Biological and Functional Analysis of the ATR Checkpoint Pathway

Biologische en functionele analyse van de ATR signaaltransductieroute

Daniël O. Warmerdam

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Contents

Scope of the thesis

In 2009 the President of the United States of America Barrack Obama developed a comprehensive plan to fight cancer. He pledged: “A new effort to conquer a disease that has touched the life of nearly every American by seeking a cure for cancer in our time” [1]. But will there ever be a cure for cancer? And if yes, when will that be? To be able to state something about these matters it is important to put cancer research in a broader perspective. To do that, we can visualize the human body as a balance. Under normal conditions the weight at both ends of the balance is equal, however due to hereditary disorders or a hazardous lifestyle the balance can become unstable, weighing more to one side, letting that side go down and thereby raising the other. The way the two sides of a balance are interconnected is, in a way, very similar to how cells, organs and all the different parts of the human body are connected to one another. Cancer is a disease that arises from accumulating genetic mutations that lead to genetic instability and the subsequent formation of a tumor due to the gain or loss of cellular functions [2]. But why does not everybody get cancer? The balance may sometimes already be unevenly distributed from the beginning, meaning less or more weight on either side of the balance. Some families carry genetic mutations that makes them more prone to developing cancer [3]. Also during the course of someone’s life the balance may get distorted. Exposure to radioactive compounds or a non-healthy lifestyle such as smoking will increase the chance of gaining mutations [4, 5]. Therefore the cells of some individuals are genetically more unstable or accumulate mutations more easily than others. The time it takes to form a tumor may vary largely in a given population for these reasons. For individuals carrying inherited genetic cancer susceptibility it may be easier to predict what cellular processes might get deregulated, although these are never the same for every person. Like for people that do not carry any known genetic risk, the course of tumor formation greatly depends on chance. Although we define cancers mainly based on location and characteristics, all tumors are distinct and cancer can therefore not be defined as one disease. The genetic component is becoming more and more important in differentiating tumors [2, 6]. Life expectancy and therapy success seem to be greatly dependent on the tumors genetic characteristics and therefore to understand cancer we must be able to identify the tumors unique ‘genetic barcode’ and act accordingly [7]. Knowing the genetic barcode may not be enough however, which processes are disrupted in the tumor, how precisely are they affected and what is the relationship of these processes to each other? In order to rebalance we need to know how much weight and at which side we have to place it, questions that can be answered only through the practice of fundamental scientific research. In addition, one cure for cancer does not exist. Instead, individual treatments can be made more efficient on the basis of the tumors genetic information [8-10]. The time it will take to get to that point is dependent on the success of biomedical research in creating more mechanistic insight into cellular processes and uncovering new connections between pathways.

Recent advancements have been made into this direction, and highlight the course of future research in the fight against cancer. An enormous effort was taken into resolving the DNA sequence of the human genome, published in 2001 [11, 12], followed by the first cancer genome in 2007 [13]. Besides unraveling the content of the healthy human and cancer genome, these efforts have also conducted to the rapid advancements in large scale DNA sequence technology for clinical use. Scientific researches creating further insight into cellular processes that are linked to cancer have been recently awarded with the Nobel

Prize. In 2001, Hartwell, Hunt and Nurse were awarded for the discovery of key regulators of the cell cycle [14-16]. And in 2009, Blackburn, Greider and Szostak received the award for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase [17-19]. Both findings generated more understanding into the process of cell division and the maintenance of genome integrity with vast implications for cancer development.

It is within the context laid out above that, in this thesis, we study the ATR-mediated checkpoint response that helps to protect cells from becoming genetically instable after DNA damage. The source of genotoxic DNA lesions that may eventually result in a mutation arise mostly from exogenous sources like ultra violet (UV) light, nuclear radiation and cigarette smoke and from endogenous cellular metabolic processes. Different cellular mechanisms have evolved, which efficiently remove most genotoxic lesions from the DNA before they become incorporated as a mutation. Important in the process of lesion removal and subsequent repair of the DNA is the detection of DNA damage. The ATR-Chk1 pathway is involved in sensing DNA alterations and the subsequent activation of this process will result in a temporal halt of the cell cycle to create time for the repair of the damaged DNA. Thereby it functions as a so called 'checkpoint', controlling the integrity of the genome. In recent years biochemical work has provided invaluable insight into the requirements, substrates and activities of proteins involved in the ATR-mediated checkpoint pathway [20-22], yet not much was known about how the ATR-Chk1 pathway operates in living cells and how the spatio and temporal behaviour of proteins in this pathway influence the DNA damage response. Here, we set out to investigate the biological and functional involvement of the ATR-ATRIP, Rad17-RFC and Rad9-Rad1-Hus1 (9-1-1) putative DNA damage sensor complexes in ATR-mediated checkpoint signalling. To study the behaviour of the involved proteins, we generated human cell lines that stably express GFP-fusion proteins from the ATR-mediated checkpoint pathway. In **chapter 3** we describe how we generated and validated these cell lines and show results showing a differential dynamic behaviour of the 9-1-1 and ATR-ATRIP damage sensor complexes and downstream effector kinase Chk1 in response to UV-induced DNA damage.

We observed that GFP-Rad9 nuclear foci induced by DNA damage were not visible in all irradiated cells, suggesting a cell cycle-dependent regulation of DNA lesion processing. To address this we took advantage of the existence of various cell cycle markers and additionally generated cells with two fluorescent-fusion proteins that enabled us to discriminate S and G1/G2 phase in living cells. Thereby we uncovered a relationship between the cell cycle, DNA repair mechanisms and Rad9 focus formation, which is discussed in **chapter 4**. In **chapter 5**, we continue to investigate the cell cycle-dependent regulation of the DNA damage response upon UV damage specifically in G2 cells. We showed that UV-induced checkpoint activation in G2 is regulated in a different manner compared to G1 and S-phase and in addition is distinct from ionizing radiation-induced checkpoint activation in G2 phase.

It is known that ATR is responsible for the phosphorylation of Chk1 in a 9-1-1-dependent manner, indicating a direct relationship between the ATR-ATRIP and 9-1-1 complexes. In **chapter 6** we investigated this relationship in more detail and showed that the phosphorylation of Rad17 by ATR is necessary for stable recruitment of the 9-1-1 complex to sites of damage. This indicates that although recruitment of the 9-1-1 and ATR-ATRIP complexes to damaged DNA might be independent, maintaining checkpoint activation is dependent on their functional interaction. Overall, these results demonstrate that protein mobility measurements are an excellent addition to classical biochemical assays because they enable us to study the effect of transient protein-protein interactions and post-

translational modifications such as phosphorylation on protein behaviour in more detail. Additionally, another type of post-translational modifications might influence 9-1-1 protein complex dynamics. In **chapter 7** we demonstrated that the Hus1 component of the 9-1-1 complex is modified by ubiquitination. Mass-spectrometry analysis identified a lysine in Hus1 that is ubiquitinated. Moreover we identified a number of potential candidate E3 ubiquitin ligases that might be involved in 9-1-1 ubiquitination. The data in this last chapter might give us new insights into the regulation of the 9-1-1 complex.

Will there ever be a cure for cancer? The work presented in this thesis reflects on a particular topic of the DNA damage response, which is linked to cancer development. What is presented here will not cure cancer, but this scientific contribution, like many others, may help to understand the basic workings of the human cell and thereby as a whole, help to fight cancer in a rational and decisive manner [23, 24].

REFERENCES

1. Obama. [cited; Available from:www.whitehouse.gov/video/EVRO22409].
2. Hahn, W.C. and R.A. Weinberg, *Modelling the molecular circuitry of cancer.* Nat Rev Cancer, 2002. **2**(5): p. 331-41.
3. Narod, S.A., *Modifiers of risk of hereditary breast and ovarian cancer.* Nat Rev Cancer, 2002. **2**(2): p. 113-23.
4. *KWF kanker bestrijding*. [cited; Available from: www.kwf.nl].
5. Weisburger, J.H., *Lifestyle, health and disease prevention: the underlying mechanisms.* Eur J Cancer Prev, 2002. **11 Suppl 2**: p. S1-7.
6. Mullenders, J. and R. Bernards, *Loss-of-function genetic screens as a tool to improve the diagnosis and treatment of cancer.* Oncogene, 2009. **28**(50): p. 4409-20.
7. van 't Veer, L.J., et al., *Gene expression profiling predicts clinical outcome of breast cancer.* Nature, 2002. **415**(6871): p. 530-6.
8. Reinhardt, H.C., et al., *Exploiting synthetic lethal interactions for targeted cancer therapy.* Cell Cycle, 2009. **8**(19): p. 3112-9.
9. Paddison, P.J., et al., *A resource for large-scale RNA-interference-based screens in mammals.* Nature, 2004. **428**(6981): p. 427-31.
10. Brummelkamp, T.R. and R. Bernards, *New tools for functional mammalian cancer genetics.* Nat Rev Cancer, 2003. **3**(10): p. 781-9.
11. Venter, J.C., et al., *The sequence of the human genome.* Science, 2001. **291**(5507): p. 1304-51.
12. Lander, E.S., et al., *Initial sequencing and analysis of the human genome.* Nature, 2001. **409**(6822): p. 860-921.
13. Greenman, C., et al., *Patterns of somatic mutation in human cancer genomes.* Nature, 2007. **446**(7132): p. 153-8.
14. Simanis, V. and P. Nurse, *The cell cycle control gene cdc2+ of fission yeast encodes a protein kinase potentially regulated by phosphorylation.* Cell, 1986. **45**(2): p. 261-8.
15. Evans, T., et al., *Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division.* Cell, 1983. **33**(2): p. 389-96.
16. Sudbery, P.E., A.R. Goodey, and B.L. Carter, *Genes which control cell proliferation in the yeast Saccharomyces cerevisiae.* Nature, 1980. **288**(5789): p. 401-4.
17. Greider, C.W. and E.H. Blackburn, *A telomeric sequence in the RNA of Tetrahymena*

telomerase required for telomere repeat synthesis. Nature, 1989. **337**(6205): p. 331-7.
18. Greider, C.W. and E.H. Blackburn, *Identification of a specific telomere terminal transferase activity in Tetrahymena extracts.* Cell, 1985. **43**(2 Pt 1): p. 405-13.
19. Szostak, J.W. and E.H. Blackburn, *Cloning yeast telomeres on linear plasmid vectors.* Cell, 1982. **29**(1): p. 245-55.
20. Zhou, B.B. and S.J. Elledge, *The DNA damage response: putting checkpoints in perspective.* Nature, 2000. **408**(6811): p. 433-9.
21. Shiotani, B. and L. Zou, *ATR signaling at a glance.* J Cell Sci, 2009. **122**(Pt 3): p. 301-4.
22. Harper, J.W. and S.J. Elledge, *The DNA damage response: ten years after.* Mol Cell, 2007. **28**(5): p. 739-45.
23. Alberts, B., *The promise of cancer research.* Science, 2008. **320**(5872): p. 19.
24. Alberts, B., *Redefining cancer research.* Science, 2009. **325**(5946): p. 1319.

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Mechanisms of ATR-mediated checkpoint signalling

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ABSTRACT

Cell cycle checkpoints maintain genomic integrity by delaying cell division in the presence of DNA damage or replication problems. A crucial player in this process is the ATR kinase. The rapid localization of ATR to sites of genotoxic stress and the central role of this kinase in the checkpoint response lead to the suggestion that ATR functions as a sensor of DNA lesions. After activation, ATR phosphorylates and activates the effector kinase Chk1, thereby causing an inhibition in cell cycle progression. However, this would not be possible without the existence of many other proteins operating in this pathway. Here we review current progress in our understanding of the regulatory factors involved in the ATR-mediated checkpoint response, as well as resumption of cell cycle progression upon repair of the damage, thereby focussing on the mechanisms in mammalian cells.

INTRODUCTION

To protect against the continuous threat of DNA damage by environmental factors and intracellular processes, eukaryotic cells have developed DNA damage checkpoint and repair mechanisms, which help to ensure transmission of an intact genome. Cell cycle checkpoints and DNA repair together determine the ultimate response of a cell to DNA damage. The importance and relevance of such surveillance mechanisms in ensuring genomic stability is underscored by the fact that checkpoint responses and DNA repair mechanisms are highly conserved throughout evolution. In addition, inherited defects in regulators of checkpoint signalling and repair pathways result in severe hereditary human genetic disorders with susceptibility to cancer. For example, Ataxia Telangiectasia (AT), Ataxia Telangiectasia-Like Disorder (AT-LD), Nijmegen Breakage Syndrome (NBS), and the recently described Seckel Syndrome are thought to be the result of defects in the checkpoint pathway [1], whereas Xeroderma Pigmentosum (XP) is caused by a deficiency in the nucleotide excision repair (NER) pathway, that removes UV-induced DNA lesions [2].

DNA damage checkpoints are signal transduction cascades, in which ATM and ATR, members of the family of phosphoinositide 3-kinase like kinases, are the master regulators. ATM and ATR are proteins of approximately 300 kDa, with a conserved C-terminal catalytic domain that preferably phosphorylates serine or threonine residues followed by a glutamine, i.e. an SQ or TQ motif [3, 4]. Both kinases respond to various forms of genotoxic stress by phosphorylating key proteins in the DNA damage response, such as p53 and the effector kinases Chk1 and Chk2. ATR rapidly relocalizes to sites of damage, seen as the formation of nuclear foci after the induction of DNA lesions [5]. ATM autophosphorylation on Ser1981 is quickly detected in response to DNA damage and can also be found in nuclear foci [6]. These data, together with their critical function upstream in the checkpoint signalling cascade, support a role for these kinases in sensing the damaged DNA.

ATM, Ataxia-Telangiectasia Mutated, was originally identified as the product of the gene that is mutated in the heritable chromosomal instability disorder AT [7] and is the main kinase required for early responses to DSBs induced by, for example, ionizing radiation (IR), such as phosphorylation of p53 and Chk2. ATR (ATM and RAD3-related) on the other hand, mainly activates Chk1 in response to UV light-induced damage and stalled replication forks [3]. Chk1 activation leads to the phosphorylation of a variety of substrates, thereby regulating different aspects of the DNA damage response such as cell cycle arrest, stabilization of stalled replication forks and DNA repair [8]. Interestingly, recent data demonstrate crosstalk between the ATM and ATR pathways. ATM was shown to be required for ATR recruitment and subsequent activation of Chk1 in response to DSBs, exclusively in S and G2 phases of the cell cycle [9-11].

In the past decade it has become clear that activation of the DNA damage checkpoint involves more than the kinase cascade initiated by ATM/ATR. A multitude of mediator proteins were discovered (including Claspin and BRCA1) that function in the recruitment of substrates to DNA lesions or as scaffolds on which complexes are assembled. The relocalization of DNA damage response proteins to sites of damage, shown as nuclear foci, was shown to be of crucial importance for downstream checkpoint events, although how, is not yet fully understood. Also regulation of proteins by ubiquitination or other post-translational modifications appeared to be an important factor in the surveillance mechanisms controlling genomic stability. Current progress in our understanding of the ATR-mediated checkpoint response and its regulatory factors, as well as resumption of cell cycle progression upon repair of the damage, is discussed in this review.

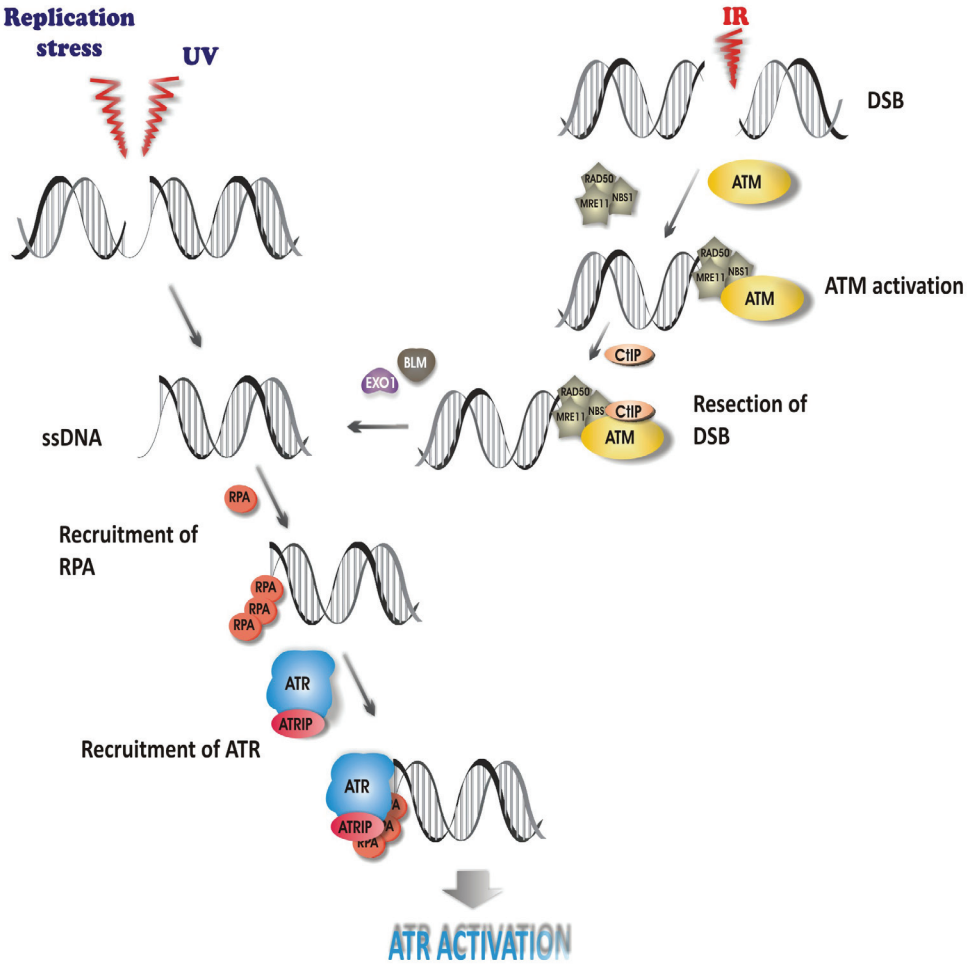

Figure 1: ATR can be activated by different types of DNA lesions.

ESSENTIAL FUNCTION OF ATR

While ATM and ATR act in concert to protect the integrity of the genome, a major difference between these two kinases is that ATR is essential for viability [12, 13], whereas mammals that lack functional ATM are viable, even though they have a limited life span. The early embryonic death in ATR knock out mice indicates that ATR is essential for cell growth and differentiation at an early stage of development [12, 13]. In addition, disruption of ATR in mouse or human cells results in cell cycle arrest or death, even in the absence of exogenous DNA damage [5, 14]. Interestingly, not only ATR is essential, but also other members of the ATR pathway. Inactivation of Chk1 in mice results in lethality at early embryonic stages [15, 16], Hus1 knock out mice are not viable [17] and mutation of mouse Rad17 leads to embryonic lethality during early/mid-gestation [18]. Together these studies suggested

that ATR-dependent signalling is not only required to protect cells against exogenous DNA damage, but also to deal with spontaneous DNA lesions or replicative stress.
Although complete inactivation of ATR is lethal, a hypomorphic mutation was found in humans suffering from the rare autosomal recessive disorder Seckel syndrome, characterised by growth retardation and microcephaly. In homozygosity, this mutation affects ATR splicing which results in a reduction of ATR protein levels to almost undetectable, yet the remaining protein is sufficient for viability [19]. A recently developed mouse model of ATR-Seckel syndrome confirms the essential role of ATR during normal embryonic development, as the diminished function of ATR results in high levels of replication stress in Seckel embryos, which is reduced to marginal amounts in tissues of the adult mouse [20]. The origin of the observed replicative stress in the ATR-Seckel embryo is not entirely clear. Several lines of evidence suggest that ATR signalling is required to stabilise replication forks and/or prevent replication fork collapse. First, conditional deletion of ATR in mouse embryonic fibroblasts (MEFs) leads to accumulation of DNA DSBs during S phase [12]. Second, both loss of ATR and Chk1 activity leads to break formation at fragile sites, which are thought to represent chromosomal regions that are particularly difficult to replicate [21, 22]. Third, Chk1-deficient cells fail to maintain viable replication forks when DNA polymerase is inhibited [23]. Studies in yeast also show that Mec1 (ATR) is required to maintain viability when cells are exposed to the alkylating agent MMS [24], confirming the fork-stabilization activity of ATR signalling. In addition to stabilising replication forks, Chk1 was also demonstrated to regulate replication initiation and fork progression [25, 26]. Together these studies suggest a general role for ATR signalling in the regulation of DNA replication.

TRIGGERING ATR SIGNALLING

Lesions that activate the ATR pathway

The initial step in ATR activation is the recognition of DNA structures that are induced by the damaging agents. As discussed above, the ATR pathway is predominantly triggered by UV lesions and stalled replication forks. A biochemical study demonstrated preferential binding of purified ATR to DNA with UV photoproducts, suggesting that ATR binding to unprocessed DNA could initiate signalling [27]. However, recent data indicate that ATR signalling is initiated by a wider range of DNA lesions, including DSBs. This raises the question how the ATR pathway sensor proteins can detect such different types of lesions. A structure that is commonly generated by genotoxic stress is single stranded DNA (ssDNA), which is, for example, generated at DNA replication forks when the coordination between DNA polymerase activity and DNA helicase activity is compromised [28]. Upon detection of DSBs, ssDNA can be formed by resection of broken ends and in addition, ssDNA gaps are introduced during several DNA repair processes such as NER and mismatch repair [29]. Interestingly, *in vitro* studies using *Xenopus* egg extracts demonstrate that ATR-dependent Chk1 phosphorylation can be initiated by single-stranded DNA (ssDNA) and ssDNA-double stranded DNA junctions [30].
In eukaryotes, DNA damage-induced ssDNA is first detected by ssDNA-binding protein complex RPA, and the resulting stretches of RPA-coated ssDNA seem to function as the key structure for initiating checkpoint signalling by ATR [31, 32]. Processing of DNA lesions into ssDNA has consequently been proposed to be involved in triggering ATR-dependent checkpoint responses. Upon IR treatment and in S and G2 phases of the cell cycle, CtIP/

scSae2, together with Mre11, is involved in nucleolytic processing of DSBs into short ssDNA overhangs, thereby recruiting ATR to sites of DNA damage [11, 33]. Recent publications show that subsequent resection by BLM/scSgs1 and scExo1 generates longer stretches of ssDNA, further promoting ATR-mediated checkpoint signalling such as Chk1 phosphorylation [34-36].

The way by which UV lesions lead to ssDNA and ATR activation seems not to involve resection. UV light induces the formation of cyclobutane pyrimidine dimers and 6-4 photoproducts which can form obstacles during DNA replication. Stretches of ssDNA at the resulting stalled replication forks were suggested to be sufficient for activation of the ATR checkpoint pathway upon exposure to UV during S phase [37, 38]. Other data suggest that ATR signalling can also be triggered outside S and in non-cycling cells. Several reports implicate NER, which involves lesion processing by nucleotide excision of the damaged DNA resulting in an ssDNA gap intermediate, in ATR checkpoint activation during such phases of the cell cycle [19, 38, 39]. From these data it can be concluded that ATR and/or other sensor proteins in the ATR pathway recognise a DNA intermediate, further supporting the evidence of RPA-coated ssDNA as initial trigger for ATR-mediated checkpoint signalling (Figure 1).

The role of ATRIP and TopBP1

ATR forms a stable complex with its co-factor ATR-Interacting Protein, ATRIP. Deletion of ATR in cells resulted in the loss of not only ATR but also ATRIP and in addition, downregulation of ATRIP caused a decrease in ATR levels, indicating that formation of the complex is important for the stability of both proteins [5]. As RPA stimulates binding of ATRIP to ssDNA *in vitro*, it was proposed that the interaction between ATRIP and RPA-coated ssDNA recruits the ATR-ATRIP complex to sites of genotoxic stress [31]. *In vitro* studies indeed identified a conserved domain in the N-terminus of ATRIP required for RPA binding and localization to sites of DNA damage [40]. Recruitment of ATR to damaged chromatin was additionally shown to depend on a C-terminal ATRIP domain that regulates its binding to ATR. Interestingly, this conserved domain was also found in Nbs1, which functions as a similar co-factor for ATM [41]. Based on the above studies the current model for initiation of ATR signalling is that ATRIP-mediated binding of ATR to RPA-coated ssDNA facilitates recognition of ATR substrates for phosphorylation.

Downregulating ATRIP leads to checkpoint defects [5] and expression of ATRIP mutants that cannot bind ATR lead to defective Chk1 phosphorylation [41, 42], demonstrating the necessity of the ATR-ATRIP interaction in this process. However, the essential function of ATRIP in binding RPA-coated ssDNA for ATR signalling was questioned as Chk1 phosphorylation is still observed under conditions in which ATRIP is not able to bind RPA-ssDNA [32, 42], indicating a more complicated model for activation of ATR in response to DNA lesions.

Reports demonstrating the involvement of TopBP1, a mediator protein containing eight BRCT phospho-recognition motifs, in the activation of ATR shed new light on this mechanism. Chk1 phosphorylation was demonstrated to be reduced in the absence of TopBP1 [43]. Kumagai and co-workers subsequently showed that the addition of recombinant TopBP1 to *Xenopus* egg extracts induces an increase in ATR kinase activity. TopBP1 binds ATR through its ATR activation domain (AAD), located between the sixth and seventh BRCT repeat, in an ATRIP-dependent manner and this interaction is required for ATR stimulation [44]. Interestingly, triggering the ATR kinase activity by TopBP1 can occur independently from the interaction of ATRIP with RPA [40]. Recent data explained the involvement of ATRIP in ATR activation by demonstrating that ATRIP contains a conserved TopBP1 interacting region, required for

the association of TopBP1 and ATR and the subsequent TopBP1-mediated triggering of ATR activity [45]. Together these data support a multistep model for ATR checkpoint signalling in which recruitment of ATR to sites of DNA lesions occurs partially through binding of ATRIP with RPA. As will be discussed later, the activation of ATR by TopBP1 depends on the assembly of TopBP1 by Rad9, which is required for ATR activation and the subsequent phosphorylation of ATR substrates (Figure 2).

Rad17 and Rad9-Rad1-Hus1

The localization of ATR-ATRIP to sites of DNA lesions, and activation by TopBP1 is not enough to activate the DNA damage checkpoint response fully. Instead, it requires additional regulators including Rad17 and the Rad9-Rad1-Hus1 (9-1-1) complex. The human Rad9, Rad1 and Hus1 proteins and their orthologs in yeast share sequence similarity with homotrimer PCNA, that is loaded onto the DNA during unperturbed DNA replication by the RFC clamp loader complex. Encircling the DNA helix and tethering DNA polymerase to the substrate increases polymerase processivity and thereby facilitates genome replication. The recently resolved crystal structure of the 9-1-1 complex demonstrates a toroidal structure with a similar architecture to PCNA [46-48], confirming earlier biochemical data that proposed a heterotrimeric ring formed by the three proteins [49, 50]. These data suggest that the 9-1-1 complex is a second PCNA-like structure that may be critical for checkpoint function.

Although MEFs lacking only Hus1 cannot be grown, inactivation of p21waf1 results in proliferating Hus1-deficient cells in culture. These cells are hypersensitive to hydroxyurea (HU) and UV light, but are only slightly sensitive to IR [17]. Also knocking out Rad9 in mouse embryonic stem cells or chicken DT40 cells resulted in increased sensitivity for these agents [51, 52]. Furthermore, Hus1 and Rad9 were shown to be required for Chk1 and Rad17 phosphorylation [52-54]. Whereas the intra-S phase checkpoint upon IR is intact in Hus1 deficient cells, these cells display problems in responding to blocked replication [55]. Together these studies suggest that the 9-1-1 complex functions in the maintenance of genome stability by facilitating ATR-signalling.

Similar to PCNA-loading by the RFC complex, Rad9 was shown to be loaded onto chromatin in response to DNA damage by Rad17, in an ATP-dependent manner [54, 56]. Rad17, and its yeast homolog Rad24, share homology with RFC1, a subunit of the RFC clamp loader complex. Rad17 forms a complex with RFC2-5, the four small subunits of the replicative RFC complex and associates with chromatin prior to damage [54, 57]. Rad17 is phosphorylated by ATR on chromatin after damage but this phosphorylation is not required for Rad9 loading onto chromatin [54]. As is the case for ATR-ATRIP, RPA-coated ssDNA is thought to be an important structure for recruitment of the Rad17 and 9-1-1 complexes to sites of DNA damage, as RPA was shown to stimulate the binding of Rad17 to ssDNA and also facilitates the recruitment of 9-1-1 by Rad17 [58].

Loading of the 9-1-1 checkpoint clamp onto chromatin by the Rad17/RFC complex in response to DNA damage was shown to be required for the phosphorylation of several ATR substrates including Rad17 and Chk1 [53, 54, 59] (Figure 2). However, both Rad17 and ATR are recruited independently to sites of DNA lesions [54]. The necessity of the 9-1-1 complex in the ATR branch was explained by showing that Rad9 recruits the ATR-activator TopBP1 near sites of DNA damage, which was consisted with earlier reports showing interaction between Rad9 and TopBP1 [60, 61]. The N-terminal region of TopBP1, consisting of BRCT regions I-II, binds the C-terminus of Rad9 in *Xenopus* egg extracts and mammalian cells. More precisely, the interaction between Rad9 and TopBP1 depends on the phosphorylation of serine 373 in the

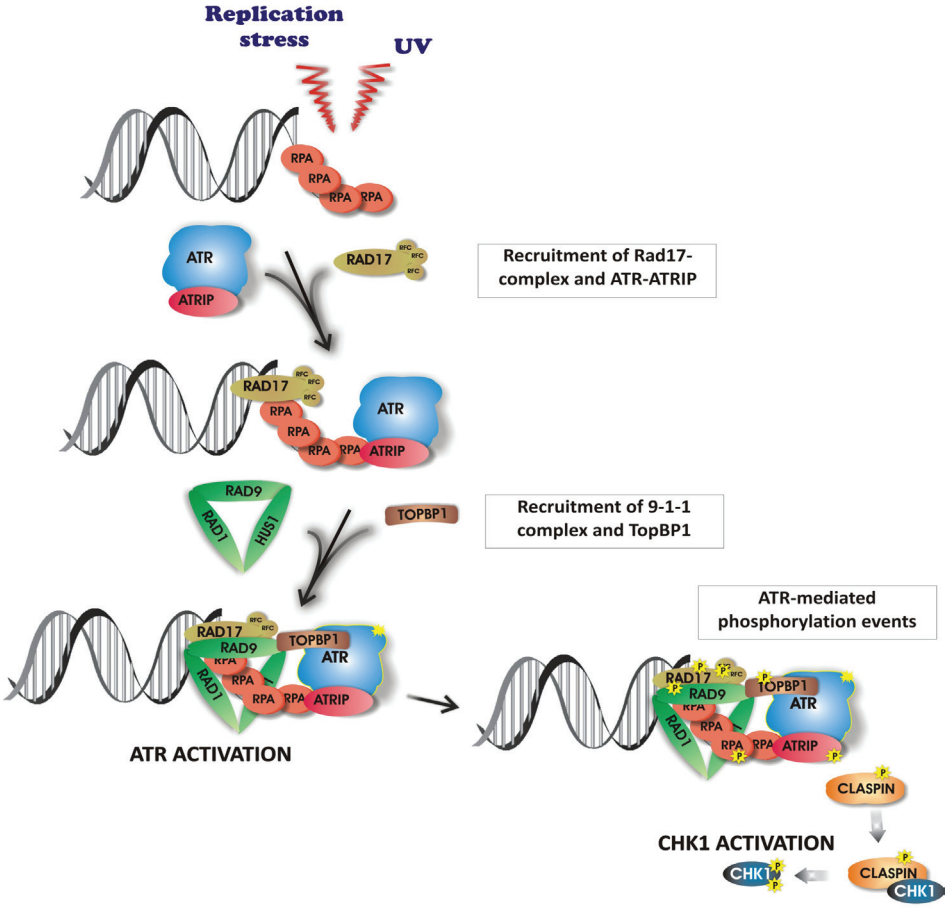

Figure 2: Factors involved in the ATR pathway, resulting in activation of effector kinase Chk1.

C-terminal tail of Rad9 [44, 62, 63]. In the current model for ATR activation, ATR-ATRIP and Rad17 are recruited to RPA-coated ssDNA appearing around DNA lesions. Rad17 mediates the loading of the 9-1-1 complex, which recruits TopBP1, resulting in further stimulation of ATR activity and the subsequent phosphorylation of ATR substrates, including RPA, ATRIP, Rad17, components of the 9-1-1 complex and effector kinase Chk1 (Figure 2). Furthermore it should be mentioned that the removal of TopBP1 from *Xenopus* chromatin preps results in less aphidicolin-induced chromatin accumulation of Rad9, suggesting a positive feedback loop in recruitment of Rad9 and TopBP1 to sites of genotoxic stress [64].

Apart from its involvement in ATR-mediated checkpoint signalling, the 9-1-1 complex is also thought to play a role in DNA repair. Data from *Schizosaccharomyces pombe* (*S. pombe)* and *Saccharomyces cerevisiae* (*S. cerevisiae)* show that the 9-1-1 clamp can physically interact with polymerases specific for translesion synthesis (TLS) [65, 66] and evidence suggests that also human 9-1-1 might be involved in this process [67]. TLS is a DNA damage tolerance process that allows the replication machinery to replicate past DNA lesions by switching from regular DNA polymerases to specialised translesion polymerases. This polymerase switching

is thought to be mediated by post-translational modification of homotrimer PCNA. Mono-ubiquitinated PCNA interacts with TLS polymerases, resulting in the recruitment of these polymerases to sites of blocked forks [68, 69]. Interestingly, regulation of the 9-1-1 complex by ubiquitination has also recently been demonstrated in yeast. Monoubiquitination of the Rad1 ortholog in *S. cerevisiae* (scRad17) was not only reported to be required for the transcriptional induction in response to DNA damage, but also for promoting the activation of downstream effector kinase scRad53, possibly through the recruitment or maintenance of the *S. cerevisiae* 9-1-1 complex at sites of DNA lesions [70]. Future experiments will show if a similar regulation mechanism exists for mammalian 9-1-1.

Claspin and other mediators implicated in ATR signalling

Another protein that binds chromatin independently of ATR or Rad17/9-1-1, and is required for the ATR-mediated activation of Chk1 in response to genotoxic stress is Claspin. Immunodepletion of Claspin abolished Chk1 activation induced by DNA templates in *Xenopus* egg extracts [71] and downregulation of Claspin protein levels in HeLa cells decreased Chk1 phosphorylation upon HU [72]. Interestingly, Claspin appears to selectively regulate phosphorylation of Chk1, but not other ATR-substrates [73].

As Claspin was shown to bind Chk1 and ATR in response to genotoxic stress in both *Xenopus* egg and human cell extracts, Claspin was proposed to function as an adaptor molecule bringing ATR and Chk1 together [71, 72]. The Claspin-Chk1 interaction depends on ATR-mediated phosphorylation of Claspin and is required for Chk1 phosphorylation by ATR [71, 72]. Subsequent studies identified repeated phosphopeptide motifs in Claspin, which are required for association with a phosphate-binding site in the N-terminal kinase domain of Chk1, resulting in full activation of Chk1 [71, 74, 75]. Next, fully phosphorylated *Xenopus* Chk1 dissociates from Claspin [74] (Figure 2).

Two other adaptor proteins that have been implicated in Chk1 activation are BRCA1 and MDC1. In BRCA1 mutant cells, less Chk1 phosphorylation upon IR is detected as compared to wild type cells [43], whereas expression of BRCA1 in such mutant cells induces Chk1 kinase activity [76]. Likewise, IR-induced Chk1 phosphorylation decreases after downregulation of BRCA1 protein levels [77]. In addition, MDC1 knock down also results in a defect in phosphorylation of Chk1 in response to DNA damage [78]. However, BRCA1 and MDC1 generally function as mediators in the ATM signalling pathway and have mainly been implicated in IR-induced phosphorylation of Chk1. It is therefore unclear at this stage whether these proteins are directly involved in Chk1 activation, or act via mediating the ATM-dependent activation of ATR in response to DSBs.

Finally, additional, yet less characterised, mediators of ATR signalling are Nbs1 [79], Tim [80], Tipin [80, 81], CEP164 [82] and hClk2 [83]. Less HU- or UV-induced Chk1 phosphorylation is observed in the absence of these proteins.

ATR SUBSTRATES AND DOWNSTREAM RESPONSES

A multitude of proteins have been identified as ATR substrates. In fact, a recent large-scale proteomic screen of proteins phosphorylated in response to DNA damage on ATM/ATR consensus sites identified over 700 proteins [84]. Among the better characterised substrates of ATR are ATRIP, Rad17, Rad9, TopBP1, Claspin, BRCA1 and p53. Accumulating evidence, as described earlier in this review, suggests that the majority of these proteins, with the

exception of p53, functions to regulate the cell cycle arrest by controlling the well-studied effector kinase Chk1 [43, 52, 73, 85]. Interestingly, also ATR-mediated phosphorylation of some of these substrates has been implicated in Chk1 activation. An example is Rad17, which is phosphorylated by ATR *in vitro* on serine 635 and serine 645. Expression of kinase-inactive ATR consequently suppresses the phosphorylation of these sites induced by HU or UV. ATR-mediated phosphorylation of Rad17 was proposed to be required for functioning of the DNA damage-induced G2 checkpoint [59]. Subsequent work showed that cells expressing a Rad17 phosphorylation mutant fail to sustain HU-induced Chk1 phosphorylation [86], which could be explained by the fact that phosphorylation of Rad17 regulates maintenance of Rad9 as sites of genotoxic stress [87], as will be discussed later. Adaptor protein Claspin contains many SQ/TQ motifs, potential phosphorylation sites of ATM/ATR. HU-induced phosphorylation of Claspin was demonstrated to be inhibited by caffeine-treatment, suggesting that this modification was indeed dependent on ATM/ATR [72] and phosphorylation of serine 864 in the phosphopeptide motif of *Xenopus* Claspin is not observed in extracts lacking ATR [75]. That phosphorylation of Claspin by ATR is thought to be involved in binding and activation of Chk1 was discussed above.

Also Chk1 has several SQ/TQ phosphorylation sites in the C-terminal regulatory domain. Of these sites, serine 317, serine 345 and the recently identified serine 366 are phosphorylated after genotoxic stress [88, 89]. Serines 317 and 345 were shown to be phosphorylated by ATR *in vitro* and phosphorylated in an ATR-dependent manner *in vivo* [15, 89]. Mutation of these residues in *S. pombe* and mammalian Chk1 compromises checkpoint arrest, indicating that ATR-mediated phosphorylation of Chk1 is required for the function of this effector kinase in the DNA damage response [26, 88, 90, 91]. Phosphorylation of Chk1 directly increases kinase activity and non-phosphorylatable mutants are poorly activated in response to DNA damage [88, 89]. Interestingly, recent data indicate that phosphorylation of serine 345 is essential for Chk1 functioning, and phosphorylation of this residue depends on the modification of serine 317 and 366, suggesting a sequential order of events [26, 88]. In addition to elevating its kinase activity, ATR-mediated Chk1 phosphorylation also directs the dissociation of Chk1 from chromatin in response to genotoxic stress [92, 93]. Chromatin release was shown to be required for an efficient DNA damage-induced checkpoint arrest and spreading of phosphorylated Chk1 throughout the nucleus, suggesting that chromatin dissociation of Chk1 provides a mechanism to ensure that active Chk1 reaches its substrates [92, 94].

Activated Chk1 slows down cell cycle progression during S phase and prohibits the G2/M transition as long as damaged or incompletely replicated DNA is present by inactivating downstream Cdc25 phosphatases. Cdc25 proteins regulate the timely activation of cyclin-dependent kinases by dephosphorylating the inhibitory phosphorylation of tyrosine 15. Early reports demonstrate that *S. pombe* or human Chk1 phosphorylates Cdc25C on serine 216, thereby creating binding sites for the 14-3-3 scaffold proteins, which is thought to subsequently promote the nuclear exclusion of the phosphatase [91, 95-97]. On the other hand, evidence for direct inhibition of Cdc25C emerged from experiments concluding that Cdc25C activity is abolished upon incubation with recombinant Chk1 [98, 99]. Studies in mammalian cells showed that Chk1 is also able to phosphorylate Cdc25A *in vitro* and *in vivo* [100], resulting in regulation of checkpoint arrest during all phases of the cell cycle. Inactivation of this phosphatase is regulated differently though, as DNA damage-induced phosphorylation of Cdc25A leads to proteasome-dependent degradation of the protein [100-102].

Chk1 seems to have functions additional to controlling cell cycle arrest by phosphorylating Cdc25 proteins. In response to DNA damage, phosphorylation of Histone H3 was shown to rapidly reduce, correlating with the repression of several genes. Phosphorylation of Histone H3 in unperturbed cells is dependent on Chk1 and the described release of Chk1 from chromatin upon genotoxic stress is thought cause the reduction in Histone H3 phosphorylation. Based on these data, Chk1 was hypothesized to function as a Histone kinase, responsible for DNA damage-induced transcriptional repression of a variety of genes related to cell cycle progression [103]. Moreover, evidence for the involvement of Chk1 in a completely different DNA damage response came from data showing that inhibition or downregulation of Chk1 results in decreased DNA repair by homologous recombination. This effect that was explained by demonstrating that Chk1 interacts with Rad51 and the fact that phosphorylation of Rad51 occurs in a Chk1-dependent manner, presumably regulating the localization of Rad51 at sites of DNA lesions [104]. An alternative explanation comes from data demonstrating that Chk1 phosphorylates BRCA2 in a domain that is essential for interaction with Rad51 and the recruitment of Rad51 into nuclear foci [105]. Finally, a role for Chk1 in regulating DNA cross-link repair was suggested by the observation that FANCE, a Fanconi Anemia core complex protein, is phosphorylated by Chk1 [106].

SPATIO-TEMPORAL ORGANISATION OF CHECKPOINT REGULATION

Perhaps unsurprisingly, numerous studies over the past years indicated that DNA damage checkpoint pathways do not function by linear signal transduction only. Apart from the crosstalk between the different pathways, it also became clear that the accumulation of DNA damage response proteins at sites of damage is of crucial importance for downstream checkpoint events. Although many proteins involved in checkpoint regulation are (partially) bound to chromatin in unperturbed cells, the majority of these relocalize at or near sites of DNA lesions to form so-called nuclear foci. Although the biological function of such large protein aggregations is still not entirely clear, the concentration of factors in the vicinity of the lesion is thought to enhance checkpoint pathways and the repair of DNA lesions, possibly by amplification of signalling. This also raised the question whether the DNA lesion might just serve as a platform to accumulate these proteins. Two elegant publications answered that question by showing that targeting of individual proteins involved in the early checkpoint responses to chromatin in the absence of DNA damage activates a DNA damage response [107, 108]. Toczyski and co-workers did so by fusing *S. cerevisiae* orthologs to ATRIP (scDdc2) and Rad9 (scDdc1) proteins to the LacI repressor and expressing them in cells harbouring Lac operator arrays. Artificial colocalization of these fusions resulted in phosphorylation of downstream effector kinase scRad53 and a cell cycle arrest. Interestingly, artificial localization of scDdc1 bypasses the requirement of the *S. cerevisiae* Rad17 (scRad24) clamp loader. From these data can be concluded that colocalization of the sensor proteins is sufficient to activate the DNA damage response [107]. Soutoglou and Misteli performed the experiment in mammalian cells using a similar approach and reached the same conclusion. Artificial targeting of ATM, sensor proteins Nbs1/Mre11 or mediator MDC1 to a Lac operator array resulted in an ATM-dependent checkpoint response as other proteins of the pathway were recruited and a G2 phase delay was triggered. In contrast, targeting Chk1 or Chk2 to chromatin did not result in a downstream checkpoint response [108].
The latter result is in accordance to the observation that the effector kinases Chk1 and Chk2

do not accumulate into nuclear foci in response to DNA damage, in contrast to ATM/ATR, TopBP1, 9-1-1 and many checkpoint mediator proteins. Instead, as mentioned earlier, Chk1 associates to chromatin in unperturbed cells and is released from the chromatin in response to DNA damage [92]. *In vivo* imaging experiments using fluorescently labelled proteins confirm the absence of a stably chromatin-bound fraction for effector kinases Chk1 and Chk2. It was shown that although these proteins are phosphorylated at or near sites of DNA lesions, this association is very transient and phosphorylated proteins are found throughout the entire nucleoplasm [92, 109] (Warmerdam et al. *in press*). The rapid disassembly of the activated proteins was hypothesized to ensure the access of additional Chk1/Chk2 molecules to ATM/ATR and the transmission of the damage signal throughout the nucleus [94].
Live cell imaging also gave insight into the dynamic aspect of the DNA damage response. Are proteins in established DNA damage-induced foci immobile or are such foci more dynamic structures existing of proteins that actively turnover? Initial studies following repair proteins of the Rad52 group of homologous recombination proteins and ATM-sensor Nbs1 by videomicroscopy revealed that even though these proteins accumulate at sites of DNA damage, as seen by the formation of nuclear foci, this assembly with damaged regions is transient. In fact, these proteins still underwent a dynamic exchange in the close proximity of DNA lesions [109, 110]. More recently, similar results were shown for Rad9. Whereas Rad9 is highly mobile in undamaged cells, in response to genotoxic stress Rad9 forms Rad17-dependent foci and turnover of Rad9 in such foci was comparatively slow. However, as for Rad52 proteins and Nbs1, the majority of the Rad9 proteins still display turnover, arguing against foci as being static protein structures [87]. Additional experiments demonstrated regulation of the mobility of Rad9 at sites of DNA lesions. First, Rad9 was shown to accumulate faster, and the proportion of immobilization was higher upon damage induction during S phase [38]. And second, the turnover of Rad9 molecules in foci was higher in the absence of ATR or the expression of a Rad17 mutant that cannot be phosphorylated by ATR [87]. These results indicate that although the initial recruitment of the 9-1-1 complex to sites of DNA lesions might be ATR-independent, ATR and Rad17 seem to collaborate in the retention of this complex at sites of genotoxic stress. This process critically depends on ATR-mediated phosphorylation of Rad17 and might have implications for checkpoint maintenance.

RECOVERY FROM CHECKPOINT ACTIVATION

Triggering checkpoint activation is of critical importance for an efficient DNA damage response and the maintenance of genome stability. Once a cell has arrested due to DNA damage, there are generally two options. When the DNA lesions cannot be repaired, the cell can undergo apoptosis or stay irreversibly arrested. Alternatively, the cell will repair the DNA and re-enter the cell cycle. In the case of cell cycle resumption, switching off the checkpoint once the damage has been repaired is an important process. The basic principles of controlling such checkpoint recovery are only just starting to emerge but indicate that active signalling is also required at this level of checkpoint regulation.

