Maintaining genome integrity

Meeting on Responses to DNA Damage

Raymond Waters

Cardiff University Medical School, Cardiff, UK

This Biochemical Society and the Royal Society of Chemistry meeting on Responses to DNA Damage took place between 19 and 21 September 2005 in Brighton, UK. The meeting was organized by P. Jeggo, A. Lehmann, A. Doherty and K. Caldecott.

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disease (Friedberg *et al.*, 2005a). This conference on Responses to DNA Damage conveyed an understanding of the complexity of repair and included reports on the intimate details of how enzymes recognize and remove certain DNA lesions. Fig 1 summarizes the DNA repair mechanisms covered in this report; it shows those that remove DNA damage and those that allow cells to tolerate damage.

Base excision repair

The meeting began with the presentation of the Nucleic Acids Award to G. Verdine (Cambridge, MA, USA), who considered the surveillance, recognition and repair of oxidative DNA lesions through base excision repair (BER; Barnes & Lindahl, 2004; Fromme & Verdine, 2004). During this process, DNA *N*-glycosylases remove damage, and Verdine focused on the 8-oxoguanine *N*-glycosylase—Ogg1—which removes 8-oxo-7,8-dihydroguanine (8-oxoGua). This lesion mispairs during DNA replication and is mutagenic. Verdine described the structural basis for discriminating 8oxo-Gua from guanine. Only 8-oxoGua enters the Ogg1 active site because guanine is excluded owing to steric repulsion. Using the *Escherichia coli* Ogg1 homologue MutM, early intermediates before base extrusion were studied. Simple bond rotations around the DNA backbone at the site of the extra-helical 8oxoGua allow the lesion to enter the MutM active site for base excision, and there is a bias against GC base pairs to reduce the time taken to interrogate the DNA. MutM diffusion is fast, and every base is sampled a thousand times every one-tenth of a second. This fascinating analysis set the foundation for subsequent presentations.

J. Cadet (Grenoble, France) reported on the spectrum and frequency of oxidative DNA damage, which is an area fraught with over-estimations owing to lesions created during DNA purification. His data show that the spontaneous incidence of 8-oxoGua is close to two lesions per ten million bases and not one per ten thousand as previously reported. These results are crucial for the correlation of specific damage to mutation frequencies. B. Dimple (Boston, MA, USA) considered how BER processes oxidize abasic sites and focused on Ape1 and DNA polymerase- β (Pol β). Ape1 is an apurinic/apyrimidinic endonuclease, but it also cleaves at the 4'-oxidation product 2-deoxypentose-4-ulose and the 1'-oxidation product 2-deoxyribonolactone. After Ape1 cleavage of 2-deoxyribonolactone residues, attempted excision by Pol β leads to protein–DNA crosslinks by amide bonds, which block BER and

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Introduction

Genomes have always been vulnerable to damage from intra- and extracellular sources such as oxidative species generated by cell metabolism, spontaneous base loss or modification, radiation and chemicals (Friedberg *et al.*, 2005a). As a result, mechanisms to cope with DNA damage developed early in evolution and have been conserved. Organisms need some genome stability, but must allow mutational changes to drive adaptation and evolution. How this is achieved has been the subject of much interest primarily because, in humans, defects in maintaining genome stability are associated with susceptibility to cancer, abnormal immune responses and genetic

Pathology Department, Medical School, Cardiff University, Heath Park, Cardiff CF14 4XN, UK
Tel: +44 2920 744 848; Fax: +44 2920 744 276;
E-mail: watersr1@cf.ac.uk

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Fig 1 | DNA-repair mechanisms. Base excision repair, nucleotide excision repair and mismatch repair all usually restore DNA to its pre-damaged state. Mechanisms such as homologous recombination and non-homologous end joining can repair DNA or result in changes in DNA sequences to varying degrees. Translesion synthesis is a way of coping with lesions instead of removing them, and specific DNA polymerases can replicate past DNA damage with varying degrees of accuracy, sometimes leading to mutation.

therefore must be removed. Importantly, Dempfle and G. Dianov (Harwell, UK) reported that Ape1 is the main enzyme in human cells that removes spontaneous phosphoglycolate residues from the 3'-ends at oxidative single-strand breaks (SSBs). Dianov used a DNA-protein crosslinking assay to show that polynucleotide kinase initiates repair of SSBs with a 3'-end phosphate, whereas Ape1 is used for those with phosphoglycolate or 8-oxoGua.

