# 7

# Perspectives

## RESPONDING TO DNA DAMAGE

The genetic material of cells is continually subjected to genotoxic attacks, raising the question how the cell can protect itself from the potentially harmful consequences of DNA damage. Decades of research have uncovered in increasing detail how cells, from bacteria to mammals, have dedicated mechanisms for repairing DNA by removing DNA damage and restoring the original sequence. It has also become apparent that the DNA damage signaling cascade, through protein phosphorylation, regulates the cell cycle and acts in concert with DNA repair to protect genomic integrity and cell viability. In addition, it has more recently become apparent that other post-translational modifications (PTM) such as poly(ADP-ribosyl)ation (PAR), ubiquitylation and possibly sumoylation have important roles in DNA repair. These modifications are likely to have a signaling function but might also affect protein function due to imposed structural changes. The stability of the nucleotide excision repair factor DDB2 is for example regulated by both ubiquitin as well as PAR modifications on the protein (chapter 3). The widespread use of PTM in cells in concert with the possibility to target the same protein with different types of PTM adds to the challenge of understanding protein function and regulation.

Despite the clear role of phosphorylation in the DNA damage response, results from unbiased phosphoproteomic screens suggest that other pathways which are not obviously related to DNA damage repair may also be modulated (Bennetzen et al., 2010;Pines et al., 2011). The biological significance of such changes in the phosphoproteome are, however, often unknown and require further study. Nevertheless, it is clear that the most prominent changes in the phosphoproteome after cisplatin or ionizing radiation exposure are due to activation of PI3 like kinases such as ATM and ATR. These kinases are known to respond to DNA damage and are important regulators of cell cycle progression following genotoxic stress. What has been surprising, however, is the abundance of ATM/ATR target proteins that have been identified in both unbiased screens (Bennetzen et al., 2010;Pines et al., 2011) and screens specifically directed towards ATM/ATR target identification (Matsuoka et al., 2007;Stokes et al., 2007). It can thus be conjectured that processes other than cell cycle checkpoints, replication and repair are also regulated through these kinases.

Many of the cell's proteins can be phosphorylated. However, the phosphoproteome is subject to variables such as cell type, phase of the cell cycle and other parameters such as DNA damage induced stress. The prevalence of phospho modification is reflected by the cohesion complex in which all components (i.e. Smc1$\alpha$, Smc3, Rad21, SA1/2, Wapl and Pds5a/b) undergo multiple phosphorylation events. Consistent with its role in DNA repair (chapter 6), DNA damage signaling (Yazdi et al., 2002) and sister chromatid cohesion, changes in the pattern of cohesin phosphorylation were observed after cisplatin treatment (chapter 5). Although most phospho sites remain unchanged a significant increase or decrease of phosphorylation was found on Smc1$\alpha$, Smc3, SA2, Pds5a/b and Wapl. The complex changes in up and down phosphorylation events, are predicted to involve multiple kinases and likely reflects cohesin's diverse role in cell biology. The observed increase in putative ATM/ATR dependent phosphorylation of Smc1$\alpha$ and Pds5a could relate to a DNA damage signaling function while decreased Wapl phosphorylation, likely due to lower

PLK activity, could reflect the decrease in mitotic cells after cisplatin treatment. The significance of other changes remains, however, elusive.

The wealth of information obtained from unbiased mass spectrometry based analyses can evidently be used to improve our understanding of the interconnectivity of processes and pathways and their regulation by phosphorylation. It will allow changes to be observed in an unbiased manner after for example treatment with cytostatic drugs or pharmacological inhibitors, many of the latter being inhibitors of kinases. Consequently, target proteins for kinases can be identified as well as their wider impact on the cell's phosphoproteome. Such a broad view of the changes will allow the identification of pathways that might unexpectedly be altered in response to drugs, potentially creating a compensatory effect thereby reducing drug efficacy.