Direct inactivation of Chk1

As many of the steps during checkpoint activation are regulated by phosphorylation, it is not surprising that phosphatases have been implicated in switching off the checkpoint once

DNA repair has been completed. Direct inactivation of effector kinase Chk1, the main target of ATR signalling, mediated by several phosphatases indeed has been reported. In human cells, phosphatase PPM1D/Wip1 binds Chk1 and dephosphorylates serine 345, resulting in inhibition of Chk1 kinase activity *in vitro*. Inducible expression of PPM1D leads to a reduced DNA damage-induced cell cycle arrest [111], suggesting that a way to control checkpoint recovery is via regulation of PPM1D. Also purified phosphatase PP2A has been shown to dephosphorylate serines 317 and 345 of Chk1 *in vitro* and knock down of this protein leads to an increased phosphorylation of Chk1 on these residues *in vivo*. Interestingly, as dephosphorylation of wild-type Chk1 occurred more rapidly than of kinase-inactive Chk1, a model was hypothesized in which Chk1 dephosphorylation is (partially) regulated by its own kinase activity [112]. Chk1 activity was also shown to regulate its own degradation as at later time points after the exposure to genotoxic stress, Chk1 levels are downregulated in a proteasome-dependent manner and this process was triggered by ATR-mediated phosphorylation of serine 345 [93]. Recently the ubiquitination of Chk1 was demonstrated to be mediated by the Fbx6-SCF ubiquitin ligase [113]. Together these data point to the existence of several ways to regulate Chk1 inactivation. However, it should be mentioned that the involvement of such phosphatases and Chk1 degradation in the recovery of a DNA damage-induced cell cycle arrest is yet to be experimentally demonstrated.

Plk1, Claspin and Wee1

As mentioned above, protein degradation is, next to direct inhibition, a way to inactivate checkpoint signalling. Several groups have recently identified the role of mitotic kinase Plk1 in mammalian checkpoint recovery by regulating the degradation of crucial cell cycle mediators. The Medema group first published that knock down of Plk1 results in a delay in mitotic entry following the recovery from a G2 DNA damage arrest. However, this effect could be rescued by depletion of Wee1, the kinase that targets the inhibitory phosphorylation of tyrosine 15 of Cdc2, indicating that Wee1 functions downstream of Plk1. Indeed, Plk1 was shown to be involved in the degradation of Wee1 at the onset of mitosis, a process that likely to be dependent on β-TrCP ubiquitin ligase [114, 115]. Three reports subsequently described Claspin as additional Plk1 target regulating checkpoint recovery. Claspin was shown to be transiently stabilised upon DNA damage, but degraded upon mitotic entry. Claspin degradation was triggered by its binding to β-TrCP. The phosphorylation of Claspin was dependent on Plk1 and essential for the interaction with this ubiquitin ligase and the degradation of Claspin. Expression of a stable Claspin mutant prolongs activation of Chk1, thereby delaying the G2/M transition after the recovery from DNA damage or replication stress [116-118]. Finally, the mechanism behind the activation of Plk1 was identified by demonstrating that Aurora A activates Plk1 by phosphorylation. Aurora A-dependent phosphorylation of Plk1 was shown to be required for promoting the G2/M transition after DNA damage checkpoint arrest [119]. How Aurora A is activated to trigger checkpoint recovery remains a subject of investigation. Together these results suggest a model for checkpoint recovery in which Aurora A activates Plk1, which leads to the degradation of both Claspin and Wee1, resulting in simultaneous downregulation of Chk1 activation and the stimulation of mitotic entry.

PERSPECTIVE

Correct functioning of DNA damage responses is of crucial importance for the maintenance of genomic stability and the mechanisms of checkpoint functioning have therefore been the focus of attention in many laboratories in the past decade. It should be mentioned that numerous studies using model systems like *S. pombe* and *S. cerevisiae* have been of major benefit for the rapid progress in our understanding of checkpoint regulation in mammalian cells. From several observations throughout the years it has become clear that the checkpoint pathways initiated by the activation of ATM or ATR cannot be seen as static protein cascades, but are dynamic and highly regulated mechanisms, and new proteins involved in this regulation are still being discovered.

First, data showing that ATM and cofactors are required for the activation of ATR in response to DSBs indicates interplay between the two pathways [9-11]. Interestingly, ATM activation in response to replication fork stalling or UV light was demonstrated to be ATR-dependent [120]. Second, although ATR signalling mainly functions as a kinase cascade, phosphorylation is not the only post-translational modification involved in regulating this pathway. Regulation of proteins by ubiquitination or modification by ubiquitin-like molecules appears to be of importance for checkpoint regulation as well [121, 122]. This involves protein turnover such as Cdc25A [101] and Chk1 [93], but also checkpoint regulation for example by ubiquitination of *S. cerevisiae* 9-1-1, a PCNA-like complex [70]. The latter observation is especially interesting since the process of SUMOylation, monoubiquitination and subsequent polyubiquitination of PCNA serves as a molecular switch between various DNA damage bypass processes [68]. Future experiments will demonstrate if similar modifications of mammalian 9-1-1 also play a key role in the ATR-dependent checkpoint response. Fourth, new technology has made it possible to study protein dynamics at sites of DNA lesions and have lead to the conclusion that the accumulations of proteins seen as foci, are actually not static structures. Instead, the majority of the proteins recruited to DNA damage sites only bind to chromatin transiently, and then actively turnover, which is hypothesized to facilitate the recruitment of new molecules to ensure activation of the entire pool of proteins. It was additionally shown that this protein turnover can be regulated by checkpoint signalling, as ATR-mediated phosphorylation of Rad17 regulates the retention time of the 9-1-1 complex at sites of genotoxic stress [87]. Finally, as the basics of recovery from a DNA damage-induced cell cycle arrest are only just starting to emerge, research will without doubt concentrate more on this subject. Protein degradation by the ubiquitin system was shown to play a crucial role in checkpoint recovery. The involvement of deubiquitin enzymes in the regulation of checkpoint initiation [123, 124] therefore raises the possibility that these enzymes might equally be important in switching off the checkpoint after DNA repair has been completed.

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REFERENCES

1. Khanna, K.K. and S.P. Jackson, *DNA double-strand breaks: signaling, repair and the cancer connection.* Nat. Genet., 2001. **27**(3): p. 247-254.
2. de Boer, J. and J.H. Hoeijmakers, *Nucleotide excision repair and human syndromes.* Carcinogenesis, 2000. **21**(3): p. 453-460.
3. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes Dev, 2001. **15**(17): p. 2177-96.
4. Kim, S.T., et al., *Substrate specificities and identification of putative substrates of ATM kinase family members.* J. Biol. Chem., 1999. **274**(53): p. 37538-37543.
5. Cortez, D., et al., *ATR and ATRIP: partners in checkpoint signaling.* Science, 2001. **294**(5547): p. 1713-6.
6. Bakkenist, C.J. and M.B. Kastan, *DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation.* Nature, 2003. **421**(6922): p. 499-506.
7. Savitsky, K., et al., *A single ataxia telangiectasia gene with a product similar to PI-3 kinase.* Science, 1995. **268**(5218): p. 1749-53.
8. Chen, Y. and Y. Sanchez, *Chk1 in the DNA damage response: conserved roles from yeasts to mammals.* DNA Repair (Amst), 2004. **3**(8-9): p. 1025-32.
9. Adams, K.E., et al., *Recruitment of ATR to sites of ionising radiation-induced DNA damage requires ATM and components of the MRN protein complex.* Oncogene, 2006. **25**(28): p. 3894-904.
10. Cuadrado, M., et al., *ATM regulates ATR chromatin loading in response to DNA double-strand breaks.* J Exp Med, 2006. **203**(2): p. 297-303.
11. Jazayeri, A., et al., *ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks.* Nat Cell Biol, 2006. **8**(1): p. 37-45.
12. Brown, E.J. and D. Baltimore, *ATR disruption leads to chromosomal fragmentation and early embryonic lethality.* Genes Dev, 2000. **14**(4): p. 397-402.
13. de Klein, A., et al., *Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice.* Curr Biol, 2000. **10**(8): p. 479-82.
14. Brown, E.J. and D. Baltimore, *Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance.* Genes Dev, 2003. **17**(5): p. 615-28.
15. Liu, Q., et al., *Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint.* Genes Dev, 2000. **14**(12): p. 1448-59.
16. Takai, H., et al., *Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice.* Genes Dev, 2000. **14**(12): p. 1439-47.
17. Weiss, R.S., T. Enoch, and P. Leder, *Inactivation of mouse Hus1 results in genomic instability and impaired responses to genotoxic stress.* Genes Dev, 2000. **14**(15): p. 1886-98.
18. Budzowska, M., et al., *Mutation of the mouse Rad17 gene leads to embryonic lethality and reveals a role in DNA damage-dependent recombination.* Embo J, 2004. **23**(17): p. 3548-58.
19. O'Driscoll, M., et al., *A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome.* Nat. Genet., 2003. **33**(4): p. 497-501.
20. Murga, M., et al., *A mouse model of ATR-Seckel shows embryonic replicative stress and accelerated aging.* Nat Genet, 2009.
21. Casper, A.M., et al., *ATR regulates fragile site stability.* Cell, 2002. **111**(6): p. 779-89.

22. Durkin, S.G., et al., *Depletion of CHK1, but not CHK2, induces chromosomal instability and breaks at common fragile sites.* Oncogene, 2006. **25**(32): p. 4381-8.
23. Zachos, G., M.D. Rainey, and D.A. Gillespie, *Chk1-deficient tumour cells are viable but exhibit multiple checkpoint and survival defects.* Embo J, 2003. **22**(3): p. 713-23.
24. Tercero, J.A., M.P. Longhese, and J.F. Diffley, *A central role for DNA replication forks in checkpoint activation and response.* Mol Cell, 2003. **11**(5): p. 1323-36.
25. Maya-Mendoza, A., et al., *Chk1 regulates the density of active replication origins during the vertebrate S phase.* Embo J, 2007. **26**(11): p. 2719-31.
26. Wilsker, D., et al., *Essential function of Chk1 can be uncoupled from DNA damage checkpoint and replication control.* Proc Natl Acad Sci U S A, 2008. **105**(52): p. 20752-7.
27. Unsal-Kacmaz, K., et al., *Preferential binding of ATR protein to UV-damaged DNA.* Proc Natl Acad Sci U S A, 2002. **99**(10): p. 6673-8.
28. Byun, T.S., et al., *Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint.* Genes Dev, 2005. **19**(9): p. 1040-52.
29. Friedberg, E.C., *DNA damage and repair.* Nature, 2003. **421**(6921): p. 436-40.
30. MacDougall, C.A., et al., *The structural determinants of checkpoint activation.* Genes Dev, 2007. **21**(8): p. 898-903.
31. Zou, L. and S.J. Elledge, *Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.* Science, 2003. **300**(5625): p. 1542-8.
32. Ball, H.L., J.S. Myers, and D. Cortez, *ATRIP binding to replication protein A-single-stranded DNA promotes ATR-ATRIP localization but is dispensable for Chk1 phosphorylation.* Mol Biol Cell, 2005. **16**(5): p. 2372-81.
33. Sartori, A.A., et al., *Human CtIP promotes DNA end resection.* Nature, 2007. **450**(7169): p. 509-14.
34. Gravel, S., et al., *DNA helicases Sgs1 and BLM promote DNA double-strand break resection.* Genes Dev, 2008. **22**(20): p. 2767-72.
35. Mimitou, E.P. and L.S. Symington, *Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing.* Nature, 2008. **455**(7214): p. 770-4.
36. Zhu, Z., et al., *Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends.* Cell, 2008. **134**(6): p. 981-94.
37. Ward, I.M., K. Minn, and J. Chen, *UV-induced ataxia-telangiectasia-mutated and Rad3-related (ATR) activation requires replication stress.* J Biol Chem, 2004. **279**(11): p. 9677-80.
38. Warmerdam, D.O., et al., *Cell cycle-dependent processing of DNA lesions controls localization of Rad9 to sites of genotoxic stress.* Cell Cycle, 2009. **8**(11): p. 1765-74.
39. Giannattasio, M., et al., *Physical and functional interactions between nucleotide excision repair and DNA damage checkpoint.* Embo J, 2004. **23**(2): p. 429-38.
40. Ball, H.L., et al., *Function of a conserved checkpoint recruitment domain in ATRIP proteins.* Mol Cell Biol, 2007. **27**(9): p. 3367-77.
41. Falck, J., J. Coates, and S.P. Jackson, *Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage.* Nature, 2005. **434**(7033): p. 605-11.
42. Kim, S.M., et al., *Phosphorylation of Chk1 by ATM- and Rad3-related (ATR) in Xenopus egg extracts requires binding of ATRIP to ATR but not the stable DNA-binding or coiled-coil domains of ATRIP.* J Biol Chem, 2005. **280**(46): p. 38355-64.
43. Yamane, K., J. Chen, and T.J. Kinsella, *Both DNA topoisomerase II-binding protein*

1 and BRCA1 regulate the G2-M cell cycle checkpoint. Cancer Res, 2003. **63**(12): p. 3049-53.
44. Kumagai, A., et al., *TopBP1 activates the ATR-ATRIP complex.* Cell, 2006. **124**(5): p. 943-55.
45. Mordes, D.A., et al., *TopBP1 activates ATR through ATRIP and a PIKK regulatory domain.* Genes Dev, 2008. **22**(11): p. 1478-89.
46. Dore, A.S., et al., *Crystal structure of the rad9-rad1-hus1 DNA damage checkpoint complex--implications for clamp loading and regulation.* Mol Cell, 2009. **34**(6): p. 735-45.
47. Sohn, S.Y. and Y. Cho, *Crystal structure of the human rad9-hus1-rad1 clamp.* J Mol Biol, 2009. **390**(3): p. 490-502.
48. Xu, M., et al., *Structure and functional implications of the human rad9-hus1-rad1 cell cycle checkpoint complex.* J Biol Chem, 2009. **284**(31): p. 20457-61.
49. St Onge, R.P., et al., *The human G2 checkpoint control protein hRAD9 is a nuclear phosphoprotein that forms complexes with hRAD1 and hHUS1.* Mol Biol Cell, 1999. **10**(6): p. 1985-95.
50. Volkmer, E. and L.M. Karnitz, *Human homologs of Schizosaccharomyces pombe rad1, hus1, and rad9 form a DNA damage-responsive protein complex.* J Biol Chem, 1999. **274**(2): p. 567-70.
51. Hopkins, K.M., et al., *Deletion of mouse rad9 causes abnormal cellular responses to DNA damage, genomic instability, and embryonic lethality.* Mol Cell Biol, 2004. **24**(16): p. 7235-48.
52. Kobayashi, M., et al., *Critical role for chicken Rad17 and Rad9 in the cellular response to DNA damage and stalled DNA replication.* Genes Cells, 2004. **9**(4): p. 291-303.
53. Weiss, R.S., et al., *Hus1 acts upstream of chk1 in a mammalian DNA damage response pathway.* Curr Biol, 2002. **12**(1): p. 73-7.
54. Zou, L., D. Cortez, and S.J. Elledge, *Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin.* Genes Dev, 2002. **16**(2): p. 198-208.
55. Weiss, R.S., P. Leder, and C. Vaziri, *Critical role for mouse Hus1 in an S-phase DNA damage cell cycle checkpoint.* Mol Cell Biol, 2003. **23**(3): p. 791-803.
56. Bermudez, V.P., et al., *Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex in vitro.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1633-8.
57. Green, C.M., et al., *A novel Rad24 checkpoint protein complex closely related to replication factor C.* Curr Biol, 2000. **10**(1): p. 39-42.
58. Zou, L., D. Liu, and S.J. Elledge, *Replication protein A-mediated recruitment and activation of Rad17 complexes.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 13827-32.
59. Bao, S., et al., *ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses.* Nature, 2001. **411**(6840): p. 969-74.
60. Greer, D.A., et al., *hRad9 rapidly binds DNA containing double-strand breaks and is required for damage-dependent topoisomerase II beta binding protein 1 focus formation.* Cancer Res, 2003. **63**(16): p. 4829-35.
61. Makiniemi, M., et al., *BRCT domain-containing protein TopBP1 functions in DNA replication and damage response.* J Biol Chem, 2001. **276**(32): p. 30399-406.
62. Delacroix, S., et al., *The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1.* Genes Dev, 2007. **21**(12): p. 1472-7.

63. Lee, J., A. Kumagai, and W.G. Dunphy, *The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR.* J Biol Chem, 2007. **282**(38): p. 28036-44.
64. Yan, S. and W.M. Michael, *TopBP1 and DNA polymerase-alpha directly recruit the 9-1-1 complex to stalled DNA replication forks.* J Cell Biol, 2009. **184**(6): p. 793-804.
65. Kai, M., et al., *Rad3-dependent phosphorylation of the checkpoint clamp regulates repair-pathway choice.* Nat Cell Biol, 2007. **9**(6): p. 691-7.
66. Sabbioneda, S., et al., *The 9-1-1 checkpoint clamp physically interacts with polzeta and is partially required for spontaneous polzeta-dependent mutagenesis in Saccharomyces cerevisiae.* J Biol Chem, 2005. **280**(46): p. 38657-65.
67. Jansen, J.G., M.I. Fousteri, and N. de Wind, *Send in the clamps: control of DNA translesion synthesis in eukaryotes.* Mol Cell, 2007. **28**(4): p. 522-9.
68. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.
69. Kannouche, P.L., J. Wing, and A.R. Lehmann, *Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage.* Mol Cell, 2004. **14**(4): p. 491-500.
70. Fu, Y., et al., *Rad6-Rad18 mediates a eukaryotic SOS response by ubiquitinating the 9-1-1 checkpoint clamp.* Cell, 2008. **133**(4): p. 601-11.
71. Kumagai, A. and W.G. Dunphy, *Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts.* Mol Cell, 2000. **6**(4): p. 839-49.
72. Chini, C.C. and J. Chen, *Human claspin is required for replication checkpoint control.* J Biol Chem, 2003. **278**(32): p. 30057-62.
73. Liu, S., et al., *Claspin operates downstream of TopBP1 to direct ATR signaling towards Chk1 activation.* Mol Cell Biol, 2006. **26**(16): p. 6056-64.
74. Jeong, S.Y., et al., *Phosphorylated claspin interacts with a phosphate-binding site in the kinase domain of Chk1 during ATR-mediated activation.* J Biol Chem, 2003. **278**(47): p. 46782-8.
75. Kumagai, A. and W.G. Dunphy, *Repeated phosphopeptide motifs in Claspin mediate the regulated binding of Chk1.* Nat Cell Biol, 2003. **5**(2): p. 161-5.
76. Yarden, R.I., et al., *BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage.* Nat Genet, 2002. **30**(3): p. 285-9.
77. Lin, S.Y., et al., *Human Claspin works with BRCA1 to both positively and negatively regulate cell proliferation.* Proc Natl Acad Sci U S A, 2004. **101**(17): p. 6484-9.
78. Stewart, G.S., et al., *MDC1 is a mediator of the mammalian DNA damage checkpoint.* Nature, 2003. **421**(6926): p. 961-6.
79. Stiff, T., et al., *Nbs1 is required for ATR-dependent phosphorylation events.* Embo J, 2005. **24**(1): p. 199-208.
80. Unsal-Kacmaz, K., et al., *The human Tim/Tipin complex coordinates an Intra-S checkpoint response to UV that slows replication fork displacement.* Mol Cell Biol, 2007. **27**(8): p. 3131-42.
81. Yoshizawa-Sugata, N. and H. Masai, *Human Tim/Timeless-interacting protein, Tipin, is required for efficient progression of S phase and DNA replication checkpoint.* J Biol Chem, 2007. **282**(4): p. 2729-40.
82. Sivasubramaniam, S., et al., *Cep164 is a mediator protein required for the maintenance of genomic stability through modulation of MDC1, RPA, and CHK1.* Genes Dev, 2008. **22**(5): p. 587-600.

83. Danielsen, J.M., et al., *HCLK2 Is Required for Activity of the DNA Damage Response Kinase ATR.* J Biol Chem, 2009. **284**(7): p. 4140-4147.
84. Matsuoka, S., et al., *ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage.* Science, 2007. **316**(5828): p. 1160-6.
85. Kumagai, A., S.M. Kim, and W.G. Dunphy, *Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1.* J Biol Chem, 2004. **279**(48): p. 49599-608.
86. Wang, X., et al., *Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress.* Mol Cell, 2006. **23**(3): p. 331-41.
87. Medhurst, A.L., et al., *ATR and Rad17 collaborate in modulating Rad9 localisation at sites of DNA damage.* J Cell Sci, 2008. **121**(Pt 23): p. 3933-40.
88. Walker, M., et al., *Chk1 C-terminal regulatory phosphorylation mediates checkpoint activation by de-repression of Chk1 catalytic activity.* Oncogene, 2009. **28**(24): p. 2314-23.
89. Zhao, H. and H. Piwnica-Worms, *ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1.* Mol Cell Biol, 2001. **21**(13): p. 4129-39.
90. Capasso, H., et al., *Phosphorylation activates Chk1 and is required for checkpoint-mediated cell cycle arrest.* J Cell Sci, 2002. **115**(Pt 23): p. 4555-64.
91. Lopez-Girona, A., et al., *Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein.* Nature, 1999. **397**(6715): p. 172-5.
92. Smits, V.A., P.M. Reaper, and S.P. Jackson, *Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response.* Curr Biol, 2006. **16**(2): p. 150-9.
93. Zhang, Y.W., et al., *Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway.* Mol Cell, 2005. **19**(5): p. 607-18.
94. Smits, V.A., *Spreading the signal: dissociation of Chk1 from chromatin.* Cell Cycle, 2006. **5**(10): p. 1039-43.
95. Furnari, B., N. Rhind, and P. Russell, *Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase.* Science, 1997. **277**(5331): p. 1495-7.
96. Peng, C.Y., et al., *Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216.* Science, 1997. **277**(5331): p. 1501-5.
97. Sanchez, Y., et al., *Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25.* Science, 1997. **277**(5331): p. 1497-501.
98. Blasina, A., et al., *A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase.* Curr Biol, 1999. **9**(1): p. 1-10.
99. Furnari, B., et al., *Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1.* Mol Biol Cell, 1999. **10**(4): p. 833-45.
100. Sorensen, C.S., et al., *Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A.* Cancer Cell, 2003. **3**(3): p. 247-58.
101. Mailand, N., et al., *Rapid destruction of human Cdc25A in response to DNA damage.* Science, 2000. **288**(5470): p. 1425-9.
102. Xiao, Z., et al., *Chk1 mediates S and G2 arrests through Cdc25A degradation in response to DNA-damaging agents.* J Biol Chem, 2003. **278**(24): p. 21767-73.
103. Shimada, M., et al., *Chk1 is a histone H3 threonine 11 kinase that regulates DNA*

damage-induced transcriptional repression. Cell, 2008. **132**(2): p. 221-32.
104. Sorensen, C.S., et al., *The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair.* Nat Cell Biol, 2005. **7**(2): p. 195-201.
105. Bahassi, E.M., et al., *The checkpoint kinases Chk1 and Chk2 regulate the functional associations between hBRCA2 and Rad51 in response to DNA damage.* Oncogene, 2008. **27**(28): p. 3977-85.
106. Wang, X., et al., *Chk1-mediated phosphorylation of FANCE is required for the Fanconi anemia/BRCA pathway.* Mol Cell Biol, 2007. **27**(8): p. 3098-108.
107. Bonilla, C.Y., J.A. Melo, and D.P. Toczyski, *Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage.* Mol Cell, 2008. **30**(3): p. 267-76.
108. Soutoglou, E. and T. Misteli, *Activation of the cellular DNA damage response in the absence of DNA lesions.* Science, 2008. **320**(5882): p. 1507-10.
109. Lukas, C., et al., *Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage.* Nat Cell Biol, 2003. **5**(3): p. 255-60.
110. Essers, J., et al., *Nuclear dynamics of RAD52 group homologous recombination proteins in response to DNA damage.* Embo J, 2002. **21**(8): p. 2030-7.
111. Lu, X., B. Nannenga, and L.A. Donehower, *PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints.* Genes Dev, 2005. **19**(10): p. 1162-74.
112. Leung-Pineda, V., C.E. Ryan, and H. Piwnica-Worms, *Phosphorylation of Chk1 by ATR is antagonized by a Chk1-regulated protein phosphatase 2A circuit.* Mol Cell Biol, 2006. **26**(20): p. 7529-38.
113. Zhang, Y.W., et al., *The F box protein Fbx6 regulates Chk1 stability and cellular sensitivity to replication stress.* Mol Cell, 2009. **35**(4): p. 442-53.
114. van Vugt, M.A., A. Bras, and R.H. Medema, *Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells.* Mol Cell, 2004. **15**(5): p. 799-811.
115. Watanabe, N., et al., *M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP.* Proc Natl Acad Sci U S A, 2004. **101**(13): p. 4419-24.
116. Mailand, N., et al., *Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress.* Mol Cell, 2006. **23**(3): p. 307-18.
117. Mamely, I., et al., *Polo-like kinase-1 controls proteasome-dependent degradation of Claspin during checkpoint recovery.* Curr Biol, 2006. **16**(19): p. 1950-5.
118. Peschiaroli, A., et al., *SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response.* Mol Cell, 2006. **23**(3): p. 319-29.
119. Macurek, L., et al., *Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery.* Nature, 2008. **455**(7209): p. 119-23.
120. Stiff, T., et al., *ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling.* Embo J, 2006. **25**(24): p. 5775-82.
121. Harper, J.W. and S.J. Elledge, *The DNA damage response: ten years after.* Mol Cell, 2007. **28**(5): p. 739-45.
122. Hofmann, K., *Ubiquitin-binding domains and their role in the DNA damage response.* DNA Repair (Amst), 2009. **8**(4): p. 544-56.
123. Faustrup, H., et al., *USP7 counteracts SCFbetaTrCP- but not APCCdh1-mediated proteolysis of Claspin.* J Cell Biol, 2009. **184**(1): p. 13-9.
124. Zhang, D., et al., *A role for the deubiquitinating enzyme USP28 in control of the DNA-damage response.* Cell, 2006. **126**(3): p. 529-42.

2

Dealing with DNA damage: relationships between checkpoint and repair pathways

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ABSTRACT

Cell cycle checkpoint activation and DNA repair pathways govern genomic stability after genotoxic stress. Genotoxic insult results in activation of an interwoven network of DNA damage checkpoints and DNA repair pathways. Post-translational modifications on a number of proteins involved in both checkpoint activation and DNA repair play an important role in this cellular response. Genotoxic stress can induce a wide variety of DNA lesions. Among these DNA alterations are double-stranded breaks and single-stranded DNA gaps. Repair of these DNA alterations requires damage recognition and resection. Here we discuss how DNA repair and DNA damage checkpoints cooperate and deal with DNA damage. Processing of DNA lesions by structure-specific nucleases results in DNA-protein intermediates, which form the basis for checkpoint activation and DNA repair. Post-translational modifications like phosphorylation and ubiquitination modulate the DNA damage response in a spatial and temporal manner. Cell cycle-dependent regulation additionally plays a key role in the regulation of both DNA repair and checkpoint activation. We highlight recent advances in *in vivo* imaging that greatly expand our knowledge on the relationships between DNA damage checkpoints and DNA repair.

INTRODUCTION

Many cells in our body divide and thereby renew the tissue they are part of. For this to happen, DNA encoding the genetic material has to be duplicated. During the cell cycle, all necessary transactions take place in order for a cell to divide into two daughter cells. Proper timing of different cell cycle phases is crucial since growth, DNA replication and cell division need to occur in the correct order. A complex regulatory network controls the cell cycle on multiple levels. Transcriptional control regulates the expression of genes in time, in a relatively slow process that operates within a timeframe of several hours. Fast responses (within minutes), are propagated through direct protein-interactions and post-translational modifications, either of which will cause activation or inhibition of specific activities of a protein or protein complexes. Cell cycle related post-translational modifications also play a key role in the initial reactions to DNA damage. Since damaged DNA is a major threat to the integrity of genetic information, fast actions need to be taken to preserve genomic integrity. Cell cycle checkpoints control the transition of the DNA through the cell cycle, thereby safeguarding the integrity of the genome. In this review we explore the relationship between activation of different DNA repair pathways by different DNA lesions and the rapid associated activation of cell cycle checkpoints.

We discriminate three phases in the cell cycle (Fig 1), before the physical separation of the two daughter cells in mitosis. Different types of Cyclins, which associate with Cyclin-dependent kinases (CDKs), drive the progression of the cell cycle [1]. During the beginning G1 phase, D type Cyclins are expressed. The Cyclin – CDK complexes positively control the release and activation of growth stimulating proteins during this phase of the cell cycle [2]. Expression of E type Cyclins during G1 phase initiates entry into S-phase [3]. During replication Cyclin A expression comes up, controlling the onset and transition of DNA replication. Cyclin B expression rises at the end of S phase. In G2, Cyclin B – CDK2 is responsible for the entry into mitosis. The activities of the Cylin – CDK complex are negatively controlled by posttranslational modification [4]. Wee1 and Myt1 phosphorylate CDK2 on two specific residues. These phosphorylation events are counteracted by the CDC25 phosphatases that remove the phosphate groups. Specific inhibitor proteins called CDK inhibitors (CKIs) inhibit Cyclin – CDK activity through direct binding to the CDK subunit [5].

During an unperturbed cell cycle, the described cell cycle events are carried out in an orderly manner. However, genotoxic environmental agents, irradiation and cellular metabolism are a constant threat for cycling and non-cycling cells [6, 7]. Cell cycle checkpoints ensure a proper and intact passing of DNA into the next cell cycle phase by regulating Cyclin – CDK activity [8]. Phosphorylation of CDK reduces activity of the Cyclin – CDK complex and therefore cell cycle progression is temporarily halted [9]. The DNA damage induces activation of the effector kinases Chk1 and Chk2. The phosphorylation of the Cdc25 phosphatase by Chk1 and/or Chk2 targets Cdc25 for proteasomal degradation, thereby enhancing CDK phosphorylation and inhibition of cell cycle progression [10]. Checkpoint proteins at the same time are able to induce and/or facilitate DNA repair and stimulate either apoptosis or checkpoint recovery.

DNA DAMAGE CHECKPOINTS

Due to the complex chemical nature of DNA the variety of structurally diverse DNA lesions that can occur is vast. Repair of a huge set of possible DNA alterations requires detection

by DNA structure-specific recognition proteins or processes that track along DNA such as transcription or replication [11]. Checkpoints however are less diverse. They do not act on the DNA lesion directly, but respond to a common DNA-protein complex that builds up at a lesion, in general after incision(s) in the DNA phosphodiester backbone have been made by nucleases in a specific DNA repair pathway [12]. In this review we concern ourselves with DNA lesions such as DNA double strand breaks (DSBs) and single-stranded DNA (ssDNA) gaps.

The DNA damage checkpoint response is under control of members of the phosphoinositide three-kinase-related kinase (PIKK) family [13, 14]. In response to DNA damage the PIKK family kinases ATM and ATR phosphorylate target proteins on serine and threonine residues, thereby activating the DNA damage checkpoint.

ATM signalling

In response to DSBs, ATM activation is necessary for checkpoint activation [15, 16]. The full activation of ATM is dependent on its autophosphorylation on Ser1981 and the interaction with the Mre11-Rad50-Nbs1 (MRN) complex at the DSB sites [17]. However it is still unclear which DNA structures stimulate ATM activation. In unperturbed cells, ATM is an inactive dimer. In response to DSBs, ATM is quickly autophosphorylated on Ser1981, which coincides with ATM forming active monomers [18-20]. Besides Ser1981, ATM is also autophosphorylated at Ser367 and Ser1893. Cells harbouring mutations in these sites are more sensitive to DSBs and show defective checkpoint signalling [21]. ATM is dephosphorylated by the Wip1 phosphatase on Ser1981 [22]. Wip1 knockout cells, in unstressed state show elevated levels of ATM Ser1981 and p53 phosphorylation.

In human cells, ATM recruitment to a specific DSB is dependent on autophosphorylation of Ser1981, the presence of Nbs1 and ATM kinase activity [23]. These data indicate the important role for ATM autophosphorylation in the checkpoint response. Therefore it was surprising that mice expressing ATM Ser1987A (human homologue of ATM Ser1981) show a normal ATM-dependent checkpoint response after DNA damage [24]. In addition, experiments performed with *Xenopus* egg extracts showed that ATM monomers are formed even in the absence of ATM autophosphorylation [25]. The reasons for these discrepancies may come from inherent differences between the species used in these studies. Another explanation is the presence of other autophosphorylation sites on ATM that may suggest a redundant function. In this context it is interesting that mice lacking H2AX, a histone variant phosporylated by PIKK kinases in response to DSBs, do not show the expected strong phenotype in response to DSBs [26, 27]. Possibly, some components of the damage induced signalling response are not crucial for checkpoint activation, potentially due to redundancy in the system.

Besides the role of Ser1981 autophosphorylation in ATM activation, the MRN complex plays an important function (Fig 3). ATM activation is hampered in Mre11 and Nbs1 mutant cell lines [28, 29]. Biochemical experiments show that MRN and ATM directly interact and thereby increase substrate affinity [19]. MRN is thought to act as a sensor for DNA damage that tethers DNA ends together before repair [30, 31]. Through binding and unwinding of DNA ends by the MRN complex ATM activation is increased [20]. The endonuclease activity of Mre11, involved in further processing of DSBs for repair, is critical for ATM activation, since cells expressing a mutant form of Mre11 that is not able to resect DSBs shows defective downstream checkpoint signalling [28, 32].

A two step model for ATM activation was proposed to explain the functional relationship

between MRN and ATM activation in response to DSBs [25]. Initially after induction of a DSB there is MRN-dependent DNA tethering, ATM recruitment and ATM monomerization. In the second step, monomeric ATM is further activated through an interaction with MRN subunit Nbs1. ATM directly interacts with the C-terminus of Nbs1, thereby recruiting ATM to sites of damage [33]. However, autophosphorylation of ATM occurs in Nbs1 mutant cells, although no damage-induced ATM Ser1981 foci are formed in these cells [34]. MRN may therefore recruit ATM to sites of damage in an already activated state, placing it in close proximity to its substrates and thereby facilitating the downstream checkpoint response [35]. These data indicate a strong network of interdependencies. However using live cell imaging techniques it was shown that even in the absence of damage the mere recruitment of DNA damage response factors to the chromatin was enough to elicit checkpoint activation [36, 37].
Further requirements for an efficient ATM-mediated checkpoint response are the phosphorylation of a number of ATM targets. A large scale screen revealed a series of target proteins involved in a wide range of cellular processes [38]. Among these are Nbs1, H2AX and Artemis. Nbs1 is phosphorylated by ATM on Ser278, Ser343 and Ser615 [39, 40]. Nbs1 mutant cells wherein the Serine434 has been mutated to alanine show a partial checkpoint defect [41, 42]. It was suggested that the phosphorylation of Nbs1 by ATM acts to recruit specific substrates to sites of damage [43]. The phosphorylation of specific substrates in a spatio-temporal manner may direct DSB processing steps in time and amplify the checkpoint when necessary. For example, the ATM-mediated phosphorylation of Artemis, a nuclease involved in one of the DSB repair pathways (see section on the processing of DNA lesions), is important for the resection of specific types of DNA lesions [44]. Chromatin-modifications also play a role in ATM activation [18], but since this is not the topic of this review the interested reader is referred to other papers for further information [18, 45, 46].

ATR signalling

After DNA damage infliction, ATR signalling is initiated in response to RPA that is loaded onto ssDNA gaps or resected DSBs (Fig 2). ATRIP, which is in complex with ATR is then recruited to these sites of damage. Independently, the Rad17-RFC complex is loaded onto these sites of damage [47-49]. The Rad17-RFC complex consists of the Rad17 subunit and four additional subunits named RFC2-RFC5. During normal replication the RFC complex, containing RFC1 instead of Rad17, plays a role in the loading of PCNA onto DNA. PCNA is a processivity factor for DNA polymerases. Both the Rad17-RFC and RFC complex require RPA for their loading onto DNA [50, 51]. However Rad17-RFC requires 5' dsDNA-ssDNA junctions, rather than the 3' ended junctions preferred by PCNA [52, 53]. These types of structures are specifically created by the resection of DSBs, stalled replication forks and UV-induced ssDNA gaps. The Rad17-RFC protein complex facilitates the loading of the Rad9-Rad1-Hus1 sliding clamp onto the DNA. The 9-1-1 sliding clamp shows high structural similarity to the PCNA clamp [53].
The PCNA and 9-1-1 sliding clamps, preferably load onto different DNA substrates. In the case of a DNA damage-induced ssDNA gap the two clamps would be placed opposite of each other at the ssDNA gap, since PCNA loads onto 3' ended junctions and 9-1-1 onto 5' ended junctions. Live cell imaging revealed that during S-phase PCNA forms replication-associated foci [54-56]. In response to DNA damage Rad9 also localizes into foci [57] indicating a specific function for the 9-1-1 complex in the DNA damage response. This is further supported by the fact that in response to damage the loading of the 9-1-1 complex onto DNA is necessary for Chk1 activation by ATR and subsequent checkpoint signalling [58].
Artificial localization of the Mec1 and Ddc1, the *S. cerevisiae* equivalents of ATR and Rad9

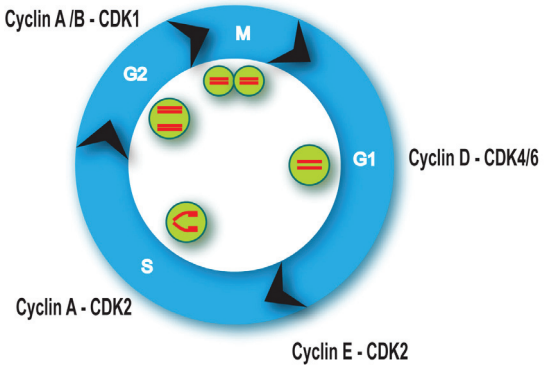

Figure 1: Cyclin - CDK regulation throughout the different phases of the cell cycle. During the G1 phase, the Cyclin D – CDK4/6 complex is responsible for cell cycle progression after which the Cyclin E – CDK2 complex come up, leading to further progression into S phase and duplication of the DNA. Cyclin A – CDK2 levels rise during S and G2 phase and finally Cyclin A/B – CDK1 drives cells into mitosis with segregation of cells into two identical daughters. The G1/S and G2/M borders together with replication (intra S phase) function as checkpoints to maintain genomic stability after DNA damage.

is enough to trigger a checkpoint response without the presence of DNA damage [37]. This suggests that ATR kinase activation and subsequent checkpoint activation is merely dependent on the close proximity of these two complexes to each other on DNA. However another protein, called TopBP1, stimulates ATR activity in human cells. Like ATRIP-ATR and the 9-1-1 complex, TopBP1 also localizes to sites of damage [57]. TopBP1 binds to the Rad9 subunit of the 9-1-1 complex, thereby locating it near the ATRIP-ATR heterodimer. ATR only becomes fully activated through an interaction with the ATR-activating domain of TopBP1 [59, 60]. Besides the phosphorylation of Chk1, other ATR targets are Rad17, TopBP1, RPA, ATRIP, 9-1-1 and Claspin (Fig 2). The phosphorylation of Rad17 is involved in the localization of Rad9 to sites of DNA damage [57]. These and other data suggest that the phosphorylation of Rad17 acts as a positive feedback loop in amplifying checkpoint activation [57, 61, 62]. A similar mechanism was proposed for the ATR-mediated phosphorylation of TopBP1 [63]. The phosphorylation of *Xenopus* TopBP1 on Serine 1131 by ATR enhances the interaction between ATRIP-ATR and TopBP1.

ATR-mediated phosphorylation of Claspin is important for Chk1 activation. Claspin mediates Chk1 phosphorylation and subsequent activation by ATR. Claspin levels are tightly regulated throughout the cell cycle by the SCF ubiquitin ligase in a Plk1-dependent manner [64]. Damage-induced ATR-mediated phosphorylation of Claspin reduces its targeting for degradation and thereby enhances Chk1 activation. The amplification of the checkpoint signal through post-translational modifications actually sustains the initial signal and broadens it in both the spatial and temporal directions [65, 66].

The effector kinases Chk1 and Chk2 are important for the spreading of the checkpoint signal throughout the cell [67]. Activated Chk1 and Chk2 phosphorylate Cdc25A during the G1/S-phase transition and Cdc25C during G2/M, thereby stopping cell cycle progression. DNA damage-induced phosphorylation of Chk1 releases the protein from chromatin [68]. Unlike the PIKK kinases these so called effector kinases are not stably recruited to sites of damage and do not localize in damage-induced foci. Furthermore live-cell studies have shown that GFP-tagged versions of Chk1 and Chk2 are highly mobile proteins even in the presence of DNA damage [69, 70].

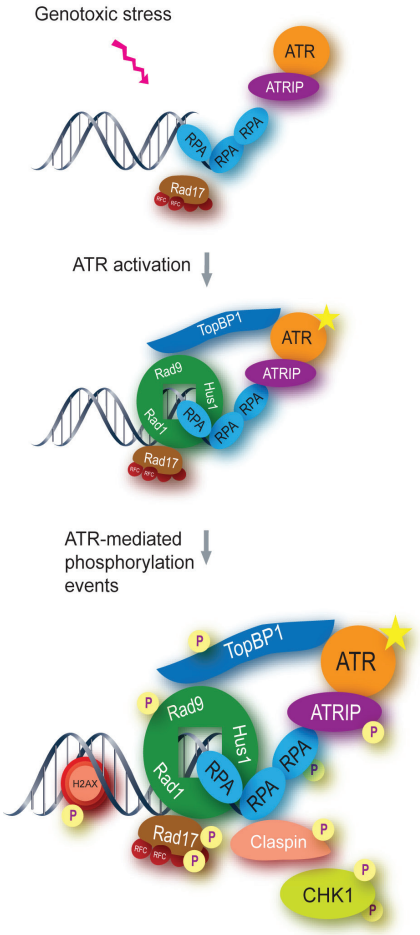

Figure 2: Genotoxic stress leads to many ATR-mediated phosphorylation events and subsequent checkpoint activation, amplification and maintenance. RPA coated ssDNA functions as the initial signal for ATR-mediated checkpoint activation, through the independent recruitment of the ATR-ATRIP and Rad17-RFC(2-5) complexes. ATR activation is dependent upon the loading of the 9-1-1 complex by Rad17-RFC(2-5) and subsequent recruitment of TopBP1. ATR activation leads to transient cell cycle arrest through the phosphorylation of effector kinase Chk1. ATR-mediated phosphorylation of Rad17 and TopBP1 are involved in checkpoint maintenance and amplification. The phosphorylation of Claspin, Rad9, ATRIP and H2AX by ATR are all suggested to play a role in checkpoint regulation, indicating the importance of signalling through phosphorylation in checkpoint control.

The DNA damage-induced checkpoint inhibits initiation of new replicons during replication, thereby slowing it down. However, at or behind the replication forks, nucleotide incorporation can continue due to translesion synthesis (TLS) polymerases [71]. These polymerases are loaded onto the DNA when damaged bases block replication forks and are able to replicate over the damaged area. Besides many phosphorylation events that relay the checkpoint signal after DNA damage, other types of post-translational modifications take place. For example, PCNA is involved in the initiation of translesion synthesis [72]. Rad6/Rad18-dependent mono-ubiquitination of PCNA leads to the recruitment of TLS polymerases at the site of damage [73, 74]. The *S. cerevisiae* Rad17 (human homologue Rad1) of the Ddc1-

Rad17-Mec3 (human homolgues Rad9-Rad1-Hus1 complex) is ubiquitinated in a Rad6/Rad18-dependent manner, similar to PCNA. Ubiquitination of *S. cerevisiae* Rad17 is involved in checkpoint regulation after DNA damage. A strain harbouring the Rad17-K197R mutation, that block the ubiquitination, shows reduced Ddc2 (homologue to human ATRIP) focus formation in response to HO-endonuclease-induced DNA breaks [75]. These data suggest a role for Rad1 ubiquitination in the recruitment of the 9-1-1 and ATR-ATRIP complex to chromatin in response to DNA damage. It has been suggested that the 9-1-1 complex has a role in TLS, during replication [76]. In yeast, the 9-1-1 clamp can physically interact with TLS polymerases [77, 78] and therefore ubiquitination of the 9-1-1 complex may likely play a role in these processes. However no evidence has been found to support this in mammalian cells. That ubiquitination plays an important role in the DNA damage response was recently shown. In response to both DSBs and UV irradiation histone H2A becomes ubiquitinated [79, 80]. These modifications are important for the recruitment of checkpoint mediators 53BP1 and MDC1 [81-83]. These data implicate ubiquitination as an important post-translational modification in the DNA damage response and also directly link chromatin modifications to the DNA damage response. Other modifications like SUMOylation and NEDDylation may very likely also play a role in the DNA damage response [84-88].

DNA REPAIR PATHWAYS

There are a wide variety of DNA repair pathways that are necessary to repair structurally diverse DNA lesions. Here we will discuss the dominant pathways involved in the repair of DSBs and ssDNA gaps.

Double strand break repair

Repair pathways that are associated with the repair of DSBs are non homologous end joining (NHEJ) and homologous recombination. In human somatic cells NHEJ is an important repair pathway for DSBs. This is surprising since NHEJ-mediated repair is more error-prone than repair via homologous recombination, which uses the genetic information on the duplicate strand for repair of the damaged bases. Since DSB repair mediated by NHEJ does not require an identical sister chromatid template it might be a fast and easy method to seal a two-ended break arising from the effects of chemicals or X-rays on DNA to counter potentially oncogenic translocations [6, 7]. Two-ended breaks that are repaired by NHEJ are first recognized by the Ku70/80 heterodimer (Fig 3). This ring-shaped complex has a high affinity for DNA ends [89]. DNA-PK_{CS} (DNA-PK catalytic subunit) locates and binds to the Ku complex at the site of damage. The binding of DNA-PK_{CS} mediates the recruitment of XRCC4, XLF and DNA ligase IV [89-92]. These latter proteins are responsible for the completion of the ligation reaction, resulting in the reannealing of the two broken DNA ends back together. Certain chemicals that induce DSBs, like topoisomerase II inhibitors (Etoposide) do not induce a directly ligatable DNA end, because of the covalent attachment of topoisomerase to the DNA end [44]. For repair through NHEJ, these types of DNA ends are cleaned up by the Artemis nuclease [93-95]. Artemis interacts directly with DNA-PK_{CS} and locates to sites of damage *in vivo*.