P. Schär gave an intriguing view of thymine DNA *N*-glycosylase (TDG), which excises uracil and thymine from G:U and G:T mispairs in the deamination of cytosine and 5-methylC, respectively. The TDG N-terminal domain is a flexible clamp that changes conformation on binding to DNA. It remains at the apurinic/aprimidinic-site after base excision and is released only after this conformation is reversed by the addition of small ubiquitin-like modifiers (SUMO). TDG interacts with transcription factors, suggesting a function in gene regulation, and is indispensable for embryonic development. Analyses of TDG-deficient mouse embryonic stem cells and fibroblasts (MEFs) revealed that some genes are differentially regulated in TDG-proficient compared with TDG-deficient MEFs, but not in embryonic stem cells. Unlike other known *N*-glycosylases, TDG probably has a role in establishing gene expression patterns during differentiation.

Base excision repair has implications for several issues related to cancer. B. Sedgwick (London, UK) described studies with anti-cancer agents that induce BER-repairable DNA methylations and proposed that inhibiting repair might sensitize tumour cells to these agents. A main cytotoxic lesion is 3-methyladenine (3-meA), which is excised from DNA by 3-meA-DNA glycosylase (AAG). Derivatives of HeLa cells with low AAG levels had increased sensitivity to the anti-cancer agents, therefore new therapies ablating AAG activity in human carcinoma cells might enhance chemotherapeutic regimes.

Thiopurine derivatives are used to suppress the immune system and to treat leukaemia. They are metabolized to 6-thioguanine (6-TG) and its incorporation into DNA is important for their impact.

P. Karran (London, UK) examined why squamous cell skin carcinoma is more frequent in transplant patients treated with thiopurine derivatives. Thiopurines absorb ultraviolet A (UVA), and DNA with 6-TG is a potential UVA chromophore. When cells with 6-TG in their DNA are exposed to UVA, mutagenic reactive oxygen species are generated, creating DNA damage. Because the skin of treated patients is UVA-photosensitive, this could explain the high cancer incidence. Reducing these events in patients treated with thiopurine derivatives should be a main goal.

Mismatch repair

DNA polymerases make replicative errors and mismatched base pairs can escape proofreading. These can be corrected by mismatch repair (MMR) to ensure mutation rates in replicating cells are not excessive (Kunkel & Erie, 2005). Exciting research by T. Sixma (Amsterdam, The Netherlands) explains important functional aspects of the *E. coli* MutS mismatch recognition protein. It relies on the instability of the mismatch, and then on verification through ATP binding to MutS, to create a sliding clamp that involves a key residue—glutamate 38. This residue forms an H-bond to one of the mispaired bases and the charge on the glutamate is important to discriminate mismatched from normally paired DNA. Crystal structures of mutated proteins complexed with mismatched DNA have revealed that this bond is important for the ATP-dependent verification of mismatch binding.

The mutation of a subset of human MMR genes causes most cases of the cancer susceptibility syndrome hereditary non-polyposis colorectal cancer. This subset consists of the MutS and MutL human homologues (MSH and MLH, or post meiotic segregation (PMS) proteins, respectively). R. Fishel (Philadelphia, PA, USA) examined their activation and signalling. There are five MSH and four MLH proteins, which seem to function as heterodimers. The MSH2–MSH3 and MSH2–MSH6 heterodimers repair a variety of DNA mispairs, whereas MSH4–MSH5 operates only in meiosis I and has no role in MMR. There are three MLH/PMS heterodimers: MLH1–PMS1, MLH1–PMS2 and MLH1–MLH3. Whereas MLH1–PMS2 seems to have a principal role in MMR, the role of the other two heterodimers remains unclear. Another role is also implied for MLH1 and PMS1, which are important for the increase in p53 phosphorylation by ataxia telangiectasia mutated (ATM) in response to DNA damage (Luo *et al*, 2004). The question of how specific DNA mismatches that activate MSH ADP–ATP exchange are recognized requires further research. DNA *N*-glycosylases recognize the same DNA lesions as MSH proteins, but the specificity of the former for a single DNA substrate is remarkable. By contrast, MSH proteins recognize a plethora of DNA mismatches that far outnumber those recognized by DNA *N*-glycosylases. Fishel showed evidence for a simple recognition process by MSH proteins and proposed a molecular switch model that satisfies the requirement that the excision tracts are directional and cover only the region between initiating strand scission to just past the mismatch.

