCF-7, T47D, and others). Since Rad51 serves as a substrate for caspase-3, caspase-activation after drug treatment was monitored by measuring caspase-3 dependent PARP degradation. However, PARP degradation started beginning at 6-8h after treatment, thus excluding Rad51 degradation by caspase-3 within the first 4h of drug treatment. Treating the cells with proteasome inhibitors proofed proteasome-dependent degradation of Rad51. We could detect neither ubiquitination nor sumoylation of Rad51 concomitant to degradation. Despite rapid degradation of Rad51, Rad51 over-expressing cells remained drug resistant for more than 64h. Thus, Rad51-dependent resistance of tumour cells towards chemotherapy is not based solely on enhanced Rad51-directed DNA-repair and does not require permanent over-expression of Rad51.

Supported by: Deutsche Forschungsgemeinschaft project Stu 178/7-1.

Alterations in the expression of DNA repair genes in response to ionizing radiation

S. S. Mello(1), C. L. Bassi(1), I. M. Merchi(2), A. L. Fachin(1), C. M. Junta(1), P. Sandrin-Garcia(1), E. A. Donadi(4), G. A. S. Passos(3,1), E. T. Sakamoto-Hojo(p)(1,2)

(1) Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.

(2) Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.

(3) Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo, SP, Brazil.

(4) Departamento de Clínica Médica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.

Ionizing radiation can interact with the genetic material, leading to cell transformation and tumorigenesis, due to induction of DNA damage. In order to face this damage, cells activate cell cycle checkpoints and specific DNA repair mechanisms, or alternatively, the induction of apoptosis. We are studying the profiles of gene expression (by cDNA microarrays) in response to the lesions caused by gamma-irradiation in different types of human cells (lymphocytes, primary and SV40-transformed fibroblast cell lines, and glioma cell line). In general, cell irradiation with doses between 10 cGy to 4 Gy modulated the expression of several genes participating in different DNA repair mechanisms: homologous recombinational repair (RAD52, RUVBL2, BRCA1, NBS1 and H2AFX), nucleotide excision repair (ERCC1, ERCC3, ERCC4, XPA, DDB2 and BRCA1), base excision repair (FEN1, ADPRTL1, APEX and LIG3), and mismatch repair (MLH3). The expression of some genes involved in signal transduction was also observed (FANCC, FANCG and ATM), but their connection with DNA repair mechanisms is not completely understood. Under conditions of low dose irradiation, we observed the induction of genes involved in the response to oxidative damage, as well as the induction of repair genes engaged in oxidative damage repair. In primary cells, a variation in dose rate (DR), between 0.5 and 2 Gy/min, was also found to induce differences in the gene expression profiles. The cell response to low DR showed higher induction of ERCC1, ERCC3, ERCC4, FEN1 and FANCC genes, when compared to high DR. As a whole, these results indicate that many types of repair processes are activated by gamma-rays induced-DNA damage in several cell types. Furthermore, low- doses, or -DR, are also effective in inducing alterations in the expression of DNA repair genes, differently from those observed for high-doses, or -DR. [Financial support: FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Proc. 99/12135-9; 02/07314-6; 01/10995-2), CNPq (fellowship to CLB), and Pró-Reitoria Pesquisa-USP (fellowship to IMM)].

**Session 9: DNA damage-triggered signaling
and apoptosis**

Antagonistic roles of the RAD17 pathway and BRCA1 in the regulation of DNA repair and homologous recombination in *Arabidopsis thaliana*

H. Puchta (p) (1, 2) , W. Reidt (1), H.H. Chu, (1), I.-P. Chen (1), F. Hartung (1), K.J. Angelis (3) and F. Heitzeberg (2)