Homologous recombination is very important in the repair of replication-associated DNA damage. ssDNA and one-ended DSBs can arise during replication and cause stalling of replication forks especially after DNA damage [98]. Because homologues recombination

uses the information on the undamaged sister chromatid, the repair process is error-free. Homologous recombination is initiated by the binding of the MRN complex to the DNA ends. Together with the CtIP nuclease MRN is responsible for the initial resection of the DNA ends to produce short 3' overhangs [96, 97]. The nucleases ExoI or Dna2 in combination with the Sgs1 helicase create larger 3' overhangs through further resection of the DNA end. These stretches of ssDNA are covered by RPA which later on is replaced by Rad51 nucleoprotein filaments (Fig 3). Homologous recombination proceeds through strand invasion of the Rad51 covered ssDNA into the homologous double-stranded DNA (dsDNA) template, producing a joint molecule. Branch migration will lead to the forming of so called Holliday junctions that will be resolved to result in an error-free repaired DSB [98, 99]. The above reactions are facilitated by many proteins among which are BRCA2, Rad51AP1, BLM and Rad54 [100, 101].

Nucleotide excision repair

UV-induced DNA lesions are typically repaired by Nucleotide Excision Repair (NER). As a result of UV-irradiation helical distortions are formed in DNA, due to covalent linkage of bases on the same strand of the DNA double helix. These do not directly induce DNA breaks but are able to stall replication forks [102]. Unrepaired UV lesions can lead to base changes which become fixed in DNA as mutations and thereby increase the risk of tumorigenesis. NER works in two distinct pathways, either via transcription-coupled NER (TC-NER) or through global genome NER (GG-NER). These two pathways differ in the way nucleotide damage is recognized. TC-NER damage recognition involves active transcription of the template and requires CSA and CSB whereas during GG-NER XPC is responsible for damage detection. The two major UV-induced DNA helical distortions are 6-4 photoproducts (6-4 PPs) and cyclobutylpyrimidine dimers (CPDs). Both lesions are recognized and processed by NER; 6-4 PPs are more rapidly removed than CPD lesions since these are recognized better and faster by the NER machinery [103]. For the recognition of CPD lesions during GG-NER, DDB1 and DDB2 are required accessory proteins involved in the damage recognition process [104, 105]. After the initial recognition step, TFIIH is recruited [106]. This large, 10-subunit protein complex unwinds the DNA in an ATP-dependent process, thereby opening up the DNA to gain further access to the damaged bases [107]. RPA and XPA bind TFIIH and the open DNA structure, respectively stabilizing and validating the DNA lesion. The 3' and 5' endonucleases named XPG and XPF/ERCC1 are recruited to either side of the lesion to perform a dual incision of approximately 35 nucleotides including the damaged bases [108]. The introduced ssDNA gap is filled and sealed with the aid of post-incision proteins including PCNA, XRCC1, DNA polymerases and DNA ligase III [109]. The NER mechanism has been extensively investigated. Live cell imaging of NER proteins revealed some of their spatial and temporal behaviour. Combining these data with the use of computer modelling will gain further insight into how the NER pathway as a whole behaves in real time in the context of the nucleus [110, 111].

PROCESSING OF DNA LESIONS

In response to DNA damage, the checkpoint is activated only when the DNA alterations are processed into intermediate structure. Consequently the lesions must be recognized and processed first, before the DNA damage checkpoint becomes fully activated. Recently

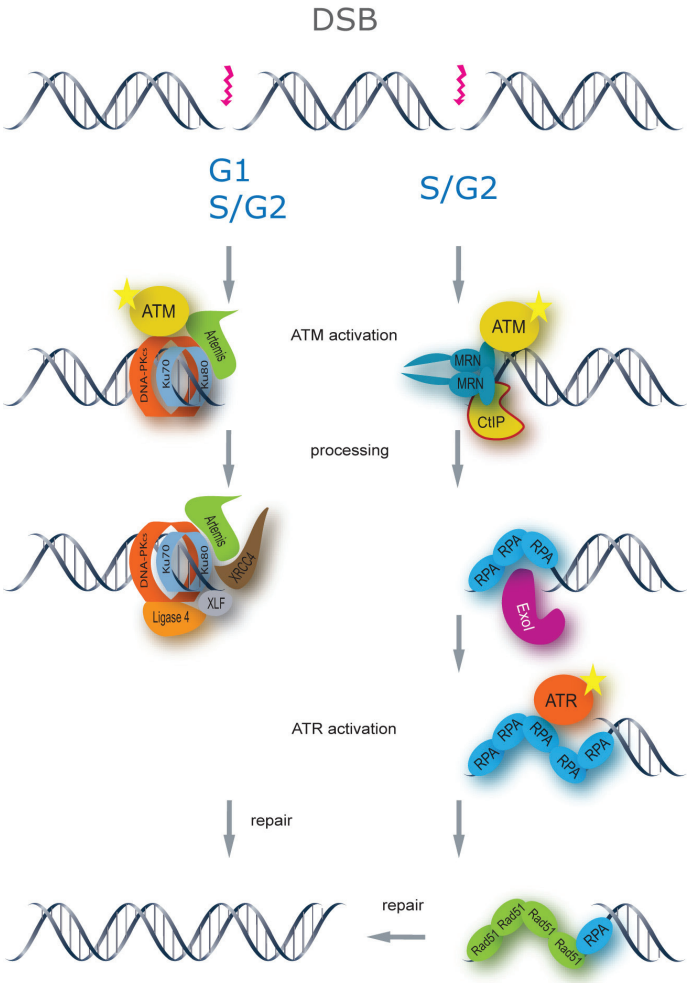

Figure 3: Cell cycle-dependent processing of DSBs and subsequent checkpoint activation. DSBs generated in G1 and S/G2 can be repaired by NHEJ. The Ku70/80 complex binds the broken DNA ends first. ATM becomes activated inducing a checkpoint response. The Artemis nuclease trims down the DNA end creating a ligatable substrate. DNA-PKcs binds Ku and thereafter functions as a platform for the recruitment of DNA ligase IV, XLF and XRCC4, which are ultimately responsible for direct ligation of the two broken DNA ends. During S and G2 phase, ATM becomes activated via the MRN complex eliciting an early checkpoint response. DSBs are processed first by the MRN complex and CtIP, creating a small stretch of ssDNA bound by RPA. Secondary processing is performed by ExoI (or Sgs1/Dna2), creating longer stretches of RPA coated ssDNA. This initiates an additional checkpoint response through ATR. Finally RPA is exchanged for Rad51 that initiates repair through homologous recombination.

a number of (structure-specific) nucleases have been implicated in the DNA damage response.. In the current model for resection of DSBs the first resection step requires MRN and CtIP, producing small ssDNA overhangs [96]. The subsequent action of ExoI or Dna2 in combination with Sgs1 (a helicase) which both act redundantly results in long stretches of ssDNA [112-114]. The long stretch of ssDNA can act as a substrate for Rad51 filament formation and further DNA repair via homologous recombination [112].

ssDNA and specifically ssDNA-dsDNA overhangs *in vitro* using *Xenopus* egg extracts induce

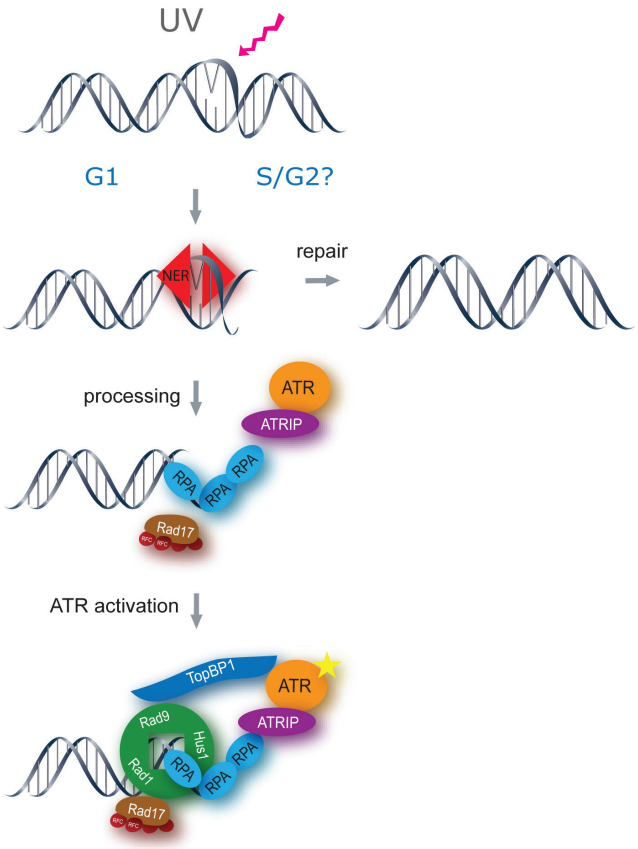

Figure 4: NER-dependent checkpoint activation after UV-irradiation. UV lesions are recognized by NER and thereafter either directly repaired or processed further to create ssDNA gaps. RPA quickly accumulates at the ssDNA gap after which the ATR-ATRIP and Rad17-RFC(2-5) complexes independently localize to the site of damage. Rad17-RFC(2-5) loads the 9-1-1 complex onto the 5' ssDNA-dsDNA junction. ATR is activated through the interaction with TopBP1, which binds to a C-terminal domain of Rad9.

ATR-dependent checkpoint activation [115]. In response to DSBs, ss/dsDNA junctions are important in the activation of ATM and in later instance ATR [116]. Using HeLa nuclear extracts Zou and co-workers showed that MRN and CtIP activate ATM in response to DNA ends that are actively resected. After the initial resection MRN is released and ExoI is responsible for further resection, which leads to a larger stretch of ssDNA and subsequent ATR activation. The larger stretch of ssDNA is responsible for the loss of activated ATM and the switch to increased ATR activation. All in all these data indicate that the DSB response *in vitro* is driven by resection. Resection and checkpoint control directly regulate each other. The nuclease ExoI for example is phosphorylated by ATR in a DNA damage-dependent manner. Moreover, its protein levels are also regulated by ubiquitination after damage, indicating a post-translational feedback mechanism for controlling ExoI activity [117, 118].
Proteins containing nuclease activity, such as XPG and ERCC1/XPF, also play roles during the later steps of repair reactions, as detailed below for UV-induced DNA damage. In addition to the role of ERCC1/XPF in NER, its nuclease activity is also crucial for repair of interstrand

DNA crosslinks [119]. Interstrand crosslink repair also requires the action of a ERCC1/XPF related structure-specific endonuclease called Mus81/Eme1 [120]. Mus81/Eme1 also plays an important role in restart of blocked DNA replication forks [121]. Replication stress can give rise to complicated DNA lesions and appears to require multiple structure-specific nucleases. The SLX4 (human homologue BTBD12) protein functions as a scaffold for the recruitment of different structure-specific endonucleases to replication-associated DNA lesions. In response to DNA damage SLX4 can bind and enhance the nuclease activity of ERCC1/XPF, Mus81/Eme1 and SLX1 [122-126]. SLX4 in combination with Mus81/Eme1 cleaves branched DNA structures, like 3' and 5' DNA flaps and replication forks. As mentioned above repair of DSBs through homologous recombination can physically join two sister chromatids through a Holliday junction. Resolution of these junctions can be performed by resolvases. Gen1 was recently identified as a Holliday junction resolvase [127]. SLX4 in complex with SLX1 can also perform Holliday junction cleavage and resolution. Interestingly SLX4 is also phosphorylated in response to DNA damage by ATM and ATR kinases, suggesting a form of regulation, possibly through a feedback mechanism [38, 123]. Thus, many endonucleases, some part of the same protein complex, have been identified that play a role in the repair of replication-associated DNA damage and recombination. It will be interesting to determine what the precise DNA substrates are for individual nucleases. Potentially there is a lot of redundancy between nucleases that will make this task an interesting challenge.

INTEGRATION AND COOPERATION BETWEEN DNA REPAIR AND CELL CYCLE CHECKPOINTS

In the previous sections we discussed the mechanism of DNA damage checkpoint activation and the functions of dedicated DNA repair pathways. These events take place in the context of the cell cycle however. In the next section we will discuss these processes in the context of three cell cycle phases.

G1/S checkpoint

The G1 phase of the cell cycle is characterized by the fact that only one copy of the DNA is present. Homologous recombination can therefore not be efficient during this time. The major DNA repair pathway for DSBs during G1 is NHEJ [128]. Since many cells in the human body are post-replicative and thus non-cycling, error-free DNA repair is often not essential for cell viability. Whether proteins involved in homologous recombination are still active in the G1 phase is a question that remains. Do they simply not fulfil their job due to the need for an identical template? Or is the homologous recombination machinery actively being shutdown during the G1 phase? Core proteins involved in homologous recombination, like Rad51 and BRCA2 do not form DSB-induced foci in G1, suggesting that homologous recombination is not active during this phase. In *S. cerevisiae*, Sae2 is involved in the resection of DSBs thereby facilitating repair via homologues recombination. The protein is under control of the Cdc28/Cdk1 complex (Cyclin B/CDK2 in humans), which is expressed during S and G2 phase. Through the phosphorylation of specific residues on Sae2 by Cdc28/Cdk1, the protein becomes active and homologous recombination becomes more favourable [129]. These CDK residues also exist in the human Sae2 homologue CtIP, for which they work according to the same mechanism [130].

The nature of the DNA lesion also facilitates the choice of repair in G1. In *S. cerevisiae* cells synchronized in G1, so called 'dirty ends' (DNA ends whose chemical composition precludes

direct ligation) are recognized more efficiently by the MRN complex and are consequently subjected to DNA end-resection. These resected breaks are then further repaired in the next round of replication by homologous recombination. HO-endonuclease induced breaks on the other hand, leaving a 3'-hydroxyl and 5'-phosphate group at the DNA end can be directly ligated. These ends are a substrate for the Ku70/80 end-binding complex and thus favour repair by NHEJ [12, 131, 132]. Thus, both the cell cycle and the type of DNA lesion that is induced control the DNA damage response.

Intra S phase checkpoint

During S phase, CtIP becomes active due to an increased CDK2 activity, thereby stimulating DNA repair via homologous recombination. BRCA2 is another example of a DNA damage response protein under cell cycle control. BRCA2 is involved in the repair of DSBs by homologous recombination, wherein it interacts directly with Rad51 via its C-terminus [133-135]. The BRCA2 C-terminus is phosphorylated in a CDK-dependent manner on Ser3291 [136]. This phosphorylation event blocks the interaction with Rad51. In unperturbed cells phosphorylation is lowest during replication after which it increases towards mitosis. After DNA damage, CDK-dependent phosphorylation of BRCA2 goes down immediately, stimulating the interaction with Rad51 and repair via homologous recombination.

In response to UV-induced DNA lesions, gap-sealing by ligase I occurs in a cell cycle-dependent manner [109, 137]. Ligase I is phosphorylated by CDK2, thereby restricting its activity to S phase. Since DNA ligase III is active throughout the cell cycle, gap-sealing after NER is able to function even in the absence of functional ligase I. The function of ligase I is critical however during DNA replication [138, 139], when it associates with the PCNA sliding clamp in joining adjacent Okazaki fragments [140, 141]. After DNA damage ligase I displays a more enhanced binding to Rad17 [142] and the 9-1-1 complex [143], suggesting a functional interaction between the checkpoint and ligase I during DNA damage.

ATR-dependent checkpoint signalling is activated in response to UV irradiation [144, 145]. The UV-induced helical distortions are recognized and processed by NER, however further widening of the NER-induced ssDNA gap may result into a longer stretch of ssDNA [146]. It is likely that nucleases are involved in the processing of these ssDNA gaps [147]. The involvement of NER in ATR-mediated checkpoint activation in reaction to UV damage has been puzzling (Fig 4). UV-induced damage stalls replication forks and induces a checkpoint response. This is due to the formation of ssDNA during the uncoupling of replicative helicases from stalled forks [148]. Nevertheless UV-irradiation induces ATR-dependent signalling also outside of S phase [149-151]. UV-induced lesions outside of S phase will also be recognized by NER. During NER-dependent processing of the DNA for nucleotide excision a small ssDNA gap is created [106]. This small ssDNA gap recruits RPA, although whether these structures are enough to trigger the checkpoint response is not known. Nonetheless, the Rad9 checkpoint protein is recruited to UV-induced lesions during G1 [152]. Additionally, Rad9 binds to chromatin in G1-enriched cells after DNA damage [153]. NER-dependent Rad9 focus formation during G1 is dependent on both XPC and XPA, whereas during replication XPA is more important [152]. A specific role for XPA outside of classical NER had been shown for replication-induced checkpoint activation [154]. XPA also directly interacts with ATR [155]. However, experiments performed with primary cells from patients with defects in NER do not support this. In these type of cells, phosphorylation of Chk1 in response to UV is dependent on NER in non-replicating and nocodazol-arrested cells and not during replication [144]. The basis for these conflicting results might originate from cell type specific effects,

and/or from the methods used in each study. Another reason could be that the degree of Chk1 phosphorylation is different during each cell cycle phase.

G2/M checkpoint

Before the cell goes into mitosis it must go through the G2/M checkpoint. When cells enter mitosis with DSBs, this can lead to gross chromosomal rearrangements and an uneven separation of chromosomes between the two daughter cells. Therefore the G2/M checkpoint must work effectively enough to turn off the cell cycle progression. In this regard it is surprising that it is not essential to repair all DSBs to continue entering into mitosis [156]. Similar results were obtained after UV-irradiation for yeast cells. These cells do not stop at the G2/M boarder after low doses of UV. High doses on the other hand can trigger a delay in cell cycle progression at the G2/M boarder [157]. It will be interesting to determine what type of DNA structures activate the checkpoint after DNA damage in G2, given that the chromosomal properties of the DNA are dramatically changed in G2. The cohesion complex may play a role here, since cohesion is required for DSB repair by homologous recombination in G2 cells [158]. Additionally cohesion subunit SMC1 is a downstream target of the ATM kinase [159].

PERSPECTIVE

Interwoven networks of DNA damage checkpoints and DNA repair pathways are of crucial importance for maintaining genomic integrity after DNA damage induction, because they enable cells to respond in both a cell cycle-dependent and lesion-specific manner. To fully understand these networks, numerous questions remain to be answered. How precisely are DNA damage checkpoints activated? Single-stranded DNA can trigger a response in a length dependent-manner and either a 3' or a 5' DNA end is required for Chk1 activation [115]. Since these are the requirements for checkpoint activation why do they not induce a checkpoint response during normal replication? 3' ends are generated during both lagging and leading strand synthesis, also seen as PCNA replication-associated foci during S phase. 5' ends are specifically generated during lagging strand synthesis. What then makes the situation different from normal replication to stalled replication forks? The first option could be that checkpoint activation requires blocking of the fork for efficient activation. Many of the proteins involved in checkpoint control, such as Claspin, ATR and Chk1, also play a role in replication fork stabilization. In the second option, the checkpoint is actively repressed during normal ongoing replication. However when the forks are stalled the repression is alleviated and the checkpoint becomes active. The third option implies a threshold for checkpoint activation. During replication the sum of the total amount of ssDNA and primed ends is not enough to significantly induce checkpoint activation. We favour a fourth option that is not necessarily incompatible with the mentioned possibilities; checkpoint activation is achieved by the build-up of specialized nucleoprotein structures at the sites of damage, thereby creating checkpoint response specificity [12]. Evidence for this hypothesis exists, since checkpoint activation can be achieved through artificial recruitment of checkpoint proteins to chromatin in an undamaged state [36, 37]. This implies that spatial distribution of proteins is important for checkpoint control. Additionally *in vivo* checkpoint activation is controlled by dynamic protein-protein interactions in a spatial and temporal manner. Namely, checkpoint regulators ATM and ATR are dependent on other proteins for their full

activation and recruitment to sites of DNA damage. ATM requires the MRN complex for its localization to DSBs and for complete kinase activation [19, 20, 33, 34]. ATR recruitment and activation on the other hand relies on RPA coated ssDNA and a loaded 9-1-1 complex at the 5' ssDNA/dsDNA junction [51, 58, 160]. These arguments suggest that checkpoint activation is simply controlled through recruitment and activation of specific proteins and protein complexes. Future work should provide further insight into these remaining questions.

What happens when DNA repair is completed? Given that DNA intermediate substrates act as the primary signal for checkpoint activation, fewer intermediates become present in time when damage is repaired. By reducing the amount of intermediates, checkpoint signalling will also be reduced, which results in a restart of the cell cycle. Experiments show, however, that cells resume cycling when breaks are still present [156]. It was suggested that cells arrest only when a damage threshold is reached and therefore also start cycling again with some DNA damage still present. A similar threshold was suggested for the activation of the checkpoint [161].

Cell cycle-dependencies influence the DNA damage response extensively. This makes the situation more complicated, as DNA damage repair and checkpoint pathways should not be seen as static protein cascades but as dynamic and highly regulated mechanisms. To study the cell cycle dependent regulation of checkpoint and repair pathways onto specific types of DNA damage in the future, we need to use and develop better ways of inducing DNA damage. For example, recent studies make use of specific types of sources and lasers to induce specific subsets of DNA lesions [162-166] and, with the use of live cell imaging and specific cell cycle markers [54, 152, 167], cell cycle-dependency can be studied in more detail.

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REFERENCES

1. Sherr, C.J., *D-type cyclins.* Trends Biochem Sci, 1995. **20**(5): p. 187-90.
2. Beijersbergen, R.L. and R. Bernards, *Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins.* Biochim Biophys Acta, 1996. **1287**(2-3): p. 103-20.
3. Koff, A., et al., *Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle.* Science, 1992. **257**(5077): p. 1689-94.
4. van den Heuvel, S. and E. Harlow, *Distinct roles for cyclin-dependent kinases in cell cycle control.* Science, 1993. **262**(5142): p. 2050-4.
5. Obaya, A.J. and J.M. Sedivy, *Regulation of cyclin-Cdk activity in mammalian cells.* Cell Mol Life Sci, 2002. **59**(1): p. 126-42.
6. Hoeijmakers, J.H., *Genome maintenance mechanisms for preventing cancer.*

Nature, 2001. **411**(6835): p. 366-74.
7. Kanaar, R., C. Wyman, and R. Rothstein, *Quality control of DNA break metabolism: in the 'end', it's a good thing.* Embo J, 2008. **27**(4): p. 581-8.
8. Zhou, B.B. and S.J. Elledge, *The DNA damage response: putting checkpoints in perspective.* Nature, 2000. **408**(6811): p. 433-9.
9. Zierhut, C. and J.F. Diffley, *Break dosage, cell cycle stage and DNA replication influence DNA double strand break response.* Embo J, 2008. **27**(13): p. 1875-85.
10. Falck, J., et al., *The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis.* Nature, 2001. **410**(6830): p. 842-7.
11. Dronkert, M.L. and R. Kanaar, *Repair of DNA interstrand cross-links.* Mutat Res, 2001. **486**(4): p. 217-47.
12. Wyman, C., D.O. Warmerdam, and R. Kanaar, *From DNA end chemistry to cell-cycle response: the importance of structure, even when it's broken.* Mol Cell, 2008. **30**(1): p. 5-6.
13. Lovejoy, C.A. and D. Cortez, *Common mechanisms of PIKK regulation.* DNA Repair (Amst), 2009.
14. Cimprich, K.A. and D. Cortez, *ATR: an essential regulator of genome integrity.* Nat Rev Mol Cell Biol, 2008. **9**(8): p. 616-27.
15. Shiloh, Y., *ATM and related protein kinases: safeguarding genome integrity.* Nat Rev Cancer, 2003. **3**(3): p. 155-68.
16. Shiloh, Y., *The ATM-mediated DNA-damage response: taking shape.* Trends Biochem Sci, 2006. **31**(7): p. 402-10.
17. Wyman, C. and R. Kanaar, *DNA double-strand break repair: all's well that ends well.* Annu Rev Genet, 2006. **40**: p. 363-83.
18. Bakkenist, C.J. and M.B. Kastan, *DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation.* Nature, 2003. **421**(6922): p. 499-506.
19. Lee, J.H. and T.T. Paull, *Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex.* Science, 2004. **304**(5667): p. 93-6.
20. Lee, J.H. and T.T. Paull, *ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex.* Science, 2005. **308**(5721): p. 551-4.
21. Kozlov, S.V., et al., *Involvement of novel autophosphorylation sites in ATM activation.* Embo J, 2006. **25**(15): p. 3504-14.
22. Shreeram, S., et al., *Wip1 phosphatase modulates ATM-dependent signaling pathways.* Mol Cell, 2006. **23**(5): p. 757-64.
23. Berkovich, E., R.J. Monnat, Jr., and M.B. Kastan, *Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair.* Nat Cell Biol, 2007. **9**(6): p. 683-90.
24. Pellegrini, M., et al., *Autophosphorylation at serine 1987 is dispensable for murine Atm activation in vivo.* Nature, 2006. **443**(7108): p. 222-5.
25. Dupre, A., L. Boyer-Chatenet, and J. Gautier, *Two-step activation of ATM by DNA and the Mre11-Rad50-Nbs1 complex.* Nat Struct Mol Biol, 2006. **13**(5): p. 451-7.
26. Celeste, A., et al., *H2AX haploinsufficiency modifies genomic stability and tumor susceptibility.* Cell, 2003. **114**(3): p. 371-83.
27. Bassing, C.H., et al., *Histone H2AX: a dosage-dependent suppressor of oncogenic translocations and tumors.* Cell, 2003. **114**(3): p. 359-70.
28. Uziel, T., et al., *Requirement of the MRN complex for ATM activation by DNA damage.* Embo J, 2003. **22**(20): p. 5612-21.

29. Carson, C.T., et al., *The Mre11 complex is required for ATM activation and the G2/M checkpoint.* Embo J, 2003. **22**(24): p. 6610-20.
30. Moreno-Herrero, F., et al., *Mesoscale conformational changes in the DNA-repair complex Rad50/Mre11/Nbs1 upon binding DNA.* Nature, 2005. **437**(7057): p. 440-3.
31. de Jager, M., et al., *Human Rad50/Mre11 is a flexible complex that can tether DNA ends.* Mol Cell, 2001. **8**(5): p. 1129-35.
32. Kanaar, R. and C. Wyman, *DNA repair by the MRN complex: break it to make it.* Cell, 2008. **135**(1): p. 14-6.
33. Falck, J., J. Coates, and S.P. Jackson, *Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage.* Nature, 2005. **434**(7033): p. 605-11.
34. Kitagawa, R., et al., *Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway.* Genes Dev, 2004. **18**(12): p. 1423-38.
35. Lee, J.H. and T.T. Paull, *Activation and regulation of ATM kinase activity in response to DNA double-strand breaks.* Oncogene, 2007. **26**(56): p. 7741-8.
36. Soutoglou, E. and T. Misteli, *Activation of the cellular DNA damage response in the absence of DNA lesions.* Science, 2008. **320**(5882): p. 1507-10.
37. Bonilla, C.Y., J.A. Melo, and D.P. Toczyski, *Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage.* Mol Cell, 2008. **30**(3): p. 267-76.
38. Matsuoka, S., et al., *ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage.* Science, 2007. **316**(5828): p. 1160-6.
39. Lim, D.S., et al., *ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway.* Nature, 2000. **404**(6778): p. 613-7.
40. Gatei, M., et al., *ATM-dependent phosphorylation of nibrin in response to radiation exposure.* Nat Genet, 2000. **25**(1): p. 115-9.
41. Stewart, G.S., et al., *Residual ataxia telangiectasia mutated protein function in cells from ataxia telangiectasia patients, with 5762ins137 and 7271T-->G mutations, showing a less severe phenotype.* J Biol Chem, 2001. **276**(32): p. 30133-41.
42. Kim, S.T., B. Xu, and M.B. Kastan, *Involvement of the cohesin protein, Smc1, in Atm-dependent and independent responses to DNA damage.* Genes Dev, 2002. **16**(5): p. 560-70.
43. Lee, J.H., et al., *Distinct functions of Nijmegen breakage syndrome in ataxia telangiectasia mutated-dependent responses to DNA damage.* Mol Cancer Res, 2003. **1**(9): p. 674-81.
44. Riballo, E., et al., *A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci.* Mol Cell, 2004. **16**(5): p. 715-24.
45. Gontijo, A.M., C.M. Green, and G. Almouzni, *Repairing DNA damage in chromatin.* Biochimie, 2003. **85**(11): p. 1133-47.
46. Misteli, T. and E. Soutoglou, *The emerging role of nuclear architecture in DNA repair and genome maintenance.* Nat Rev Mol Cell Biol, 2009. **10**(4): p. 243-54.
47. Zou, L., D. Cortez, and S.J. Elledge, *Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin.* Genes Dev, 2002. **16**(2): p. 198-208.
48. Kondo, T., et al., *Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms.* Science, 2001. **294**(5543): p. 867-70.

49. Melo, J.A., J. Cohen, and D.P. Toczyski, *Two checkpoint complexes are independently recruited to sites of DNA damage in vivo.* Genes Dev, 2001. **15**(21): p. 2809-21.
50. Ellison, V. and B. Stillman, *Biochemical characterization of DNA damage checkpoint complexes: clamp loader and clamp complexes with specificity for 5' recessed DNA.* PLoS Biol, 2003. **1**(2): p. E33.
51. Zou, L., D. Liu, and S.J. Elledge, *Replication protein A-mediated recruitment and activation of Rad17 complexes.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 13827-32.
52. Majka, J., et al., *Replication protein A directs loading of the DNA damage checkpoint clamp to 5'-DNA junctions.* J Biol Chem, 2006. **281**(38): p. 27855-61.
53. Dore, A.S., et al., *Crystal structure of the rad9-rad1-hus1 DNA damage checkpoint complex--implications for clamp loading and regulation.* Mol Cell, 2009. **34**(6): p. 735-45.
54. Essers, J., et al., *Nuclear dynamics of PCNA in DNA replication and repair.* Mol Cell Biol, 2005. **25**(21): p. 9350-9.
55. Leonhardt, H., et al., *Dynamics of DNA replication factories in living cells.* J Cell Biol, 2000. **149**(2): p. 271-80.
56. Somanathan, S., et al., *Targeting of PCNA to sites of DNA replication in the mammalian cell nucleus.* J Cell Biochem, 2001. **81**(1): p. 56-67.
57. Medhurst, A.L., et al., *ATR and Rad17 collaborate in modulating Rad9 localisation at sites of DNA damage.* J Cell Sci, 2008. **121**(Pt 23): p. 3933-40.
58. Majka, J., A. Niedziela-Majka, and P.M. Burgers, *The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint.* Mol Cell, 2006. **24**(6): p. 891-901.
59. Lee, J., A. Kumagai, and W.G. Dunphy, *The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR.* J Biol Chem, 2007. **282**(38): p. 28036-44.
60. Delacroix, S., et al., *The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1.* Genes Dev, 2007. **21**(12): p. 1472-7.
61. Bao, S., et al., *ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses.* Nature, 2001. **411**(6840): p. 969-74.
62. Wang, X., et al., *Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress.* Mol Cell, 2006. **23**(3): p. 331-41.
63. Yoo, H.Y., et al., *Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM.* J Biol Chem, 2007. **282**(24): p. 17501-6.
64. Mamely, I., et al., *Polo-like kinase-1 controls proteasome-dependent degradation of Claspin during checkpoint recovery.* Curr Biol, 2006. **16**(19): p. 1950-5.
65. Essers, J., W. Vermeulen, and A.B. Houtsmuller, *DNA damage repair: anytime, anywhere?* Curr Opin Cell Biol, 2006. **18**(3): p. 240-6.
66. Lukas, J., C. Lukas, and J. Bartek, *Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time.* DNA Repair (Amst), 2004. **3**(8-9): p. 997-1007.
67. Smits, V.A., *Spreading the signal: dissociation of Chk1 from chromatin.* Cell Cycle, 2006. **5**(10): p. 1039-43.
68. Smits, V.A., P.M. Reaper, and S.P. Jackson, *Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response.* Curr Biol, 2006. **16**(2): p. 150-9.

69. Lukas, C., et al., *Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage.* Nat Cell Biol, 2003. **5**(3): p. 255-60.
70. Kramer, A., et al., *Centrosome-associated Chk1 prevents premature activation of cyclin-B-Cdk1 kinase.* Nat Cell Biol, 2004. **6**(9): p. 884-91.
71. Lehmann, A.R., et al., *Translesion synthesis: Y-family polymerases and the polymerase switch.* DNA Repair (Amst), 2007. **6**(7): p. 891-9.
72. Davies, A.A., et al., *Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a.* Mol Cell, 2008. **29**(5): p. 625-36.
73. Kannouche, P.L., J. Wing, and A.R. Lehmann, *Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage.* Mol Cell, 2004. **14**(4): p. 491-500.
74. Lehmann, A.R., *Translesion synthesis in mammalian cells.* Exp Cell Res, 2006. **312**(14): p. 2673-6.
75. Fu, Y., et al., *Rad6-Rad18 mediates a eukaryotic SOS response by ubiquitinating the 9-1-1 checkpoint clamp.* Cell, 2008. **133**(4): p. 601-11.
76. Jansen, J.G., M.I. Fousteri, and N. de Wind, *Send in the clamps: control of DNA translesion synthesis in eukaryotes.* Mol Cell, 2007. **28**(4): p. 522-9.
77. Sabbioneda, S., et al., *The 9-1-1 checkpoint clamp physically interacts with polzeta and is partially required for spontaneous polzeta-dependent mutagenesis in Saccharomyces cerevisiae.* J Biol Chem, 2005. **280**(46): p. 38657-65.
78. Kai, M., et al., *Rad3-dependent phosphorylation of the checkpoint clamp regulates repair-pathway choice.* Nat Cell Biol, 2007. **9**(6): p. 691-7.
79. Stewart, G.S., et al., *The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage.* Cell, 2009. **136**(3): p. 420-34.
80. Bergink, S., et al., *DNA damage triggers nucleotide excision repair-dependent monoubiquitylation of histone H2A.* Genes Dev, 2006. **20**(10): p. 1343-52.
81. Mailand, N., et al., *RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins.* Cell, 2007. **131**(5): p. 887-900.
82. Huen, M.S., et al., *RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly.* Cell, 2007. **131**(5): p. 901-14.
83. Kolas, N.K., et al., *Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase.* Science, 2007. **318**(5856): p. 1637-40.
84. Muller, S., et al., *SUMO, ubiquitin's mysterious cousin.* Nat Rev Mol Cell Biol, 2001. **2**(3): p. 202-10.
85. Bergink, S. and S. Jentsch, *Principles of ubiquitin and SUMO modifications in DNA repair.* Nature, 2009. **458**(7237): p. 461-7.
86. Ulrich, H.D., *Preface. Ubiquitin, SUMO and the maintenance of genome stability.* DNA Repair (Amst), 2009. **8**(4): p. 429.
87. Hu, J., et al., *Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in response to DNA damage.* Nat Cell Biol, 2004. **6**(10): p. 1003-9.
88. Yang, X., et al., *The COP9 signalosome inhibits p27(kip1) degradation and impedes G1-S phase progression via deneddylation of SCF Cul1.* Curr Biol, 2002. **12**(8): p. 667-72.
89. Lieber, M.R., et al., *Mechanism and regulation of human non-homologous DNA end-joining.* Nat Rev Mol Cell Biol, 2003. **4**(9): p. 712-20.
90. Wilson, T.E., U. Grawunder, and M.R. Lieber, *Yeast DNA ligase IV mediates non-homologous DNA end joining.* Nature, 1997. **388**(6641): p. 495-8.

91. Lees-Miller, S.P. and K. Meek, *Repair of DNA double strand breaks by non-homologous end joining.* Biochimie, 2003. **85**(11): p. 1161-73.
92. Ahnesorg, P., P. Smith, and S.P. Jackson, *XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining.* Cell, 2006. **124**(2): p. 301-13.
93. Ma, Y., et al., *Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination.* Cell, 2002. **108**(6): p. 781-94.
94. Ma, Y., K. Schwarz, and M.R. Lieber, *The Artemis:DNA-PKcs endonuclease cleaves DNA loops, flaps, and gaps.* DNA Repair (Amst), 2005. **4**(7): p. 845-51.
95. Goodarzi, A.A., et al., *DNA-PK autophosphorylation facilitates Artemis endonuclease activity.* Embo J, 2006. **25**(16): p. 3880-9.
96. Sartori, A.A., et al., *Human CtIP promotes DNA end resection.* Nature, 2007. **450**(7169): p. 509-14.
97. Paull, T.T. and M. Gellert, *The 3' to 5' exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks.* Mol Cell, 1998. **1**(7): p. 969-79.
98. Eppink, B., C. Wyman, and R. Kanaar, *Multiple interlinked mechanisms to circumvent DNA replication roadblocks.* Exp Cell Res, 2006. **312**(14): p. 2660-5.
99. Agarwal, S., A.A. Tafel, and R. Kanaar, *DNA double-strand break repair and chromosome translocations.* DNA Repair (Amst), 2006. **5**(9-10): p. 1075-81.
100. Modesti, M., et al., *RAD51AP1 is a structure-specific DNA binding protein that stimulates joint molecule formation during RAD51-mediated homologous recombination.* Mol Cell, 2007. **28**(3): p. 468-81.
101. Essers, J., et al., *Disruption of mouse RAD54 reduces ionizing radiation resistance and homologous recombination.* Cell, 1997. **89**(2): p. 195-204.
102. Friedberg, E.C., et al., *DNA repair: from molecular mechanism to human disease.* DNA Repair (Amst), 2006. **5**(8): p. 986-96.
103. Hoogstraten, D., et al., *Versatile DNA damage detection by the global genome nucleotide excision repair protein XPC.* J Cell Sci, 2008. **121**(Pt 17): p. 2850-9.
104. Nishi, R., et al., *UV-DDB-dependent regulation of nucleotide excision repair kinetics in living cells.* DNA Repair (Amst), 2009. **8**(6): p. 767-76.
105. Sugasawa, K., et al., *UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex.* Cell, 2005. **121**(3): p. 387-400.
106. de Laat, W.L., N.G. Jaspers, and J.H. Hoeijmakers, *Molecular mechanism of nucleotide excision repair.* Genes Dev, 1999. **13**(7): p. 768-85.
107. Giglia-Mari, G., et al., *A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A.* Nat Genet, 2004. **36**(7): p. 714-9.
108. Huang, J.C., et al., *Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' to the photodimer.* Proc Natl Acad Sci U S A, 1992. **89**(8): p. 3664-8.
109. Moser, J., et al., *Sealing of chromosomal DNA nicks during nucleotide excision repair requires XRCC1 and DNA ligase III alpha in a cell-cycle-specific manner.* Mol Cell, 2007. **27**(2): p. 311-23.
110. Dinant, C., et al., *Assembly of multiprotein complexes that control genome function.* J Cell Biol, 2009. **185**(1): p. 21-6.
111. Politi, A., et al., *Mathematical modeling of nucleotide excision repair reveals efficiency of sequential assembly strategies.* Mol Cell, 2005. **19**(5): p. 679-90.

112. Mimitou, E.P. and L.S. Symington, *Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing.* Nature, 2008. **455**(7214): p. 770-4.
113. Gravel, S., et al., *DNA helicases Sgs1 and BLM promote DNA double-strand break resection.* Genes Dev, 2008. **22**(20): p. 2767-72.
114. Zhu, Z., et al., *Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends.* Cell, 2008. **134**(6): p. 981-94.
115. MacDougall, C.A., et al., *The structural determinants of checkpoint activation.* Genes Dev, 2007. **21**(8): p. 898-903.
116. Shiotani, B. and L. Zou, *Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks.* Mol Cell, 2009. **33**(5): p. 547-58.
117. El-Shemerly, M., et al., *ATR-dependent pathways control hEXO1 stability in response to stalled forks.* Nucleic Acids Res, 2008. **36**(2): p. 511-9.
118. El-Shemerly, M., et al., *Degradation of human exonuclease 1b upon DNA synthesis inhibition.* Cancer Res, 2005. **65**(9): p. 3604-9.
119. Niedernhofer, L.J., et al., *The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks.* Mol Cell Biol, 2004. **24**(13): p. 5776-87.
120. Hanada, K., et al., *The structure-specific endonuclease Mus81-Eme1 promotes conversion of interstrand DNA crosslinks into double-strands breaks.* Embo J, 2006. **25**(20): p. 4921-32.
121. Hanada, K., et al., *The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks.* Nat Struct Mol Biol, 2007. **14**(11): p. 1096-104.
122. Munoz, I.M., et al., *Coordination of structure-specific nucleases by human SLX4/BTBD12 is required for DNA repair.* Mol Cell, 2009. **35**(1): p. 116-27.
123. Svendsen, J.M., et al., *Mammalian BTBD12/SLX4 assembles a Holliday junction resolvase and is required for DNA repair.* Cell, 2009. **138**(1): p. 63-77.
124. Klein, H.L. and L.S. Symington, *Breaking up just got easier to do.* Cell, 2009. **138**(1): p. 20-2.
125. Andersen, S.L., et al., *Drosophila MUS312 and the vertebrate ortholog BTBD12 interact with DNA structure-specific endonucleases in DNA repair and recombination.* Mol Cell, 2009. **35**(1): p. 128-35.
126. Fekairi, S., et al., *Human SLX4 is a Holliday junction resolvase subunit that binds multiple DNA repair/recombination endonucleases.* Cell, 2009. **138**(1): p. 78-89.
127. Ip, S.C., et al., *Identification of Holliday junction resolvases from humans and yeast.* Nature, 2008. **456**(7220): p. 357-61.
128. Hinz, J.M., et al., *Influence of double-strand-break repair pathways on radiosensitivity throughout the cell cycle in CHO cells.* DNA Repair (Amst), 2005. **4**(7): p. 782-92.
129. Huertas, P., et al., *CDK targets Sae2 to control DNA-end resection and homologous recombination.* Nature, 2008. **455**(7213): p. 689-92.
130. Huertas, P. and S.P. Jackson, *Human CtIP mediates cell cycle control of DNA end resection and double strand break repair.* J Biol Chem, 2009. **284**(14): p. 9558-65.
131. Barlow, J.H., M. Lisby, and R. Rothstein, *Differential regulation of the cellular response to DNA double-strand breaks in G1.* Mol Cell, 2008. **30**(1): p. 73-85.
132. Lisby, M., et al., *Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins.* Cell, 2004. **118**(6): p. 699-713.
133. Wong, A.K., et al., *RAD51 interacts with the evolutionarily conserved BRC motifs in*

the human breast cancer susceptibility gene brca2. J Biol Chem, 1997. **272**(51): p. 31941-4.

134. Chen, P.L., et al., *The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment.* Proc Natl Acad Sci U S A, 1998. **95**(9): p. 5287-92.
135. West, S.C., *Molecular views of recombination proteins and their control.* Nat Rev Mol Cell Biol, 2003. **4**(6): p. 435-45.
136. Esashi, F., et al., *CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair.* Nature, 2005. **434**(7033): p. 598-604.
137. Ferrari, G., et al., *Cell cycle-dependent phosphorylation of human DNA ligase I at the cyclin-dependent kinase sites.* J Biol Chem, 2003. **278**(39): p. 37761-7.
138. Montecucco, A., et al., *DNA ligase I is recruited to sites of DNA replication by an interaction with proliferating cell nuclear antigen: identification of a common targeting mechanism for the assembly of replication factories.* Embo J, 1998. **17**(13): p. 3786-95.
139. Cardoso, M.C., et al., *Mapping and use of a sequence that targets DNA ligase I to sites of DNA replication in vivo.* J Cell Biol, 1997. **139**(3): p. 579-87.
140. Tomkinson, A.E. and D.S. Levin, *Mammalian DNA ligases.* Bioessays, 1997. **19**(10): p. 893-901.
141. Martin, I.V. and S.A. MacNeill, *ATP-dependent DNA ligases.* Genome Biol, 2002. **3**(4): p. REVIEWS3005.
142. Song, W., et al., *A conserved physical and functional interaction between the cell cycle checkpoint clamp loader and DNA ligase I of eukaryotes.* J Biol Chem, 2007. **282**(31): p. 22721-30.
143. Song, W., et al., *The DNA binding domain of human DNA ligase I interacts with both nicked DNA and the DNA sliding clamps, PCNA and hRad9-hRad1-hHus1.* DNA Repair (Amst), 2009.
144. Marini, F., et al., *DNA nucleotide excision repair-dependent signaling to checkpoint activation.* Proc Natl Acad Sci U S A, 2006. **103**(46): p. 17325-30.
145. Stiff, T., et al., *ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling.* Embo J, 2006. **25**(24): p. 5775-82.
146. Lazzaro, F., et al., *Checkpoint mechanisms at the intersection between DNA damage and repair.* DNA Repair (Amst), 2009.
147. Nakada, D., Y. Hirano, and K. Sugimoto, *Requirement of the Mre11 complex and exonuclease 1 for activation of the Mec1 signaling pathway.* Mol Cell Biol, 2004. **24**(22): p. 10016-25.
148. Byun, T.S., et al., *Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint.* Genes Dev, 2005. **19**(9): p. 1040-52.
149. Marti, T.M., et al., *H2AX phosphorylation within the G1 phase after UV irradiation depends on nucleotide excision repair and not DNA double-strand breaks.* Proc Natl Acad Sci U S A, 2006. **103**(26): p. 9891-6.
150. O'Driscoll, M., et al., *A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome.* Nat Genet, 2003. **33**(4): p. 497-501.
151. Costanzo, V., et al., *An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication.* Mol Cell, 2003. **11**(1): p. 203-13.

152. Warmerdam, D.O., et al., *Cell cycle-dependent processing of DNA lesions controls localization of Rad9 to sites of genotoxic stress.* Cell Cycle, 2009. **8**(11).
153. Roos-Mattjus, P., et al., *Genotoxin-induced Rad9-Hus1-Rad1 (9-1-1) chromatin association is an early checkpoint signaling event.* J Biol Chem, 2002. **277**(46): p. 43809-12.
154. Bomgarden, R.D., et al., *Opposing effects of the UV lesion repair protein XPA and UV bypass polymerase eta on ATR checkpoint signaling.* Embo J, 2006. **25**(11): p. 2605-14.
155. Shell, S.M., et al., *Checkpoint kinase ATR promotes nucleotide excision repair of UV-induced DNA damage via physical interaction with XPA.* J Biol Chem, 2009.
156. Deckbar, D., et al., *Chromosome breakage after G2 checkpoint release.* J Cell Biol, 2007. **176**(6): p. 749-55.
157. Callegari, A.J. and T.J. Kelly, *Shedding light on the DNA damage checkpoint.* Cell Cycle, 2007. **6**(6): p. 660-6.
158. Watrin, E. and J.M. Peters, *The cohesin complex is required for the DNA damage-induced G2/M checkpoint in mammalian cells.* Embo J, 2009.
159. Yazdi, P.T., et al., *SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint.* Genes Dev, 2002. **16**(5): p. 571-82.
160. Zou, L. and S.J. Elledge, *Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.* Science, 2003. **300**(5625): p. 1542-8.
161. Buscemi, G., et al., *Activation of ATM and Chk2 kinases in relation to the amount of DNA strand breaks.* Oncogene, 2004. **23**(46): p. 7691-700.
162. Dinant, C., et al., *Activation of multiple DNA repair pathways by sub-nuclear damage induction methods.* J Cell Sci, 2007. **120**(Pt 15): p. 2731-40.
163. Aten, J.A., et al., *Dynamics of DNA double-strand breaks revealed by clustering of damaged chromosome domains.* Science, 2004. **303**(5654): p. 92-5.
164. Stap, J., et al., *Induction of linear tracks of DNA double-strand breaks by alpha-particle irradiation of cells.* Nat Methods, 2008. **5**(3): p. 261-6.
165. Bekker-Jensen, S., et al., *Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks.* J Cell Biol, 2006. **173**(2): p. 195-206.
166. Mone, M.J., et al., *Local UV-induced DNA damage in cell nuclei results in local transcription inhibition.* EMBO Rep, 2001. **2**(11): p. 1013-7.
167. Jazayeri, A., et al., *ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks.* Nat Cell Biol, 2006. **8**(1): p. 37-45.