Nucleotide excision repair

Nucleotide excision repair (NER) operates on a spectrum of DNA lesions, including those caused by UV. Defects in the NER system promote non-variant forms of the cancer-prone recessive genetic disorder xeroderma pigmentosum, in which high frequencies of melanoma are often observed in areas exposed to sunlight (Friedberg *et al*, 2005a).

Most Y-family DNA polymerases are thought to be used for translesion synthesis. An unexpected role of the Y-family DNA polymerase Pol κ in NER was reported by T. Ogi. Although Pol κ cannot bypass UV photoproducts, Pol κ -deficient MEFs are UV-sensitive and have reduced NER. This raises the intriguing possibility that either only certain lesions need this polymerase during NER, or that Pol κ is used specifically for NER in certain parts of the genome.

B. den Dulk (Leiden, The Netherlands) used budding yeast to describe the DNA-damage response protein Rad32, which stabilizes Rad4 and YDR314C. Rad4 combines with Rad32 to recognize DNA damage in non-transcribed sequences for NER. The YDR314C protein is essential for preferential repair of the transcribed strand of RNA Pol I-transcribed ribosomal DNA. For Pol II-transcribed genes in the *rad32* mutant, repair of the non-transcribed strand is absent and lesions in the transcribed strand are less efficiently repaired, whereas repair of both strands of rDNA is absent. The amounts of Rad4 and YDR314C are strongly reduced in the absence of Rad32, thus Rad32 probably protects them from degradation. In the same yeast, R. Waters (Cardiff, UK) showed how chromatin remodelling influences NER (Waters & Smerdon, 2005). After UV treatment, histones H3 in nucleosomes at the repressed mating pheromone α -factor 2 (*MFA2*) promoter are hyperacetylated and chromatin in the promoter becomes accessible to restriction. Both events return to the pre-UV state through NER. Histone hyperacetylation and chromatin remodelling depend on Gcn5 and partly on Swi2, respectively, but not on damage recognition by Rad4 or Rad14. In the absence of NER, the remodelled chromatin does not return to its pre-UV state. Gcn5 also optimizes transcription at *MFA2*, but when Gcn5 functions in repair of the repressed gene there is no transcription, and the TATA box-binding protein is excluded. At the same nucleosomes at *MFA2* there was little change in histone H4 acetylation after UV. However, acetylation increased after UV at H3 and H4 in the total histones in the cell, even in mutants totally deficient in Gcn5. Therefore, to facilitate NER there are probably different specific enzymes that modify chromatin in different regions of the yeast genome. Which proteins facilitate access to certain chromosomal domains, the types of modifications required in each domain, what the domain boundaries are and how much overlap there is with proteins needed to activate transcription, are intriguing questions that remain unanswered.

Double-strand DNA break repair

Recombination facilitates DNA repair, creates diversity for evolution, and enables the production of antibodies, whereas defects in this process are linked to cancer-prone conditions. Recombination can be subdivided into homologous recombination (HR; Liu & West, 2004) and non-homologous end joining (NHEJ), both of which are crucial for genome stability (Shiloh, 2004; Friedberg *et al.*, 2005a).

S.C. West (London, UK) reviewed the mechanisms of double-strand break (DSB) repair by HR. Mutations in the breast cancer 2 (*BRCA2*) gene are associated with early-onset breast cancer and are linked to defects in DSB repair by HR. The C-terminal BRCA2 region interacts with the essential recombination protein RAD51 and contains a site (S3291) that is phosphorylated by cyclin-dependent kinases (CDKs). When this residue is phosphorylated, it blocks C-terminal interactions between BRCA2 and RAD51. Phosphorylation at this site is low in S-phase when HR is active, but increases as cells progress to mitosis. DNA damage overcomes cell-cycle regulation by reducing the phosphorylation.

This stimulates the interaction between BRCA2 and RAD51 and might be a switch to regulate RAD51 recombination activity, which could explain why BRCA2 C-terminal deletions lead to radiation sensitivity and cancer predisposition.