## FROM UV LESION TO DAMAGE SIGNALING

The most prominent response to DNA damage when considering phospho modifications relates to the activation of the ATR/ATM family of kinases. Responding to aberrant DNA configurations, i.e. double stranded DNA breaks or single stranded DNA, their activation upon exposure to DNA damaging agents is expected. Indeed these aberrant DNA structures can be induced, either directly or through processing, by a variety of DNA damaging agents such as cisplatin, UV or ionizing radiation. The manners by which UV lesions can promote ATM/ATR activation are manifold. Although it has been suggested on the basis of *in vitro* studies that UV lesions are directly recognized by components of the ATR signaling cascade (Unsal-Kacmaz et al., 2002), there is no evidence *in vivo* that ATR kinase is activated directly by UV lesions (chapter 4). It should nevertheless be stressed that kinase activation depends on processing of the UV lesions such that a DNA configuration is created that supports signaling.

A process that does lead to UV-induced ATM/ATR activation is DNA replication. As UV lesions cannot be passed by replicative polymerases, the presence of UV photolesions during replication can potentially lead to uncoupling of the polymerase from the replication fork helicase, resulting in extended single stranded DNA regions (Cotta-Ramusino et al., 2005). It is highly likely that these expanses of ssDNA lay at the base of ATR dependent UV mediated signaling during S phase (Byun et al., 2005), although other processes will also contribute. While most lesions are likely to be taken care of by DNA repair or translesion synthesis the possibility exists that the replication fork cannot recover leading to its collapse, a process that is associated with the formation of DSBs. UV lesions can therefore albeit indirectly activate ATM signaling.

Signaling is, however, not restricted to cycling cells as various mechanisms exist that evoke an ATR response in G0/G1 cells as well. One route to signaling proceeds via NER itself. Once a damage containing oligo is removed by NER the resulting structure, an approximately 35 nucleotide single stranded DNA gap bound by a single RPA moiety, should in principle be able to support ATR signaling. There are, however, two caveats: the time that such structures exist and the size of the formed gap. During the process of NER the gapped intermediates are expected to exist only transiently as they are removed by the

replicative polymerases δ or ε. Nevertheless, under certain conditions the process of gap filling might be attenuated. The abundance of factors required for gap filling such as PCNA, DNA polymerase δ/ε or ligase I is reduced in non-cycling cells (Zeng et al., 1994;Kurki et al., 1986;Moser et al., 2007). It can therefore be considered that upon high DNA lesion induction there might be an insufficient level of post-incision factors to complete repair, thus stabilizing the gapped DNA intermediates. A similar effect has previously been demonstrated in primary lymphocytes in which inhibition of repair was observed due to very low level of deoxyribonucleosides (Green et al., 1996).

A gap created by NER and covered by a single RPA unit would comply with the known structural requirements for ATR activation. Given that ssDNA patch size is a determinant for ATR activity, such minimal gaps as generated by NER are expected to promote signaling less efficiently then the large ssDNA regions formed after replication stalling (MacDougall et al., 2007). In fact, much of the ATR signaling after UV exposure of quiescent cells is dependent on the exonuclease EXO1 suggesting extension of the ssDNA gap (Sertic et al., 2011). It is unclear whether the conversion from a persistent NER gap to a resected gap is a ubiquitous event or whether it affects merely a small subset of NER gaps. The mechanism by which EXO1 would participate and is recruited to these gaps has also not been established. One possibility lies in the presence of a PCNA interaction domain (PIP-box) on EXO1 which perhaps could facilitate the recruitment to persistent gaps provided that PCNA, or any upstream components essential for PCNA loading, are not rate limiting factors.

Activation of ATR through the resection of NER induced gaps engages the G1/S checkpoint, preventing cells from entering S-phase. Such a safeguard would prevent the formation of toxic DSBs that are likely to form when replication forks encounter these gaps. It can, however, be envisioned that even in non-replicating cells the resection of gaps can lead to DSBs i.e. if during resection a ssDNA gap is encountered on the opposite DNA strand. The frequency of such events would be predicted to increase exponentially with higher DNA damage loads and could potentially lead to chromosomal rearrangements.