3

Differential dynamics of ATR-mediated checkpoint regulators

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ABSTRACT

The ATR-Chk1 checkpoint pathway is activated by UV-induced DNA lesions and replication stress. Little was known about the spatio and temporal behaviour of the proteins involved and we therefore examined the behaviour of the ATRIP-ATR and Rad9-Rad1-Hus1 putative DNA damage sensor complexes and the downstream effector kinase Chk1. We developed assays for the generation and validation of stable cell lines expressing GFP-fusion proteins. Photobleaching experiments in living cells expressing these fusions indicated that after UV-induced DNA damage, ATRIP associates more transiently with damaged chromatin than members of the Rad9-Rad1-Husus1 complex. Interestingly, ATRIP directly associated with locally induced UV damage, whereas Rad9 bound in a cooperative manner, which can be explained by the Rad17-dependent loading of Rad9 onto damaged chromatin. Although Chk1 dissociates from the chromatin upon UV damage, no change in the mobility of GFP-Chk1 was observed, supporting the notion that Chk1 is a highly dynamic protein.

INTRODUCTION

Eukaryotic cells are continuously threatened by DNA damage caused by environmental factors and intracellular metabolic processes. To protect themselves against these potential threats, cells have developed DNA damage checkpoint and repair mechanisms, which help to ensure transmission of an intact genome. Cell cycle checkpoints and DNA repair mechanisms together determine the ultimate faith of the cell after suffering DNA damage. Activation of the DNA damage checkpoint involves the activation of transducer kinases ATR/ATM and subsequently the effector kinases Chk1/Chk2 [1]. So called mediator proteins, including Claspin and BRCA1, were additionally discovered, and function either in the recruitment of substrates to DNA lesions or as scaffolds on which protein complexes are assembled [2, 3]. In response to a variety of DNA damaging agents like UV light and replication stress, the ATR-mediated checkpoint pathway is activated. Biochemical data indicates that ATRIP, in complex with ATR, binds to RPA-coated single stranded DNA (ssDNA) [4]. Independently, the Rad17-RFC complex is also recruited to sites of damage. The Rad17-RFC protein complex facilitates the loading of the Rad9-Rad1-Hus1 (9-1-1 complex) sliding clamp onto the DNA [5-7]. Subsequently, TopBP1 is recruited to DNA lesions by binding to the Rad9 subunit of the 9-1-1 complex, thereby locating near the ATRIP-ATR heterodimer. Through an interaction with TopBP1, ATR becomes fully active, resulting in the activation of effector kinase Chk1 and subsequent checkpoint arrest [8-10].

Detection of DNA alterations after genotoxic stress is essential for the survival of cells and gaining more insight into the early events of the DNA damage response will give a better understanding of how DNA damage checkpoints function, how genome stability is achieved and how cancer can develop. In recent years, biochemical work has provided invaluable insight into the requirements, substrates and activities of proteins involved in the ATR-mediated checkpoint pathway [11-13]. Although the importance for protein localization after DNA damage induction has been reported for proteins involved in the response to double-stranded breaks (DSBs), not much is known about how the ATR-Chk1 pathway operates in living cells and moreover, how the spatio and temporal behaviour of proteins in this pathway influence the DNA damage response [14, 15]. We set out to study the behaviour of ATRIP-ATR and 9-1-1 DNA damage sensor complexes by creating cDNA constructs expressing GFP (green fluorescent protein)-tagged proteins in human cells. The use of GFP-fusion proteins creates advantages over using standard immunofluorescence techniques as it avoids fixation methods and antibody artefacts. In addition, GFP-labelled proteins can be followed in time using live cell video microscopy. Furthermore, due to the spectral qualities of GFP and its variants it has become possible to perform quantitative fluorescent analysis [16-18]. The use of GFP-labelled proteins opens up a number of new possibilities in the DNA damage response field. First, the localization GFP-fusion proteins can be directly followed into DNA damage-induced nuclear foci. Second, the ability to measure in time makes it possible to establish an order of events occurring directly after DNA damage induction. Third, the existence of multiple spectral GFP-variants allows for the simultaneous detection of several fluorescently labelled proteins in a single cell [19-21]. Forth, live cell video microscopy in combination with GFP-photobleaching experiments can be applied to quantitatively determine changes in protein mobility in response to DNA damage. Collectively, these tools help to increase our understanding of cellular mechanisms involved in DNA damage response.

The accumulation of DNA damage response proteins at sites of damage, shown in cells as

nuclear foci, is essential for downstream checkpoint events, although how, is not yet fully understood [22-24]. Many, if not most, proteins in the ATR-mediated checkpoint pathway are recruited to sites of damage into nuclear foci. As a result a proportion of the total amount of free proteins becomes immobilized in these foci due to interactions with either the damaged chromatin or other proteins at the DNA lesion. To gain more understanding of the DNA damage-induced behaviour of a protein, its localization and mobility can be determined. The DNA damage-induced change in protein mobility has been studied for several proteins involved in the DNA damage response, mainly in DNA repair and in response to double stranded breaks (DSBs) [17, 25-27]. The studies performed on proteins involved in nucleotide excision repair (NER) for example, have been important in understanding this DNA repair process and helped explain some of the phenotypic characteristics seen in patients that harbour mutations in proteins in this pathway. This indicates the importance of these types of studies for research in cancer and related diseases associated with genome instability [28, 29].

A method was setup for the generation of stable cell lines expressing GFP-fusion proteins and different assays to validate and characterize GFP-expressing cell lines were developed. We analyzed the dynamic behaviour of multiple proteins involved in the ATR checkpoint pathway including all three components of the 9-1-1 complex, ATRIP and of the downstream kinase Chk1. We compared the mobility changes of these proteins after UV-irradiation and together the results indicated a distinct dynamic behaviour of the studied proteins. Whereas effector kinase Chk1 was highly mobile and did not immobilize upon DNA damage, Rad9 and ATRIP associated to sites of DNA damage. ATRIP directly bound to DNA lesions but in a more transient manner than Rad9, which displayed a relatively slow exchange with damaged chromatin. Together these data demonstrate the importance of spatio and temporal protein regulation in the cells response to DNA damage.

MATERIALS & METHODS

Cell culture

U2OS, Hela and 293T cells were grown using standard procedures. U2OS and Hela cells stably expressing eGFP-fusion proteins were grown in standard medium supplemented with 350-700 μg/ml of G418 (gentamicin).

Antibodies

Antibodies obtained from commercial sources were as follows: Orc2 (BD Pharmingen), Grb2 (BD Transduction), RPA (Ab2, Oncogene), Rad9 (Novus), γH2AX (Upstate), Chk1-pS317 (Cell Signaling), Chk1 (Abcam). The following antibodies were obtained from Santa Cruz Biotechnology: CENP-F (H-260), Cyclin A (H-432), Cyclin B1 (GNS1), Ku86 (C-20), Chk1 (G-4), Rad1 (N-18), Rad9 (C-20) and ATR (N-19).

Rabbit polyclonal anti-Rad9, anti-Hus1 and anti-GFP were described before and a kind gift by R. Freire (Tenerife, Spain) [30, 31]. Rabbit polyclonal anti-ATRIP was a kind gift by P.M. Reaper (UK) [32].

Transfection

Plasmid DNA was transfected into cells using the calcium phosphate transfection method.

siRNA oligonucleotides (Dharmacon Research) were transfected into cells using

Oligofectamine (Invitrogen) according to the manufacturer's instructions and as previously described [33]. Sequences of oligonucleotides were as follows:
Luc (CGUACGCGGAAUACUUCGAdTdT),
Chk1 (UCGUGAGCGUUUGUUGAACdTdT)
Rad9 (ACCACUAUAGGCAAUGAGGdTdT)
Rad9 3'UTR (CCAAGAACCUGAAGCCUGUUU / GAAUCCAGCUUUGACCUUUU)

Colony survival assays
Cells were counted and 1000 cells were seeded onto 60 mm diameter dishes where after left for 12 hours to attach. Cells were treated with different doses of UV (5, 10, 15, 20 J/m^2) and incubated for 7-14 days, after which the colonies were fixed, stained and counted. All experiments were performed in triplicate.

Immunofluorescence
For immunostaining, cells were fixed in 2% paraformaldehyde containing 0.2% Triton X-100 for 20 minutes at RT and then permeabilized with 0.1% Triton X-100 for 5 minutes at RT. Samples were blocked in 1% FCS and immunostained with antibodies as indicated. For detection of GFP-tagged proteins in U2OS cells, living cells were studied, or cells were fixed and permeabilized as above.
Images were made using a Cell Observer fluorescent microscope equipped with Axiovision software (Zeiss) or a Confocal Laser Scanning Microscope LSM 510 (Zeiss), equipped with a 488 nm Ar-laser and a 505-530 nm band pass filter for green fluorescence. Red fluorescence was detected using a 543 nm laser and 560 nm long pass filter. For strip-FRAP experiments and time lapse imaging after UV laser induction, GFP-tagged proteins were detected in living cells using a Confocal Laser Scanning Microscope LSM 510 (Zeiss), equipped with a 488 nm Ar-laser and a 505-530 nm band pass filter for green fluorescence.

Whole cell extracts and cell fractionation
For whole cell extracts, cells were washed in cold PBS, after which the cells were resuspended in Laemmli sample buffer (4% SDS, 20% glycerol, 120 mM Tris pH 6.8) and boiled for 5 min. Protein concentrations were determined using the Lowry protein assay. Chromatin fractionation was performed as previously described [34, 35].

Generation of DNA damage and photobleaching techniques
UV-irradiation was performed using a 254 nm UV-C lamp (Philips) at 20 J/m^2 and cells were processed 1 hour post treatment. Cells were incubated with 5 μg/ml of aphidicolin (Sigma) for 20 hours prior to processing of samples. Cells were incubated with 200 nM camptothecin (Sigma) for 20 hours before fixation. IR (10 Gy) was induced using a ^{137}Cs source or a linear accelerator (LINAC) and cells were processed 2 hours after treatment. Local UV laser induction was performed as previously described [36]. In short, cells were grown on a 25 mm quartz coverslip. A small area inside the nucleus was exposed for ~1 second to a 2 mW pulsed (7.8 kHz) diode laser emitting at 266 nm. Time-lapse images were made every minute immediately starting after local UV induction. The time course measurements were normalized to the plateau phase. Start of UV irradiation was defined as t=0. Assembly curves were normalized to 1 and processed as previously described [26].
In the FRAP experiments, a strip spanning the nucleus was photobleached for 20 milliseconds using an Ar-laser (488 nm) at 100% laser intensity, which irreversibly bleaches

all GFP molecules in that area. Subsequently, the redistribution of fluorescence in the strip was monitored by taking confocal images every 20 milliseconds for a total of 24 seconds at low laser intensity to avoid further bleaching. The fluorescence before bleaching ($I_{t<0}$) was set to 1 and the intensity immediately after bleaching (I_0) was set to 0. The recovery of fluorescence was plotted against time.
In the FLIP experiments, half of the nucleus was continuously bleached using an Ar-laser (488 nm) at 50 % laser intensity. Subsequently, the loss of fluorescence in the unbleached area was monitored taking confocal images every 10 seconds for a total of 300 seconds at low laser intensity. The fluorescence before bleaching ($I_{t<0}$) was set to 1. The loss of fluorescence was plotted against time. FLIP-FRAP experiments were performed as previously described [33, 37].

RESULTS

Generation of cell lines stably expressing GFP-tagged proteins

The limitations of conventional fixation methods were overcome by the introduction of GFP, the green fluorescent protein derived from *Aequorea Victoria*, a species of jellyfish [38, 39]. Through the cloning of the cDNA encoding GFP, it became possible to fluorescently label proteins and express them, thereby creating the opportunity for monitoring proteins in living cells. Different fluorescent versions of the GFP exist such as the red fluorescent (RFP), yellow fluorescent (YPF) and cyan fluorescent (CFP) protein, and many more spectral variants have been created since [40]. The expression of GFP and derivatives itself in mammalian cells is not harmful although the protein originates from a different species. Nonetheless, labelling of proteins with (fluorescent) epitopes can potentially influence the behaviour of the labelled protein. Validation and functional analysis of a GFP-fusion protein construct therefore is essential for the interpretation of experimentally obtained data.
The amount of fluorescence signal in a cell population derived from a single cell clone is in principle identical in all cells and therefore comparing fluorescence intensities after different treatments becomes reliable. We setup a method for the creation, generation and validation of GFP-fusion proteins for the purpose of live cell imaging. The approach is schematically depicted in Figure 1A and discussed below.

Creation of GFP-tagged protein constructs

After the discovery of GFP and its potential usefulness in biology, the protein itself became the subject of intensive research. Certain mutations in the GFP gene lead to different spectral variants and improvements in the protein characteristics, such as an increase in brightness (fluorescent intensity) and photo stability [41]. These qualities can be found in, for example, the GFP variant called ‘enhanced GFP’ (eGFP). Vectors encoding for eGFP and fluorescent derivates are commercially available (Clontech) and have been published [40]. In the studies presented here we made use of vectors containing eGFP or eGFP with the addition of two other, non-fluorescent epitopes, namely an HA- and His-tag (eGFP will hereafter be referred to as GFP). The additional epitope creates the possibility to detect the GFP-fusion protein with antibodies directed against the HA- and/or His-tags. Additionally, these tags can be used for immunoprecipitation experiments to identify interaction partner proteins.
For proper cellular function a protein needs to be folded correctly upon its expression. Since GFP and the cDNA of interest encode for two proteins that are attached, proper protein

folding might be influenced by sterical hindrance. As a possible solution to this problem, adding a small DNA sequence linker in between the sequence encoding for GFP and the cDNA this can be minimized [38, 42]. In addition, such a linker might help to restore the functionality of the protein, as a rigid tag might interfere with for example binding to (endogenous) partners.
In addition to a neomycin resistance cassette for selection, the GFP vectors contain a CMV promoter that is compatible with expression in immortalized cells, such as the human osteosarcoma (U2OS) and epithelial cervical cancer (Hela) cell lines used in this study. As adding a tag might influence protein functionality, it is advisable to construct both N- and C-terminal labelled versions of the proteins. cDNA was cloned into the GFP-vectors via PCR and/or restriction enzyme cutting and religating by T4 DNA ligase, resulting in plasmids encoding an in frame sequence of GFP and the cDNA of interest. After obtaining the right plasmid DNA the functionality of the GFP-fusion protein was tested by determining the interaction with its known binding partners. In this way a reasonable choice can be made to continue with either the N- or C-terminally tagged version of the protein, or both. After transient expression of the cDNA encoding the GFP-fusion protein and the known binding partner, cell extracts were prepared for immunoprecipitation using antibodies against GFP. The interaction with the known binding partners was determined by western blotting. The N- and C-terminally labelled proteins from the Rad9-Rad1-Hus1 complex were tested in this manner. Labelling of Rad1 at either site of the protein did not influence the interaction with endogenous Hus1 (Figure 1B). GFP-labelling of Rad9 at the C-terminus completely abrogated the interaction with its binding partners, whereas Hus1 labelling at the N-terminus decreased the binding to its partners (data not shown). When binding partners of a GFP-fusion protein are not known or do not exist and/or antibodies are not available testing for functionality must be performed in a different manner. Later in this section different assays that can be used to validate the function of the GFP-tagged protein in living cells are described.

Expression of GFP-tagged proteins in human cells
After obtaining cDNA encoding a GFP-fusion protein cells were transfected to achieve random integration of the GFP-fusion construct. We used the well studied human U2OS and Hela cells for the expression of the GFP-fusion protein since these cells are well suited for both live cell imaging and siRNA-mediated downregulation of proteins. We obtained a transient transfection efficiency of ~70% in both Hela and U2OS cells using the calcium-phosphate transfection method, where after we started with antibiotic selection. Importantly, multiple copies of the cDNA can be incorporated in the genome and the site(s) of integration additionally may create artefacts and influence cellular behaviour. In human cells this problem can only be tackled by using retroviral constructs that integrate only once. To minimize these possible artefacts we only selected individual clones expressing low amounts of GFP-fusion proteins.

Selection of GFP-positive cells using an antibiotic selection marker
To obtain cells that incorporated at least one copy of the GFP-fusion cDNA we used antibiotic selection through the neomycin-resistance cassette present in the GFP vector. To achieve stable integration of the cDNA in the genome of the transfected cells, cells were seeded in different densities and selected using 700 μg/ml G418 for at least two weeks, refreshing the medium with antibiotics every 3 days.

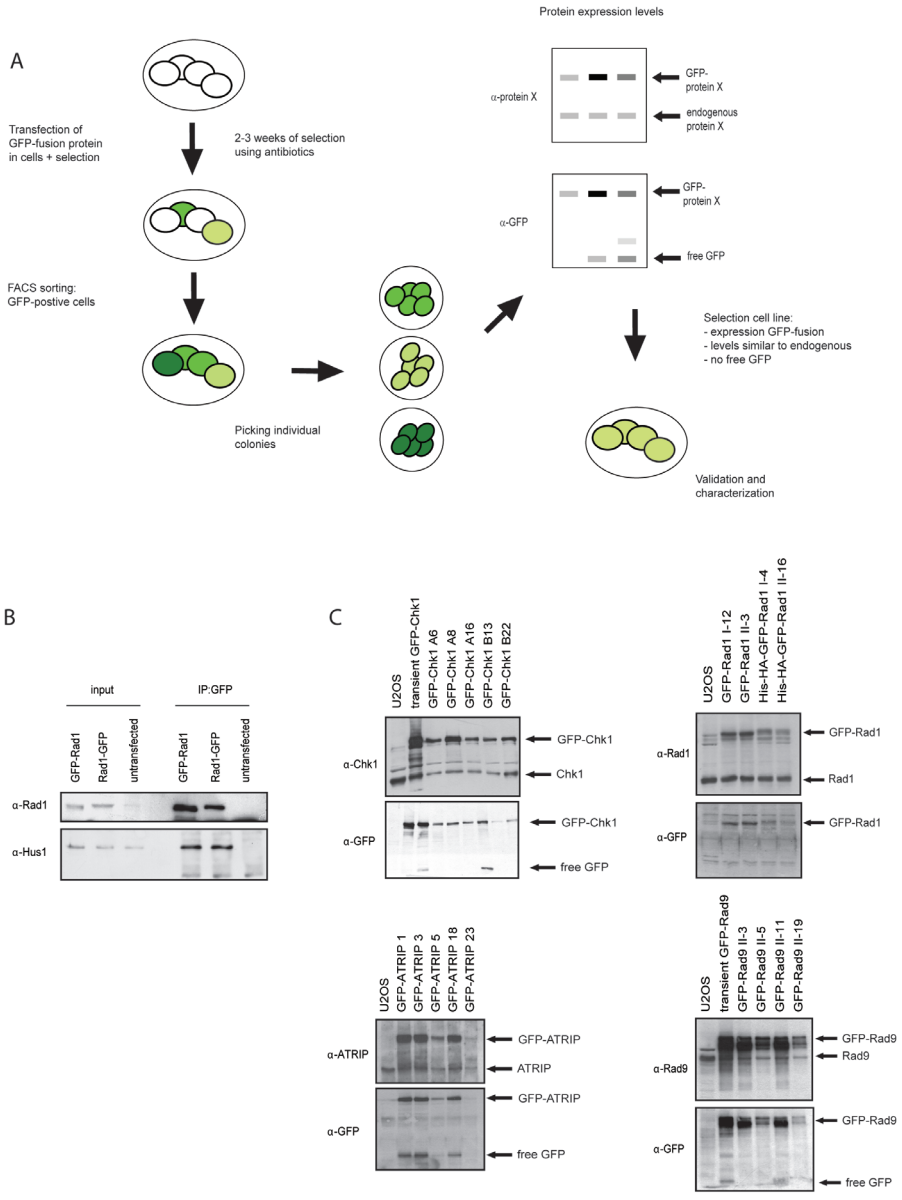

Figure 1: Generation of stable cell lines expressing GFP-tagged proteins. (A) Stepwise protocol for stable expression of GFP-fusion proteins in cells. (B) 293T cells were transfected with GFP-Rad1, Rad1-GFP or mock transfected, after which cells were lysed and immunoprecipitations were carried out using anti-GFP antibody. Western blot analysis of the immunoprecipitates using the indicated antibodies. (C) Protein expression analysis of different stable clones. Western blot analysis of whole cell extracts using the indicated antibodies.

FACS sorting of GFP-positive cells
G148 selection caused cell death of cells that did not integrate the cDNA construct in their genome. GFP-positive cells were sorted from the total population by FACS. Approximately 5-10% of the cells became GFP-positive after two weeks of G418 selection. This small percentage is most likely due to an incorrect integration the GFP-fusion cDNA causing expression of only part of the construct including the G418 resistance cassette. The sorted GFP-positive cells were replated in different dilutions so that single cells could grow into discrete colonies. Cells were kept in similar conditions of antibiotic selection as previously used and medium was refreshed at least twice a week.
Picking of individual cell colonies
After the multiplication of a single cell into a colony of around 50-100 cells, the colonies were randomly picked under sterile conditions using an inverted light microscope. At least 24 colonies were picked for every cell line. The selected colonies were kept in individual wells and were cultured until completely confluent, where after split into two plates. One plate was frozen and the other one was used to prepare cell lysates for expression analysis.

Determining protein expression levels of the GFP-fusion
The cell lysates from individual clones were analyzed by SDS-PAGE and western blotting using antibodies specifically against the protein of interest and GFP. Expression levels of the endogenous compared to the GFP-fusion protein was determined as well as the amount of free GFP or the existence of degradation products.

Selecting clones expressing relatively low amounts of GFP-tagged protein
Clones were selected on the basis of the following criteria: low expression levels of the GFP-fusion protein (not higher than that of the endogenous protein) and the absence of free GFP or other undefined products, based on the western blotting analysis. A number of 4 clones that meet these criteria were selected and thawed for further validation. Clones were cultured in medium containing 350 μg/ml G418 to maintain expression of the GFP-fusion protein.
With the method described above stable cell lines were obtained that express a GFP-fusion from the ATR-Chk1 pathway, namely ATRIP, Rad17, Rad9, Rad1, Hus1 and Chk1 (Figure 1C and data not shown). We also generated control cell lines expressing GFP and H2B-GFP-Chk1.

Validation and characterization of the selected stable cell lines
To validate the functionality of the GFP-fusion protein and characterize the stable cell lines we utilized a number of assays:
Immunoprecipitations (IP) of the GFP-fusion protein were performed to confirm the interaction of the GFP-fusion with its known endogenous protein partners. We published before that GFP-Rad9 interacts with its endogenous partners Rad1 and Hus1 [33]. A similar experiment was performed with GFP-ATRIP, which demonstrated that the GFP-fusion was able to bind endogenous ATR (Figure 2A).
To check if stable expression of the GFP-fusion protein influences the cells response to DNA damage by functioning as a dominant negative, we determined the sensitivity of our cell lines to genotoxic stress. We compared the colony survival in response to UV light of the stable clones to the parental untransfected cell line. As shown in Figure 2B, the expression of the GFP-Rad9 in both Hela and U2OS cells did not alter the sensitivity to UV damage,

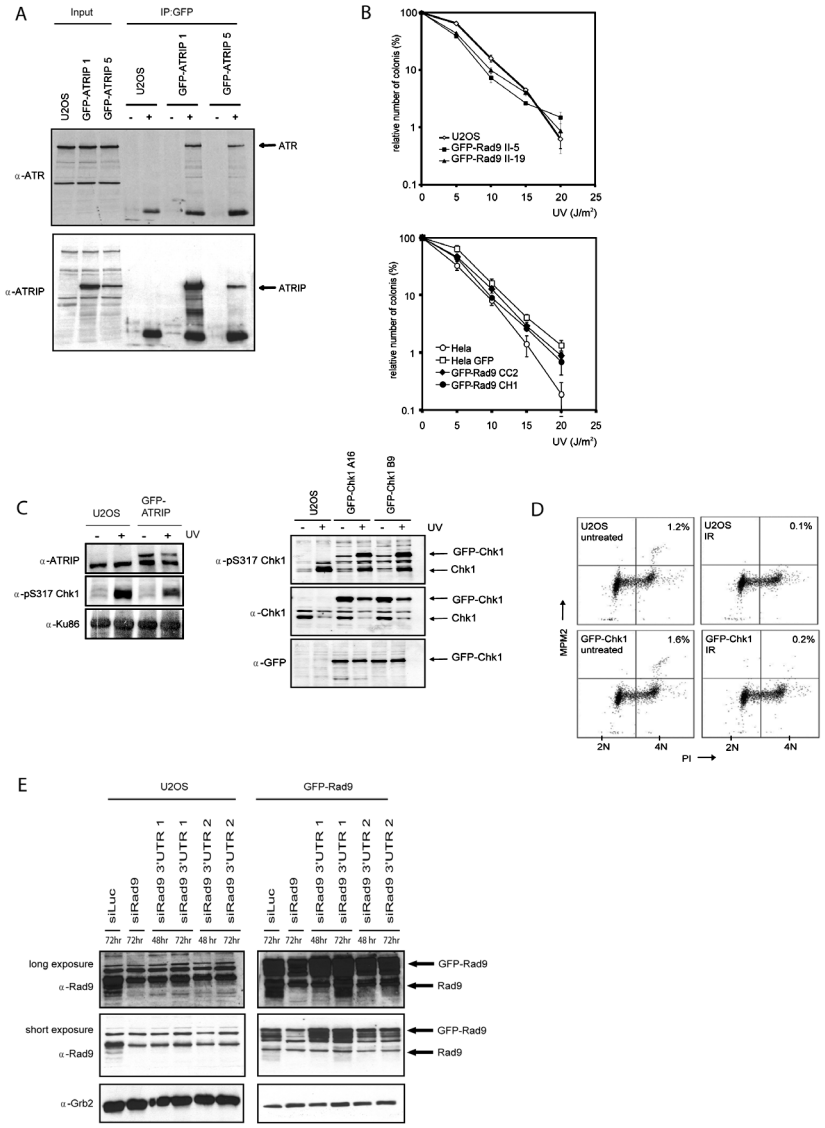

Figure 2: Validation and functionality of stable cells expressing GFP-fusions. (A) Two independent U2OS clones expressing GFP-ATRIP were lysed after which immunoprecipitations were carried out using anti-GFP antibody. Western blot analysis of the immunoprecipitates using the indicated antibodies. (B) U2OS and Hela cells expressing GFP-Rad9 were seeded in low density and treated with different doses of UV. The number of surviving colonies after 10 days was counted. (C) Left panel: cells expressing GFP-ATRIP or control U2OS cells were left untreated or treated with UV. One hour later, cells were lysed and analyzed by western blotting with the indicated antibodies. Right panel: as left panel, but with two clones expressing GFP-Chk1. (D) Control U2OS cells or cells expressing GFP-Chk1 were left untreated or treated with 10 Gy IR. After 2 hours, cells were fixed and stained with PI and MPM2 and analyzed by FACS. The percentage of mitotic cells is indicated. (E) U2OS cells expressing GFP-Rad9 were transfected with siRNA oligos directed against Rad9 or the 3'UTR of Rad9 for the indicated time periods. Cell extracts were prepared and western blot analysis was performed using the indicated antibodies.

indicating that GFP-Rad9 does not function as a dominant negative. The expression of GFP alone in Hela cells also did not influence sensitivity to UV-irradiation.

The ATR-dependent checkpoint pathway is triggered in response to genotoxic stress and induces the ATR-mediated phosphorylation of Chk1, which results in a temporal cell cycle arrest [11]. To test if expression of the GFP-fusion proteins influences checkpoint activation we monitored the UV-induced phosphorylation of Chk1. Expression of GFP-ATRIP and GFP-Chk1 did not influence the levels of phosphorylated Chk1 (Figure 2C). GFP-Chk1 is phosphorylated in response to UV damage, indicating the GFP-tagged Chk1 behaves similarly to the endogenous one. To study the G2 phase arrest induced by DNA damage, we determined the mitotic index of cells expressing GFP-Chk1 and untransfected cells. A similar decrease in mitotic cells upon ionizing radiation (IR) was observed in GFP-Chk1 expressing and control cells, indicating that expression of GFP-Chk1 does not interfere with the DNA damage-induced G2 checkpoint (Figure 2D).

Although these assays demonstrate the absence of a dominant negative effect of the GFP-fusions, to definitively rule out problems due to GFP-tagging, complementation of a mutant or knock out cell line is required. Cells derived from Seckel syndrome patients that harbour a mutation in the ATR gene, leading to an abrogated expression of the ATR protein, are available [43], but most proteins in this pathway are essential for cell viability making a direct complementation impossible in human cells. In addition, knock outs of Rad9 and Hus1 in mouse embryonic stem cells are lethal, although the lethality of Hus1 disruption can be rescued by knocking out p21 [44-46]. Chicken DT40 cells can be successfully used for knocking out genes that are essential in mammalian cells, like Chk1, but are less suitable for the generation of stable GFP-fusion cell lines and live cell imaging, since these cells are genomically unstable and can only be cultured in suspension [47]. Another approach is siRNA-mediated downregulation of the endogenous protein and subsequent complementation by expression of the GFP-tagged protein. We favour this option since in this way; we are able to study both the functionality of the GFP-fusion protein as well as its mobility in the same cell line.

To specifically downregulate the endogenous protein, different siRNA-mediated approaches are available. First, as the cDNA encoding for the GFP-fusion protein does not contain a 3'UTR, the use of siRNA oligos that specifically target this region of the protein of interest should result in knock down of the endogenous protein only. We applied this approach in cells expressing GFP-Rad9. Figure 2E demonstrates that 48 hours after transfection with siRNA oligo number 1, endogenous Rad9 is more efficiently downregulated than the GFP-labelled Rad9 protein. At 72 hours after transfection the efficiency of downregulation became less, demonstrating that knock down is optimal 48 hours after transfection with siRNA oligo number 1. Transfection with siRNA oligo number 2 also lowered endogenous Rad9 levels more than the GFP-labelled Rad9 protein levels and the efficiency of downregulation was similar between 48 and 72 hours after transfection. In an alternative approach a silent mutation is made in the cDNA of the GFP-tagged protein, thereby leaving the encoded protein unchanged. By using siRNA oligos that recognize the mRNA from the endogenous but not the GFP-fusion protein, the endogenous protein can be specifically downregulated, such that the functionality of the GFP-fusion protein can be assessed [33]. Collectively the performed validation assays indicate that the introduction and stable expression of GFP-fusions of Chk1, ATRIP, Rad9, Rad1 and Hus1 did not change the response of these cells to DNA damage. These GFP-fusion proteins can therefore be seen as functional equivalents of their endogenous counterparts.

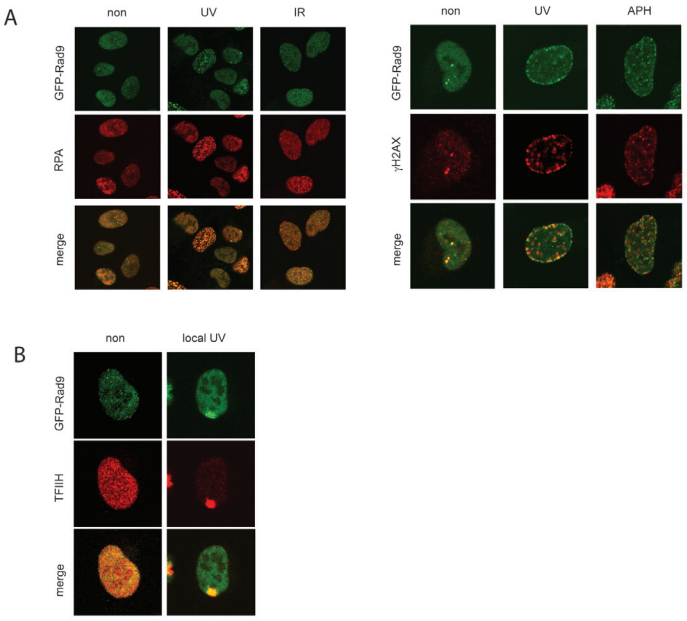

Figure 3: DNA damage-induced foci formation of GFP-Rad9. (A) GFP-Rad9 expressing cells were left untreated or exposed to UV, IR or aphidicolin. GFP-Rad9 was detected by direct fluorescence and RPA and γH2AX by immunofluorescence. (B) GFP-Rad9 expressing cells were treated with 40 J/m^2 local UV-irradiation using an isopore filter. One hour later the cells were fixed and stained. GFP-Rad9 was detected by direct fluorescence and TFIIH by immunofluorescence.

Cellular localization of GFP-fusion proteins in response to DNA damage

Numerous studies over the past years indicated that the accumulation of DNA damage response proteins at sites of damage is of crucial importance for downstream checkpoint events [14, 22, 24, 48, 49]. Although for many proteins involved in checkpoint regulation a (relatively small) fraction of the total pool of proteins is bound to chromatin in unperturbed cells, the majority of these proteins relocalize and accumulate at or near sites of DNA lesions to form so-called nuclear foci [50]. GFP-Rad9 for example, localizes into foci after the treatment with different DNA damaging agents (Figure 3A).

To investigate the specific DNA substrate onto which a protein is recruited, different DNA damaging agents can be tested, as well as colocalization with other proteins involved in the DNA damage response. Biochemical experiments indicated that the Rad17-RFC and ATRIP-ATR protein complexes are recruited to DNA damaged-induced RPA-coated ssDNA and this localization induces the activation of the downstream kinase Chk1 [4, 51, 52]. Rad9, a component of the 9-1-1 complex, is loaded onto the DNA by Rad17-RFC in response to DNA damage [5, 53]. We observed colocalization of GFP-Rad9 and RPA upon treatment of cells with both UV light and IR, suggesting that Rad9 is recruited to sites of RPA-coated ssDNA (Figure 3A). In response to DNA damage, histone H2AX is phosphorylated at Ser139 by ATM/ATR [54]. Although phosphorylation of H2AX (γH2AX) is commonly used to identify DSBs, γH2AX is also induced in response to UV light or treatment with other agents that do not directly induce DSBs, like the replication inhibitor aphidicolin (Figure 3A) [55]. We observed colocalization of GFP-Rad9 and γH2AX in all UV-induced foci, whereas treatment of cells

with aphidicolin or IR did not result in a complete colocalization of the two proteins (Figure 3A and data not shown). Close inspection demonstrated that foci induced by aphidicolin either contained both GFP-Rad9 and γH2AX or γH2AX only. Since the phosphorylation of H2AX is triggered in response to a wide variety of DNA lesions, we conclude that GFP-Rad9 is only recruited to a subset of aphidicolin-induced DNA lesions.

Not all proteins accumulate into foci, even though they are recruited to sites of DNA damage. Proteins involved in nucleotide excision repair for example, do not form foci in response to genotoxic stress. The accumulation of these proteins onto damaged DNA can be visualized by applying local UV damage in the nucleus. By irradiating the cells while covered with an isopore filter, the UV light can only penetrate the pores and as a consequence, only a small part(s) of the nucleus contains damaged DNA [56, 57]. As shown in Figure 3B, proteins involved in the ATR-mediated checkpoint pathway, such as Rad9, also accumulate onto local UV damage. This method requires fixation of cells and is therefore unsuitable for live cell imaging. The development of laser-induced DNA damage circumvented this problem [26, 36, 58]. Laser-induced DNA damage can be easily combined with live cell imaging and different types of lasers are commonly available [15, 59]. For example, recent studies make use of specific types of sources and lasers, specifically inducing subsets of DNA lesions such as CPDs and 6-4P induced by UV light, and DSBs [14, 36, 56, 60, 61]. Nonetheless caution is required, as often sources and lasers also produce other, unwanted, types of lesions, that may influence protein behaviour adversely [36, 62].

We did not observe the accumulation of the effector kinase Chk1 into nuclear foci in response to DNA damage nor onto locally applied UV-induced DNA damage using a filter (data not shown), in contrast to ATR, TopBP1, 9-1-1 members and many other checkpoint mediator proteins. In this sense Chk1 behaves similarly to Chk2, the effector kinase for the cellular response to DSBs [15]. In order to trigger a cell cycle arrest in response to DNA damage, these effector kinases phosphorylate a range of substrates that are present throughout the nucleus. Although both Chk1 and Chk2 do not associate to chromatin after DNA damage, Chk1 associates to chromatin in unperturbed cells and is released from the chromatin in response to DNA damage [35]. In the light of this, the absence of accumulated Chk1 at sites of DNA lesions was to be expected.

Quantitative GFP-fusion protein dynamics

By measuring the change in mobility of a protein in response to DNA damage a number of parameters can be determined. First, the time the protein spends at the site of a DNA lesion and second, the fraction of the total amount of protein engaged in that process. Fluorescent Redistribution After Photobleaching (FRAP) is applied to study the mobility of GFP-fusion proteins in living cells. In a defined region (strip) of the nucleus the fluorescence is irreversibly photobleached through brief illumination at high laser intensity (strip-FRAP). Redistribution or recovery of the total fluorescence in the bleached area is then measured in time, indicative of protein mobility [16, 63, 64].

To gain further understanding of how the proteins in the ATR-pathway sense DNA lesions and subsequently respond to trigger the checkpoint-mediated cell cycle arrest, the dynamic behaviour of these proteins was studied. We demonstrated that GFP-Rad1, GFP-ATRIP and GFP-Rad9 form foci and are recruited to chromatin in response to DNA damage, as shown by chromatin fractionation (Figure 4A, data not shown) [21]. To address whether these proteins become immobilized at sites of DNA lesions, we determined the change in mobility of GFP-Rad1, GFP-Rad9, Hus1-GFP and GFP-ATRIP upon UV-irradiation. Importantly, the

total amount of GFP-labelled molecules could be different between cells and between GFP-fusion protein cell lines. These two discrepancies could potentially influence photobleaching results since relative immobilization is dependent on the total amount of protein available. To adjust for cell to cell differences in a population we normalized the obtained measurements as described in materials and methods. Furthermore we used saturating amounts of UV-irradiation to be sure that all possible molecules would be engaged (data not shown). Although we did not measure the exact amount of GFP-fusion molecules per cell, western blotting analysis indicates that the different GFP-fusion proteins are expressed to a similar extent (Figure 1C).

In response to UV light, 20-40% of the 9-1-1 complex proteins become immobilized. (Figure 4B-E). The GFP-Rad1 and Hus1-GFP FRAP curves show more variability compared to the GFP-Rad9 FRAP curve, which is probably due to the fluorescent variety of the GFP-Rad1 and Hus1-GFP cell lines and the amount of cells that were measured (Figure 4B-D). Although the ATRIP-ATR complex is also recruited to sites of DNA damage, relatively less GFP-ATRIP is immobilized (5-10%) in response to UV damage as compared to either of the proteins of the 9-1-1 complex (Figure 4E). These data demonstrate that the 9-1-1 and ATRIP-ATR complexes behave differently after DNA damage and suggest that ATRIP displays a higher turnover at sites of DNA lesions.

To further investigate the possible distinct behaviour between these two putative DNA damage sensor complexes Fluorescent Loss After photobleaching (FLIP) experiments were performed. During FLIP half of the nucleus is constantly bleached and the loss of fluorescence in the unbleached half of the nucleus is measured, representing the rate of dissociation. As shown in Figure 4F, non-damaged cells expressing either GFP-Rad9 or GFP-ATRIP completely lost fluorescence 150 seconds after start of bleaching ($k_{1/2}$ *~15 seconds*). After UV irradiation the dissociation rates were less fast. GFP-ATRIP completely lost fluorescence after 250 seconds ($k_{1/2}$ *~25 seconds*), whereas GFP-Rad9 after more than 300 seconds ($k_{1/2}$ *~40 seconds*). These FLIP experiments indicate that after DNA damage induction both ATRIP and Rad9 become less mobile, but the release (dissociation) of ATRIP from sites of damage is faster than Rad9. These data are in accordance to the FRAP experiments, that show relatively less immobilization of ATRIP upon UV irradiation as compared to the proteins of the 9-1-1 complex.

The rate at which proteins associate to sites of DNA lesions was addressed by measuring the accumulation of proteins upon local UV laser irradiation. Briefly, a small area inside the nucleus is irradiated with a 266 nm UV laser. GFP-Rad9 and GFP-ATRIP fluorescence in the locally damaged area was measured every 60 seconds until a plateau was reached. GFP-ATRIP quickly started accumulating at the damaged area and reaches a plateau ~20 minutes after damage induction ($k_{1/2}$ *~6 minutes*) (Figure 4G). GFP-Rad9 accumulation in the first 8 minutes is relatively slow, but rises faster thereafter and reaches a plateau after ~30 minutes ($k_{1/2}$ ~12 minutes) (Figure 4G). These results indicate that ATRIP and Rad9 associate very differently to UV-damaged DNA and suggest that ATRIP is recruited directly to the sites of damage in an exponential manner, following Michaelis-Menten kinetics [65], whereas the accumulation kinetics of Rad9 follow a sigmoidal curve (non Michaelis-Menten kinetics), representing cooperative binding [66]. Rad17-dependent loading of Rad9 onto the DNA might explain this cooperative binding of GFP-Rad9 after DNA damage induction[67], compared to ATRIP-ATR which binds directly to RPA-coated ssDNA [4].

Chk1 is phosphorylated at or near the DNA lesion and thereafter released from the chromatin [35] and consequently, the amount of immobilized GFP-Chk1 measured by photobleaching

experiments might become lower after DNA damage induction. To test this hypothesis, we performed strip-FRAP on cells expressing GFP-Chk1 treated with UV light. We compared the GFP-Chk1 mobility with that of free GFP and an artificially immobile form of Chk1, where GFP-Chk1 was fused to histone H2B (H2B-GFP-Chk1) [35]. Upon photobleaching GFP-Chk1 demonstrated a fast recovery of fluorescence both before and after UV damage (Figure 5A). The recovery was similar to that of free GFP, arguing against the existence of a stably bound GFP-Chk1 fraction. Since GFP-Chk1 was present in relatively high levels due to overexpression in addition to endogenous Chk1, we reasoned that the amount of Chk1 might be too high to observe any change in protein mobility upon DNA damage induction. Therefore, we lowered the total amount of Chk1 by RNA interference. Cells expressing GFP-Chk1 were transfected with either control luciferase or Chk1 siRNA oligos. Twenty four hours later cells were treated with UV light and chromatin fractions were prepared. As shown in Figure 5B, both endogenous and GFP-labelled Chkl were released from chromatin upon DNA damage induction in control downregulated cells, although the GFP-tagged version less efficiently than endogenous Chk1 (endogenous 64%, GFP-labelled 16%). Transfection of Chk1 siRNA oligos resulted in downregulation of endogenous Chk1 and did not influence the release of Chk1 from chromatin. Although GFP-Chk1 is less efficiently released from the chromatin than endogenous Chk1, a small but considerable fraction of GFP-Chk1 (~15%) is released in response to DNA damage, which is comparable to the previous experiment without the depletion of endogenous Chk1.

Next we lowered the total amount of Chk1 in GFP-Chk1 expressing cells by siRNA-mediated downregulation. Subsequent strip-FRAP analysis indicated that non-damaged GFP-Chk1 expressing cells did not contain more immobile GFP-Chk1 molecules compared to UV-treated cells (Figure 5C). Downregulation of Chk1 did not influence the recovery of residual GFP-Chk1 fluorescence in untreated cells and accordingly it did not change GFP-Chk1 mobility after UV-treatment.

The strip-FRAP experiments demonstrated the absence of a stably bound Chk1 pool in these conditions. Nonetheless, these experiments were performed in a relatively short period of time (20 seconds). Since Chk1 released from the chromatin in response to UV-induced DNA damage could be a more gradual and thus time-dependent process, we additionally addressed GFP-Chk1 mobility by determining its residence time on DNA. Chk1 was downregulated in GFP-Chk1 expressing cells as previously described and subsequently the cells were irradiated with UV-light. Thereafter, half of the nucleus was bleached once and redistribution of GFP-Chk1 fluorescence was measured in the bleached and unbleached area over time. The time it takes to reach full fluorescent redistribution determines the proteins residence time. The results shown in Figure 5D indicated that GFP-Chk1 reached redistribution after 40 seconds, which is outside the timeframe of our original experiment. However, during the 40 seconds directly after bleaching we did not observe any difference in the mobility using this method. These results further indicate that Chk1 is a highly mobile protein and suggests that it only very transiently interacts with the chromatin.