Cells defective in any RAD51 paralogue (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) are defective in HR. Extracts from cells with mutations in *RAD51C* and *XRCC3* have reduced levels of Holliday junction resolvase activity and RAD51C is essential for branch migration and Holliday junction resolution *in vitro*. Therefore, RAD51C and XRCC3 are key components of the HJ resolvosome. L. Pellegrini (Cambridge, UK) showed the crystal structure of the ATPase domain of human RAD51 bound to a BRCA2 BRC4 peptide. This explains the *in vivo* and *in vitro* inhibitory properties of isolated BRC repeats towards RAD51 nucleoprotein filament formation. Analysis of the structure uncovered a general polymerization mechanism shared by all members of the RecA-superfamily of proteins essential for HR. This mechanism received experimental confirmation by the determination of the structure of several archaeal and eukaryotic recombinases. The challenge now is to provide a description at the atomic level of all of the steps in the process of BRCA2-dependent regulation of RAD51 function.

H.E. Bryant (Sheffield, UK) investigated HR in mammalian fibroblasts at replication forks that have collapsed owing to endogenous DNA SSBs. She compared these forks with those produced when replication runs into camptothecin (CPT)-stabilized DNA SSBs. SSB repair-defective cells had increased spontaneous γ H2AX and RAD51 foci, suggesting that endogenous SSBs collapse replication forks to trigger HR. γ H2AX, DSBs and RAD51 foci are synergistically induced by CPT in these cells, therefore the lack of SSB repair probably causes more collapsed forks and more HR. This implies that DSBs are rare substrates for spontaneous HR. Multiple HR events occurred in some clones, with no genome-wide increase in HR. Thus, intriguingly, an initial HR event frequently triggers a second one at the same locus.

People with the recessive genetic cancer-prone disorder ataxia telangiectasia have weakened immune systems and are defective in NHEJ (reviewed in Shiloh, 2004). S.P. Jackson (Cambridge, UK) explained how the kinases ATM, ATM and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) are recruited to DSBs to phosphorylate downstream targets. Mutation of ATM is also linked to loss of both cell-cycle control and the temporary arrest of DNA replication after ionizing radiation. Recruitment of a protein complex to DSBs is mediated through the interaction of ATM, ATR and the catalytic subunit of DNA-PK (DNA-PK_{cs}) with conserved, related motifs in their partner proteins NBS1, ATRIP and Ku80, respectively. ATM and ATR, once viewed as functioning independently, cooperate in response to γ -rays. ATR binds to replication protein A (RPA)-coated single-stranded DNA, the generation of which requires ATM and NBS1. Accordingly, ATM activation and NBS1 phosphorylation precede phosphorylation of Chk1—which governs cell-cycle arrest after γ -ray exposure—and recruitment of NBS1 to DNA damage precedes recruitment of ATR. ATM-dependent Chk1 phosphorylation, RPA-coated ssDNA generation and ATR recruitment are restricted to the S and G2 phases. These data provide valuable insight into the temporal regulation of cell-cycle checkpoints and the co-operative relationship between ATM and ATR in response to DSBs.

L. Pearl (London, UK) elegantly described the molecular events associated with NHEJ. DNA-PK_{cs} is recruited to DSBs by interaction

with the Ku70/Ku80 heterodimer, which is a primary sensor of free DNA ends, and the complex processes the breaks. Pearl purified the human DNA-PK_{cs}/Ku70/Ku80 holo-enzyme complex assembled on a defined DNA molecule and determined its three-dimensional structure using electron microscopy. The Ku dimer associates with the N-terminal HEAT-repeats of DNA-PK_{cs} to hold and position the DNA. Three-dimensional reconstruction revealed a symmetrical dimer in which two DNA-PK_{cs}/Ku70/Ku80 holo-enzymes orientate along a common longitudinal axis, with dimer interactions involving the 'claw' domains in the N-terminal HEAT-repeats. The proximity of the contacts to the probable positions of the DNA ends indicate that the dimers are synaptic complexes with broken ends held in close proximity by self-association of DNA-PK_{cs}/Ku70/Ku80.