While repair of UV lesions via GG-NER contributes to signaling it has also become apparent that a failure to swiftly remove lesions equally is a cause for ATR activation. As already mentioned it is not the lesion per se, but rather a processed form of the lesion that underlies activation. Both in the presence and absence of GG-NER DNA damage signaling correlates with the formation of DNA breaks and in the latter case is controlled, at least in part, through APE1 dependent processing. Why APE1 acts on UV lesions is unclear. It is known that cytosine residues within UV photolesions are more prone to deamination events (Peng and Shaw, 1996) which might activate UNG glycosylase and subsequently APE1. Arguing against this, however, is the observation that XPE deficient cells that lack the capacity to remove CPD, fail to activate ATR when other GG-NER deficient cells do. Alternatively it is possible that aberrant nucleotides are a direct target for APE1 endonuclease activity as has been demonstrated *in vitro* (chapter 4, Ischenko and Saparbaev, 2002).

## THE ENIGMA OF RNA POLYMERASE ARREST AND SIGNALING

An additional mechanism that activates DNA damage signaling exists which is mediated by RNA polymerase II (RNAPII) arrest (Yamaizumi and Sugano, 1994). This signaling is particularly pronounced in TC-NER deficient cells with transcription stalling lesions like UV photoproducts and is characterized by high induction of p53. It is more than 10 years ago that RNA polymerase stalling, by either DNA damage or RNAPII inhibitors, was shown to control signaling yet a mechanistic explanation for this phenomenon has remained elusive. Although it is generally assumed that the stalling of RNA polymerase itself is a determinant for signaling this has not formally been demonstrated. It would be equally possible that indirect effects of transcriptional arrest such as imbalances in RNA transcript levels, the release of incomplete transcripts or disruption of RNAPII associated processes such as splicing, are causal for the observed p53 activation. However, it has been recently been demonstrated that these signaling events are mediated by the ATR kinase (Derheimer et al., 2007) although the mechanistic basis for its activation remains unclear. The archetype structure for ATR activation based on replication stalling and *in vitro* studies is an ssDNA gap coated with RPA. The formation of such gaps would be predicted to be independent of NER mediated incisions given that XPA deficient cells strongly respond to RNAPII stalling. Moreover, the activation of p53 in CS-B cells does not necessarily coincide with the presence of DNA breaks (chapter 4) or RPA (S. Lagerwerf personal communication). Although it cannot be excluded that low frequencies of single strand breaks, not detected by the assays used, can support signaling in the context of a stalled RNA polymerase, it was also observed that p53 activation did not coincide with phosphorylation of H2AX in CS-B cells (chapter 4). When ATR is activated, either upon replication stalling or through GG-NER induced ssDNA gaps, it is capable of phosphorylating H2AX. These differences between canonical ATR activation and activation through RNA polymerase arrest suggest a mechanistically distinct mode for ATR activation in the latter situation.