DISCUSSION

The initial response to DNA damage is of crucial importance for cell functioning and viability, since an improper DNA damage response can eventually lead to cancer and other human diseases [1, 68]. In response to UV light, the putative DNA sensors Rad17-RFC and ATRIP-ATR

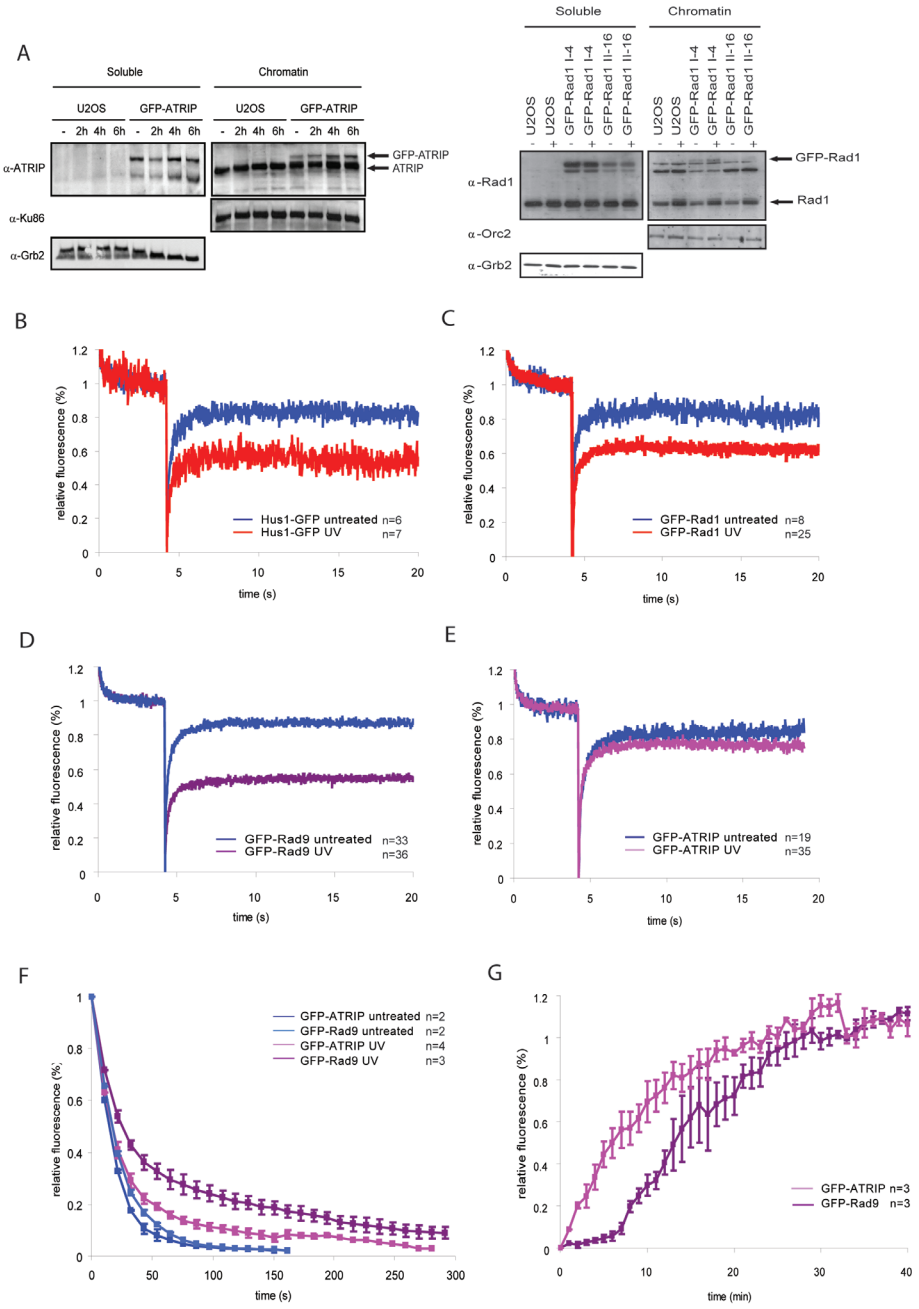

Figure 4: UV-induced changes in mobility of checkpoint proteins. (A) U2OS cells expressing GFP-ATRIP (left panel) and GFP-Rad1 (right panel) were left untreated or treated with UV. Two hours later, soluble and chromatin-bound proteins were isolated and analyzed by western blotting using the indicated antibodies. (B-E) Hus1-GFP (B), GFP-Rad1 (C), GFP-Rad9 (D) and GFP-ATRIP (E) expressing cells were left untreated or treated with UV and analyzed by strip-FRAP (see materials & methods for technical details). (F) U2OS cells expressing GFP-Rad9 or GFP-ATRIP were left untreated or treated with UV. After 1 hour cells were analyzed by FLIP (see materials & methods for technical details). Plotted is the loss of fluorescence during continuous bleaching. (G) U2OS cells expressing GFP-Rad9 or GFP-ATRIP were locally irradiated using a 266 nm UV laser. Plotted is the association of fluorescent signal at the locally damaged area.

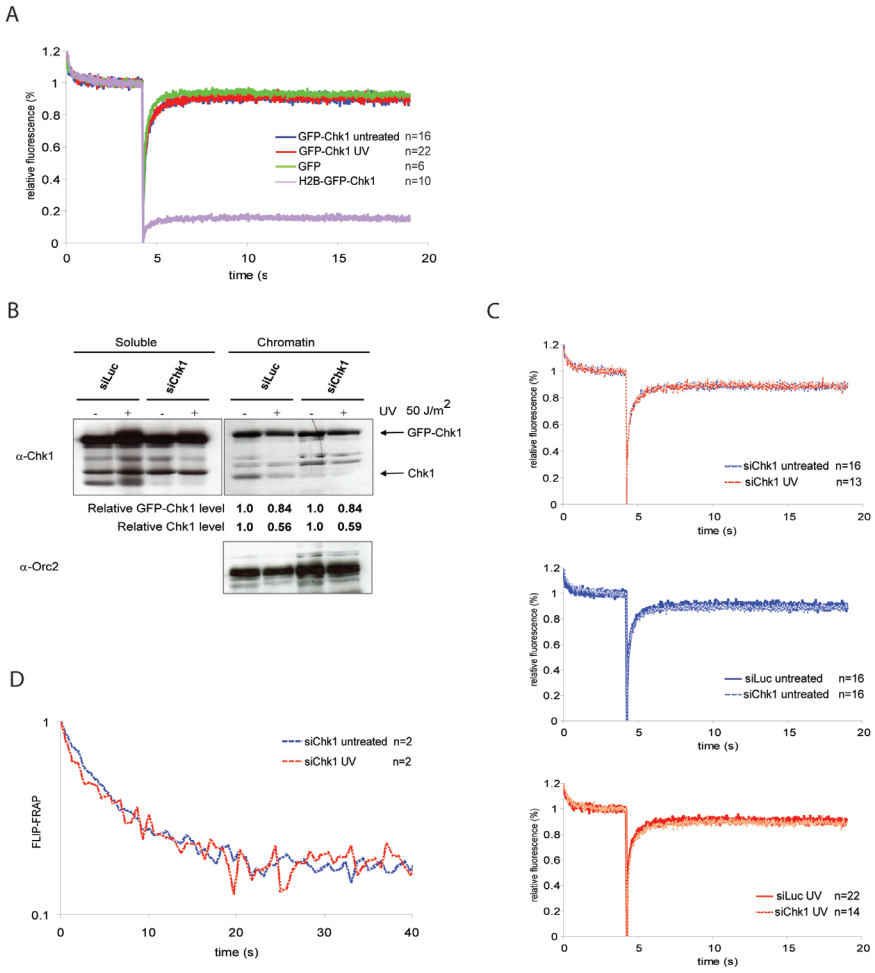

Figure 5: Mobility of GFP-Chk1 in response to DNA damage. (A) U2OS cells expressing GFP, H2B-GFP-Chk1 or GFP-Chk1 were left untreated or treated with UV and analyzed by strip-FRAP (see materials & methods for technical details). (B) Chromatin fractionation of GFP-Chk1 expressing cells transfected with siRNA oligos directed against luciferase or Chk1 for 24 hours before UV treatment and cell fractionation. Relative Chk1 levels as compared to untreated controls are indicated. Western blot analysis of the fractions was performed using the indicated antibodies. (C) GFP-Chk1 expressing cells were transfected with siRNA oligos against luciferase or Chk1 for 24 hours and left untreated or treated with UV. 1 h later cells were analyzed by strip-FRAP. (D) U2OS cells expressing GFP-Chk1 were transfected with Chk1 siRNA oligos for 24 hours and left untreated or treated with UV and subsequently analyzed by FLIP-FRAP (see materials & methods for technical details).

are independently recruited to DNA regions containing RPA-coated ssDNA [4, 52]. Rad17-RFC is responsible for the loading of the 9-1-1 complex onto the DNA [5]. These events are required for the subsequent activation of Chk1, which results in a cell cycle arrest [12]. To gain further insight into the events that take place after a genotoxic insult in living cells, we GFP-labelled several proteins of the ATR pathway and expressed these fusion proteins in human cells. We discussed how to generate and validate stable GFP-fusion cell lines.

The quantitative analysis of GFP-fusion proteins in living cells using video microscopy allowed us to determine the dynamic behaviour of different individual proteins of the pathway in response to DNA damage. With the use of photobleaching techniques (strip-FRAP, FLIP, FLIP-FRAP, local UV laser) we determined multiple parameters of the proteins, including the rate of accumulation onto locally induced UV laser damage, rate of diffusion, immobile fraction, and the average time of immobilization (residence time) before and after global UV-irradiation. These methods are useful tools to investigate the dynamics of proteins involved in the DNA damage response, as shown in this paper, since many of the involved proteins bind DNA or proteins present at the site of damage and become immobilized to a certain extent.

GFP-Rad9, GFP-Rad1, Hus1-GFP and GFP-ATRIP accumulate into nuclear foci in response to DNA damage. However, a relatively larger fraction of the 9-1-1 complex proteins become immobilized in response to UV as compared to ATRIP. Biochemical experiments show that the 9-1-1 complex is loaded onto the 5' ssDNA/dsDNA by Rad17-RFC, therefore its association to DNA lesions might take some time and is the result of cooperative binding [51]. In contrast, ATRIP interacts directly with RPA-coated ssDNA, explaining its exponential binding behaviour [4]. The results obtained by FLIP suggest that a loaded 9-1-1 complex cannot easily dissociate from the DNA. This dissociation might be slower because the 9-1-1 complex forms a ring around the DNA. It therefore has to either slide off a DNA end or actively dissociate, which likely is a more time consuming process [69]. In addition, the FRAP experiments show that 20-40% of the 9-1-1 complex molecules become immobile, whereas only 5-10 % of the ATRIP molecules is immobilized after DNA damage. These results also indicate a relatively more stable association of the 9-1-1 complex with damaged chromatin which is consistent with the FLIP results.

All together, the putative DNA damage sensors Rad9-Rad1-Hus1 and ATRIP-ATR show a different dynamic behaviour directly after induction and during the presence of UV-induced DNA damage. Nonetheless, both GFP-Rad9 and GFP-ATRIP molecules are exchanging rapidly in UV-induced foci since all the mobility measurements performed on both GFP-Rad9 and GFP-ATRIP DNA damage induced-foci do not indicate a stable immobilized fraction, suggesting that these foci are not static but highly dynamic structures.

Recruitment of the ATRIP-ATR heterodimer to sites of DNA damage results in ATR kinase activation which leads to the phosphorylation and subsequent activation of the Chk1 kinase [70, 71]. To investigate the spatio and temporal behaviour of downstream effector Chk1 in living cells, we studied the dynamic behaviour of GFP-Chk1 in response to DNA damage. *In vivo* imaging experiments confirm the absence of a stable chromatin-bound Chk1 fraction. The data presented here signify that Chk1 is a very mobile protein both in the absence or presence of DNA damage. The observed chromatin binding of Chk1 that is observed in biochemical experiments is not indicative of a stably bound Chk1 chromatin fraction [35] (Figure 5B). This high nuclear mobility of Chk1 was hypothesized to ensure the access of additional Chk1 molecules to ATR and the transmission of the damage signal throughout the nucleus [15, 35, 72] .

Measuring protein mobility is not always as straightforward as it seems. Problems that are commonly faced when performing these types of experiments are monitor bleaching, influx of proteins from the cytoplasm and blinking. Monitor bleaching is caused by observing the cells before, during and after photobleaching or time lapse experiments. By using low amounts of laser power this can be avoided, although with low fluorescent protein levels this could be problematic. Lowering the laser power could result in a low resolution,

which in turn results in a large variability between measurements. Monitor bleaching can be corrected for by measuring the amount of bleaching in an unbleached area of the cell, but this only applies for freely diffusion proteins [63, 73]. Another potential problem is the presence of a cytoplasmic protein fraction that could influence nuclear measurements due through the influx of fluorescence from the cytoplasm. Before the start of the actual measurement, the cytoplasmic fraction can be bleached, thereby eliminating this potential influx. Blinking is the property of fluorescent molecules to be reactivated, which can also influence photobleaching results. By keeping experimental conditions similar in all conditions the influence of blinking is constant in all measurements and therefore insignificant. When comparing results obtained from different fluorescent variants, on the other hand, its needs to be taken into account that the blinking properties are different and therefore the amount of blinking needs to be determined for every variant and corrected for in the final analysis [63, 73].

To overcome the problem of GFP-fusion protein overexpression and the (competitive) presence of the endogenous counterpart, a GFP knock-in strategy using mice can be utilized [74] (Jeroen Essers, personal communication). This method places the GFP-tag before the endogenous gene of interest in the mice genome, thereby being regulated by the endogenous promoter. As a result the expression levels of the GFP-fusion protein are identical to non-targeted protein in wild type cells, resulting in better quantitative measurements. The behaviour of proteins can additionally be studied in different tissues and cell types under physiological conditions [75]. Performing a similar approach for proteins involved in the ATR-Chk1 pathway may result in a more precise determination of the dynamic behaviour of this DNA damage checkpoint pathway. The ATR gene may act as a good candidate to start a GFP knock in approach, since it localizes to sites of damage and thereby becomes immobilized. Furthermore, ATR interacts with and phosphorylates a number of key target proteins.

Conclusively, we show that the GFP-labelling of ATRIP, Chk1 and the 9-1-1 complex proteins results in functional equivalents that can be studied by live cell video microscopy and quantitative fluorescence photobleaching. We compared the mobility changes of GFP-ATRIP, GFP-Chk1, GFP-Rad9, GFP-Rad1 and Hus1-GFP after UV-irradiation and collectively the results indicate a distinct dynamic behaviour between some of the studied proteins. Whereas effector kinase Chk1 is highly mobile and does not immobilize upon DNA damage, Rad9 and ATRIP stably associate to sites of DNA lesions. ATRIP directly binds to DNA lesions but more transiently than Rad9, which in contrast displays a more stable association with damaged chromatin. Together these data demonstrate the importance of spatio and temporal protein regulation in the DNA damage-induced ATR-Chk1 pathway.

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REFERENCES

1. Shiloh, Y., *ATM and related protein kinases: safeguarding genome integrity.* Nat Rev Cancer, 2003. **3**(3): p. 155-68.
2. Huen, M.S., S.M. Sy, and J. Chen, *BRCA1 and its toolbox for the maintenance of genome integrity.* Nat Rev Mol Cell Biol. **11**(2): p. 138-48.
3. Huen, M.S. and J. Chen, *Assembly of checkpoint and repair machineries at DNA damage sites.* Trends Biochem Sci. **35**(2): p. 101-108.
4. Zou, L. and S.J. Elledge, *Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.* Science, 2003. **300**(5625): p. 1542-8.
5. Zou, L., D. Cortez, and S.J. Elledge, *Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin.* Genes Dev, 2002. **16**(2): p. 198-208.
6. Kondo, T., et al., *Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms.* Science, 2001. **294**(5543): p. 867-70.
7. Melo, J.A., J. Cohen, and D.P. Toczyski, *Two checkpoint complexes are independently recruited to sites of DNA damage in vivo.* Genes Dev, 2001. **15**(21): p. 2809-21.
8. Lee, J., A. Kumagai, and W.G. Dunphy, *The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR.* J Biol Chem, 2007. **282**(38): p. 28036-44.
9. Delacroix, S., et al., *The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1.* Genes Dev, 2007. **21**(12): p. 1472-7.
10. Majka, J., A. Niedziela-Majka, and P.M. Burgers, *The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint.* Mol Cell, 2006. **24**(6): p. 891-901.
11. Zhou, B.B. and S.J. Elledge, *The DNA damage response: putting checkpoints in perspective.* Nature, 2000. **408**(6811): p. 433-9.
12. Shiotani, B. and L. Zou, *ATR signaling at a glance.* J Cell Sci, 2009. **122**(Pt 3): p. 301-4.
13. Harper, J.W. and S.J. Elledge, *The DNA damage response: ten years after.* Mol Cell, 2007. **28**(5): p. 739-45.
14. Bekker-Jensen, S., et al., *Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks.* J Cell Biol, 2006. **173**(2): p. 195-206.
15. Lukas, C., et al., *Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage.* Nat Cell Biol, 2003. **5**(3): p. 255-60.
16. Essers, J., A.B. Houtsmuller, and R. Kanaar, *Analysis of DNA recombination and repair proteins in living cells by photobleaching microscopy.* Methods Enzymol, 2006. **408**: p. 463-85.
17. Essers, J., et al., *Nuclear dynamics of RAD52 group homologous recombination proteins in response to DNA damage.* Embo J, 2002. **21**(8): p. 2030-7.
18. van Veelen, L.R., et al., *Analysis of ionizing radiation-induced foci of DNA damage repair proteins.* Mutat Res, 2005. **574**(1-2): p. 22-33.
19. Lippincott-Schwartz, J. and G.H. Patterson, *Development and use of fluorescent protein markers in living cells.* Science, 2003. **300**(5616): p. 87-91.
20. Easwaran, H.P., H. Leonhardt, and M.C. Cardoso, *Cell cycle markers for live cell analyses.* Cell Cycle, 2005. **4**(3): p. 453-5.
21. Warmerdam, D.O., et al., *Cell cycle-dependent processing of DNA lesions controls*

localization of Rad9 to sites of genotoxic stress. Cell Cycle, 2009. **8**(11).
22. Bonilla, C.Y., J.A. Melo, and D.P. Toczyski, *Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage.* Mol Cell, 2008. **30**(3): p. 267-76.
23. Lukas, J., C. Lukas, and J. Bartek, *Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time.* DNA Repair (Amst), 2004. **3**(8-9): p. 997-1007.
24. Soutoglou, E. and T. Misteli, *Activation of the cellular DNA damage response in the absence of DNA lesions.* Science, 2008. **320**(5882): p. 1507-10.
25. Houtsmuller, A.B., et al., *Action of DNA repair endonuclease ERCC1/XPF in living cells.* Science, 1999. **284**(5416): p. 958-61.
26. Zotter, A., et al., *Recruitment of the nucleotide excision repair endonuclease XPG to sites of UV-induced dna damage depends on functional TFIIH.* Mol Cell Biol, 2006. **26**(23): p. 8868-79.
27. Luijsterburg, M.S., et al., *Dynamic in vivo interaction of DDB2 E3 ubiquitin ligase with UV-damaged DNA is independent of damage-recognition protein XPC.* J Cell Sci, 2007. **120**(Pt 15): p. 2706-16.
28. Giglia-Mari, G., et al., *A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A.* Nat Genet, 2004. **36**(7): p. 714-9.
29. Giglia-Mari, G., et al., *Dynamic interaction of TTDA with TFIIH is stabilized by nucleotide excision repair in living cells.* PLoS Biol, 2006. **4**(6): p. e156.
30. Toueille, M., et al., *The human Rad9/Rad1/Hus1 damage sensor clamp interacts with DNA polymerase beta and increases its DNA substrate utilisation efficiency: implications for DNA repair.* Nucleic Acids Res, 2004. **32**(11): p. 3316-24.
31. Danielsen, J.M., et al., *HCLK2 Is Required for Activity of the DNA Damage Response Kinase ATR.* J Biol Chem, 2009. **284**(7): p. 4140-4147.
32. Jones, G.G., et al., *The ATR-p53 pathway is suppressed in noncycling normal and malignant lymphocytes.* Oncogene, 2004. **23**(10): p. 1911-21.
33. Medhurst, A.L., et al., *ATR and Rad17 collaborate in modulating Rad9 localisation at sites of DNA damage.* J Cell Sci, 2008. **121**(Pt 23): p. 3933-40.
34. Mendez, J. and B. Stillman, *Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis.* Mol Cell Biol, 2000. **20**(22): p. 8602-12.
35. Smits, V.A., P.M. Reaper, and S.P. Jackson, *Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response.* Curr Biol, 2006. **16**(2): p. 150-9.
36. Dinant, C., et al., *Activation of multiple DNA repair pathways by sub-nuclear damage induction methods.* J Cell Sci, 2007. **120**(Pt 15): p. 2731-40.
37. Essers, J., et al., *Nuclear dynamics of PCNA in DNA replication and repair.* Mol Cell Biol, 2005. **25**(21): p. 9350-9.
38. Tsien, R.Y., *The green fluorescent protein.* Annu Rev Biochem, 1998. **67**: p. 509-44.
39. Tsien, R.Y. and A. Miyawaki, *Seeing the machinery of live cells.* Science, 1998. **280**(5371): p. 1954-5.
40. Giepmans, B.N., et al., *The fluorescent toolbox for assessing protein location and function.* Science, 2006. **312**(5771): p. 217-24.
41. Heim, R., A.B. Cubitt, and R.Y. Tsien, *Improved green fluorescence.* Nature, 1995.

373(6516): p. 663-4.
42. Prescott, M., et al., *The length of polypeptide linker affects the stability of green fluorescent protein fusion proteins.* Anal Biochem, 1999. **273**(2): p. 305-7.
43. O'Driscoll, M., et al., *A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome.* Nat Genet, 2003. **33**(4): p. 497-501.
44. Weiss, R.S., T. Enoch, and P. Leder, *Inactivation of mouse Hus1 results in genomic instability and impaired responses to genotoxic stress.* Genes Dev, 2000. **14**(15): p. 1886-98.
45. Weiss, R.S., P. Leder, and C. Vaziri, *Critical role for mouse Hus1 in an S-phase DNA damage cell cycle checkpoint.* Mol Cell Biol, 2003. **23**(3): p. 791-803.
46. Hopkins, K.M., et al., *Deletion of mouse rad9 causes abnormal cellular responses to DNA damage, genomic instability, and embryonic lethality.* Mol Cell Biol, 2004. **24**(16): p. 7235-48.
47. Zachos, G., M.D. Rainey, and D.A. Gillespie, *Chk1-dependent S-M checkpoint delay in vertebrate cells is linked to maintenance of viable replication structures.* Mol Cell Biol, 2005. **25**(2): p. 563-74.
48. Kitagawa, R., et al., *Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway.* Genes Dev, 2004. **18**(12): p. 1423-38.
49. Bekker-Jensen, S., et al., *Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD1.* J Cell Biol, 2005. **170**(2): p. 201-11.
50. Bonner, J.A. and T.S. Lawrence, *Doxorubicin decreases the repair of radiation-induced DNA damage.* Int J Radiat Biol, 1990. **57**(1): p. 55-64.
51. Majka, J., et al., *Replication protein A directs loading of the DNA damage checkpoint clamp to 5'-DNA junctions.* J Biol Chem, 2006. **281**(38): p. 27855-61.
52. Zou, L., D. Liu, and S.J. Elledge, *Replication protein A-mediated recruitment and activation of Rad17 complexes.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 13827-32.
53. Majka, J. and P.M. Burgers, *Function of Rad17/Mec3/Ddc1 and its partial complexes in the DNA damage checkpoint.* DNA Repair (Amst), 2005. **4**(10): p. 1189-94.
54. Modesti, M. and R. Kanaar, *DNA repair: spot(light)s on chromatin.* Curr Biol, 2001. **11**(6): p. R229-32.
55. Marteijn, J.A., et al., *Nucleotide excision repair-induced H2A ubiquitination is dependent on MDC1 and RNF8 and reveals a universal DNA damage response.* J Cell Biol, 2009. **186**(6): p. 835-47.
56. Mone, M.J., et al., *Local UV-induced DNA damage in cell nuclei results in local transcription inhibition.* EMBO Rep, 2001. **2**(11): p. 1013-7.
57. Volker, M., et al., *Sequential assembly of the nucleotide excision repair factors in vivo.* Mol Cell, 2001. **8**(1): p. 213-24.
58. Mone, M.J., et al., *In vivo dynamics of chromatin-associated complex formation in mammalian nucleotide excision repair.* Proc Natl Acad Sci U S A, 2004. **101**(45): p. 15933-7.
59. Mari, P.O., et al., *Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4.* Proc Natl Acad Sci U S A, 2006. **103**(49): p. 18597-602.
60. Aten, J.A., et al., *Dynamics of DNA double-strand breaks revealed by clustering of*

damaged chromosome domains. Science, 2004. **303**(5654): p. 92-5.
61. Stap, J., et al., *Induction of linear tracks of DNA double-strand breaks by alpha-particle irradiation of cells.* Nat Methods, 2008. **5**(3): p. 261-6.
62. Williams, E.S., et al., *DNA double-strand breaks are not sufficient to initiate recruitment of TRF2.* Nat Genet, 2007. **39**(6): p. 696-8; author reply 698-9.
63. Houtsmuller, A.B., *Fluorescence recovery after photobleaching: application to nuclear proteins.* Adv Biochem Eng Biotechnol, 2005. **95**: p. 177-99.
64. Houtsmuller, A.B. and W. Vermeulen, *Macromolecular dynamics in living cell nuclei revealed by fluorescence redistribution after photobleaching.* Histochem Cell Biol, 2001. **115**(1): p. 13-21.
65. Stroppolo, M.E., et al., *Superefficient enzymes.* Cell Mol Life Sci, 2001. **58**(10): p. 1451-60.
66. Ricard, J. and A. Cornish-Bowden, *Co-operative and allosteric enzymes: 20 years on.* Eur J Biochem, 1987. **166**(2): p. 255-72.
67. Zou, L. and S.J. Elledge, *Sensing and signaling DNA damage: roles of Rad17 and Rad9 complexes in the cellular response to DNA damage.* Harvey Lect, 2001. **97**: p. 1-15.
68. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
69. Dore, A.S., et al., *Crystal structure of the rad9-rad1-hus1 DNA damage checkpoint complex--implications for clamp loading and regulation.* Mol Cell, 2009. **34**(6): p. 735-45.
70. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes Dev, 2001. **15**(17): p. 2177-96.
71. Kumagai, A., et al., *TopBP1 activates the ATR-ATRIP complex.* Cell, 2006. **124**(5): p. 943-55.
72. Smits, V.A., *Spreading the signal: dissociation of Chk1 from chromatin.* Cell Cycle, 2006. **5**(10): p. 1039-43.
73. van Royen, M.E., et al., *Fluorescence Recovery After Photobleaching (FRAP) to Study Nuclear Protein Dynamics in Living Cells.* Methods Mol Biol, 2009. **464**: p. 363-85.
74. Giglia-Mari, G., et al., *Differentiation driven changes in the dynamic organization of Basal transcription initiation.* PLoS Biol, 2009. **7**(10): p. e1000220.
75. Tan, T.L., et al., *Mouse Rad54 affects DNA conformation and DNA-damage-induced Rad51 foci formation.* Curr Biol, 1999. **9**(6): p. 325-8.

4

Cell cycle-dependent processing of DNA lesions controls localization of Rad9 to sites of genotoxic stress

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ABSTRACT

The Rad9/Rad1/Hus1 complex functions to facilitate the ATR-mediated phosphorylation of several substrates that control the checkpoint arrest induced by DNA damage. Here we show that in response to genotoxic stress induced by different types of damaging agents, Rad9 rapidly relocalized to sites of single stranded DNA, as visualized by discrete nuclear foci that co-localize with RPA. UV light-induced Rad9 foci also colocalized with TopBP1 and γH2AX. Interestingly, Rad9 foci were predominately formed in G1 and S phase after UV light, while treatment of cells with ionizing radiation (IR) resulted in accumulation of Rad9 into foci in S and G2. Photobleaching experiments in living cells revealed that the Rad9 protein is highly mobile in undamaged cells. However, genotoxic stress induced the immobilization of a large proportion of the protein. The proportion of Rad9 immobilization was larger in S phase and the accumulation to sites of locally damaged areas induced by UV-laser irradiation was faster during DNA replication. Inactivation of nucleotide excision repair by knock down of XPA and XPC resulted in a decrease of G1 phase cells that displayed Rad9 foci in response to UV light, whereas IR-induced Rad9 foci were not affected. In contrast, downregulation of CtIP, which promotes DSB resection, abrogated the IR-induced Rad9 foci. These findings show that due to processing of DNA lesions into a common intermediate, which occurs in a cell cycle-dependent manner, Rad9 is able to respond to different types of genotoxic stress.

INTRODUCTION

To maintain genomic stability, eukaryotic cells have developed DNA damage checkpoint and repair mechanisms, which help to ensure the transmission of an intact genome. The master regulators of the mammalian DNA damage response are ATM and ATR, members of the family of phosphoinositide 3-kinase like kinases. ATM and ATR respond to various genotoxic stresses by phosphorylating key proteins in the checkpoint pathway. ATM is the main kinase required for early responses to ionizing radiation (IR), such as phosphorylation of p53 and Chk2. ATR on the other hand, activates Chk1 in response to UV light-induced damage and stalled replication forks [1]. Chk1 subsequently phosphorylates a variety of substrates, thereby regulating different aspects of the DNA damage response such as cell cycle arrest, stabilization of stalled replication forks and DNA repair [2].

A number of other protein complexes facilitate the correct functioning of the checkpoint cascade initiated by ATM/ATR. Mediator proteins such as MDC1 and 53BP1 act in the recruitment of substrates or as scaffolds upon which complexes are assembled [3]. For the ATR-Chk1 pathway, loading of the Rad9/Rad/Hus1 (9-1-1) checkpoint clamp onto chromatin by the Rad17/RFC complex in response to DNA damage is required for phosphorylation of several substrates including Rad17 and Chk1 [4-6]. The recruitment of the 9-1-1 complex to sites of DNA lesions can additionally be visualized by the accumulation of Rad9 into nuclear foci. The requirement of the 9-1-1 complex in the ATR branch was explained by showing that Rad9 recruits the ATR-activator TopBP1 near sites of DNA damage [7-10]. Recent data demonstrate that the subsequent phosphorylation of Rad17 by ATR helps to retain Rad9 at sites of DNA damage, thereby maintaining checkpoint signalling [11].

Initially it was thought that ATM and its regulatory factors are responsible for checkpoint signalling in response to double strand breaks (DSBs) induced by IR, whereas ATR and the Rad17/9-1-1 complexes do not have a key function in the response to such lesions. Recent data however, demonstrate crosstalk between the ATM and ATR pathways and their regulating proteins. In response to IR, ATR activation and recruitment to sites of DNA damage depends on ATM [12, 13]. Rad17 associates with ATM upon IR treatment and under these conditions, Rad17 is phosphorylated in an ATM-dependent manner [4]. Furthermore, Rad9 deficient ES cells are not only sensitive to UV light and hydroxyurea, but also to IR [14]. Finally, Rad9 is phosphorylated in response to IR in an ATM-dependent manner and this phosphorylation is required for the IR-induced checkpoint arrest [15]. Together these data show that the ATR pathway and the 9-1-1 complex in particular, can respond to a wider variety of DNA lesions than originally anticipated, which raises the question, how checkpoint sensor proteins can detect such different types of lesions. An explanation comes from experiments showing that the proteins of the ATR pathway detect stretches of single stranded DNA (ssDNA) coated by RPA, which seems to function as a common intermediate that triggers the checkpoint [16, 17]. RPA-bound ssDNA is formed at stalled replication forks and several proteins have been implicated in the formation of RPA-coated ssDNA in response to different types of DNA lesions. Mre11 and CtIP are involved in nucleolytically processing DSBs to reveal ssDNA during S and G2 phases of the cell cycle upon IR treatment, thereby recruiting ATR to sites of DNA damage [13, 18]. How stretches of ssDNA are generated after UV light is still a matter of debate. Some reports suggest that the ATR pathway can be activated during nucleotide excision repair (NER) [19-21], whereas other studies propose that UV lesions are only processed during DNA replication [22].

Here we study the possible involvement of these and other factors in Rad9 localization to

sites of damage and show that Rad9 is able to respond to different types of genotoxic stress because diverse DNA lesions are processed into a common intermediate in a cell cycle-specific manner.

MATERIALS AND METHODS

Cell culture

U2OS cells were grown using standard procedures. U2OS cells stably expressing EGFP-HA-Rad9 (GFP-Rad9) were grown in standard medium supplemented with 700 μg/ml of geneticin (G418). For stable expression of mCherry-PCNA, GFP-Rad9 expressing U2OS cells were transfected with mCherry-PCNA (kindly provided by A. Inagaki, Erasmus MC Rotterdam, The Netherlands) together with a pBabe puro plasmid. Positive clones were selected after a selection with 20 μg/ml of puromycin for one week. Thereafter, U2OS cells stably expressing GFP-Rad9 and mCherry-PCNA were grown in medium supplemented with geneticin and puromycin.

Antibodies

Antibodies obtained from commercial sources were as follows: Orc2 (BD Pharmingen), Grb2 (BD Transduction), RPA (Ab2, Oncogene), Rad9 (Novus), γH2AX (Upstate), CtIP (Abcam). The following antibodies were obtained from Santa Cruz Biotechnology: CENP-F (H-260), Cyclin A (H-432), Cyclin B1 (GNS1), Ku86 (C-20) and XPA (FL-273).
Rabbit polyclonal anti-Rad9 and anti-TopBP1 have been described previously [23, 24]. Rabbit polyclonal EGFP antibody was raised against recombinant His-tagged EGFP full length protein. Recombinant protein was obtained by cloning the cDNA corresponding in pET-28 (Novagen) expressed in E. coli and soluble protein was purified and used to immunize.
Rabbit polyclonal anti-XPC was a gift from N. Wijgers and W. Vermeulen (Erasmus MC Rotterdam, The Netherlands).

Transfection

Plasmid DNA was transfected into cells using the calcium phosphate transfection method.
siRNA oligonucleotides (Dharmacon Research) were transfected into cells using Oligofectamine (Invitrogen) according to the manufacturers instructions, and as previously described [11]. To downregulate CtIP, a mix of 2 different oligonucleotides was used. Cells were incubated for 72 hours after transfection prior to further analysis. Sequences of oligonucleotides were as follows:
Luc (CGUACGCGGAAUACUUCGAdTdT),
CtIP (GCUAAAACAGGAACGAAUCdTdT and UCCACAACAUAAUCCUAAUdTdT),
XPC (CUGGAGUUUGAGACAUAUCUU) and
XPA (CUGAUGAUAAACACAAGCUUAUU).

Immunofluorescence

For immunostaining, cells were fixed in 2% paraformaldehyde containing 0.2% Triton X-100 for 20 minutes and then permeabilized with 0.1% Triton X-100 for 5 minutes. Samples were blocked in 1% FCS and immunostained with antibodies as indicated. For detection of GFP-Rad9 in U2OS cells, living cells were studied, or cells were fixed and permeabilized as above. Cells containing >10 Rad9 foci were scored as positive. In all instances, error bars on

graphs represent the standard error of the mean (SEM) from at least three independent experiments.
Images were made using a Cell Observer fluorescent microscope equipped with Axiovision software (Zeiss) or a Confocal Laser Scanning Microscope LSM 510 (Zeiss), equipped with a 488 nm Ar-laser and a 505-530 nm band pass filter for green fluorescence. Red fluorescence was detected using a 543 nm laser and 560 nm long pass filter. For strip-FRAP experiments and time lapse imaging after UV laser induction, GFP-Rad9 and mCherry-PCNA were detected in living cells using a Confocal Laser Scanning Microscope LSM 510 (Zeiss), equipped with a 488 nm Ar-laser and a 505-530 nm band pass filter for green fluorescence. Red fluorescence was detected using a 543 nm laser and 575-615 nm band pass filter.

Cell fractionation and Western blotting
For whole cell extracts, cells were washed in cold PBS, after which the cells were resuspended in Laemmli sample buffer (4% SDS, 20% glycerol, 120 mM Tris pH 6.8). Protein concentrations were determined using the Lowry protein assay. Bromophenol blue and β-mercaptoethanol were added and samples were boiled for 5 minutes before loading.
Chromatin fractionation was performed as previously described [25].

Generation of DNA damage and photobleaching techniques
UV-irradiation was performed using a 254 nm UV-C lamp (Philips) at 20 J/m^2 and cells were processed 1 hour post treatment. Cells were incubated with 5 μg/ml of aphidicolin (Sigma) for 20 hours prior to processing of samples. IR (10 Gy) was induced using a ^{137}Cs source or a linear accelerator (LINAC) and cells were processed 2 hours after treatment.
Local UV laser induction was performed as previously described [26]. In short, cells stably expressing GFP-Rad9 and mCherry-PCNA were grown on a 25 mm quartz coverslip. A small area inside the nucleus was exposed for ~1 second to a 2 mW pulsed (7.8 kHz) diode laser emitting at 266nm. Time-lapse images were made every minute immediately starting after local UV induction. The time course measurements were normalized to the plateau phase. Start of UV irradiation was defined as t=0. Assembly curves were normalized to 1 and processed as previously described [27].
In the FRAP experiments, a strip spanning the nucleus was photobleached for 20 milliseconds using an Ar-laser (488 nm) at 100% laser intensity, which irreversibly bleaches all GFP-Rad9 molecules in that area. Subsequently, the redistribution of fluorescence in the strip was monitored taking confocal images every 20 milliseconds for a total of 24 seconds at low laser intensity to avoid further bleaching. The fluorescence before bleaching ($I_{t<0}$) was set to 1 and the intensity immediately after bleaching (I_0) was set to 0. The recovery of fluorescence was plotted against time.

RESULTS

Rad9 rapidly relocalizes to sites of DNA damage in response to genotoxic stress
Recently we reported that ATR and Rad17 collaborate in regulating the localization and maintenance of Rad9 to sites of DNA damage [11]. To gain insight into additional factors involved in the localization of Rad9 to sites of DNA lesions, we studied DNA damage-induced Rad9 focus formation in more detail, using previously described U2OS cells stably expressing Rad9 tagged with EGFP-HA [11]. As shown in Figure 1A, Rad9 is homogenously distributed

in the nucleus in unperturbed cells. In response to UV light, Rad9 rapidly relocalizes into discrete nuclear foci in the majority of the cells. These foci can be observed from 10 minutes up to 24 hours after irradiation of the cells (Figure 1A and data not shown).
To determine the nature of the DNA lesions to which Rad9 localizes, we examined colocalization of GFP-Rad9 with several proteins involved in DNA damage checkpoint regulation by immunofluorescence. UV light-induced Rad9 foci colocalized with ssDNA-binding protein RPA and ATR-activator TopBP1 (Figure 1B). Rad9 also formed nuclear foci in response to IR, which colocalized with RPA (Figure 1C), indicating that Rad9 is recruited to sites of ssDNA generated in response to DSBs. Interestingly, UV light-induced Rad9 foci displayed clear colocalization with the phosphorylated form of H2AX (γH2AX), an early marker of DNA damage [28]. In contrast, Rad9 and γH2AX form distinct, non-overlapping foci in response to IR (Figure 1D), demonstrating the complexity of lesions induced by this treatment.

Recruitment of Rad9 to chromatin in response to DNA damage and replication stress leads to immobilization of the protein

In response to DNA damage, Rad9 is recruited to chromatin in a Rad17-dependent manner [6]. Upon fractionation of GFP-Rad9 expressing U2OS cells into a soluble and a chromatin-enriched fraction, Rad9 was only present in the soluble fraction in unperturbed cells, but the protein was recruited to chromatin after treatment of the cells with UV light or aphidicolin, a DNA polymerase α inhibitor that blocks replication progression (Figure 2A). Interestingly, whereas GFP-Rad9 runs as multiple bands in the soluble fraction, which partially represents the constitutive phosphorylation of the protein (data not shown and refs. 24-26), in the chromatin-enriched fraction only the slow mobility form of GFP-Rad9 was detected. This phenomenon was also observed by others [6, 29] and indicates that only the higher phosphorylated form of Rad9 is able to bind to chromatin in response to genotoxic stress.
To investigate the behaviour of Rad9 in response to DNA damage in living cells, we studied protein mobility in real time by fluorescence redistribution after photobleaching (FRAP). In this experiment, U2OS cells expressing GFP-Rad9 were left untreated, exposed to UV light or treated with aphidicolin. Subsequently, a narrow strip in the centre of the nucleus was photobleached, cells were followed in real time by video microscopy and the recovery of fluorescence within this region was monitored at low laser intensity (strip-FRAP). In undamaged cells, GFP fluorescence quickly recovered to ~85% of pre-bleach level, which was similar to free GFP in our experimental setup (data not shown), indicating that all GFP-Rad9 molecules are highly mobile (Figure 2B). In contrast, treating the cells with UV light or aphidicolin resulted in the immobilization of a significant proportion of the protein, as demonstrated by recovery of fluorescence to only ~55% or ~70% of pre-bleach level, respectively, even when the measurement were performed over an extended period of time (Figure 2B and data not shown). Lowering the UV light dose from 20 J/m^2 to 5 J/m^2 did not change the amount of immobile Rad9 (data not shown), indicating that the fraction of immobilized Rad9 is saturated at a low dose of UV light-induced DNA damage. This suggests that the difference in immobile fraction of Rad9 induced by UV light and aphidicolin treatment is due to the different nature of DNA lesions induced rather than the amount of lesions.
Next, we investigated the possibility that UV light-induced immobilization of Rad9 is influenced by the cell cycle phase in which the damage is detected. For this purpose, we generated U2OS cells stably expressing GFP-Rad9 and functional mCherry-PCNA (Figure

3A) [30]. Given that PCNA forms specific patterns of accumulation at different stages of S phase, [31, 32] we used subnuclear localization of PCNA to distinguish S phase cells from G1/G2 cells in asynchronously growing cultures. Interestingly, when performing strip-FRAP analysis for GFP-Rad9 after UV light treatment, we observed a clear difference between the proportion of immobilized Rad9 in S phase versus G1/G2 phase cells. Whereas UV light-induced damage resulted in a significant proportion of immobilized protein in G1/G2 cells, the amount of immobile Rad9 was considerably larger when cells were exposed to UV light during DNA replication (Figure 2C).

Finally, we determined whether the kinetics of GFP-Rad9 accumulation onto sites of DNA lesions was also influenced by the cell cycle phase by inducing DNA damage in subnuclear regions in living cells by low dose UVC (266 nm) laser irradiation, which induces only UV light-specific DNA photo-lesions [26]. As shown in Figure 2D, local UV-irradiation led to accumulation of GFP-Rad9 at the damaged area in living cells and maximum accumulation was reached around 17 minutes with $t_{1/2}$~500 seconds after damage induction during DNA replication. However, it took twice as long (36 minutes, $t_{1/2}$~1100 seconds) to reach maximal accumulation of GFP-Rad9 on locally damaged regions in cells in G1/G2 phase of the cell cycle, demonstrating that recruitment of Rad9 to sites of damage under such conditions is considerably slower. The time-lapse images of individual cells additionally showed faster accumulation of GFP-Rad9 to the locally irradiated area in the S phase cell as compared to the cell in G1/G2 phase of the cell cycle. The images also show that in S phase, most of GFP-Rad9 was translocated to the damaged area whereas outside S phase, part of GFP-Rad9 remained distributed throughout the nucleus, also at later time points (Figure 2D, left panel), confirming the higher level of immobilization of Rad9 to sites of DNA damage during S phase.

DNA lesion-specific focus formation of Rad9 during the cell cycle

While studying GFP-Rad9 focus formation in the cells that also stably expressed mCherry-PCNA a few aspects of Rad9 localization caught our attention. First, unlike PCNA [31, 32]. Rad9 did not relocalize into foci during DNA replication, but instead, was equally distributed over the nucleus throughout the cell cycle in unperturbed cells (Figure 3A). Second, Rad9 foci in response to UV light were localized either throughout the nucleus, or situated at the nuclear periphery, thereby partially overlapping with PCNA (Figure 3A, B and data not shown). Finally, Rad9 did not only form UV light-induced foci during S phase (Figure 3A), but also in G1 phase cells. This was confirmed when the levels of Cyclin A were monitored in GFP-Rad9 cells (Figure 3B). Cyclin A is expressed in increasing levels during S and G2 phases of the cell cycle and can therefore be used to distinguish between cell cycle phases in an asynchronous culture [13].

To investigate the pattern of Rad9 foci in more detail, we treated cells with aphidicolin or UV light and analyzed GFP-Rad9 foci in S phase cells by selecting cells with intermediate Cyclin A levels. Treatment with aphidicolin resulted in the arrest of all cells at the beginning of S phase (data not shown) and 80% of these cells had Rad9 foci that localize throughout the nucleus (Figure 3C). One hour after exposing the cells to UV light, cells were still equally distributed over the different phases of the cell cycle (data not shown). Most of the cells that were exposed to UV light during S phase displayed Rad9 foci, which were located either throughout the nucleus or at the nuclear periphery (Figure 3C). Together these results indicate that Rad9 foci at the nuclear border are formed at mid- or late S phase, which, given the resemblance and overlap with PCNA replication foci during mid-S phase [31,

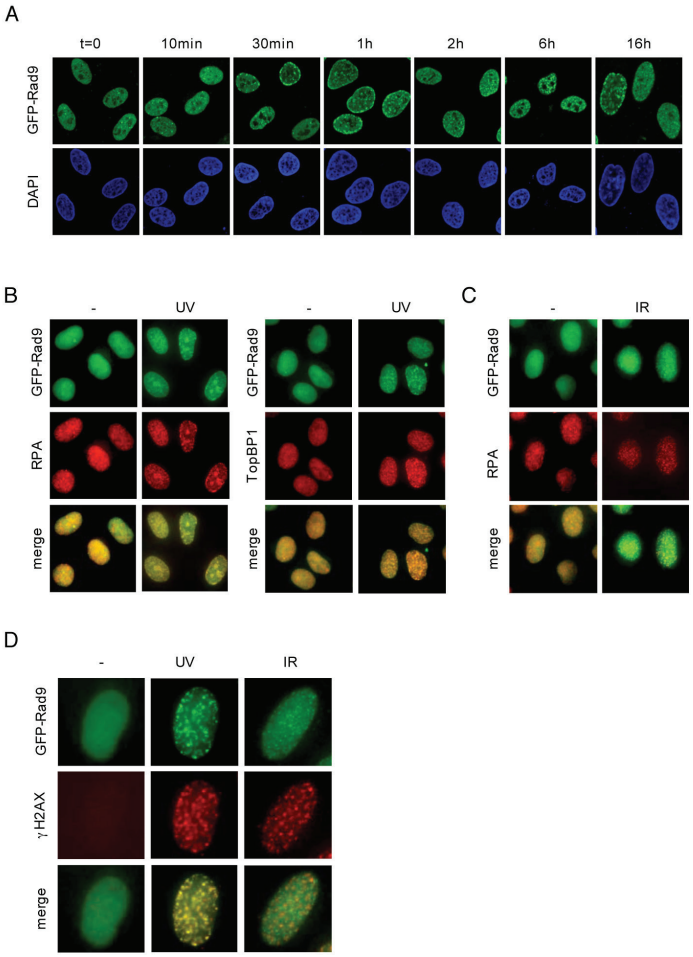

Figure 1: Rad9 rapidly accumulates into nuclear foci in response to genotoxic stress. (A) U2OS cells expressing GFP-Rad9 were treated with UV light. At the indicated time points, cells were fixed and analyzed for fluorescence. (B) GFP-Rad9 expressing U2OS cells were left untreated or exposed to UV light. GFP-Rad9 was detected by direct fluorescence and RPA34 and TopBP1 by immunofluorescence. (C) GFP-Rad9 expressing U2OS cells were left untreated or treated with IR. GFP-Rad9 was detected by direct fluorescence and RPA34 by immunofluorescence. (D) GFP-Rad9 expressing U2OS cells were left untreated or exposed to UV or IR. GFP-Rad9 was detected by direct fluorescence and γH2AX by immunofluorescence.