In budding yeast, loss of telomeric DNA-binding factor Rap1 leads to telomere fusions by NHEJ. S. Marcand (Fontaney aux Roses, France) reported that these fusions were absent in a *pol4-Δ* mutant, which was surprising, because the telomeric 3' strands are made only of thymine and guanine and cannot act as primers for each other by conventional base pairing. Pol4 seems to act in a template-dependent manner to join two ends with TGTG-3' single-strand extensions from a 3' base that is un- or mispaired when NHEJ fuses telomeres, whereas Rap1 normally functions to block these fusions. NHEJ, previously assumed to be restricted to eukarya, has a functionally homologous prokaryotic NHEJ apparatus that has been characterized by A. Doherty (Brighton, UK). It seems to require only two proteins—Ku and a multifunctional DNA ligase—that form a complex at DSBs, which has break-recognition, end-processing and ligation activities required for repair. His findings lay the foundation for understanding the molecular mechanisms of NHEJ and provide a conceptual framework for delineating the reactions in eukaryotes.

Translesion DNA synthesis

Some DNA polymerases replicate damaged DNA templates with varying degrees of accuracy by translesion synthesis (TLS; Friedberg *et al*, 2005b). Xeroderma pigmentosum variant cancer-prone patients have a mutation in one such polymerase, DNA polymerase ζ (Pol ζ).

R.P. Fuchs (Strasbourg, France) gave a fascinating description of how DNA polymerases trade places during TLS in *E. coli*. DNA polymerase V (Pol V) is the main polymerase responsible for bypass and induced mutagenesis. Efficient Pol V-mediated TLS was reconstituted using the replicative Pol III holoenzyme (Pol III HE) and Pol V in the presence of β -clamp and RecA as accessory factors. When Pol III HE disconnects from the template in the vicinity of a replication-blocking lesion, Pol V binds the replication intermediate and forms a moderately stable complex through an interaction with the tip of the RecA filament and the β -clamp—the processivity factor donated by the blocked Pol III HE. These interactions enable Pol V to synthesize a TLS patch long enough to support subsequent extension by Pol III HE; it needs to extend at least four nucleotides beyond the lesion to be successfully elongated. In the absence of the accessory factors, the patch synthesized by Pol V is too short and is degraded by the Pol III-proofreading exonuclease that senses the distortion induced by the lesion, and thus bypass is aborted.

Y-family DNA polymerases undertake TLS, but errors are up to 10,000-fold higher than with replicative polymerases. The latter achieve high fidelity through an editing function and an induced-fit active site that requires a Watson–Crick base pair. W. Yang (Bethesda, MD, USA) presented insight into the Y-family

polymerases that have no editing function. An open, preformed active site seems ready to accept lesion-containing DNA substrates to non-discriminately incorporate mismatched incoming nucleotides. The structures of an archaeal Y-family polymerase, Dpo4, in a complex with damaged DNA partly support this. Although Dpo4 can accommodate lesions, catalysis does not occur efficiently with thymine dimers and the errors are 100-fold higher than predicted if incoming nucleotide selection is based purely on H bonds between the nucleotide and a template base. Structural studies of a complex of Dpo4 with a mismatched incoming nucleotide show that a mismatched base pair leads to poor base stacking. Therefore an incorrect incoming nucleotide is not properly stacked with the primer terminus. This dislocates Mg²⁺ ions, which are essential for the phosphoryl transfer reaction. When Mg²⁺ ions are replaced with Mn²⁺, which has relaxed coordination requirements, Dpo4 shows an increase in errors and reduced substrate specificity. Yang proposed metal-ion coordination as the rate-limiting step for synthesis by replicative and translesion polymerases.

M. Lopes (Zurich, Switzerland) used electron microscopy to study the *in vivo* structure of *Saccharomyces cerevisiae* replication forks encountering irreparable UV lesions. When reaching lesions, forks rapidly uncouple nascent leading and lagging strand replication. This generates long ssDNA regions at one side of the fork—the leading strand where delayed repriming occurs. Proper repair of the resulting ssDNA gaps on the replicated duplexes requires the DNA-damage checkpoint, TLS and HR. In fact, 2D-gel data intriguingly suggest that most TLS and HR events occur behind the moving replication forks.

Pol ζ associates with replication foci in S phase. A. Zlatanou (Paris, France) reported that there is a marked increase of these foci in human cells that have been exposed to UV, owing to stalled replication forks at the DNA damage and proliferating cell nuclear antigen (PCNA), which becomes mono-ubiquitinated (mUBI-PCNA). After UV exposure, Pol ζ interacts physically with mUBI-PCNA, but not with unmodified PCNA, suggesting that this interaction is the 'key' to 'unlock' stalled replication forks for TLS.