## REFERENCE LIST


1. Bennetzen,M.V., D.H.Larsen, J.Bunkenborg, J.Bartek, J.Lukas, and J.S.Andersen. 2010. Site-specific phosphorylation dynamics of the nuclear proteome during the DNA damage response. *Mol. Cell Proteomics.* **9**: 1314-1323.
2. Byun,T.S., M.Pacek, M.C.Yee, J.C.Walter, and K.A.Cimprich. 2005. Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes Dev.* **19**: 1040-1052.
3. Cotta-Ramusino,C., D.Fachinetti, C.Lucca, Y.Doksani, M.Lopes, J.Sogo, and M.Foiani. 2005. Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells. *Mol. Cell* **17**: 153-159.
4. Derheimer,F.A., H.M.O'Hagan, H.M.Krueger, S.Hanasoge, M.T.Paulsen, and M.Ljungman. 2007. RPA and ATR link transcriptional stress to p53. *Proc. Natl. Acad. Sci. U. S. A* **104**: 12778-12783.
5. Green,M.H., A.P.Waugh, J.E.Lowe, S.A.Harcourt, P.H.Clingen, J.Cole, and C.F.Arlett. 1996. Protective effect of deoxyribonucleosides on UV-irradiated human peripheral blood T-lymphocytes: possibilities for the selective killing of either cycling or non-cycling cells. *Mutat. Res.* **350**: 239-246.
6. Ischenko,A.A. and M.K.Saparbaev. 2002. Alternative nucleotide incision repair pathway for oxidative DNA damage. *Nature* **415**: 183-187.
7. Kurki,P., M.Vanderlaan, F.Dolbeare, J.Gray, and E.M.Tan. 1986. Expression of proliferating cell nuclear antigen (PCNA)/cyclin during the cell cycle. *Exp. Cell Res.* **166**: 209-219.
8. MacDougall,C.A., T.S.Byun, C.Van, M.C.Yee, and K.A.Cimprich. 2007. The structural determinants of checkpoint activation. *Genes Dev.* **21**: 898-903.
9. Matsuoka,S., B.A.Ballif, A.Smogorzewska, E.R.McDonald, III, K.E.Hurov, J.Luo, C.E.Bakalarski, Z.Zhao, N.Solimini, Y.Lerenthal, Y.Shiloh, S.P.Gygi, and S.J.Elledge. 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316**: 1160-1166.
10. Moser,J., H.Kool, I.Giakzidis, K.Caldecott, L.H.Mullenders, and M.I.Fousteri. 2007. Sealing of chromosomal DNA nicks during nucleotide excision repair requires XRCC1 and DNA ligase III alpha in a cell-cycle-specific manner. *Mol. Cell* **27**: 311-323.
11. Peng,W. and B.R.Shaw. 1996. Accelerated deamination of cytosine residues in UV-induced cyclobutane pyrimidine dimers leads to CC-->TT transitions. *Biochemistry* **35**: 10172-10181.
12. Pines,A., C.D.Kelstrup, M.G.Vrouwe, J.C.Puigvert, D.Typas, B.Misovic, G.A.de, S.L.von, B.van de Water, E.H.Danen, H.Vrieling, L.H.Mullenders, and J.V.Olsen. 2011. Global phosphoproteome profiling reveals unanticipated networks responsive to cisplatin treatment of embryonic stem cells. *Mol. Cell Biol.* **31**: 4964-4977.
13. Sertic,S., S.Pizzi, R.Cloney, A.R.Lehmann, F.Marini, P.Plevani, and M.Muzi-Falconi. 2011. Human exonuclease 1 connects nucleotide excision repair (NER) processing with checkpoint activation in response to UV irradiation. *Proc. Natl. Acad. Sci. U. S. A* **108**: 13647-13652.
14. Stokes,M.P., J.Rush, J.Macneill, J.M.Ren, K.Sprott, J.Nardone, V.Yang, S.A.Beausoleil, S.P.Gygi, M.Livingstone, H.Zhang, R.D.Polakiewicz, and M.J.Comb. 2007. Profiling of UV-induced ATM/ATR signaling pathways. *Proc. Natl. Acad. Sci. U. S. A* **104**: 19855-19860.
15. Unsal-Kacmaz,K., A.M.Makhov, J.D.Griffith, and A.Sancar. 2002. Preferential binding of ATR protein to UV-damaged DNA. *Proc. Natl. Acad. Sci. U. S. A* **99**: 6673-6678.
16. Yamaizumi,M. and T.Sugano. 1994. U.v.-induced nuclear accumulation of p53 is evoked through DNA damage of actively transcribed genes independent of the cell cycle. *Oncogene* **9**: 2775-2784.
17. Yazdi,P.T., Y.Wang, S.Zhao, N.Patel, E.Y.Lee, and J.Qin. 2002. SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. *Genes Dev.* **16**: 571-582.
18. Zeng,X.R., H.Hao, Y.Jiang, and M.Y.Lee. 1994. Regulation of human DNA polymerase delta during the cell cycle. *J. Biol. Chem.* **269**: 24027-24033.