32], suggests that upon exposure to UV light during DNA replication, the 9-1-1 complex relocalizes to sites of stalled replication forks.

Interestingly, in addition to S phase cells, a significant proportion of G1 cells displayed UV-induced GFP-Rad9 foci, whereas the amount of foci observed in G2 phase was lower (Figure 3D). In contrast, the majority of the Rad9 foci in response to IR were present in S and G2 phase cells (Figure 3D). This result of DNA lesion-specific regulation of Rad9 focus formation

during the cell cycle was confirmed using CENP-F as a marker for S and G2 phases of the cell cycle in GFP-Rad9 expressing cells (Figure 3E) or by examining the distribution of endogenous Rad9 in response to UV light or IR throughout the cell cycle, in which cytoplasmic Cyclin B1 was used as a marker for G2 phase (Figure 3F). Both experiments showed that in response to UV light, Rad9 predominantly forms foci in S and G1, while IR-induced focus formation occurs in S and G2 phase cells. Together these data indicate that the localization of Rad9 to sites of DNA damage is cell cycle-regulated.

DNA-damage induced focus formation of Rad9 depends on processing of DNA lesions

The ATR pathway is thought to respond to a diverse range of DNA lesions through the generation of RPA-coated ssDNA as a common intermediate [17]. For example, processing of DSBs into ssDNA in S and G2 phases of the cell cycle leads to ATR-dependent checkpoint signalling. Mre11/CtIP have recently been implicated in facilitating such DNA end resection [13, 18]. In addition, some studies propose that processing of UV light-induced lesions during DNA replication is sufficient to trigger ATR activation, [22] whereas other reports suggest that instead, the ATR pathway can be activated during NER [19-21].

To determine whether processing of DNA lesions contributes to the recruitment of Rad9 to sites of damage, we downregulated XPC, XPA and CtIP by siRNA in cells expressing GFP-Rad9. XPC and XPA are involved in the early steps of damage recognition after UV light exposure and facilitate the assembly of additional factors in NER [33]. CtIP is specifically involved in the processing of DSBs. The efficiency of the downregulations is shown in Figure 4A. Downregulation of both XPA and XPC resulted in the decrease from 63% to 38% of cells that displayed UV-induced Rad9 foci compared to cells transfected with control oligos, whereas it has no effect on Rad9 focus formation upon exposure to IR (Figure 4B). In contrast, knock down of CtIP did not affect the formation of GFP-Rad9 foci in response to UV light, but almost completely abrogated (58% to 16%) the recruitment of Rad9 to sites of damage when cells were exposed to IR (Figure 4B).

As inactivation of NER by the downregulation of XPA or XPC only partially affected the UV-induced focus formation of Rad9, we wondered whether the contribution of the NER machinery to the recruitment of Rad9 to sites of genotoxic stress was restricted to a certain phase of the cell cycle. To investigate this possibility, we scored GFP-Rad9 foci in each of the cell cycle phases after knock down of XPA and XPC. Interestingly, inactivation of NER predominantly affected UV-induced focus formation in G1 phase cells, but had a smaller effect during DNA replication (Figure 4C). This differential effect was most obvious with XPC, when focus formation in G1 drops from 68% in control cells to 19% in XPC downregulated cells, while knock down of XPC did not have a significant effect on the amount of GFP-Rad9 foci in S phase (Figure 4C). As the amount of Rad9 foci in G2 was very low, we could not draw conclusions about the involvement of NER in Rad9 focus formation in this phase.

Scoring IR-induced GFP-Rad9 foci throughout the cell cycle after downregulation of CtIP showed that the loss of CtIP resulted in a dramatic decrease in Rad9 foci during all cell cycle phases (Figure 4D). Together these results indicate that recruitment of Rad9 to sites of DNA damage is partially controlled by processing of UV light-induced lesions by NER or during DNA replication. In contrast, focus formation of Rad9 in response to IR during S and G2 phase depends on CtIP-mediated resection of DSBs.

DISCUSSION

Accumulating evidence suggests that the ATR pathway is responsible for activating a cell cycle checkpoint upon a variety of DNA lesions, but how exactly the sensor proteins in this pathway can recognize these lesions is not clear. Here we show that putative sensor protein Rad9 is able to respond to different types of genotoxic stress, including IR (Figure 1). Recently, IR-induced phosphorylation of effector kinase Chk1 was shown to be dependent on both ATM and ATR and in addition, ATM was shown to be required for IR-induced focus formation of ATR [12, 13]. Our data demonstrating Rad9 focus formation in response to IR is in accordance with the fact that the ATR pathway responds to these type of lesions. Interestingly, the cell cycle phase in which Rad9 relocalizes to sites of damage depends on the type of DNA lesion. Using Cyclin A, Cyclin B1 and CENP-F as markers of different phases in the cell cycle, both endogenous Rad9 and GFP-Rad9 were shown to accumulate into nuclear foci in S and G2 phase in response to IR treatment. Strikingly, Rad9 foci predominantly appear in G1 and S phase cells after exposure to UV light, whereas we only observe a small amount of such foci in G2 cells (Figure 3). A possible explanation for this observation is that the recognition of UV light-induced lesions is not very efficient in G2, and instead, such lesions are predominantly repaired during the G1 or S phase of the next cell cycle.

Our data studying the dynamic behaviour of GFP-Rad9 by live-cell imaging show that a significant proportion of Rad9 is immobilized in response to DNA damage, and the fraction of immobile Rad9 is highest upon UV light damage in S phase cells (Figure 2C). In addition, the recruitment of GFP-Rad9 to sites of UV lesions in living cells is much faster when cells are in S phase at the time of damage induction (Figure 2D). Together these data indicate that the 9-1-1 complex is more vigilant when cells are undergoing DNA replication.

UV light produces pyrimidine dimers and other photoproducts, which are basically bulky adducts that can lead to replication fork stalling. Rad9 foci in S phase cells display several patterns, either equally distributed throughout the nucleus or situated at the nuclear periphery (Figure 3A). A similar foci pattern is seen for TopBP1 (Figure 1B), which activates ATR near sites of damage by interaction with Rad9 [7]. Given the resemblance and partial colocalization of these foci with replication foci marked by PCNA [31, 32] this indicates that the 9-1-1 complex localizes to sites of stalled replication forks in response UV light and suggests that 9-1-1 acts as sensor of DNA damage during replication.

Although UV-induced ATR activation was reported to require replication stress [22]. others have shown that Rad9 binds chromatin after damage in a G1-enriched cell population [34]. In agreement with this, we observe Rad9 foci after exposure to UV light in a proportion of G1 cells (Figure 3), which demonstrates that processing of UV light lesions per se and not just UV light lesions that result in a block in replication fork progression, trigger a response by the 9-1-1 complex. However, UV light-induced Rad9 relocalization does seem to be cell cycle regulated, as we observe little cells with foci in G2 (Figure 3).

As the ATR pathway is triggered by a broad spectrum of DNA lesions, the ATR kinase is thought to sense a common DNA intermediate. The principle DNA architecture recognized by ATR is ssDNA coated by the ssDNA protein binding complex RPA. RPA-coated ssDNA facilitates the recruitment of ATR to sites of damage through an interaction with ATR-associated protein ATRIP [16, 17]. Even though RPA-coated ssDNA induced phosphorylation of Rad17 by the ATR-ATRIP complex *in vitro*, [17] it seems likely that RPA and ssDNA additionally facilitate activation of ATR in other ways, for example by the recruitment of other checkpoint proteins. The observed co-localization of Rad9 with RPA in response to UV light and IR

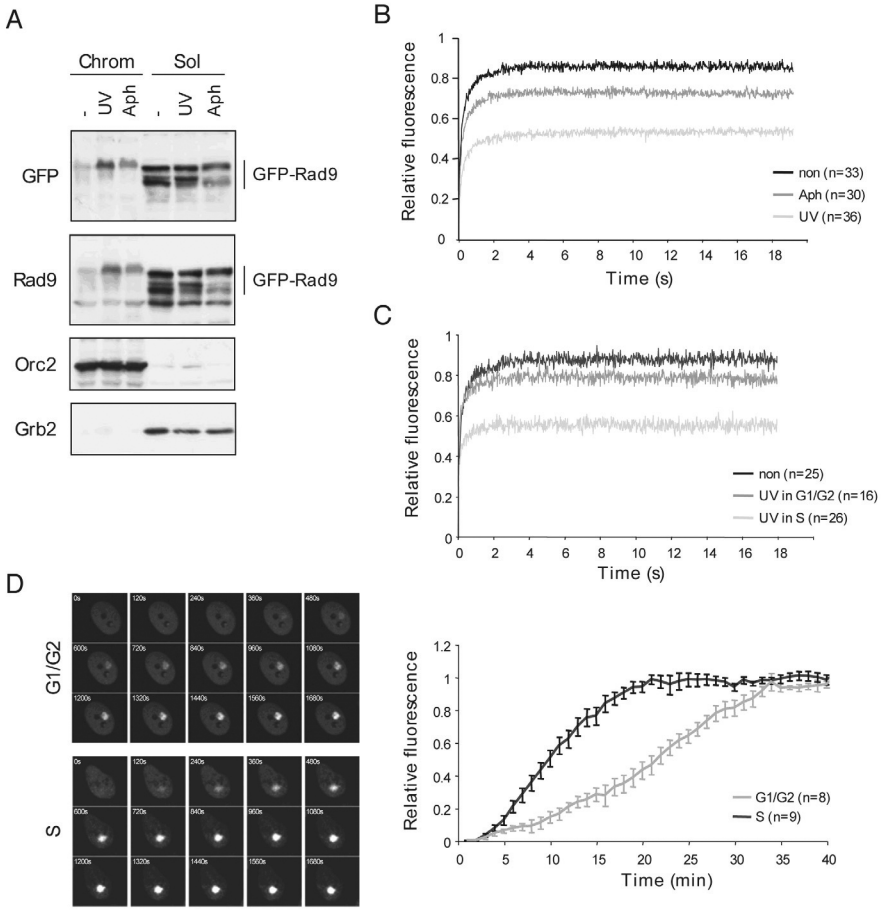

Figure 2: Genotoxic stress-induced chromatin binding of Rad9 leads to immobilization of the protein. (A) GFP-Rad9 expressing cells were left untreated or exposed to UV or aphidicolin, after which cells were fractionated as described in materials and methods. Soluble and chromatin fractions were analyzed using the indicated antibodies. (B) GFP-Rad9 expressing cells were left untreated or exposed to UV light or aphidicolin. A small strip of the nucleus was bleached for 20 milliseconds at 100% laser intensity, after which redistribution of fluorescence was monitored every 20 milliseconds. (C) Cells expressing GFP-Rad9 and mCherry-PCNA were left untreated or treated with UV light. Then FRAP analysis was performed for GFP-Rad9 as in (B). Cells were divided into the group of S or G1/G2 phase cells based on the mCherry-PCNA pattern. (D) A small area of the nucleus of cells expressing GFP-Rad9 and mCherry-PCNA was exposed to UV light using a 266 nm laser for ~1 second, after which the fluorescence was monitored by recording images every 60 seconds. Note that fluorescence at time of irradiation was normalized to 0 and steady-state level to 1 (right panel) for individual measurements. Nuclear distribution patterns of PCNA were used to determine the cell cycle phase.

(Figure 1B and C) is in accordance with this idea, which is supported by biochemical data showing that the 9-1-1 complex is loaded onto DNA in an RPA-dependent manner *in vitro,* [35] and interaction studies of Rad9 and RPA in mammalian cells [36, 37]. In addition, RPA bound to ssDNA replication intermediates during unperturbed replication might enhance the efficiency of recognition of genotoxic stress by sensor proteins, which could explain the

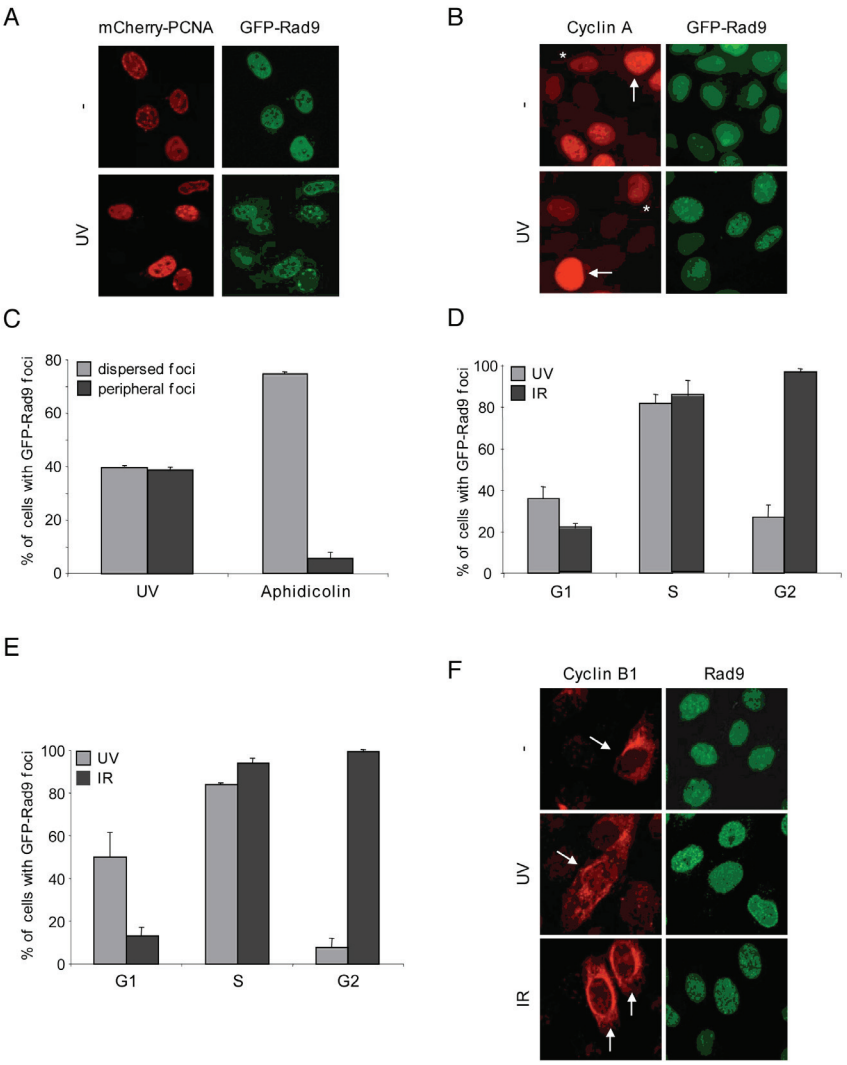

Figure 3: DNA damage-induced focus formation of Rad9 during different phases of the cell cycle. (A) U2OS cells expressing GFP-Rad9 and mCherry-PCNA were left untreated or treated with UV light. One hour later, cells were fixed and analyzed by direct fluorescence. Note that PCNA localizes into subnuclear foci during S phase. (B) GFP-Rad9 expressing cells were left untreated or exposed to UV light. One hour later, cells were fixed. GFP-Rad9 was detected by direct fluorescence and Cyclin A by immunofluorescence. Cells without Cyclin A are in G1 phase, cells with intermediate levels of Cyclin A in S (asterisk) and G2 phase cells display high Cyclin A levels (arrow). (C) GFP-Rad9 expressing cells were treated with UV light or aphidicolin. After fixing the cells, GFP-Rad9 was detected by direct fluorescence and Cyclin A by immunofluorescence. Only S phase cells were scored, depending on Cyclin A levels, and the total number of S phase cells was set to 100%. Shown is the percentage of cells with >10 GFP-Rad9 accumulations, either distributed throughout the nucleus ('dispersed foci') or localized at the nuclear periphery ('peripheral foci'). Error bars represent the SEM of three independent experiments. (D) GFP-Rad9 expressing cells were exposed to UV light or IR. After fixing the cells, GFP-Rad9 was detected by direct fluorescence and Cyclin A by immunofluorescence. Cell cycle phase was judged on levels of Cyclin A and the total number of cells in each phase was set to 100%. (E). As in (D), but with staining for CENP-F instead of Cyclin A. (F). U2OS cells were left untreated or treated with UV light or IR. After fixing the cells, endogenous Rad9 and Cyclin B1 were analyzed after immunostaining. Arrows point to G2 cells that express Cyclin B1.

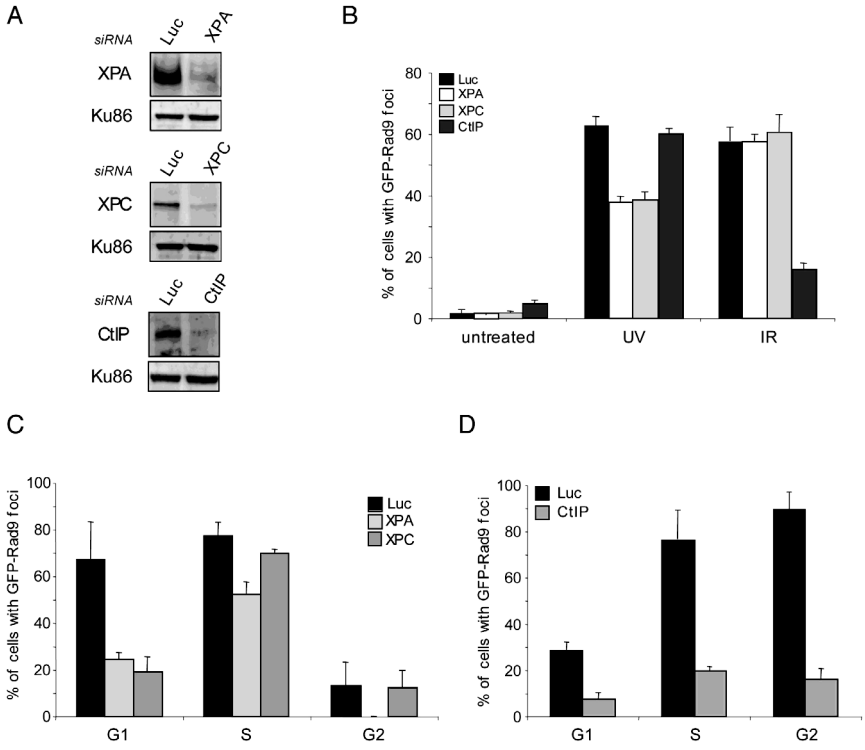

Figure 4: Rad9 focus formation is dependent on processing of DNA lesions. (A) U2OS cells expressing GFP-Rad9 were transfected with luciferase (Luc), XPA, XPC or CtIP siRNA oligonucleotides. Seventy two hours after transfection, cells were lysed and western blot analysis was performed with the indicated antibodies. (B) Cells were transfected as in (A). After 72 hours, cells were left untreated or exposed to UV light or IR. GFP-Rad9 foci were detected by direct fluorescence. Shown is the percentage of cells with >10 GFP-Rad9 foci with error bars representing the SEM of at least three independent experiments. Total number of cells in each cell cycle phase was set to 100%. (C) GFP-Rad9 expressing cells were transfected with luciferase, XPA or XPC siRNA oligos. Cells were treated with UV light, fixed and stained for Cyclin A. Cell cycle phase was determined on Cyclin A levels and foci were quantified as in (B). (D) GFP-Rad9 expressing cells were transfected with luciferase or CtIP siRNA oligos. After 72 hours, cells were exposed to IR and further treated as described as in (C).

observed faster accumulation of Rad9 to sites of damage during S phase (Figure 2D).
RPA-coated ssDNA is generated by uncoupling of the replicative helicase from stalled replication forks [38] but is also formed during the repair of DNA lesions. During NER for example, lesion processing involves base excision of the damaged DNA resulting in an ssDNA gap intermediate [33]. However, conflicting results have been reported for the involvement of NER in checkpoint activation. Whereas some suggested that UV light-induced ATR activation only requires replication stress, [22] others implied the involvement of NER in triggering ATR checkpoint signalling, either during replication [21] or outside S phase [19, 39]. Our data demonstrate that upon downregulation of XPA and XPC, proteins involved in early stages of NER, UV light-induced Rad9 focus formation is decreased (Figure 4B), indicating that processing of UV light lesions during NER indeed generates a signal that is recognized by Rad9. NER has an important role during G1, although its activity is not

thought to be restricted to this phase of the cell cycle [40]. Indeed, focus formation in G1 was more affected than in S phase cells, mainly after knockdown of XPC (Figure 4C), which could explain why UV-induced focus formation of Rad9 is not completely abrogated in an asynchronous population, even though downregulation of XPA and XPC was quite efficient (Figure 4A). Our results suggest a model that could explain the apparently contradictory results, in which a fail-safe mechanism ensures triggering the ATR pathway in several ways: RPA-coated ssDNA can be generated during the repair of UV light-induced damage by NER but also when replication forks encounter UV light lesions. Consequently, NER plays a more important role in this process outside S phase. In contrast, resection of DSBs into ssDNA, which, after the subsequent coating by RPA enhances ATR recruitment, is promoted by CtIP [18]. Our data demonstrate that CtIP also facilitates the recruitment of the 9-1-1 complex to sites of damage upon the induction of DSBs, as downregulation of CtIP protein levels abrogate IR-, but not UV-induced Rad9 foci (Figure 4B). As for ATR, CtIP is required for Rad9 recruitment in both S and G2 phases of the cell cycle (Figure 4D). Together these results indicate that each type of DNA lesion is processed into an intermediate DNA structure, by a different cellular process.

Here we show that the response of checkpoint protein Rad9 is cell cycle-regulated. Even though Rad9 can localize to sites of DNA lesions throughout the cell cycle, the immobilization is larger and the accumulation faster when cells are undergoing replication. Our data demonstrate that proteins involved in NER and processing of DNA DSB lesions are required for localization of Rad9 to sites of damage, most likely by the generation of RPA-coated ssDNA, which makes this the first report investigating factors outside the ATR checkpoint pathway that are involved in the localization of Rad9 to sites of damage and shows that DNA repair mechanisms and checkpoint signalling are interconnected. Concomitant localization of ATR and the 9-1-1 complex at sites of DNA damage leads to the recruitment of ATR-activating protein TopBP1 by Rad9, [7, 10] thereby ensuring full activation of the ATR pathway and downstream checkpoint response.

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REFERENCES

1. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes Dev, 2001. **15**(17): p. 2177-96.
2. Chen, Y. and Y. Sanchez, *Chk1 in the DNA damage response: conserved roles from yeasts to mammals.* DNA Repair (Amst), 2004. **3**(8-9): p. 1025-32.
3. Riches, L.C., A.M. Lynch, and N.J. Gooderham, *Early events in the mammalian*

response to DNA double-strand breaks. Mutagenesis, 2008. **23**(5): p. 331-9.
4. Bao, S., et al., *ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses.* Nature, 2001. **411**(6840): p. 969-74.
5. Weiss, R.S., et al., *Hus1 acts upstream of chk1 in a mammalian DNA damage response pathway.* Curr Biol, 2002. **12**(1): p. 73-7.
6. Zou, L., D. Cortez, and S.J. Elledge, *Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin.* Genes Dev, 2002. **16**(2): p. 198-208.
7. Delacroix, S., et al., *The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1.* Genes Dev, 2007. **21**(12): p. 1472-7.
8. Kumagai, A., et al., *TopBP1 activates the ATR-ATRIP complex.* Cell, 2006. **124**(5): p. 943-55.
9. Kumagai, A. and W.G. Dunphy, *How cells activate ATR.* Cell Cycle, 2006. **5**(12): p. 1265-8.
10. Lee, J., A. Kumagai, and W.G. Dunphy, *The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR.* J Biol Chem, 2007. **282**(38): p. 28036-44.
11. Medhurst, A.L., et al., *ATR and Rad17 collaborate in modulating Rad9 localisation at sites of DNA damage.* J Cell Sci, 2008. **121**(Pt 23): p. 3933-40.
12. Adams, K.E., et al., *Recruitment of ATR to sites of ionising radiation-induced DNA damage requires ATM and components of the MRN protein complex.* Oncogene, 2006. **25**(28): p. 3894-904.
13. Jazayeri, A., et al., *ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks.* Nat Cell Biol, 2006. **8**(1): p. 37-45.
14. Roos-Mattjus, P., et al., *Phosphorylation of human Rad9 is required for genotoxin-activated checkpoint signaling.* J Biol Chem, 2003. **278**(27): p. 24428-37.
15. Chen, M.J., et al., *ATM-dependent phosphorylation of human Rad9 is required for ionizing radiation-induced checkpoint activation.* J Biol Chem, 2001. **276**(19): p. 16580-6.
16. Ball, H.L., J.S. Myers, and D. Cortez, *ATRIP binding to replication protein A-single-stranded DNA promotes ATR-ATRIP localization but is dispensable for Chk1 phosphorylation.* Mol Biol Cell, 2005. **16**(5): p. 2372-81.
17. Zou, L. and S.J. Elledge, *Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.* Science, 2003. **300**(5625): p. 1542-8.
18. Sartori, A.A., et al., *Human CtIP promotes DNA end resection.* Nature, 2007. **450**(7169): p. 509-14.
19. O'Driscoll, M., et al., *A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome.* Nat. Genet., 2003. **33**(4): p. 497-501.
20. Giannattasio, M., et al., *Physical and functional interactions between nucleotide excision repair and DNA damage checkpoint.* Embo J, 2004. **23**(2): p. 429-38.
21. Bomgarden, R.D., et al., *Opposing effects of the UV lesion repair protein XPA and UV bypass polymerase eta on ATR checkpoint signaling.* Embo J, 2006. **25**(11): p. 2605-14.
22. Ward, I.M., K. Minn, and J. Chen, *UV-induced ataxia-telangiectasia-mutated and Rad3-related (ATR) activation requires replication stress.* J Biol Chem, 2004. **279**(11): p. 9677-80.
23. Toueille, M., et al., *The human Rad9/Rad1/Hus1 damage sensor clamp interacts*

with DNA polymerase beta and increases its DNA substrate utilisation efficiency: implications for DNA repair. Nucleic Acids Res, 2004. **32**(11): p. 3316-24.

24. Danielsen, J.M., et al., *HCLK2 Is Required for Activity of the DNA Damage Response Kinase ATR.* J Biol Chem, 2009. **284**(7): p. 4140-4147.
25. Smits, V.A., P.M. Reaper, and S.P. Jackson, *Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response.* Curr Biol, 2006. **16**(2): p. 150-9.
26. Dinant, C., et al., *Activation of multiple DNA repair pathways by sub-nuclear damage induction methods.* J Cell Sci, 2007. **120**(Pt 15): p. 2731-40.
27. Zotter, A., et al., *Recruitment of the nucleotide excision repair endonuclease XPG to sites of UV-induced dna damage depends on functional TFIIH.* Mol Cell Biol, 2006. **26**(23): p. 8868-79.
28. Fernandez-Capetillo, O., A. Celeste, and A. Nussenzweig, *Focusing on foci: H2AX and the recruitment of DNA-damage response factors.* Cell Cycle, 2003. **2**(5): p. 426-7.
29. Burtelow, M.A., S.H. Kaufmann, and L.M. Karnitz, *Retention of the human Rad9 checkpoint complex in extraction-resistant nuclear complexes after DNA damage.* J Biol Chem, 2000. **275**(34): p. 26343-8.
30. Inagaki, A., et al., *Dynamic localization of human RAD18 during the cell cycle and a functional connection with DNA double-strand break repair.* DNA Repair (Amst), 2009. **8**(2): p. 190-201.
31. Essers, J., et al., *Nuclear dynamics of PCNA in DNA replication and repair.* Mol Cell Biol, 2005. **25**(21): p. 9350-9.
32. Leonhardt, H., et al., *Dynamics of DNA replication factories in living cells.* J Cell Biol, 2000. **149**(2): p. 271-80.
33. de Laat, W.L., N.G.J. Jaspers, and J.H.J. Hoeijmakers, *Molecular mechanisms of nucleotide excision repair.* Genes Dev., 1999. **13**: p. 768-785.
34. Roos-Mattjus, P., et al., *Genotoxin-induced Rad9-Hus1-Rad1 (9-1-1) chromatin association is an early checkpoint signaling event.* J Biol Chem, 2002. **277**(46): p. 43809-12.
35. Ellison, V. and B. Stillman, *Biochemical Characterization of DNA Damage Checkpoint Complexes: Clamp Loader and Clamp Complexes with Specificity for 5' Recessed DNA.* PLoS Biol, 2003. **1**(2): p. E33.
36. Wu, X., S.M. Shell, and Y. Zou, *Interaction and colocalization of Rad9/Rad1/Hus1 checkpoint complex with replication protein A in human cells.* Oncogene, 2005. **24**(29): p. 4728-35.
37. Xu, X., et al., *The basic cleft of RPA70N binds multiple checkpoint proteins, including RAD9, to regulate ATR signaling.* Mol Cell Biol, 2008. **28**(24): p. 7345-53.
38. Byun, T.S., et al., *Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint.* Genes Dev, 2005. **19**(9): p. 1040-52.
39. Marti, T.M., et al., *H2AX phosphorylation within the G1 phase after UV irradiation depends on nucleotide excision repair and not DNA double-strand breaks.* Proc Natl Acad Sci U S A, 2006. **103**(26): p. 9891-6.
40. Branzei, D. and M. Foiani, *Regulation of DNA repair throughout the cell cycle.* Nat Rev Mol Cell Biol, 2008. **9**(4): p. 297-308.

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UV-induced G2 arrest is regulated by p38 and independent of the ATR-Chk1 pathway

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ABSTRACT

Genotoxic stress activates DNA damage checkpoints that cause a delay at multiple points during the cell cycle. The highly conserved G2 checkpoint is the last critical barrier before the physical separation of the cell into two daughter cells and prevents cells with DNA damage from entering mitosis. The ATR tranducer kinase is activated after the generation of single-stranded DNA intermediates that are formed in response to ultra violet light and replication types of DNA damage, and was thought to play an important role in the UV-induced G2 checkpoint. However, upon UV irradiation, Rad9, a component of the ATR-Chk1 pathway, localizes to sites of damage in G1 and S phase cells but not in G2 cells, unlike G2 cells treated with ionizing radiation. We set out to investigate why UV-induced DNA damage does not result in Rad9 focus formation in G2 cells and what are the implications of this observation. Like Rad9, RPA also does not localize to sites of DNA lesions in UV irradiated G2 cells, suggesting that single-stranded DNA intermediates are not generated or recognized properly. Further analysis of UV-treated G2 cells indicate that PIKK-dependent DNA damage signalling does not occur. On the contrary, the p38 MAP kinase, a protein involved in the checkpoint response, is phosphorylated in G2 phase cells after UV damage. PIKK-dependent signalling is dispensable for the UV-induced cell cycle arrest in G2 cells, unlike p38, which is partially responsible for the UV-induced G2 cell cycle arrest. However, the combined inhibition of the PIKK- and p38-dependent checkpoint pathways results in a complete abrogation of the G2/M cell cycle arrest after UV-irradiation, suggesting that these two pathways act redundantly, thereby regulating the G2/M transition after UV-induced damage.

INTRODUCTION

In response to genotoxic stress eukaryotic cells activate DNA damage checkpoint and repair pathways that ensure an intact transmission of the DNA into the next cell cycle phase [1-3]. Inefficient repair of DNA lesions leads to genomic instability, a source of cancer [4]. Exogenous DNA damaging agents cause different types of DNA alterations that will lead to activation of specific DNA repair and checkpoint mechanisms [5, 6]. The ATM and ATR kinases, members of the phosphatidyl inositol 3' *kinase*-related *(PIKK) kinases,* are the master regulators of the DNA damage-induced checkpoint response [7]. In response to double-stranded breaks (DSBs) ATM is activated leading to the phosphorylation and subsequent activation of effector kinase Chk2 [8]. In response to genotoxic stress induced by replication fork stalling, ultra violet (UV) damage and after the resection of DSBs, the ATR kinase is activated [9-12].

The single-stranded DNA (ssDNA) intermediates that are formed after these types of DNA damage are coated rapidly by RPA [13]. Thereafter, the Rad17-RFC and ATRIP-ATR complexes are independently recruited to sites of RPA-coated ssDNA and Rad17-RFC(2-4) then loads the Rad9-Rad1-Hus1 (9-1-1) PCNA-like sliding clamp onto chromatin [14-16]. Subsequently, TopBP1 binds to the C-terminus of Rad9 and activates ATR [17, 18]. An active ATR kinase phosphorylates a number of protein including the effector kinase Chk1, which results into a temporal halt of the cell cycle to gain time for DNA repair [7].

The recruitment of proteins to sites of DNA damage can be observed as the localization of these proteins into nuclear foci. Artificial localization of the Mec1 and Ddc1, the *S. cerevisiae* equivalents of ATR and Rad9 is enough to trigger a checkpoint response in the absence of DNA damage [19]. This suggests that ATR activation and subsequent checkpoint triggering is dependent on the close proximity of these two complexes. We previously reported that in response to DNA damage mammalian Rad9 localizes to sites of damage in a cell cycle-dependent manner, depending on the type of DNA lesion. In response to ionizing radiation (IR), Rad9 localizes into foci in S and G2 cells [12, 20, 21], whereas UV light induces Rad9 foci in G1 and S phase cells [22]. Interestingly, UV-induced Rad9 focus formation does not occur in G2 cells [22]. We were interested in why Rad9 does not localize to sites of UV damage in G2 cells and what the implications are for checkpoint activation. Therefore we analyzed the nuclear localization of different DNA damage response proteins in G2 cells upon UV-irradiation. We show that PIKK kinase-mediated signalling is dispensable for UV-induced damage signalling and subsequent cell cycle arrest in G2 cells, probably because RPA-coated ssDNA intermediates are not formed. Conversely, we show that the p38 MAP kinase is activated in G2 cells in response UV irradiation, and (partially) required to prevent cells from entering mitosis. Interestingly, combined inhibition of PIKK and p38 kinases leads to a complete loss of the UV-induced G2 checkpoint arrest suggesting that ATM/ATR might act redundantly in the absence of p38 and thereby functioning in parallel to the p38 pathway. Collectively these results suggest that checkpoint activation in G2 after UV-induced DNA lesions mostly depends on p38.

MATERIALS AND METHODS

Cell culture

U2OS, HeLa and T24 cells were grown using standard procedures. U2OS cells stably expressing eGFP-HA_2-Rad9 (GFP-Rad9) were grown in standard medium supplemented with 700 μg/ml G418. For stable expression of Cyclin B-mCherry, GFP-Rad9 expressing U2OS cells were transfected with Cyclin B-mCherry (kindly provided by R. Wolthuis, NKI, Amsterdam, The Netherlands) together with a pBabepuro plasmid. Positive clones were picked after a selection with 20 μg/ml of puromycin for two weeks. Thereafter, stable clones expressing GFP-Rad9 and Cyclin B-mCherry were grown in medium supplemented with G418 and puromycin.

Transfection and plasmids

Plasmid DNA was transfected into cells using the calcium phosphate transfection method. pCI neo Flag-Chk1 wt and S317A/S345A were a kind gift from Jiri Bartek (Institute of Cancer Biology and Centre for Genotoxic Stress Research, Danish Cancer Society, Copenhagen, Denmark).

Cell synchronization

To synchronize T24 cells in G0, cells were kept confluent for 5 days, changing the medium every day. Cells were released from the arrest by trypsinization and replating in low dilutions and then fixed at the indicated time points.

Antibodies and inhibitors

Antibodies obtained from commercial sources were as following: RPA (Ab2, Oncogene), Flag (Sigma), MPM2, γH2AX and pATM (Upstate Biotechnology), 53BP1, SMC1, pS1083 SMC3 (Bethyl), pThr180/Tyr182 p38, pS317 and pS345 Chk1 (Cell Signaling), the following antibodies were obtained from Santa Cruz Biotechnology: Cyclin A (H-432), Cyclin B1 (GNS1), Ku86 (C-20), p38 (C-20), Cdc25A (F-6) and Chk1 (G-4).
Caffeine (Sigma), p38 inhibitor BIRB 796 (Axon Medchem) and Chk1 inhibitor SB 218078 (Calbiochem) were administrated 30 minutes before UV treatment and directly after the treatment the medium including the inhibitors was added back to the cells.

G2 checkpoint assay

U2OS cells were UV- or mock-irradiated with 20 J/m^2 and incubated for 1 hr at 37°C. Subsequently, they were collected by trypsinization and fixed in 70% ethanol overnight at 4°C. After fixation, cells were washed with PBS and incubated with MPM2 antibody for 1 hr at 4°C. After washing with PBS, cells were incubated with a Cy5-labeled secondary antibody (Jackson Immunoresearch) for 30 min at 4°C, followed by staining the DNA with propidium iodide (PI). MPM2 and PI staining were analyzed by flow cytometry with FACSCaliber and CellQuest Pro software (Becton Dickinson).

Unscheduled DNA synthesis (UDS) assay

The UDS assay was performed as described previously [23]. Briefly, U2OS cells were untreated or UV-irradiated with 16 J/m^2 and then incubated in medium containing EdU (5-ethynyl-2'-deoxyuridine) for 1 hour before fixation with PFA. EdU incorporation was visualized using Click-iT Alexa Fluor 488 according to the manufacturer's protocol (Invitrogen).

Immunofluorescence

For immunostaining, cells were fixed in 2% paraformaldehyde containing 0.2% Triton X-100 for 20 minutes at RT and then permeabilized with 0.1% Triton X-100 for 5 minutes. Samples were blocked in 1% FCS and immunostained with antibodies as indicated. For detection of GFP-Rad9 or Cyclin B-mCherry in U2OS cells, living cells were studied, or cells were fixed and permeabilized as above.

In all instances, error bars on graphs represent the standard error of the mean of three independent experiments.

Images were made using a Cell Observer fluorescent microscope equipped with Axiovision software (Zeiss) or a Confocal Laser Scanning Microscope LSM 510 (Zeiss), equipped with a 488 nm Ar-laser and a 505-530 nm band pass filter for green fluorescence. Red fluorescence was detected using a 543 nm laser and 560 nm long pass filter. For strip-FRAP experiments and time lapse imaging after UV laser induction, GFP-Rad9 and Cyclin B-mCherry were detected in living cells using a Confocal Laser Scanning Microscope LSM 510 (Zeiss), equipped with a 488 nm Ar-laser and a 505-530 nm band pass filter for green fluorescence. Red fluorescence was detected using a 543 nm laser and 575-615 nm band pass filter.

Generation of DNA damage and photobleaching techniques

UV-irradiation was performed using a 254 nm UV-C lamp (Philips) at 20 J/m^2 and cells were processed 1 hour post treatment. IR (10 Gy) was induced using a ^{137}Cs source or a linear accelerator (LINAC) and cells were processed 1 or 2 hours after treatment.

In the FRAP experiments, a strip spanning the nucleus was photobleached for 20 milliseconds using an Ar-laser (488 nm) at 100% laser intensity, which irreversibly bleaches all GFP molecules in that area. Subsequently, the redistribution of fluorescence in the strip was monitored taking confocal images every 20 milliseconds for a total of 25 seconds at low laser intensity to avoid further bleaching. The fluorescence before bleaching ($I_{t<0}$) was set to 1 and the intensity immediately after bleaching (I_0) was set to 0. The recovery of fluorescence was plotted against time.

RESULTS

Less RPA focus formation and Rad9 immobilization after UV damage in G2

We previously reported that in response to DNA damage Rad9, a component of the Rad9-Rad1-Hus1 putative DNA damage sensor complex, localizes to sites of damage in a cell cycle-dependent manner [22]. Rad9 localization after DNA damage is dependent on the formation of RPA-coated ssDNA intermediates that are generated after many types of genotoxic stress including UV light and IR [16]. Therefore we investigated if RPA localizes to sites of UV-induced damage in G2 cells and determined RPA focus formation together with a Cyclin A immunofluorescence staining (Figure 1A). Cyclin A protein levels increase from S to G2 phase enabling us to discriminate all phases of the cell cycle [12, 22]. RPA localized into foci in S and G2 cells after IR, whereas UV-treatment induced RPA focus formation in G1 and S, but much less in G2 phase cells. These results suggest that RPA-coated ssDNA intermediates are not formed in UV-irradiated G2 cells.

The most common UV-induced DNA lesions are 6-4 photoproducts and cyclobutane pyrimidine dimers that are mainly repaired by nucleotide-excision repair (NER). ATR-dependent checkpoint activation after UV in G1 phase is (partially) dependent on NER

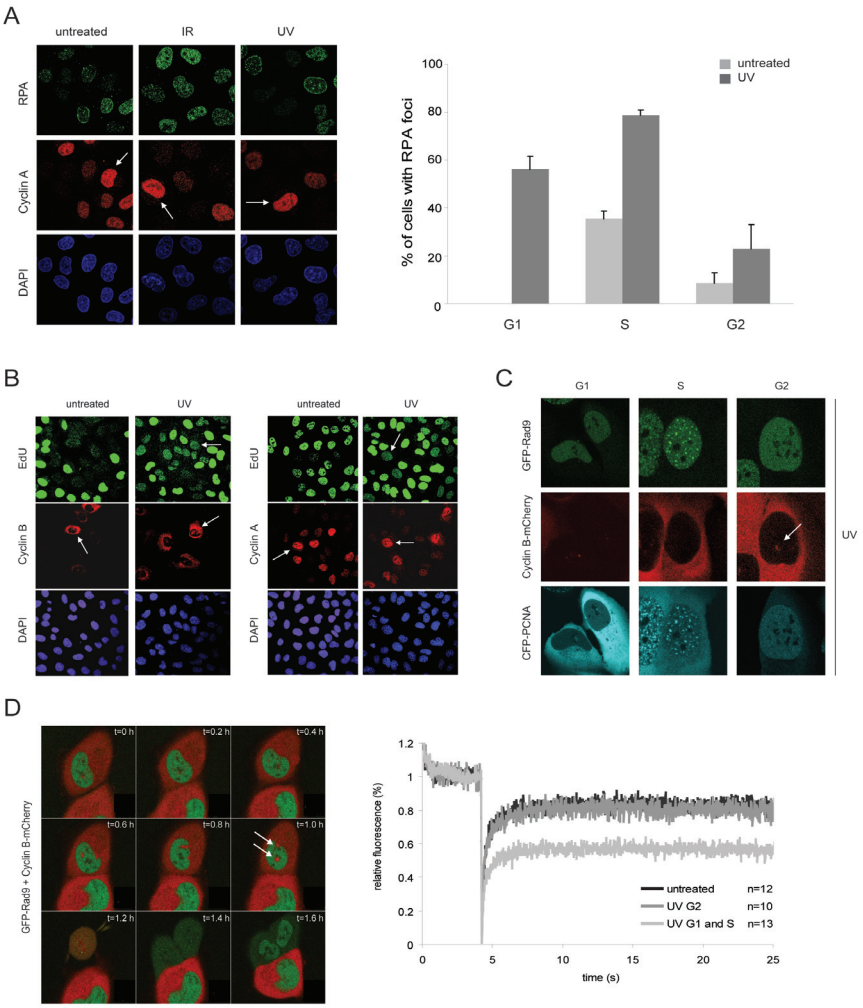

Figure 1: Cell cycle-dependent RPA focus formation and Rad9 immobilization (A) U2OS cells were untreated or treated with IR (10 Gy) or UV (20 J/m^2). Two or one hour later, respectively. cells were fixed and stained using immunofluorescence with antibodies against RPA and Cyclin A. Cells without Cyclin A are in G1 phase, with intermediate levels in S and G2 phase cells display high Cyclin A levels (arrow). Right panel: the percentage of cells with RPA foci in the different phases of the cell cycle. (B) U2OS cells were incubated with nucleotide analogue EdU as describe in materials and methods. Cells were left untreated or UV-irradiated, fixed after one hour and stained using immunofluorescence with the indicated antibodies. G2 cells are indicated with an arrow. (C) U2OS cells stably expressing GFP-Rad9 and Cyclin B-mCherry were transiently transfected with CFP-PCNA and 48 hours later UV-irradiated, fixed after one hour and analyzed by direct immunofluorescence. The arrow indicates centrometric Cyclin B-mCherry. (D) Time-lapse analysis of stable GFP-Rad9 and Cyclin B-mCherry expressing cells, indicating that during G2, Cyclin B-mCherry localizes into centromeres. Cells expressing GFP-Rad9 and Cyclin B-mCherry were left untreated or treated with UV light. Then FRAP analysis was performed. Briefly, a small strip of the nucleus was bleached for 20 msec. at 100% laser intensity, after which redistribution of fluorescence was monitored every 20 msec. Cells were divided into the group of G1/S or G2 phase cells based on the presence of centromeric Cyclin B-mCherry.

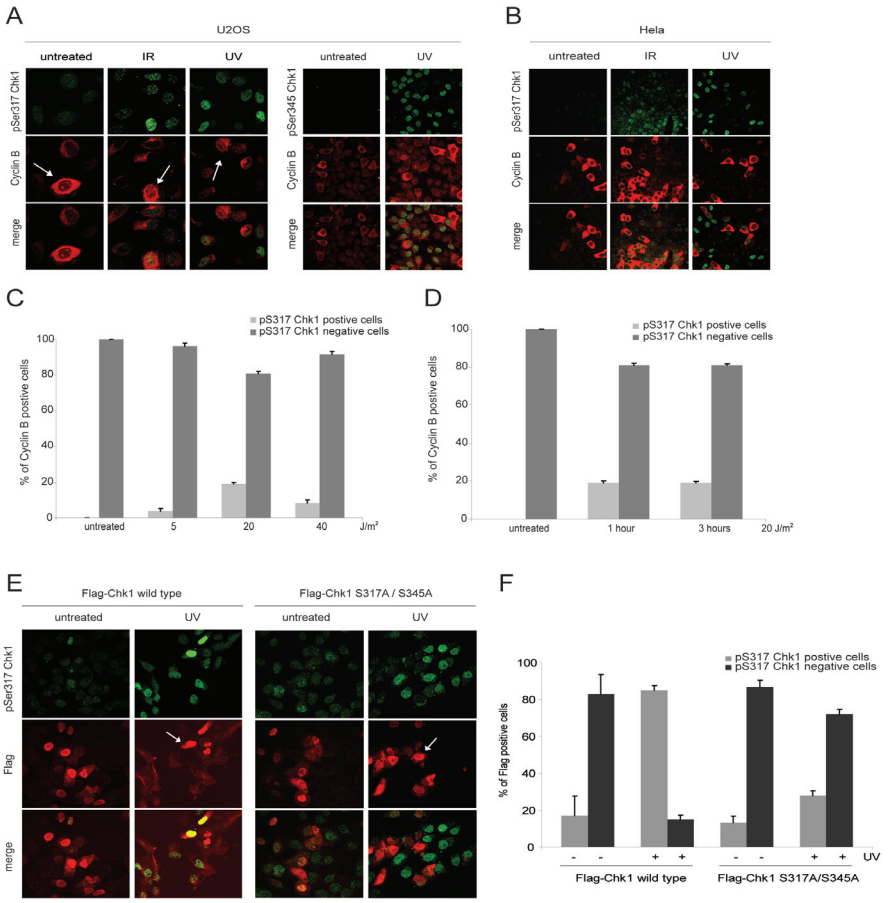

Figure 2: UV-induced ATR-mediated checkpoint activation is absent in G2 cells (A) U2OS cells were untreated or exposed to IR (10 Gy) or UV (20 J/m^2), fixed two or one hour later respectively and analyzed by immunofluorescence with the indicated antibodies. G2 cells are indicated with an arrow. (B) As performed in (A) but with HeLa cells. (C) U2OS cells were treated with different doses of UV light (5, 20, 40 J/m^2) and fixed one hour later. Shown is the percentage of Cyclin B-positive cells that were either negative or positive for phosphorylated Ser317 Chk1. Total number of cells in each cell cycle phase was set to 100%. (D) Similar as in (C) but now after one and three hours of UV (20 J/m^2). (E) U2OS cells were transfected with wild type or S317A/S345A Flag-labelled Chk1. 48 hours after transfection cells were UV-treated, fixed one hour later and analyzed by immunofluorescence using the indicated antibodies. (F) As performed in (E), shown is the percentage of Flag-positive cells that were either negative or positive for phosphorylated Ser317 Chk1.