(1) Botanical Institute II, University Karlsruhe, D-76128 Karlsruhe, Germany,

(2) IPK, D-06466 Gatersleben, Germany,

(3) Institute of Experimental Botany ASCR, 160 00 Praha 6, Czech Republic

Rad17 is involved in DNA damage checkpoint control in human cells. A homologue of this gene as well as other genes of the pathway (e.g. RAD9) are present in *Arabidopsis*. AtRAD17 mutants show increased sensitivity to DNA damaging chemicals, which can be reversed by complementation. AtRAD17 seems to be epistatic to AtRAD9 as the double mutant is not more sensitive than the single mutants. The mutants show a delay in the general repair of double-strand breaks (DSBs). However, frequencies of intrachromosomal homologous recombination (HR) are enhanced. The mutants are proficient for a further induction of HR by genotoxic stress. Thus, a mutant Rad17 pathway is associated with a deregulation of DNA repair due to a deficiency in non-homologous DSB repair. Plants also contain a homologue of the BRCA1 gene, the mutation of which is correlated with breast cancer in humans. Mutation of this gene in *Arabidopsis* results in a deficiency in the induction of HR after genotoxic stress. However, no significant change in DSB repair could be detected. Here, the deregulation of DNA repair seems to be correlated with a defect in HR but not non-homologous DSB repair. Thus, AtRAD17 and AtBRCA1 seem to have antagonistic roles in the regulation of DNA repair in plants.

Apoptosis triggered by O⁶-methylguanine in primary human lymphocytes: role of DNA replication, DNA double-strand breaks, p53 and death receptor activation

W. P. Roos (p), M. Baumgartner and B. Kaina

Institute of Toxicology, University Mainz, Obere Zahlbacher Str. 67, D-55131 Mainz, Germany

Chemotherapeutic drugs that alkylate DNA induce O⁶-methylguanine (O⁶MeG), which provokes cell death by apoptosis. We examined apoptosis triggered by O⁶MeG in human peripheral lymphocytes that were not proliferating or proliferating upon CD3/CD28 stimulation. In these cell populations O⁶benzylguanine (O⁶BG) was also used to inactivate the DNA repair enzyme O⁶-methylguanine-DNA methyltransferase (MGMT). Apoptosis upon treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or the anticancer drug temozolomide was only observed in proliferating cells. This was not the case with extremely high alkylation doses ($\geq 15 \mu\text{M}$ of MNNG) where apoptosis was also observed in resting lymphocytes although this was not affected by O⁶BG, which shows that replication-independent apoptosis is independent of O⁶MeG. A wave of DNA double-strand breaks (DSBs) preceded O⁶MeG-triggered apoptosis in proliferating lymphocytes, which coincided with p53 and Fas receptor upregulation. Treatment with anti-Fas neutralizing antibody inhibited MNNG-induced apoptosis in MGMT depleted proliferating lymphocytes, showing the importance of Fas for the signaling to apoptosis. In proliferating lymphocytes apoptosis was observed only at late time points. The data suggests that O⁶MeG is converted by mismatch repair (MMR) and DNA replication into DSBs that trigger apoptosis by p53 stabilization and Fas/CD95/Apo-1 upregulation. This indicates that tumor proliferation rate, Fas responsiveness, MGMT and MMR status are all important prognosis parameters for cancer chemotherapy using O⁶-methylating agents.

Roos W., Baumgartner M., Kaina B. *Oncogene* (2004) 23, 359-367.

The ‘regulatory’ β -subunit of protein kinase CK2 negatively influences p53-mediated allosteric effects on CHK2 activity towards p53 activation.

M. Bjørling-Poulsen (1), S. Siehler (2), L. Wiesmüller (2), D., Meek (3) and Karsten Niefind (4), and O.-G. Issinger (p)(1).

(1) Institut for Biokemi og Molekylærbiologi, Syddansk Universitet, Odense, Denmark, (2) Universitätsfrauenklinik, Universität Ulm, Ulm, Germany,

(3) Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Scotland, UK.

(4) Institut für Biochemie, Universität zu Köln, Köln, Germany.