Interstrand DNA crosslink repair

Patients with Fanconi's Anaemia (FA) have increased cancer predisposition and their cells are hypersensitive to agents that cause inter-strand cross-links (ICLs). It is a genetically heterogeneous disease, and 11 complementation groups have been identified (Thompson *et al*, 2005a). The repair of ICLs is poorly understood and involves excision and recombination (Friedberg *et al*, 2006). Mutations in *BRCA1* are associated with early-onset familial breast and ovarian cancer but the molecular defect associated with loss of *BRCA1* was unclear. However, we know that cells from these patients are also hypersensitive to crosslinking agents.

Fortunately, responses to DNA damage in mammalian cells are conserved in cells such as the avian B-cell lymphoma line DT40, which offers the advantage of efficient homologous gene targeting. K. Hiom (Cambridge, UK) used this to examine *BRCA1* function in the DNA-damage response. DT40 cells knocked out for either *BRCA1* or its partner protein *BRCA1*-associated RING domain 1 (*BARD1*) are defective in HR repair (HRR) and are sensitive to agents that induce ICLs. Whereas the C-terminal domains of *BRCA1* (*BRCT*) and *BARD1* are crucial for HRR, the E3 ubiquitin ligase activity of the *BRCA1*–*BARD1* complex is dispensable. The *BRCA1*-interacting

protein (BRIP1) interacts with the BRCT domain of BRCA1, but after DNA damage, the phenotype of a *BRIP1* DT40 knockout differs from that for *BRCA1*—showing a profound sensitivity to cisplatin and an acute cell-cycle arrest in late S–G2. BRIP1 functions independently of BRCA1 in the FA pathway for ICL repair and is the previously unassigned FA group J (*FANCL*) gene. When *BRCA1* is mutated in combination with the *FANCL* or *FANCC* genes, the FA-like cell-cycle arrest after cisplatin is compromised by defects in BRCA1. Therefore, BRCA1 probably has a dual role in the repair of ICLs by promoting HRR and enforcing a cell-cycle checkpoint.

N.J. Jones (Liverpool, UK) showed that FANCD2 interacts with a conserved C-terminal site in BRCA2 and that these immunoprecipitate together. BRCA2 interacts with the monoubiquitinated and non-monoubiquitinated forms of FANCD2, and these interactions depend on FANCG, but not on other FA core complex proteins. This co-immunoprecipitation occurs in human cells from complementation groups FA-A, FA-C, FA-E and FA-F, and hamster *FANCL* mutant cells that express only the non-monoubiquitinated form of FANCD2. Conversely, co-immunoprecipitation is absent in FA-D2 and is restored by wild-type *FANCD2* or mutant K561R *FANCD2* cDNAs. Therefore, D2-S interacts with BRCA2 and the ubiquitinated D2-L isoform. There was no interaction between BRCA2 and D2 in FA-G cells. Thus, interaction of BRCA2 with the isoforms of D2 requires FANCG and is independent of other FA proteins. FANCG also interacts with BRCA2 and XRCC3, but does not require exogenous DNA damage. The data indicate a role for some FA proteins in HRR and for a function of the FANCG protein that is independent of the FA core complex.

NER has an early role in ICL repair in *S. cerevisiae* by incising crosslinked DNA and releasing the covalently linked strands. P.J. McHugh (Oxford, UK) examined the events that occur downstream of NER and *Pso2*, a yeast gene required specifically for the repair of crosslinked DNA. These downstream events are thought to rely on HR or TLS, or both. He showed that the TLS polymerase ζ (Pol ζ ; encoded by *REV3* and *REV7*) with *REV1*, a member of the Y-family polymerases, maintains viability in the presence of ICLs. Consistent with the sequential incision by NER followed by TLS past the incised ICL repair intermediate, Pol ζ is found associated with chromatin when ICLs are induced, and PCNA is monoubiquitinated to enable the action of Pol ζ during repair.

Concluding remarks

Many advances in our knowledge of DNA repair were highlighted at this meeting, but we still know little about the detailed structural chemistry of the molecular reactions behind most repair mechanisms, and how components interact either with DNA or with each

other. Inroads have been made through elegant approaches to explain some mechanisms such as BER and MMR. However, inter-relationships between DNA repair pathways and how they might vary with the cell cycle or with chromatin structure remain elusive. There is no doubt that these aspects will be the topic of intense efforts and many future meetings.

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