[22, 24, 25]. NER is most probably required for the formation of small ssDNA gaps that can trigger ATR-mediated checkpoint activation. We investigated if NER is cell cycle regulated, which could explain why we observe less RPA focus formation in G2 cells after UV irradiation. UV-treated cells were incubated with nucleotide analogue EdU, which is incorporated in newly synthesized DNA in S phase and during NER [23]. The cell cycle phase was determined by staining with antibodies for Cyclin A or Cyclin B. The latter localizes in the cytoplasm during G2 phase and can therefore be used as a marker for G2 cells. In untreated cells EdU incorporation was only present in replicating cells, whereas after UV irradiation EdU

incorporation was also present in G1 and G2 cells indicating that NER is active during all cell cycle phases (Figure 1B). This indicates that in G2 cells NER is proficient but is not sufficient for the formation of extended RPA-coated ssDNA intermediates that trigger ATR signalling.
In response to UV damage GFP-Rad9 is recruited to sites of damage seen as the formation of nuclear foci and thereby GFP-Rad9 becomes immobilized on the chromatin [26]. GFP-Rad9 immobilization is transient since there is a constant turnover of GFP-Rad9 molecules into DNA damage-induced GFP-Rad9 foci, which makes these foci highly dynamic structures [22]. To specifically address GFP-Rad9 focus formation and its subsequent immobilization in G2 cells, we generated a cell line stably expressing GFP-Rad9 together with Cyclin B-mCherry (Figure 1C). During S and G2, Cyclin B-mCherry protein levels rise and Cyclin B localizes in the cytoplasm. Shortly before mitosis Cyclin B1 appears on centrosomes seen as one or two nuclear spots [27, 28] (Figure 1C and 1D left panel). We transiently co-expressed a CFP-labelled version of PCNA to determine GFP-Rad9 focus formation and centromeric Cyclin B1-mCherry after UV-irradiation in all three cell cycle phases. As shown in Figure 1C, cells with centromeric Cyclin B-mCherry do not show CFP-PCNA foci indicating these cells are in G2.
GFP-Rad9 immobilization was determined by Fluorescent Recovery After Photobleaching (FRAP) in cells co-expressing Cyclin B-mCherry [29]. GFP-Rad9 showed a 20-30% immobilization of GFP-Rad9 after UV damage in G1 and S-phase cells. In contrast, UV-treated G2 cells did not show any immobilized GFP-Rad9 as the recovery of fluorescence was similar to untreated cells (Figure 1D). Additionally, time-lapse video microscopy of UV-treated cells did not show formation of GFP-Rad9 foci in G2 cells over a 24 hour period (data not shown). Collectively these experiments suggest that during G2, GFP-Rad9 does not become immobilized by UV-induced DNA damage, possibly due to the absence of RPA-coated ssDNA intermediates that are long enough to elicit a checkpoint response [30].

UV-induced ATR-mediated Chk1 phosphorylation is absent in G2 cells

The observed decreased RPA focus formation and the high mobility of Rad9 after UV damage in G2 cells prompted us to study the consequences for ATR-mediated checkpoint signalling. We therefore investigated the phosphorylation of effector kinase Chk1 after UV and IR-treatment by immunofluorescence. G2 cells were identified by staining for endogenous Cyclin B1. In response to UV damage in U2OS cells, Chk1 was not phosphorylated on Ser317 in cells positive for cytoplasmic Cyclin B, whereas treatment with IR did result in Chk1 phosphorylation (Figure 2A left panel). Similar results were obtained in HeLa cells and using antibodies against phosphorylated Ser345, the other ATR-mediated phosphorylation site of Chk1 (Figure 2A right panel, 2B). Moreover, Chk1 phoshorylation did not change after treatments with higher (40 J/m^2) and lower (5 and 10 J/m^2) doses of UV (Figure 2C). Experiments performed 3 hour after UV irradiation showed similar result as 1 hour after UV-treatment, indicating that UV dose and time after irradiation did not influence the amount of phosphorylated Chk1 in G2 cells (Figure 2D). To address the specificity of the antibody against phosphorylated Ser317 of Chk1 we transfected U2OS cells with a mutant form of Chk1 that cannot be phosphorylated on Ser317 and Ser345. Transient expression of this Flag-tagged version of Chk1 worked as a dominant-negative since cells staining positive for Flag did not show phosphorylation of Chk1 [31] (Figure 2E). Hereby we show that effector kinase Chk1 is not phosphorylated in G2 cells after UV-irradiation.

PIKK-dependent UV response is absent in G2 cells

Our data show that UV-induced DNA lesions did not induce the phosphorylation of Chk1 in G2 cells. We investigated the localization and activation of other DNA damage signalling components in G2 phase after UV damage. Cells were treated with UV or IR and immunofluorescence was performed for several proteins involved in the PIKK-dependent DNA damage response, namely 53BP1, SMC1 and the phosphorylated forms of ATM and H2AX. Additionally we stained cells for the phosphorylated form of the p38 MAP kinase, which is implicated in the UV-induced G2 arrest [32]. Cells were co-stained for Cyclin A or B to access the cell cycle phases. Figure 3A shows that in response to IR, 53BP1 and SMC1 formed foci and ATM and H2AX were phosphorylated and also localized to sites of damage in G2 cells, unlike p38, which was not phosphorylated (Figure 3A). In contrast, in response to UV light, p38 was phosphorylated during all phases of the cell cycle, whereas 53BP1, SMC1, γH2AX and pS1981 ATM did not localize into foci during the G2 phase in response to this type of DNA damage.

Next, the experiments were performed in synchronized T24 cells to validate the absence of PIKK-dependent signalling after UV damage and confirm the presence of phosphorylated p38. At different stages of the cell cycle, cells were UV-irradiated and PIKK-dependent signalling was analyzed by western blotting. FACS analysis demonstrated that 30 to 31 hours after the release from G_0 approximately 50% of the total cell population was in G2/M (Figure 3B left panel). Cells treated with UV damage at this time point showed decreased levels of phopshorylated Chk1, H2AX, ATM and SMC3 when compared to asynchronous or S-phase cell populations (t=26h) (Figure 3B right panel). Also the DNA damage-induced degradation of Cdc25A was decreased at the 30 and 31 hour time points. Together these results demonstrate the absence of a PIKK-mediated DNA damage signalling in G2 after UV damage. Alternatively, p38 is phoshorylated at every time point in this experiment in response to UV damage. The presence of phosphorylated p38 confirms a possible role for this kinase in the UV-induced cell cycle arrest in G2 cells.

p38-dependent cell cycle arrest in G2 upon UV irradiation

After DNA damage the G2 checkpoint is the last critical barrier before the physical separation of the cell into two daughter cells [33, 34]. After IR, PIKK-dependent targeting of the Cdc25A phosphatase inhibits the dephosphorylation of Cdk1 resulting in a temporal cell cycle arrest [35, 36]. After UV damage the p38 MAP kinase is thought to control a cell cycle arrest via Cdc25B [32, 37-40]. However it remained unclear if ATM/ATR also play a role in this checkpoint, possibly by regulating p38 [41]. Alternatively, PIKKs and p38 signalling might be parallel pathways controlling the UV-induced G2 arrest. To investigate how the UV-induced cell cycle arrest in G2 cells is regulated we analyzed the percentage of cells that enters mitosis after UV-irradiation. After treatment with UV light, cells were incubated with a low dose of aphidicolin to inhibit S phase cells to progress into mitosis during the time course of the experiment. FACS analysis demonstrated that one hour after UV-irradiation cells stopped entering mitosis (Figure 4A, left panels). In addition, time-lapse analysis of GFP-Rad9 and Cyclin B-mCherry expressing cells showed that G2 cells do not enter mitosis after this dose of UV-irradiation (data not shown). To investigate if the UV-induced cell cycle arrest was dependent on ATM and ATR, cells were incubated with caffeine, thereby inhibiting PIKK kinase activation. Caffeine-treatment did not rescue the G2 arrest in response to UV light (Figure 4A). However, treatment of cells with BIRB 796, a specific p38 inhibitor, partially abrogated (50%) the UV-induced G2 arrest. Interestingly, combined treatment with

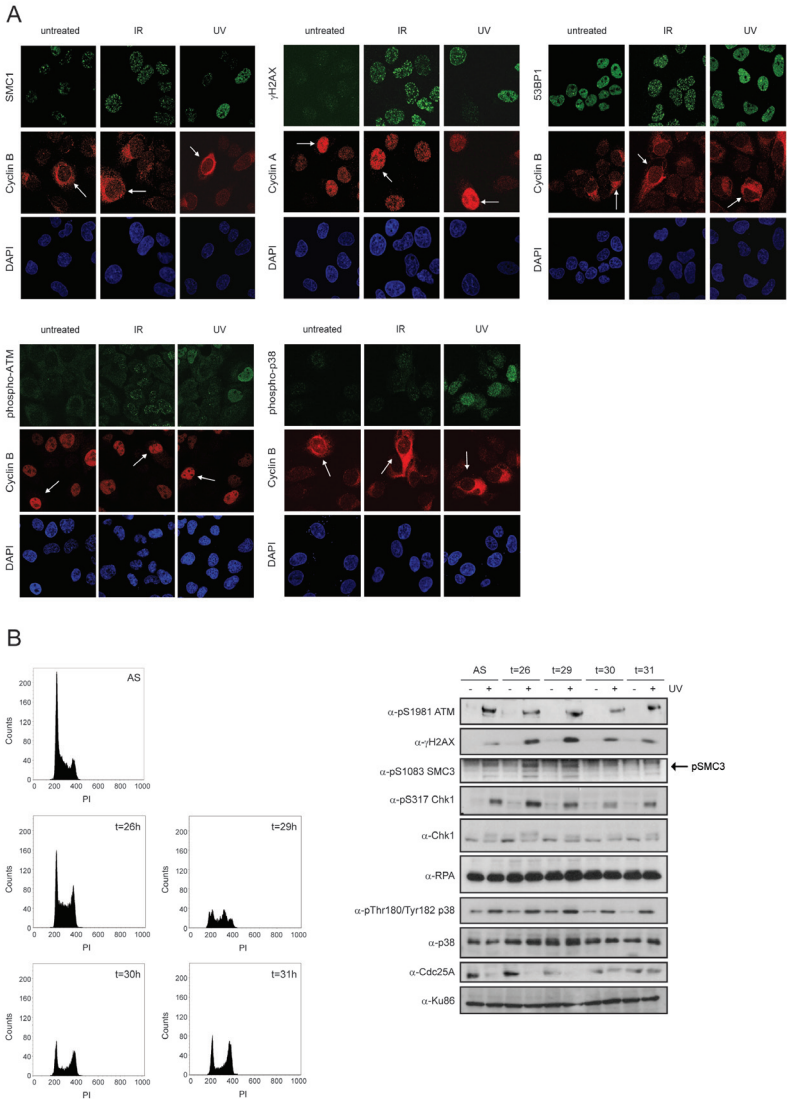

Figure 3: UV-induced DNA damage response of the ATR pathway is absent in G2 cells (A) U2OS cells were either untreated, treated with IR (10 Gy) and fixed after two hours or UV (20 J/m^2) irradiated and fixed one hour later. Cells were analyzed by immunofluorescence using the indicated antibodies. (B) T24 cells were synchronized, released and at the indicated time points, left untreated or UV-treated and fixed or lysed one hour after the treatment. (Left panel) Shown are the (PI) cell cycle profiles of the cells before UV-irradiation .(Right panel) Western blot analysis was performed using the indicated antibodies.

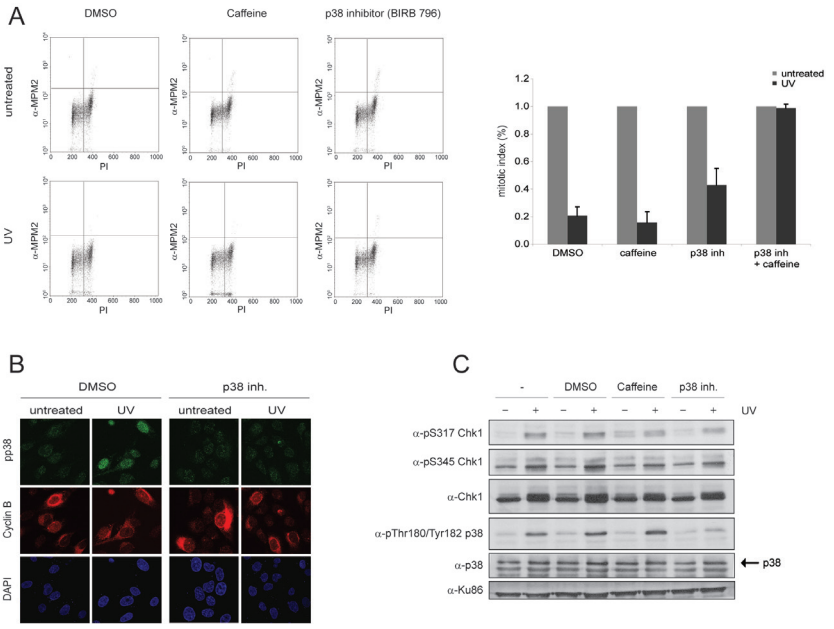

Figure 4: UV-induced cell cycle arrest in G2 (A) U2OS cells were incubated with DMSO, caffeine (5mM) or BIRB 796 (40 μM) for 30 minutes before UV-irradiation (20 J/m^2). One hour later cells were fixed, stained for MPM2 and analyzed by FACS (Left panel). Shown is the percentage of mitotic cells after the different treatments, error bars represent the SEM of at least three independent experiments. The percentage of mitotic cells in the untreated situation was set to 100% (Right panel). (B) U2OS cells were incubated with DMSO or BIRB 796 (40 μM) for 30 minutes before UV-irradiation (20 J/m^2), fixed and analyzed by immunofluorescence with the indicated antibodies. (C) Cells were treated as in (A) but now lysed one hour after UV irradiation and analyzed by western blotting using the indicated antibodies.

caffeine and p38 inhibitor led to a complete loss of the UV-induced arrest in G2 cells (Figure 4A). To test the specificity of the p38 inhibitor, the phosphorylation of p38 was analyzed by immunofluorescence. Incubation of the cells with BIRB 796 completely abrogates the UV-induced phosphorylation of p38 (Figure 4B). Western blotting gave the same result: UV-induced p38 phosphorylation is inhibited by pre-incubation with BIRB 796. In contrast, caffeine treatment only mildly reduced Chk1 phosphorylation after UV (Figure 4C). Although these results show that caffeine did not completely inhibit PIKK kinase activity after UV under these conditions, it was enough to abrogate the G2-mediated cell cycle arrest after IR (data not shown). In addition, caffeine treatment did not inhibit UV-induced p38 phosphorylation, indicating that ATM/ATR are not involved in p38 regulation in response to UV light (Figure 4C).

Taken together, these results suggest that the p38 kinase is primarily required for the G2 phase arrest after UV light. ATM/ATR might act redundantly in the absence of p38 and thereby functioning in parallel to the p38 pathway.

DISCUSSION

Several studies have previously reported that the ATR-Chk1 pathway is required for the G2 checkpoint response after DNA damage. Chk1 deficient vertebrate DT40 cells show a G2 checkpoint defect after IR [42] and Takai et al. demonstrated that Chk1 deficient embryos do not arrest at the G2/M border after exposure to UV light [43], indicating that the ATR-Chk1 pathway is involved in the G2 checkpoint after different types of genotoxic stress. We observed however that Rad9, a component of the Rad9-Rad1-Hus1 complex in the ATR-mediated checkpoint pathway, did not localize to sites of damage in G2 cells after UV-irradiation. Additionally ATRIP, Hus1 and Rad1 also did not localize into UV-induced foci specifically in G2 cells, unlike after IR (data not shown). Moreover we observed less localization of RPA into foci in G2. The binding of RPA to ssDNA, generated after genotoxic stress, is required for the recruitment of the 9-1-1 complex and ATRIP to damaged chromatin therefore we suggest that the absence of Rad9 immobilization and RPA focus formation after UV-irradiation is because ssDNA intermediates cannot be formed efficiently or are inaccessible to RPA molecules. The formation of UV-induced ssDNA intermediates in G1 cells is partially dependent on NER [22, 25]. But as our data show that NER is active during all phases of the cell cycle (Figure 1B), defective NER cannot be responsible for the absence of ssDNA intermediates. IR-induced DSBs in G2 are efficiently repaired by homologous recombination, and specifically require ATM and the nuclease Artemis, due to the inaccessibility of heterochromatin during this phase of the cell cycle [44]. We therefore speculate that nucleases that are normally involved in the resection of UV types of DNA lesions might be unable to access UV damage in G2.

Athough our data suggest that RPA-coated ssDNA intermediates cannot be formed efficiently, UV-induced DNA damage leads to a cell cycle arrest in G2 cells. The underlying mechanism for the UV-induced G2 checkpoint arrest was unclear though, since a number of studies published seemingly conflicting results. PIKK-dependent checkpoint signalling is required for the G2 checkpoint after IR and also in response to UV damage ATR signalling was suggested to play a role [45, 46]. In addition, the p38 MAP kinase pathway is implicated in the UV-induced checkpoint response at the G2-M transition by regulating Cdc25B protein levels [32, 37-39]. The question remained if the PIKK- and p38-dependent checkpoint pathways operate independently or together and separate or in parallel? Stiff et al. reported that the UV-induced G2 arrest requires NER-dependent ATR activation [45]. This was supported by a study that determined the phosphorylation of Chk1 in different NER deficient cell lines, thereby showing the phosphorylation of Chk1 was NER-dependent [25]. Although we observed that NER is active in G2 cells treated with UV, we did not detect the localization of RPA, SMC1 and 53BP1 into UV-induced foci. Moreover we were unable to detect the phosphorylation ATM, SMC3, H2AX and downstream effector kinase Chk1 in G2 cells treated with UV. Furthermore we show that PIKK-dependent signalling is dispensable for the G2 checkpoint-mediated cell cycle arrest directly after UV, but show that this response is partially dependent on the p38 MAPK. In support of our findings, Reinhardt et al. and Bulavin et al. have shown that the UV-induced damage response, unlike other types of genotoxic stress, is independent of the PIKK kinases but requires the p38 MAP kinase signalling pathway [32, 39, 47]. The discrepancy between these results and those from Stiff et al. could be coming from the different cell types that are used. It would therefore be interesting to study the UV-induced p38 activation in untransformed cells.

A model was proposed suggesting that the p38 MAP kinase might play a role in the initial

response to UV damage in G2, whereas the PIKK kinases maintain checkpoint activation at a longer time period [41]. Therefore the p38 and PIKK-mediated checkpoint responses may act in parallel. This would explain our observation that inhibition of p38 alone partially inhibits the G2 arrest whereas combined inhibition using caffeine and p38 inhibitor completely abrogates UV-induced cell cycle arrest. Thereby the PIKK-dependent response may take over G2 checkpoint regulation after p38 inhibition indicating that the ATM/ATR-dependent pathway acts redundantly. Future studies specifically investigating the initiation and maintenance of the UV-induced G2 arrest are required to verify this hypothesis.

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REFERENCES

1. Shiloh, Y., *ATM and related protein kinases: safeguarding genome integrity.* Nat Rev Cancer, 2003. **3**(3): p. 155-68.
2. Kanaar, R., C. Wyman, and R. Rothstein, *Quality control of DNA break metabolism: in the 'end', it's a good thing.* Embo J, 2008. **27**(4): p. 581-8.
3. Hoeijmakers, J.H., *Genome maintenance mechanisms for preventing cancer.* Nature, 2001. **411**(6835): p. 366-74.
4. Jeggo, P.A. and M. Lobrich, *Contribution of DNA repair and cell cycle checkpoint arrest to the maintenance of genomic stability.* DNA Repair (Amst), 2006. **5**(9-10): p. 1192-8.
5. Lazzaro, F., et al., *Checkpoint mechanisms at the intersection between DNA damage and repair.* DNA Repair (Amst), 2009.
6. Warmerdam, D.O. and R. Kanaar, *Dealing with DNA damage: Relationships between checkpoint and repair pathways.* Mutat Res, 2009.
7. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes Dev, 2001. **15**(17): p. 2177-96.
8. Shiloh, Y., *The ATM-mediated DNA-damage response: taking shape.* Trends Biochem Sci, 2006. **31**(7): p. 402-10.
9. Callegari, A.J. and T.J. Kelly, *Shedding light on the DNA damage checkpoint.* Cell Cycle, 2007. **6**(6): p. 660-6.
10. Cimprich, K.A. and D. Cortez, *ATR: an essential regulator of genome integrity.* Nat Rev Mol Cell Biol, 2008. **9**(8): p. 616-27.
11. Shiotani, B. and L. Zou, *ATR signaling at a glance.* J Cell Sci, 2009. **122**(Pt 3): p. 301-4.
12. Jazayeri, A., et al., *ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks.* Nat Cell Biol, 2006. **8**(1): p. 37-45.
13. Callegari, A.J., et al., *Postreplication gaps at UV lesions are signals for checkpoint activation.* Proc Natl Acad Sci U S A. **107**(18): p. 8219-24.
14. Shiotani, B. and L. Zou, *Single-stranded DNA orchestrates an ATM-to-ATR switch at*

DNA breaks. Mol Cell, 2009. **33**(5): p. 547-58.
15. Zou, L. and S.J. Elledge, *Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.* Science, 2003. **300**(5625): p. 1542-8.
16. Zou, L., D. Liu, and S.J. Elledge, *Replication protein A-mediated recruitment and activation of Rad17 complexes.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 13827-32.
17. Kumagai, A., et al., *TopBP1 activates the ATR-ATRIP complex.* Cell, 2006. **124**(5): p. 943-55.
18. Lee, J., A. Kumagai, and W.G. Dunphy, *The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR.* J Biol Chem, 2007. **282**(38): p. 28036-44.
19. Bonilla, C.Y., J.A. Melo, and D.P. Toczyski, *Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage.* Mol Cell, 2008. **30**(3): p. 267-76.
20. Huertas, P., et al., *CDK targets Sae2 to control DNA-end resection and homologous recombination.* Nature, 2008. **455**(7213): p. 689-92.
21. Huertas, P. and S.P. Jackson, *Human CtIP mediates cell cycle control of DNA end resection and double strand break repair.* J Biol Chem, 2009. **284**(14): p. 9558-65.
22. Warmerdam, D.O., et al., *Cell cycle-dependent processing of DNA lesions controls localization of Rad9 to sites of genotoxic stress.* Cell Cycle, 2009. **8**(11).
23. Nakazawa, Y., et al., *A semi-automated non-radioactive system for measuring recovery of RNA synthesis and unscheduled DNA synthesis using ethynyluracil derivatives.* DNA Repair (Amst).
24. Giannattasio, M., et al., *Physical and functional interactions between nucleotide excision repair and DNA damage checkpoint.* Embo J, 2004. **23**(2): p. 429-38.
25. Marini, F., et al., *DNA nucleotide excision repair-dependent signaling to checkpoint activation.* Proc Natl Acad Sci U S A, 2006. **103**(46): p. 17325-30.
26. Medhurst, A.L., et al., *ATR and Rad17 collaborate in modulating Rad9 localisation at sites of DNA damage.* J Cell Sci, 2008. **121**(Pt 23): p. 3933-40.
27. Hagting, A., et al., *Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal.* Curr Biol, 1999. **9**(13): p. 680-9.
28. Jackman, M., et al., *Active cyclin B1-Cdk1 first appears on centrosomes in prophase.* Nat Cell Biol, 2003. **5**(2): p. 143-8.
29. Houtsmuller, A.B., *Fluorescence recovery after photobleaching: application to nuclear proteins.* Adv Biochem Eng Biotechnol, 2005. **95**: p. 177-99.
30. MacDougall, C.A., et al., *The structural determinants of checkpoint activation.* Genes Dev, 2007. **21**(8): p. 898-903.
31. Smits, V.A., P.M. Reaper, and S.P. Jackson, *Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response.* Curr Biol, 2006. **16**(2): p. 150-9.
32. Bulavin, D.V., et al., *Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase.* Nature, 2001. **411**(6833): p. 102-7.
33. Lobrich, M. and P.A. Jeggo, *The impact of a negligent G2/M checkpoint on genomic instability and cancer induction.* Nat Rev Cancer, 2007. **7**(11): p. 861-9.
34. O'Connell, M.J. and K.A. Cimprich, *G2 damage checkpoints: what is the turn-on?* J Cell Sci, 2005. **118**(Pt 1): p. 1-6.
35. Falck, J., et al., *The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis.* Nature, 2001. **410**(6830): p. 842-7.
36. Mailand, N., et al., *Rapid destruction of human Cdc25A in response to DNA damage.*

Science, 2000. **288**(5470): p. 1425-9.
37. Lemaire, M., B. Ducommun, and A.R. Nebreda, *UV-induced downregulation of the CDC25B protein in human cells.* FEBS Lett. **584**(6): p. 1199-204.
38. Lemaire, M., et al., *CDC25B phosphorylation by p38 and MK-2.* Cell Cycle, 2006. **5**(15): p. 1649-53.
39. Manke, I.A., et al., *MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation.* Mol Cell, 2005. **17**(1): p. 37-48.
40. Mikhailov, A., M. Shinohara, and C.L. Rieder, *The p38-mediated stress-activated checkpoint. A rapid response system for delaying progression through antephase and entry into mitosis.* Cell Cycle, 2005. **4**(1): p. 57-62.
41. Bulavin, D.V., S.A. Amundson, and A.J. Fornace, *p38 and Chk1 kinases: different conductors for the G(2)/M checkpoint symphony.* Curr Opin Genet Dev, 2002. **12**(1): p. 92-7.
42. Zachos, G., M.D. Rainey, and D.A. Gillespie, *Chk1-deficient tumour cells are viable but exhibit multiple checkpoint and survival defects.* Embo J, 2003. **22**(3): p. 713-23.
43. Takai, H., et al., *Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice.* Genes Dev, 2000. **14**(12): p. 1439-47.
44. Beucher, A., et al., *ATM and Artemis promote homologous recombination of radiation-induced DNA double-strand breaks in G2.* Embo J, 2009. **28**(21): p. 3413-27.
45. Stiff, T., et al., *Replication independent ATR signalling leads to G2/M arrest requiring Nbs1, 53BP1 and MDC1.* Hum Mol Genet, 2008. **17**(20): p. 3247-53.
46. Stiff, T., et al., *ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling.* Embo J, 2006. **25**(24): p. 5775-82.
47. Reinhardt, H.C., et al., *p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage.* Cancer Cell, 2007. **11**(2): p. 175-89.

6

ATR and Rad17 collaborate in modulating Rad9 localization at sites of DNA damage

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ABSTRACT

The cell cycle checkpoint kinase Chk1 is phosphorylated and activated by ATR in response to DNA damage and is critical for initiating the DNA damage response. A number of factors act in concert with ATR to facilitate Chk1 phosphorylation including Rad17-RFC, the Rad9-Rad1-Hus1 complex, TopBP1 and Claspin. Rad17 is required for loading of Rad9-Rad1-Hus1 (9-1-1) onto sites of DNA damage. Although phosphorylation of Rad17 by ATR is required for checkpoint function, how this impacts on 9-1-1 regulation remains unclear. We report that exposure of cells to DNA damage or replication stress results in Rad17-dependent immobilization of Rad9 into nuclear foci. Furthermore, expression of mutant Rad17 that cannot be phosphorylated by ATR ($Rad17^{AA}$), or downregulation of ATR, results in a decreased number of cells that display Rad9 foci. Photobleaching experiments reveal an increase in the dynamic behaviour of Rad9 within remaining foci in the absence of ATR or following expression of $Rad17^{AA}$. Together these data suggest a model in which Rad17 and ATR collaborate in regulating Rad9 localization and association at sites of DNA damage.

INTRODUCTION

Proteins involved in signalling DNA damage are critical for maintaining genome stability and guard against a variety of disease states including a predisposition to malignancy [1]. Consequently, the cell has evolved a comprehensive and intricate network of pathways that act in concert to detect and signal DNA damage for subsequent processing and repair. The phosphatidylinosotol (PI)-3 kinase related kinases (PIKKs) ATM and ATR function in distinct but partially overlapping pathways activated in response to genotoxic stress. ATM and ATR are recruited to sites of DNA damage, become activated, and subsequently phosphorylate a number of proteins that regulate various aspects of the DNA damage response (DDR). Whilst ATM is the principle kinase responsible for detecting and signalling DNA double strand breaks (DSBs), ATR responds to a wider variety of DNA damage architectures including UV-induced base damage, replication stress and DNA DSBs [2-4]. ATR phosphorylates and activates the Chk1 kinase in response to genotoxic stress. Chk1 then proceeds to phosphorylate a variety of proteins that regulate aspects of the DDR including cell cycle arrest, stabilization of stalled replication forks, and DNA repair [5]. As such, the ATR-Chk1 axis is central to the DDR and critical for maintaining genome integrity. For example, hypomorphic mutations in the *ATR* gene result in Seckel syndrome, a rare disease characterised by developmental abnormalities and genome instability [6].

The principle DNA architecture recognised by ATR is single stranded DNA (ssDNA) generated by either nucleases processing sites of DNA damage [3, 4, 7], or uncoupling of the replicative helicase from stalled replication forks [8]. Regions of ssDNA are subsequently coated by the ssDNA protein binding complex RPA that facilitates the recruitment of ATR to sites of DNA damage through an interaction with the ATR associated protein ATRIP [7, 9]. Although recognition of ssDNA by ATR is critical in allowing this kinase to phosphorylate and activate Chk1, a number of other factors are required for this event. For example, disruption of either Rad17 or components of the Rad9-Rad1-Hus1 (9-1-1) complex results in defective phosphorylation of Chk1 in response to DNA damage [10]. Rad17 along with the 4 small subunits of replication factor C (RFC2-5) acts as a clamp loader of 9-1-1 at or near sites of DNA damage [11-13]. Similar to ATR, Rad17/9-1-1 is also recruited to ssDNA coated with RPA [14]. However, Rad17/9-1-1 and ATR are recruited to DNA damage independently of each other arguing against these two DNA damage sensors acting in a linear pathway with regard to activation of Chk1 [13, 15, 16]. Instead, 9-1-1 activates ATR by recruiting the ATR-activating protein TopBP1 to DNA damage [17-19]. The final step in Chk1 activation is phosphorylation of Claspin by ATR that provides docking sites for Chk1 recruitment and subsequent phosphorylation by ATR [20].

Although a unified view of how Rad17, 9-1-1 and ATR access sites of DNA damage and facilitate Chk1 activation is beginning to emerge, how these proteins act in concert to propagate and maintain the checkpoint response remains unclear. Phosphorylation of Rad17 by ATR on serines 635 and 645 is required for cells to initiate cell cycle arrest following DNA damage [21]. However, the molecular basis of how ATR-mediated Rad17 phosphorylation influences the 9-1-1 complex once it has accessed sites of DNA damage remains largely unexplored. Here we assess the impact of ATR on 9-1-1 complex regulation once it has accumulated at sites of DNA damage and provide data that support a role for ATR-mediated Rad17 phosphorylation in maintaining Rad9 at sites of genotoxic stress.

MATERIALS AND METHODS

Cell culture and maintenance

HeLa and Swiss 3T3 cells, obtained from the European Collection of Cell Cultures (Health Protection Agency, Porton Down, Wiltshire, UK), and U2OS cells were grown using standard procedures. U2OS cells stably expressing eGFP-HA_2-Rad9 were grown in standard medium supplemented with 700 μg/ml of geneticin (G418).

The ATM inhibitor KU-55933 [22] and DNA-PK inhibitor KU00557788 [23] were pre-incubated with cells for 1 hour before UV treatment, and used at concentrations of 10 μM and 1 μM respectively. UV-light was administered using a StrataLinker 2400 source (Stratagene) or a 254 nm UV-C lamp (Philips) at 20 J/m^2 and cells processed 1 hour post treatment. To stall replication forks, asynchronously growing cells were incubated in 5 μg/ml of aphidicolin (Sigma) for 20 hours prior to processing of samples.

Antibodies

Antibodies obtained from commercial sources were as follows: Chk1 (Cell Signalling Technology), Chk1 pS317 (Bethyl), FLAG (M2; Sigma), Orc2 (BD Pharmingen), Rad9 (Novus Biologicals), Rad17 pS645 (Cell Signalling Technology), RPA (Neomarkers) and RPA (Oncogene). The following antibodies were obtained from Santa Cruz Biotechnology: ATR (N-19), Chk1 (G-4), Ku86 (C-20), Rad1 (N-18), Rad9 (C-20), Rad9 (M-389) and Rad17 (H-300). Rabbit polyclonal anti-Hus1, anti-Rad9 and anti-GFP antibodies were a gift from R. Freire (Unidad de Investigación HUC, Tenerife, Spain).

Plasmids and site-directed mutagenesis

pmCherry-C1 was kindly provided by R.Y. Tsien (Department of Pharmacology and Howard Hughes Medical Institute, University of California, San Diego, USA). pPCMV eGFP-spectrin has been previously described [24].

hRad9 cDNA was digested from pGEX-4T3-GST-Rad9, kindly provided by R. Freire (Unidad de Investigación HUC, Tenerife, Spain), and cloned into the MCS of pcDNA3.1$^+$ (Invitrogen). eGFP-HA was amplified by PCR from pEGFP-C2 (Clontech) and inserted in frame into pcDNA3.1$^+$Rad9 to generate pcDNA3.1$^+$eGFP-HA-Rad9.

A full-length Rad17 clone was purchased from Open Biosystems (Clone ID: 5170876, code: MHS1010-7508047). Rad17 was subcloned into the EcoR1 and Kpn1 sites of the pCMV-FLAG-2 plasmid (Sigma). The full-length cDNA and linker regions were confirmed to be correct by sequencing. The pCMV-FLAG-Rad17 construct was subjected to PCR amplification with the following primers to incorporate the S635A mutation: $^{5'}$TCTCTTCCTTTGGCTCAGAATAGTGCC$_{3'}$ and $^{5'}$GGCACTATTCTGAGCCAAAGGAAGAGA$_{3'}$. PCR products were digested with the methylation sensitive restriction enzyme Dpn1 to eliminate any original plasmid. To create the second mutation (S645A) we followed the same process using the following primers: $^{5'}$GAACTGCCTGCTGCCCCAGCCCCAGC$_{3'}$ and $^{5'}$GCTGGGGCTGGGCAGCAGGCAGTTC$_{3'}$.

Generation of FLAG-Rad17 and FLAG-Rad17^{AA} constructs that are resistant to siRNA was performed using the same methodology to introduce conservative mutations in the third nucleotide of each codon contained within the Rad17 siRNA target sequence. Primers used in site directed mutagenesis were as follows:
$^{5'}$CCAGAAACCCAACACGAGCTAGCAGTGCATAAAAAGAAAATTG$_{3'}$

$^{5'}$TATGCACTGCTAGCTCGTGTTGGGTTTCTGGTTTATATTTATCC$_{3'}$
Resulting Rad17 open reading frames, including the linker regions, were fully sequenced to confirm mutations of the relevant sites.

Transfection
siRNA oligonucleotides (Dharmacon Research) were transfected into cells using Oligofectamine (Invitrogen) according to the manufacturers instructions, and as previously described [4, 25, 26]. Cells were incubated for 72 hours after transfection prior to further analysis. Sequences of oligonucleotides were as follows:
NT, UGCGACUAAACACAUCAAUUdTdT
Luc, CGUACGCGGAAUACUUCGAdTdT
ATR, CCUCCGUGAUGUUGCUUGAdTdT
Rad9, GUCUUUCCUGUCUGUCUUCdTdT
Rad17, CAGACUGGGUUGACCCAUCdTdT

Plasmid DNA was transfected into cells using lipofectamine (Figure 2B) or the calcium phosphate transfection method (all other experiments). For stable expression of eGFP-HA-Rad9 in U2OS cells, positive clones were selected after a selection with G418 (1400 μg/ml) for two weeks.

For downregulating endogenous Rad17 while expressing siRNA resistant FLAG-Rad17 constructs, we transfected plasmid DNA into cells using FuGENE HD transfection reagent (Roche) according to the manufacturers instructions. The next day siRNA oligos were transfected as described above and cells were harvested 72 hours after transfection.

Immunofluorescence
Cells were grown as monolayers on glass coverslips. For detection of endogenous Rad9, cells were pre-extracted in Buffer 1 (1% Triton X-100, 10 mM HEPES (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$) for 5 minutes at 4^0C. Cells were then fixed in 4% paraformaldehyde for 10 minutes at 4°C and finally permeabilised further in Buffer 2 (0.5% Triton X-100, 20 mM HEPES pH (pH 7.4), 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose) for 5 minutes at 4°C. Cells containing >10 foci were scored as Rad9 positive. This number reflects the DNA damage inducible population within experiments. In all instances, error bars on graphs represent the standard error of the mean (SEM) from three independent experiments. For detection of RPA, cells were fixed in 4% paraformaldehyde for 10 minutes at 4°C followed by permeabilization in 0.5% Triton X-100 for 5 minutes at 4°C.
Immunofluorescence was performed using a Axioscope 2 fluorescent microscope equipped with Axiovision imaging software (Zeiss).
For detection of GFP-Rad9 in U2OS cells, living cells were studied, or cells were fixed in 2% paraformaldehyde containing 0.2% Triton X-100 for 20 minutes at RT and then permeabilised with 0.1% Triton X-100 for 5 minutes at RT.
Imaging and FLIP/FRAP of GFP-Rad9 were performed on a Confocal Laser Scanning Microscope LSM 510 (Zeiss), equipped with a 488 nm Ar-laser and a 505-550 nm band pass filter. In Figure 5, cells were co-transfected with mCherry-C1, kindly provided by R.Y. Tsien (Department of Pharmacology/Department of Chemistry & Biochemistry, University of California, San Diego, USA). Red fluorescence was selected using a 514 nm Ar laser and 575-615 nm band pass filter, after which the FLIP/FRAP measurements of GFP-Rad9 were performed, as described, in mCherry-positive cells.

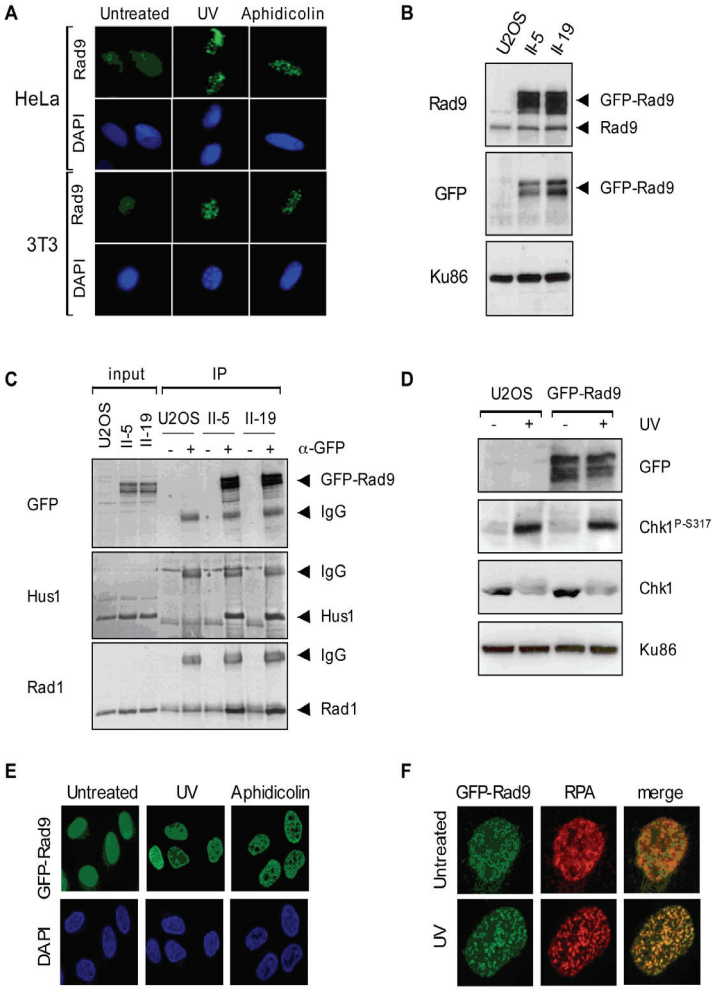

Figure 1: Rad9 accumulates to nuclear foci in response to genotoxic stress. (A) HeLa and Swiss 3T3 cells were left untreated, exposed to UV-light, or treated with aphidicolin. Rad9 was visualised by immunofluorescence. (B) Western blot analysis of untransfected U2OS cells or different U2OS clones stably expressing GFP-Rad9 (II-5 and II-19), using the indicated antibodies. (C) U2OS cells or clones expressing GFP-Rad9 (II-5 and II-19) were lysed and GFP-Rad9 immunoprecipitated using anti-GFP antibodies. The presence of associated proteins was analysed by immunoblotting with the indicated antibodies. (D) U2OS cells or cells expressing GFP-Rad9 were either left untreated or exposed to UV-light. After 1 hour, cells were lysed, and western blot analysis was performed using the indicated antibodies. (E) U2OS cells expressing GFP-Rad9 were left untreated, exposed to UV-light, or treated with aphidicolin. GFP-Rad9 was visualised by direct fluorescence. (F) GFP-Rad9 expressing U2OS cells were left untreated or exposed to UV-light. GFP-Rad9 was detected by direct fluorescence and RPA (p34 subunit) by immunofluorescence.

Cell fractionation and Western blotting

Whole-cell extracts were prepared by washing cultures in PBS before boiling cells in Laemmli buffer for 10 minutes. Protein concentrations were determined using the Lowry protein assay.

Biochemical fractionation of cells was performed as previously described [25, 27].

Cell cycle analysis

For cell cycle analysis, cells were collected by trypsinization and fixed in 70% ethanol at 4˚C for minimal 2 hours. After fixation, cells were washed with PBS and the DNA was stained with propidium iodide. Cell cycle profiles were analyzed by flow cytometry using an Epics XL-MCL from Beckman Coulter.

Photobleaching experiments

In the simultaneous FLIP/FRAP experiments, half of the nucleus was bleached for 2.7 seconds at 100% laser intensity, which irreversibly bleaches all GFP-Rad9 molecules. Subsequently, the redistribution of fluorescence in the nucleoplasm and the exchange of bleached and unbleached molecules between foci and nucleoplasm were monitored taking confocal images at fixed time intervals (60 seconds for Figure 4A and 30 seconds for Figs 4B and C, and Figure 5A and B). The relative intensities I_R of the bleached and unbleached halves of the cell were calculated as $I_R = (I_t - I_0)/(I_{t<0} - I_0)$, where I_t is the intensity measured at consecutive time points, I_0 is the intensity of the bleached part of the nucleus immediately after bleaching and $I_{t<0}$ is the intensity before bleaching (Figs 4B and 5A). The fluorescence before bleaching ($I_{t<0}$) was set to 1 and the intensity immediately after bleaching (I_0) was set to 0. The difference in relative fluorescence in bleached and unbleached parts of the nucleus was plotted against time (Figs 4C and 5B).

RESULTS

A fraction of Rad9 is converted into an immobile pool within nuclei in response to DNA damage and replication stress

To gain a greater understanding of the events required for regulation of 9-1-1, we examined the enrichment of Rad9 into nuclear foci in response to DNA damage or replication stress. When examining endogenous Rad9 by immunofluorescence, we observed a diffuse nuclear distribution of this protein in untreated human and mouse cells that relocated to discrete nuclear retained foci in response to UV-light and aphidicolin treatment (Figure 1A). To assess formation of Rad9 foci in live cells, we generated a cell line that stably expressed Rad9 tagged with eGFP-HA (GFP-Rad9; Figure 1B). GFP-Rad9 runs as multiple bands on SDS-PAGE, as observed by others [13], which partially represents the phosphorylation status of the protein (data not shown). GFP-Rad9 co-immunoprecipitated with endogenous Hus1 and Rad1 (Figure 1C). Furthermore, cells expressing GFP-Rad9 retained the ability to phosphorylate Chk1 in response to UV-light-induced DNA damage (Figure 1D) and did not display an increased sensitivity to DNA damaging agents (data not shown). Together these data indicate that GFP-Rad9 interacts with its functionally important partners and does not interfere with the DDR. Like endogenous Rad9, GFP-Rad9 was equally distributed throughout the nucleus in unperturbed cells and relocated into nuclear foci in response to UV-light or aphidicolin treatment. The majority of these foci appeared throughout the nucleus, but in a subset of the cells the foci were localized at the nuclear periphery (Figure 1E). UV-light-induced GFP-Rad9 foci became apparent between 10-30 minutes following irradiation (data not shown) and colocalized with RPA (Figure 1F), indicating that GFP-Rad9 is recruited to sites of ssDNA generated in response to DNA damage.