The tumor suppressor gene product p53 has been shown to be a target for multisite phosphorylation. But beside serving as a substrate, p53 also has been shown to interact with proteins and protein kinases exerting allosteric changes in the interacting partners. One such example is the recently described allosteric effect on CHK2 kinase. p53 has been shown to be a substrate for protein kinase CK2 holoenzyme (S392 phosphorylation), but also interaction with the regulatory CK2 β subunit has been demonstrated, where CK2 β binds to amino acids 325-344 in the p53 molecule. Since CK2 β has been shown to interact with protein kinases such as A-RAF, Mos, p90RSK, Lyn, and CHK1 we first wanted to test whether CK2 β also can interact with CHK2. We could show in vitro, using recombinant subunits of CK2 and CHK2 that only the β -subunit from CK2 can specifically interact with CHK2. When COS1 cells were transfected with two constructs expressing CHK2 and CK2, respectively, it was again only CK2 β which interacted with CHK2. Finally we could show by immunoprecipitation analyses that also endogenous CK2 β interacts with endogenous CHK2. Using recombinant CHK2, p53 and CK2 β subunit we could demonstrate that increasing amounts of CK2 β led to a decrease in CHK2-mediated p53 phosphorylation. Since the box V domain of p53 (aa 272-286) seems to be responsible to increase CHK2 activity towards T18 phosphorylation in p53 (Craig et al., 2003), the binding of CK2 β to p53 residues 325-344 could influence the domain's activating function on CHK2, alternatively one might argue that binding of CK2 β to CHK2 negatively influences its activation by the box V domain in p53.

Alkylation-induced DNA damage contributes to the activation of stress-activated protein kinases/c-Jun N-terminal kinases (SAPK/JNK)

G. Fritz(p) and B. Kaina

Institute of Toxicology, Johannes-Gutenberg University of Mainz, Obere Zahlbacher Straße 67, D-55131 Mainz, Germany

DNA damaging agents provoke a variety of cellular functions that can be subgrouped into early and late responses. A prominent early cellular response to genotoxic stress is the activation of stress-activated protein kinases (SAPK/JNK), controlling gene expression and apoptosis. It is unclear, however, whether DNA damage itself contributes to the activation of SAPK/JNK. In the present study we show that the potent DNA alkylator methyl methanesulfonate (MMS) stimulates the SEK1-dependent dual phosphorylation of SAPK/JNK (Thr183/Tyr185) in a dose and time dependent manner. Phosphorylation of SAPK/JNK was quantitatively related to both DNA strand break formation and inhibition of DNA synthesis. This indicates that MMS-induced DNA damage might generate a signal leading to an increase in SAPK/JNK phosphorylation. The level of MMS-triggered SAPK/JNK phosphorylation which was observed 4-8 h after exposure was reduced in DNA-PKCS deficient SCID cells as well as in Cockayne's syndrome B knock-out mouse cells (Csb^{-/-}). SAPK/JNK phosphorylation occurring early (up to 2h) after MMS treatment however was similar in wild-type, SCID and Csb^{-/-} cells. Notably, late MMS-induced phosphorylation of SAPK/JNK was not impaired in other repair deficient cell lines such as ATM^{-/-} or PARP^{-/-} cells. Also, p53^{-/-} cells did not differ from corresponding wild-type cells regarding dual phosphorylation of SAPK/JNK provoked by MMS treatment. UV-C-triggered phosphorylation of SAPK/JNK was similar in all cell types investigated, indicating the effects observed to be specific for alkylating compounds. The data indicate that both DNA-damage independent and -dependent mechanisms contribute to the activation of SAPK/JNK by MMS. Based on the data we suggest that early (up to 2 h after treatment) signaling to SAPK/JNK is independent from DNA damage-related mechanisms, whereas late (> 4 h after exposure) phosphorylation of SAPK/JNK originates predominantly from MMS-induced DNA damage processed in a DNA-PK CS and Csb-dependent manner.