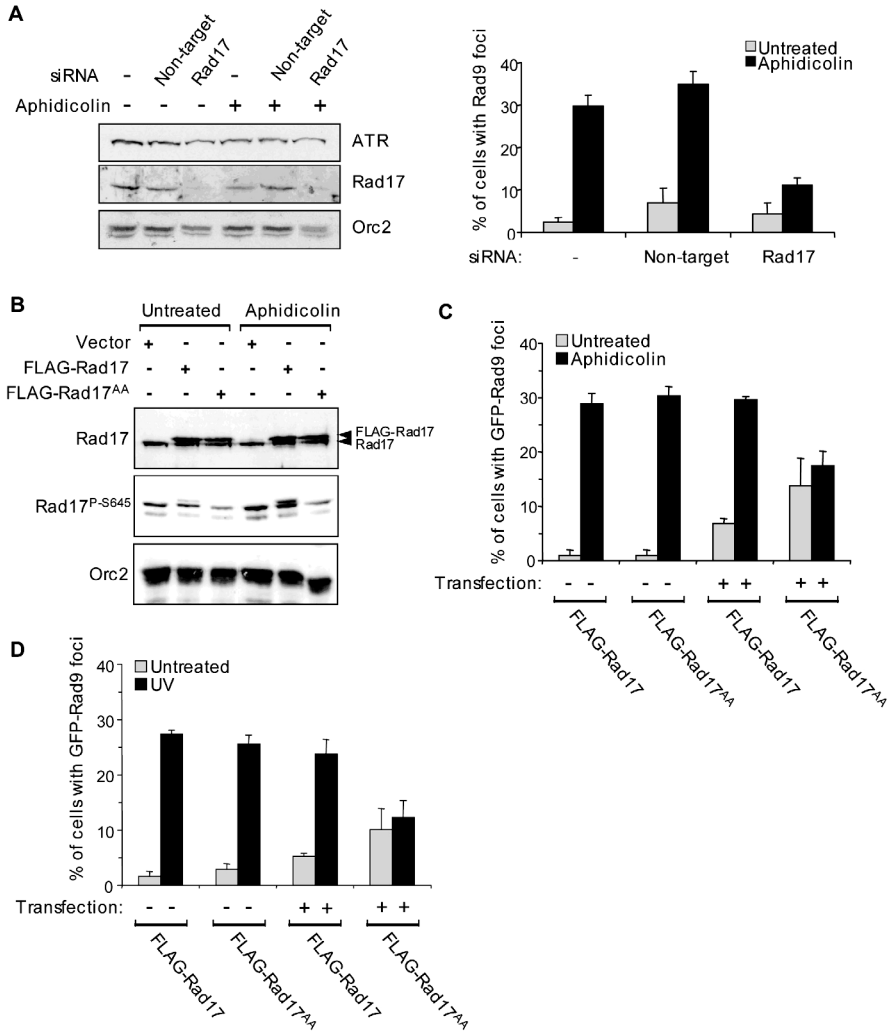

Figure 2: Rad17 is required for Rad9 to form foci in response to DNA damage. (A) HeLa cells were transfected with siRNA oligonucleotides as indicated and were left untreated or exposed to aphidicolin. Whole cell extracts were prepared for western blotting (left panel) and the percentage of cells exhibiting >10 Rad9 foci were determined (right panel). (B) U2OS cells expressing GFP-Rad9 were transfected with empty vector, or vector containing FLAG-Rad17 or FLAG-Rad17AA. Cells were left untreated or exposed to aphidicolin. Chromatin fractions were prepared and extracts subjected to western blotting using antibodies as indicated.(C) U2OS cells stably expressing GFP-Rad9 were transfected with FLAG-Rad17 or FLAG-Rad17AA constructs prior to treating cells with aphidicolin. FLAG-positive (transfected) or FLAG-negative (untransfected) cells were identified by immunofluorescence and cells scored for GFP-Rad9 foci.(D) U2OS cells stably expressing GFP-Rad9 were transfected as in (C), after which cells were treated with UV-light. FLAG-positive (transfected) or FLAG-negative (untransfected) cells were identified by immunofluorescence and cells scored for GFP-Rad9 foci.

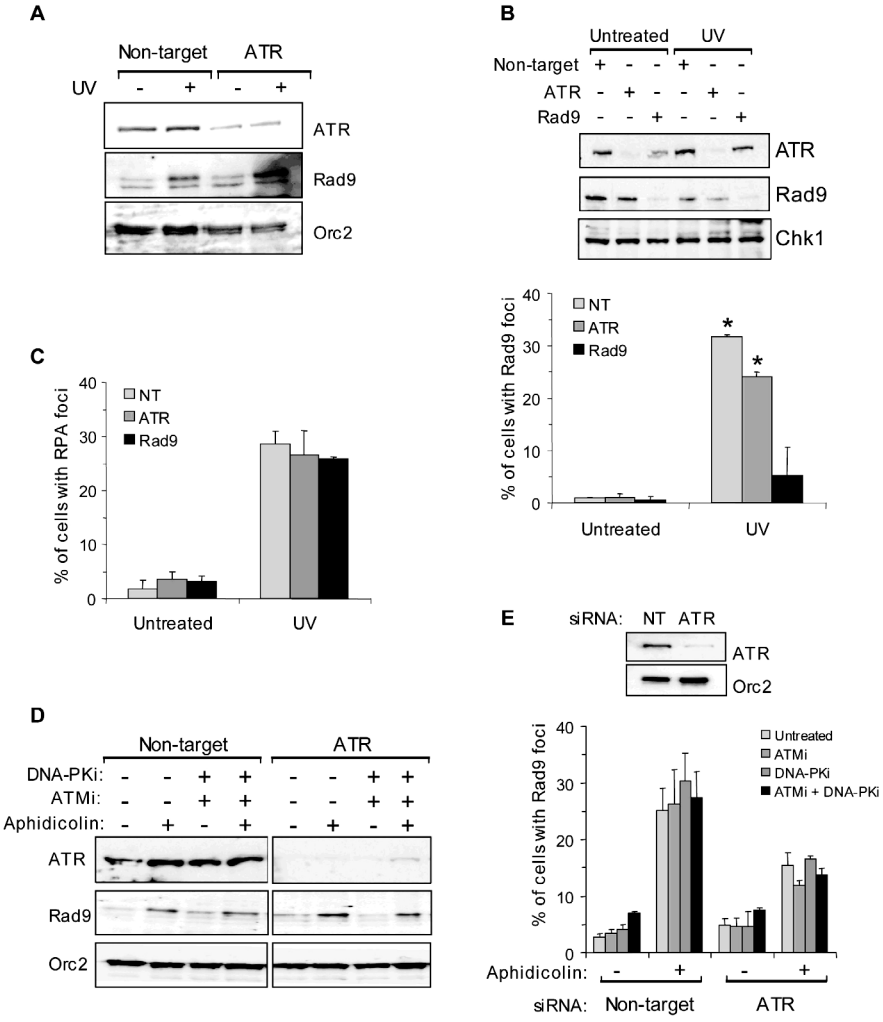

Figure 3: ATR is required for formation of a subset of UV-light and aphidicolin-induced Rad9 foci. (A) HeLa cells were transfected with siRNA oligonucleotides as indicated, exposed to UV-light and chromatin fractions prepared as described in materials and methods. Western blot analysis was performed using the indicated antibodies. (B) HeLa cells were transfected with siRNA oligonucleotides as indicated. Cells were treated with UV-light and harvested for western blotting (upper panel). The percentage of cells exhibiting >10 Rad9 foci was determined by immunoflourescence (lower panel). * represents a P value of 0.017 between these two data points indicating statistical significance at the 95% confidence level. (C) Cells were subjected to siRNA and treated as in (B). The percentage of cells exhibiting >10 RPA (p34 subunit) foci was determined by immunoflourescence. (D) HeLa cells were transfected with siRNA oligonucleotides as indicated and at 72 hours after transfection treated with a combination of ATM (10 mM) and DNA-PK (1 mM) inhibitors, or left untreated for 1 hour prior to exposure to aphidicolin. Cells were fractionated to obtain chromatin-enriched proteins and western blotting performed using the indicated antibodies. (E) HeLa cells were transfected with siRNA oligonucleotides as indicated and whole cell extracts prepared for western blotting (upper panel). In parallel cells were treated with ATM (10 mM) and/or DNA-PK (1 mM) inhibitors, or left untreated prior to exposure to aphidicolin. The percentage of cells exhibiting >10 Rad9 foci was determined.

Rad17 phosphorylation is required for Rad9 to form foci in response to DNA damage and replication stress

Although ATR-mediated phosphorylation of Rad17 on serines 635 and 645 is required for checkpoint activation [21], how this impacts on the ability of the 9-1-1 complex to access and be retained at sites of DNA damage remains unclear. For example, loading of 9-1-1 onto sites of DNA damage occurs independently of ATR, arguing that ATR-mediated Rad17 phosphorylation is not required for 9-1-1 to access sites of DNA damage [13, 15, 16, 28, 29]. Conversely, DNA damage-induced phosphorylation of Rad17 has been reported to promote the interaction of this protein with the 9-1-1 complex [21], suggesting that ATR may affect the ability of 9-1-1 to interact with sites of DNA damage.

To further assess how Rad17 contributes towards the ability of 9-1-1 to interact with sites of DNA damage, we examined the requirement for ATR-mediated phosphorylation of Rad17 in allowing Rad9 to accumulate in nuclear foci following genotoxic stress. Repression of Rad17 expression by siRNA resulted in a decrease from 35% to 10% of HeLa cells that displayed aphidicolin-induced Rad9 foci when compared to cells transfected with a control oligonucleotide (Figure 2A). A similar dependence on Rad17 was observed for UV-light or aphidicolin-induced GFP-Rad9 foci formation (data not shown). To assess how phosphorylation of Rad17 by ATR impacts on Rad9 foci formation, we generated FLAG-Rad17 and FLAG-Rad17 in which the S635 and S645 ATR phosphorylation sites were substituted with alanine (FLAG-Rad17^{AA}). When introduced into U2OS cells expressing GFP-Rad9, FLAG-Rad17 and FLAG-Rad17^{AA} were present in chromatin-enriched cellular fractions independently of replication stress, confirming that the FLAG-tag does not interfere with the DNA binding properties of these proteins (Figure 2B). Neither siRNA-mediated knockdown of Rad17, or overexpression of FLAG-Rad17^{AA} dramatically impacted on cell cycle progression in U2OS cells (Supplemental Figure 1). In cells transfected with empty expression vector, phosphorylation of endogenous Rad17 at S645 increased in response to aphidicolin (Figure 2B). Whilst aphidicolin-induced phosphorylation of Rad17 at S645 remained apparent in cells expressing FLAG-Rad17, this was reduced in cells transfected with FLAG-Rad17^{AA} (Figure 2B), confirming the dominant negative nature of this mutant [21].

To assess Rad9 foci in cells that express FLAG-Rad17 or FLAG-Rad17^{AA}, we introduced these proteins into U2OS cells stably expressing GFP-Rad9 using a low efficiency transfection procedure. Scoring FLAG-negative or FLAG-positive cells for GFP-Rad9 foci allowed a comparison of transfected and untransfected cells within the same population. Untransfected cells exhibited an induction of GFP-Rad9 foci formation in response to aphidicolin or UV-light and this remained largely unaffected by expression of FLAG-Rad17 (Figure 2C and D). However, FLAG-Rad17^{AA} expressing cells displayed a reduced ability to form aphidicolin and UV-light-induced GFP-Rad9 foci when compared to control cells expressing FLAG-Rad17 (Figure 2C and D). Expression of FLAG-Rad17^{AA} resulted in an increased number of cells that exhibit Rad9 foci in the absence of damaging agents. Given that Rad17^{AA} expression induces genomic instability in the absence of exogenous stress (Wang *et al.*, 2006), the increased number of FLAG- Rad17^{AA} cells that display Rad9 foci in the absence of UV-light or aphidicolin likely reflects recruitment of Rad9 to sites of endogenous DNA damage induced by expression of FLAG-Rad17^{AA}.

In order to discount the possibility that the reduced ability to form GFP-Rad9 foci in FLAG-Rad17^{AA} cells is a consequence of expressing mutant Rad17 in the presence of endogenous Rad17, we expressed siRNA resistant wild-type or mutant versions of FLAG-Rad17 in cells that had been subjected to Rad17 siRNA. Staining with FLAG antibodies identified cells

that expressed siRNA resistant FLAG-Rad17 in the presence of Rad17 knockdown. Whereas expression of siRNA resistant FLAG-Rad17 restored DNA damage-induced GFP-Rad9 foci formation in Rad17 downregulated cells, expression of siRNA resistant FLAG-Rad17^{AA} failed to restore the defect caused by downregulation of endogenous Rad17 (Supplemental Figure 2). Together, these data argue that the observed phenotype of FLAG-Rad17^{AA} expression is not due to the presence of endogenous Rad17 and illustrate that phosphorylation of Rad17 on S635 and/or S645 impacts on the ability of Rad9 to form foci in response to endogenous or exogenous genotoxic stress.

Disruption of ATR function results in a decreased number of cells that exhibit Rad9 foci in response to UV-light and replication stress

ATR and the 9-1-1 complex are recruited independently of each other to sites of DNA damage [13, 15, 16, 28, 29]. However, in vertebrates much of the literature describing the relationship between these two DNA damage sensors has focussed on examining enrichment of proteins in chromatin fractions isolated from cells following exposure to genotoxic stress [13, 28]. Consistent with these reports, we also observed that repression of ATR levels using siRNA did not affect enrichment of Rad9 in chromatin-containing fractions following exposure of cells to UV-light (Figure 3A).

Our observations that FLAG-Rad17^{AA} expression results in a reduced number of cells that exhibit DNA damage or replication stress-induced Rad9 foci led us to assess the requirement for ATR in Rad9 foci formation following genotoxic stress. As predicted, repression of Rad9 expression by siRNA abrogated formation of UV-light-induced Rad9 foci formation (Figure 3B). Consistent with our biochemical data (Figure 3A), UV-light-induced Rad9 foci formation was still apparent in cells that had been subjected to ATR siRNA. However, in these experiments we observe a modest but nonetheless significant decrease from 32% to 24% of cells that exhibited UV-light-induced Rad9 foci in the absence of ATR (Figure 3B). Importantly, UV-light-induced RPA foci remained intact following transfection with ATR or Rad9 siRNA (Figure 3C), arguing that the reduction of Rad9 foci is not a consequence of inefficient ssDNA formation and/or recruitment of RPA to sites of DNA damage. Although we observe a reproducible decrease in efficiency of Rad9 foci formation in ATR siRNA cells, this is not as dramatic as that achieved by overexpression of FLAG-Rad17^{AA}. Therefore, we cannot formally rule out additional effects of Rad17 phosphorylation on Rad9 foci formation in response to UV-light. Alternatively, these observations could be explained by increased genomic instability cells lacking ATR [30].

Similar to UV-light-induced DNA damage, repression of ATR using siRNA did not affect enrichment of Rad9 in chromatin-containing fractions following administration of aphidicolin (Figure 3D). However, similar to UV-light-induced damage, we also detected a decrease in the number of cells that exhibited aphidicolin-induced Rad9 or GFP-Rad9 foci formation in the absence of ATR (Figure 4E and data not shown).

To establish whether the absence of an absolute requirement for ATR in loading Rad9 onto chromatin or formation of Rad9 foci following administration of aphidicolin could be explained by a functional redundancy between ATR, ATM and/or DNA-PK, we combined siRNA of ATR with ATM [22] and DNA-PK [23] specific inhibitors prior to assessing either enrichment of Rad9 in chromatin-containing cellular fractions, or formation of Rad9 foci following administration of aphidicolin. Loading of Rad9 onto chromatin following administration of aphidicolin remained intact in cells subjected to ATR siRNA either in the absence or presence of ATM and DNA-PK specific inhibitors (Figure 3D). Similarly, addition of ATM and/or DNA-PK inhibitors to cells that had been treated with ATR siRNA

oligonucleotides did not result in a further dramatic decrease in the number of cells that exhibited Rad9 foci (Figure 3E). Together, these data argue against a functional redundancy being the explanation for a lack of total dependency of Rad9 foci formation on ATR.

ATR and Rad17 phosphorylation influence Rad9 retention at DNA damage sites

Our findings that the number of cells that display Rad9 foci is reduced by the absence of ATR prompted us to consider whether ATR might have an alternative effect on 9-1-1 function other than facilitating its ability to be recruited to sites of DNA damage. For example, should ATR be required to amplify or retain Rad9 at sites of DNA damage, it is conceivable that Rad9 may still transiently interact with DNA lesions in the absence of this kinase. Therefore, we assessed whether Rad9 proteins within established foci are immobile or whether these foci are more dynamic structures in which Rad9 proteins turnover, a process that could possibly be affected by ATR. To assess this, we used simultaneous fluorescence-loss-in-photobleaching (FLIP)/fluorescence-recovery-after-photobleaching (FRAP) technology, an assay employed in living cells that has been successfully used to monitor protein redistribution in real-time [31-35]. In these experiments, GFP-Rad9 expressing U2OS cells were transfected with control or ATR siRNA oligonucleotides. Subsequently, cells were treated with either UV-light or aphidicolin, half the nucleus containing the resulting GFP-Rad9 foci was photobleached, and cells were followed in real-time by video-microscopy (Figure 4A). The redistribution of fluorescence was monitored by measuring FRAP in the bleached part and FLIP in the unbleached part of the nucleus and the change in fluorescence versus time, indicative of GFP-Rad9 turnover, was quantified (Figure 4B).

Whereas GFP-Rad9 was highly mobile in undamaged cells and cells without foci, turnover of GFP-Rad9 within DNA damage-induced foci was comparatively slow. In addition, the FLIP and FRAP curves of control-transfected cells did not converge during the time span of our experiments (800-1000 seconds), suggesting the presence of a stably associated Rad9 fraction in damaged areas (Figure 4B and data not shown). Similar to luciferase-transfected control cells, UV-light and aphidicolin-induced foci formation also resulted in a decrease in the mobility of GFP-Rad9 in cells transfected with ATR siRNA oligonucleotides. However, in this case, the FLIP/FRAP curves almost fully converged, suggesting the absence of a long-lived immobile fraction of GFP-Rad9 in foci when ATR protein levels are repressed (Figure 4B). The time-lapse images in Figure 4A additionally demonstrate faster redistribution of fluorescence within damage-induced foci upon repression of ATR protein levels, as compared to control cells, which is confirmed by the slopes of the FLIP/FRAP curves (Figure 4B).

In addition, the difference in relative fluorescence intensities between the FRAP and FLIP areas was plotted against time and the time it took to reach full redistribution (>90%) was determined as a measure for mobility. Full redistribution of GFP-Rad9 within DNA damage-induced foci was not reached during the time course of our experiments (800-1000 seconds; Figure 4C and data not shown). However, following repression of ATR levels using siRNA, full redistribution was reached within 450-500 seconds after bleaching, indicating an increase in protein mobility upon downregulation of ATR (Figure 4C). Together these results demonstrate that in cells in which DNA damage-induced foci formation of GFP-Rad9 is not affected by the absence of ATR, Rad9 proteins in such foci display a higher turnover.

Our results suggest that the effect of ATR on the stability of GFP-Rad9 in damage-induced foci is mediated by phosphorylation of Rad17. To test if this is indeed the case, we performed simultaneous FLIP/FRAP measurements on UV-induced GFP-Rad9 foci in cells expressing FLAG-Rad17 or FLAG-Rad17^{AA}. Although wild-type FLAG-Rad17 did not interfere with the

slow dynamic behaviour of GFP-Rad9 within foci, expression of FLAG-Rad17^{AA} resulted in a similar phenotype as observed upon downregulation of ATR, an increased mobility of GFP-Rad9 within foci (Figure 5A). Furthermore, full redistribution of GFP-Rad9 in UV-induced foci was reached within 450-500 seconds after the bleach pulse upon FLAG-Rad17^{AA} expression, whereas this equilibrium was not reached in control cells (Figure 5B). Taken together, these data demonstrate that Rad17 impacts on the turnover of Rad9 molecules in foci, a process that critically depends on ATR-mediated phosphorylation.

DISCUSSION

In this report we present data that suggest a model in which Rad17 and ATR collaborate in regulating the localization of the 9-1-1 complex at sites of damage. The ability of Rad9 to form DNA damage-induced nuclear foci in the absence of ATR has not previously been reported in mammalian cells. However, analysis of *Saccharomyces cerevisiae* 9-1-1 (Ddc1-Rad17-Mec3) has revealed that formation of DNA DSB-induced Ddc1-Rad17-Mec3 nuclear foci can still occur in the absence of functional ATR (Mec1p) [15, 29]. In accordance with these findings, we also observe Rad9 foci formation in cells that have been treated with ATR siRNA following UV-light-induced DNA damage or replication stress (Figs 3 and 4). Taken together with our biochemical data that illustrates ATR is not required for Rad9 enrichment on chromatin in response to DNA damage or replication stress (Figs. 3A and D), our data support the currently accepted dogma that ATR is not required for 9-1-1 to access sites of DNA damage. Importantly, however, our experiments studying the dynamic behaviour of GFP-Rad9 foci by live-cell imaging suggest that whilst ATR does not influence the initial recruitment of Rad9 to DNA lesions, it does impact on the ability of this protein complex to be maintained within nuclear foci.

As the number of reports examining DNA damage checkpoint regulation in living cells is limited [35-37], our experiments provide valuable insights into understanding this complex process, which appears to be more dynamic than originally anticipated [38]. Whilst the Chk2 and Chk1 effector kinase are phosphorylated at sites of damage, induction of genotoxic stress does not lead to their immobilization, demonstrating the dynamic nature of these proteins [26, 35]; VAJS and DOW, unpublished). In contrast, several checkpoint proteins involved in the early stages of the checkpoint response, such as NBS1, MDC1 and 53BP1, are transiently immobilized in DNA damaged areas [35-37]. The observed immobilization of GFP-Rad9 in DNA damage-induced foci is in accordance with the suggested function of Rad9 as a DNA damage sensor protein, that upon recruitment to sites of damage contributes to activation of the checkpoint. However, the observed function of ATR in Rad9 turnover in established foci was unexpected in light of previous reports [13, 15, 16, 28, 29], and demonstrates a more subtle control of checkpoint regulation than initially envisaged.

These findings are of particular interest given the emerging theme that signalling DNA damage is not a purely linear pathway and that a variety of parallel mechanisms exists that contribute towards maintenance or amplification of the signal. For example, although histone H2AX phosphorylation is not essential for the initial recruitment of certain DNA damage signalling proteins to DNA DSBs, it is required for these proteins to persist at break sites and form DNA damage-induced nuclear foci [39]. Similarly, ATM-mediated phosphorylation of H2AX in response to DNA DSBs has been proposed to amplify the initial signal generated at a break site by recruiting additional ATM via an interaction with the

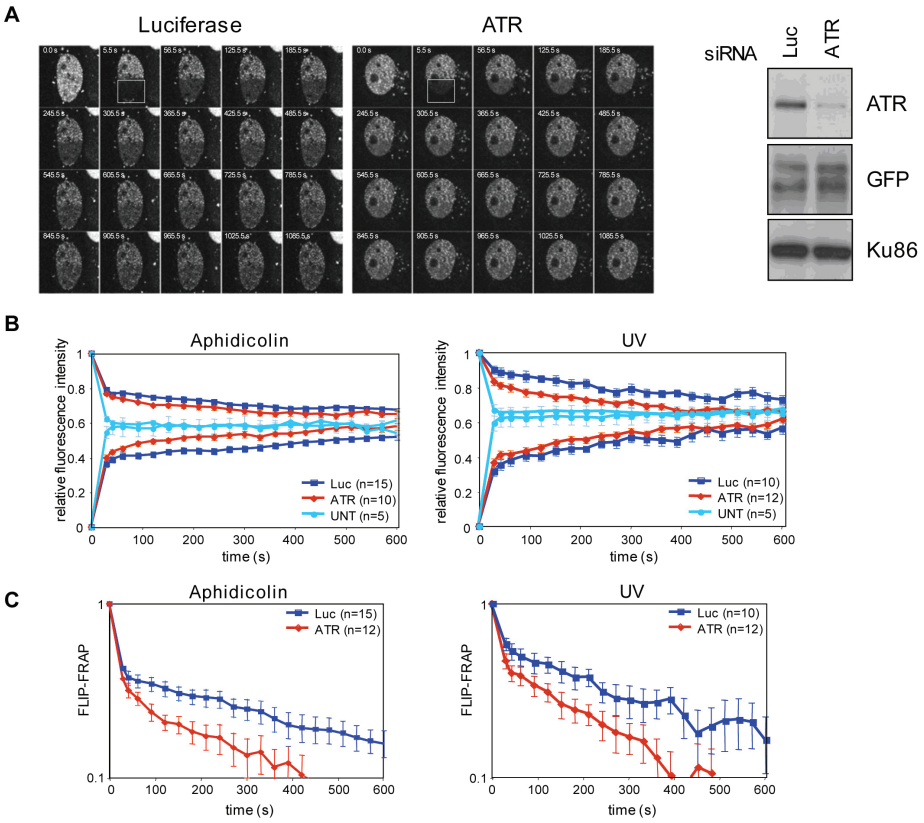

Figure 4: ATR influences the retention time of GFP-Rad9 in damage-induced foci. (A) GFP-Rad9 U2OS expressing cells were transfected with ATR or Luciferase siRNA oligonucleotides, and treated with aphidicolin. Half the nucleus containing GFP-Rad9 foci (see rectangle) was bleached for 2.7 seconds at 100% laser intensity, after which redistribution of fluorescence was monitored by recording images every 60 seconds. Western blot analysis of cell extracts from a representative experiment is illustrated (right panel). (B) Quantification of simultaneous FLIP/FRAP experiment as in A, with the exception that the cells were imaged every 30 seconds. Cells were treated with aphidicolin (left panel) or UV (right panel), after which FLIP was measured in the unbleached part of the cell and FRAP was measured in the bleached part of the cell. Control cells (UNT) are either left untreated or treated with damaging agents but not displaying GFP-Rad9 foci. The mean of the data points of individual cells analyzed is illustrated (n=number of cells) with error bars representing the SEM. (C) Difference in relative fluorescence in bleached and unbleached parts of the nucleus from (B), plotted against time. Error bars represent twice the SEM.

Mre11/Rad50/Nbs1/Mdc1 protein complex [40]. Therefore, one possible interpretation of our data is that activation of ATR serves as a signal to 9-1-1 that subsequently stabilises the interaction of this protein complex with sites of DNA damage resulting in amplification or maintenance of the checkpoint signal.

A link between ATR and the ability of 9-1-1 to be maintained at sites of genotoxic stress is further supported by our observations that expression Rad17 that is unable to be phosphorylated by ATR results in a similar increased dynamic behaviour of Rad9 within foci.

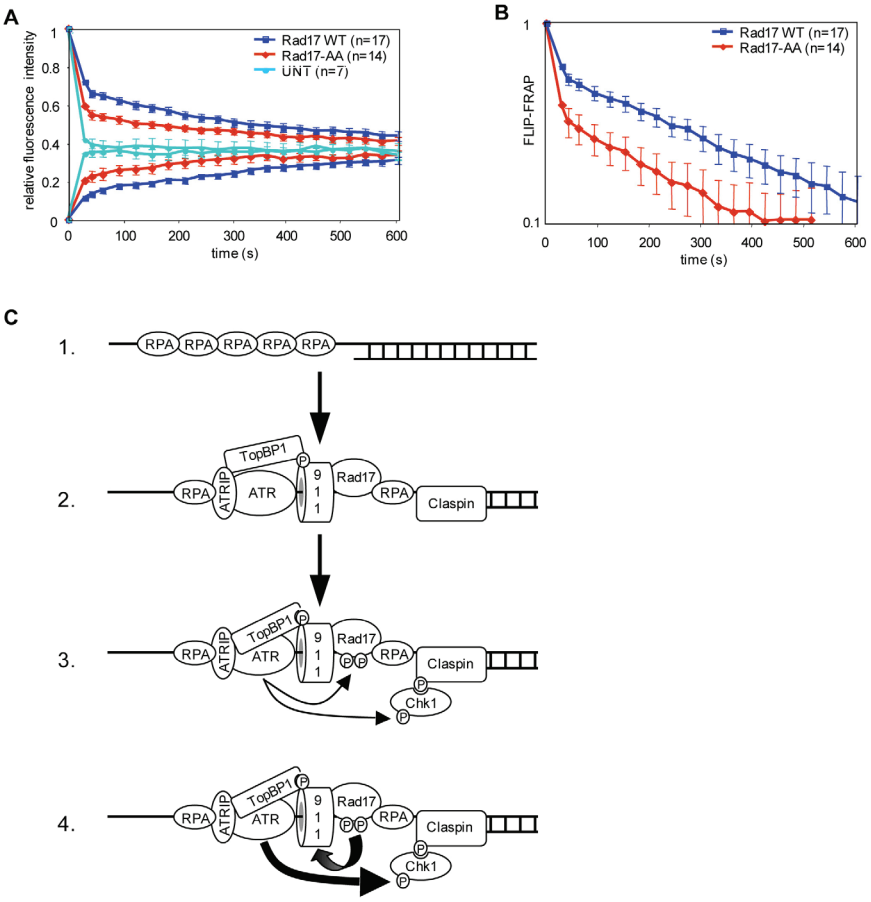

Figure 5: Rad17 phosphorylation at serines 635 and 645 decreases the mobility of GFP-Rad9 in damage-induced foci. GFP-Rad9 expressing U2OS cells were transfected with FLAG-Rad17 or FLAG-Rad17AA constructs, together with mCherry-C1, to detect transfected cells. Cells were left untreated (UNT) or treated with UV-light and FLIP/FRAP analysis was performed on red cells. (A) Quantification of FLIP and FRAP as described in Figure 5B. (B) Difference in relative fluorescence, plotted against time, as described in Figure 5C. (C) ATR and Rad17 collaborate in modulating Rad9 localization at sites of DNA damage. ssDNA generated as a result of DNA damage or replication stress is recognized by RPA (1). Recognition of ssDNA by RPA leads to the independent recruitment of ATR and Rad17 to DNA lesions. Rad17 loads the 911 complex at sites of ssDNA and facilitates activation of ATR through an interaction with TopBP1 (2). ATR subsequently phosphorylates Claspin that acts to recruit Chk1 and promote its phosphorylation by ATR. ATR also phosphorylates Rad17 (3). ATR-mediated phosphorylation of Rad17 stabilizes the 911 complex at sites of DNA lesions. This in turn could result in the maintenance of activated ATR and continued checkpoint signalling until DNA damage is repaired (4).

How DNA damage-induced phosphorylation of Rad17 contributes towards the regulation of 9-1-1 has been a matter of debate. The observation that ATR is not required for enrichment of 9-1-1 on chromatin in response to genotoxic stress argues against phosphorylation of Rad17 contributing towards regulating the access of 9-1-1 to sites of DNA damage [13, 28]. Conversely, phosphorylation of Rad17 on serines 635 and 645 promotes an interaction with the 9-1-1 complex, raising the possibility that ATR-mediated phosphorylation of Rad17 can influence the ability of 9-1-1 to interact with sites of DNA damage [21]. Together our results unify these findings and demonstrate that whilst phosphorylation of Rad17 by ATR is not required for 9-1-1 to access DNA damage sites *per se*, it does contribute to retention of 9-1-1 within the vicinity of the DNA lesion.

The 9-1-1 complex facilitates activation of ATR not through promoting its interaction with DNA damage sites, but by recruiting the ATR activating protein TopBP1 to sites of DNA damage [17-19]. It is interesting to speculate, therefore, that the initial activation of ATR by TopBP1 results in phosphorylation of Rad17 and subsequent stabilization of the Rad17/9-1-1/TopBP1 complex at DNA lesions. This in turn could result in the maintenance of activated ATR and continued checkpoint signalling until DNA damage is repaired (Figure 5C). This hypothesis is particularly interesting in light of the recent observations illustrating that ATR-mediated phosphorylation of Rad17 is not absolutely required for activation of Chk1 in response to replication fork stalling, but is instead required for the maintenance of the checkpoint following removal of stress [41]. Taken together, our findings may be indicative a biphasic response to genotoxic stress that constitutes an initial recruitment of signalling proteins to DNA damage followed by subsequent regulation of checkpoint proteins at damage sites in order to maintain the checkpoint.

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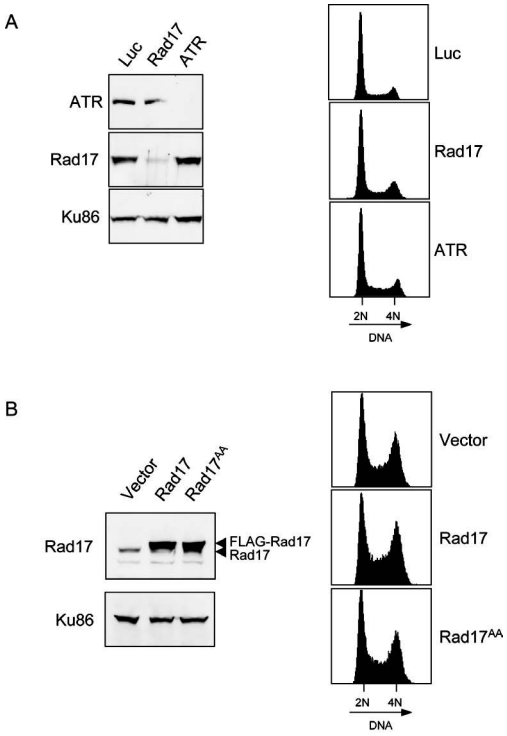
REFERENCES

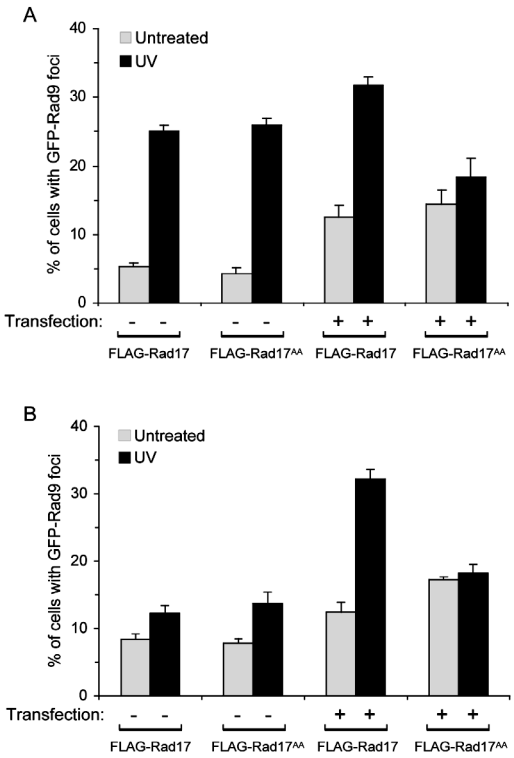
1. Kastan, M.B. and J. Bartek, *Cell-cycle checkpoints and cancer.* Nature, 2004. **432**(7015): p. 316-23.
2. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes and Development, 2001. **15**: p. 2177-2196.
3. Jazayeri, A., et al., *ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks.* Nat Cell Biol, 2006. **8**(1): p. 37-45.
4. Adams, K.E., et al., *Recruitment of ATR to sites of ionising radiation-induced DNA damage*

requires ATM and components of the MRN protein complex. Oncogene, 2006. **25**(28): p. 3894-904.
5. Chen, Y. and Y. Sanchez, *Chk1 in the DNA damage response: conserved roles from yeasts to mammals.* DNA Repair (Amst), 2004. **3**(8-9): p. 1025-32.
6. O'Driscoll, M., et al., *A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome.* Nat Genet, 2003. **33**(4): p. 497-501.
7. Zou, L. and S.J. Elledge, *Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.* Science, 2003. **300**(5625): p. 1542-8.
8. Byun, T.S., et al., *Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint.* Genes Dev, 2005. **19**(9): p. 1040-52.
9. Ball, H.L., J.S. Myers, and D. Cortez, *ATRIP binding to replication protein A-single-stranded DNA promotes ATR-ATRIP localization but is dispensable for Chk1 phosphorylation.* Mol Biol Cell, 2005. **16**(5): p. 2372-81.
10. Melo, J. and D. Toczyski, *A unified view of the DNA-damage checkpoint.* Curr Opin Cell Biol, 2002. **14**(2): p. 237-45.
11. Bermudez, V.P., et al., *Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex in vitro.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1633-8.
12. Ellison, V. and B. Stillman, *Biochemical Characterization of DNA Damage Checkpoint Complexes: Clamp Loader and Clamp Complexes with Specificity for 5' Recessed DNA.* PLoS Biol, 2003. **1**(2): p. E33.
13. Zou, L., D. Cortez, and S.J. Elledge, *Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin.* Genes Dev, 2002. **16**(2): p. 198-208.
14. Zou, L., D. Liu, and S.J. Elledge, *Replication protein A-mediated recruitment and activation of Rad17 complexes.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 13827-32.
15. Melo, J.A., J. Cohen, and D.P. Toczyski, *Two checkpoint complexes are independently recruited to sites of DNA damage in vivo.* Genes Dev, 2001. **15**(21): p. 2809-21.
16. Kondo, T., et al., *Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms.* Science, 2001. **294**(5543): p. 867-70.
17. Kumagai, A., et al., *TopBP1 activates the ATR-ATRIP complex.* Cell, 2006. **124**(5): p. 943-55.
18. Delacroix, S., et al., *The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1.* Genes Dev, 2007. **21**(12): p. 1472-7.
19. Lee, J., A. Kumagai, and W.G. Dunphy, *The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR.* J Biol Chem, 2007. **282**(38): p. 28036-44.
20. Kumagai, A. and W.G. Dunphy, *Repeated phosphopeptide motifs in Claspin mediate the regulated binding of Chk1.* Nat Cell Biol, 2003. **5**(2): p. 161-5.
21. Bao, S., et al., *ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses.* Nature, 2001. **411**(6840): p. 969-74.
22. Hickson, I., et al., *Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM.* Cancer Res, 2004. **64**(24): p. 9152-9.
23. Veuger, S.J., et al., *Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-ribose) polymerase-1.* Cancer Res, 2003. **63**(18): p. 6008-15.
24. Kalejta, R.F., T. Shenk, and A.J. Beavis, *Use of a membrane-localized green fluorescent*

protein allows simultaneous identification of transfected cells and cell cycle analysis by flow cytometry. Cytometry, 1997. **29**(4): p. 286-91.
25. Dart, D.A., et al., *Recruitment of the cell cycle checkpoint kinase ATR to chromatin during S-phase.* J Biol Chem, 2004. **279**(16): p. 16433-40.
26. Smits, V.A., P.M. Reaper, and S.P. Jackson, *Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response.* Curr Biol, 2006. **16**(2): p. 150-9.
27. Mendez, J. and B. Stillman, *Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis.* Mol Cell Biol, 2000. **20**(22): p. 8602-12.
28. You, Z., L. Kong, and J. Newport, *The role of single-stranded DNA and polymerase alpha in establishing the ATR, Hus1 DNA replication checkpoint.* J Biol Chem, 2002. **277**(30): p. 27088-93.
29. Lisby, M., et al., *Choreography of the DNA Damage Response; Spatiotemporal Relationships among Checkpoint and Repair Proteins.* Cell, 2004. **118**(6): p. 699-713.
30. Paulsen, R.D. and K.A. Cimprich, *The ATR pathway: fine-tuning the fork.* DNA Repair (Amst), 2007. **6**(7): p. 953-66.
31. Essers, J., et al., *Nuclear dynamics of PCNA in DNA replication and repair.* Mol Cell Biol, 2005. **25**(21): p. 9350-9.
32. Hoogstraten, D., et al., *Rapid switching of TFIIH between RNA polymerase I and II transcription and DNA repair in vivo.* Mol Cell, 2002. **10**(5): p. 1163-74.
33. Mattern, K.A., et al., *Dynamics of protein binding to telomeres in living cells: implications for telomere structure and function.* Mol Cell Biol, 2004. **24**(12): p. 5587-94.
34. Pryde, F., et al., *53BP1 exchanges slowly at the sites of DNA damage and appears to require RNA for its association with chromatin.* J Cell Sci, 2005. **118**(Pt 9): p. 2043-55.
35. Lukas, C., et al., *Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage.* Nat Cell Biol, 2003. **5**(3): p. 255-60.
36. Lukas, C., et al., *Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention.* Embo J, 2004. **23**(13): p. 2674-83.
37. Bekker-Jensen, S., et al., *Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD1.* J Cell Biol, 2005. **170**(2): p. 201-11.
38. Lukas, J., C. Lukas, and J. Bartek, *Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time.* DNA Repair (Amst), 2004. **3**(8-9): p. 997-1007.
39. Celeste, A., et al., *Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks.* Nat Cell Biol, 2003. **5**(7): p. 675-9.
40. Stucki, M., et al., *MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks.* Cell, 2005. **123**(7): p. 1213-26.
41. Wang, X., et al., *Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress.* Mol Cell, 2006. **23**(3): p. 331-41.



Supplementary Figure 1: Downregulation of Rad17 and ATR, or overexpression of FLAG-Rad17AA does not affect cell cycle progression. (A) U2OS cells were transfected with control Luciferase (Luc), Rad17 or ATR siRNA oligonucleotides as indicated. 72 hours after transfection, cells were either subjected to western blotting using the indicated antibodies (left panel), or the cell cycle profiles analyzed by flow cytometry (right panel). (B) U2OS cells were transfected with empty vector, FLAG-Rad17 or FLAGRad17AA in conjunction with a GFP-spectrin expression vector. 36 hours after transfection, cells were either subjected to western blotting using the indicated antibodies (left panel), or the cell cycle profiles of GFP-positive cells analyzed by flow cytometry (right panel).



Supplementary Figure 2: Expression of siRNA resistant FLAG-Rad17AA fails to restore UV-light-induced GFP-Rad9 foci formation. U2OS cells expressing GFPRad9 were transfected with siRNA resistant FLAG-Rad17 or FLAG-Rad17AA constructs and subsequently with either control (A) or Rad17 (B) siRNA oligonucleotides. Cells were left untreated or exposed to UV. Cells were fixed and stained with FLAG antibodies to identify those expressing recombinant siRNA resistant FLAG-Rad17. FLAG-positive (transfected) or FLAG-negative (untransfected) cells were scored for GFP-Rad9 foci. As would be predicted, Rad17 siRNA abrogates GFP-Rad9 foci formation following exposure to UV-light, illustrating the efficiency of Rad17 knockdown in these cells. FLAG-Rad17AA expressing cells exhibit a reduced ability to restore GFP-Rad9 foci formation when compared to those expressing FLAG-Rad17.

7

Ubiquitination of the 9-1-1 complex

Implications for checkpoint regulation

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ABSTRACT

Critical in the response to genotoxic stress is the capability of mammalian cells to activate DNA repair and checkpoint pathways. Failures associated with these responses may lead to genomic instability and cancer predisposition. The ATR-mediated checkpoint response is essential for cell viability and the cellular response to UV light and replication-associated DNA damage. A common intermediate in the repair of these lesions is RPA-coated single-stranded DNA. Proteins recruited to this intermediate include the Rad9-Rad1-Hus1 (9-1-1) sliding clamp. This protein complex shares high structural similarity with PCNA and is necessary for proficient genotoxic stress-induced checkpoint activation. In response to DNA damage, the 9-1-1 complex is regulated by post translation modifications. Here we show that the human 9-1-1 complex is modified by ubiquitination. Mass spectrometry analysis indicates that Hus1 is ubiquitinated on lysine 136 and identifies novel proteins involved in the regulation of the 9-1-1 complex. Our data indicates that the Hus1 ubiquitination is regulated by the E3 ligase EDD, whose loss results in a decrease in UV-induced Chk1 phosphorylation. We additionally demonstrate that the Rad18 E3 ligase is involved in ATR-mediated checkpoint signalling by stabilizing the 9-1-1 complex after DNA damage induction, possibly through ubiquitination. Furthermore, depletion of Rad18 negatively influences UV-induced foci formation of Hus1. Collectively, these results suggest that there are different mechanisms involved in 9-1-1 ubiquitination.

INTRODUCTION

Genotoxic stress in eukaryotic cells originates from endogenous metabolic cellular processes and exogenous DNA damaging agents, both of which lead to the activation of DNA damage checkpoint and repair pathways [1-3]. The activation of DNA damage response pathways ensures an intact transmission of the DNA to the daughter cells and failures associated with these processes can lead to genomic instability, which is a common cause of cancer [4, 5]. The DNA damage-induced checkpoint response temporally halts cell cycle progression to create the necessary time for DNA repair to remove genotoxic DNA lesions that might be present [6]. The major regulators of the DNA damage-induced checkpoint response are the transducer kinases ATM and ATR [7]. Whereas ATM is activated in response to DNA damaging agents that induce DNA double-stranded breaks (DSBs), the ATR-mediated response is mainly triggered by ultraviolet (UV) light and replication stress [7]. The latter types of genotoxic stress lead to the formation of single-stranded DNA (ssDNA) intermediates that become coated by RPA, subsequently leading to the recruitment of the ATR-ATRIP heterodimer and Rad17-RFC complexes to these sites of damage [8, 9]. Rad17-RFC is required for the loading of the Rad9-Rad1-Hus1 (9-1-1) PCNA-like sliding clamp onto damaged chromatin [10, 11]. After loading of the 9-1-1 clamp, TopBP1 binds to the N-terminal region of Rad9 and triggers the activation of the ATR kinase which subsequently leads to the phosphorylation and activation of effector kinase Chk1 responsible for cell cycle arrest [12-15]. The 9-1-1 complex was proposed to be critical in the response to genotoxic stress since Hus1-deficient mouse embryonic fibroblasts (MEFs) are hypersensitive to hydroxyurea (HU) and UV-irradiation [11, 16-18]. Knocking out Rad9 in mouse embryonic stem cells or chicken DT40 cells also results in increased sensitivity to DNA damaging agents [19, 20]. This can be explained partially by the requirement of Hus1 and Rad9 in the DNA damage-induced phosphorylation of Chk1and the subsequent cell cycle arrest [11, 17, 20, 21].

Post-translational modifications are of crucial importance for the regulation of the cellular response to DNA damage. Phosphorylation activates kinases in the pathway, leading to the recruitment of proteins to DNA lesion and to the regulation of the retention of factors in DNA damage-induced nuclear foci [22-24]. Besides the phosphorylation of Chk1, Rad17 and Rad9, ATR is also responsible for the phosphorylation of a number of other proteins, including RFC, RPA, TopBP1, ATRIP, and Claspin [7, 22-25]. In addition, ubiquitination has recently been implicated in checkpoint regulation, specifically for Claspin, RFC2 and TopBP1 [26-29].

The 9-1-1 complex is also suggested to be regulated by ubiquitination. Hus1 is degraded in a proteasome-dependent manner [30, 31]. More recently it was reported that *S. cerevisiae rad17* (homologue of human Rad1) is ubiquitinated in response to DNA damage [32]. Cells harbouring a mutation in lysine 197 of *rad17* show reduced *ddc1* (homologue of human Rad9) focus formation in response to DSBs and show decreased *rad53* (functional homologue of human Chk1) phosphorylation levels after DNA damage induction. Collectively these results indicate the importance of *rad17* ubiquitination in checkpoint activation. Interestingly, the E3 ubiquitin ligase *rad18*, necessary for DNA damage-induced ubiquitination of PCNA, is required for *rad17* ubiquitination [33-35].

We set out to investigate if the members 9-1-1 complex are ubiquitinated in human cells and if so what the functional relevance of this modification might be. We performed mass-spectrometry analysis of purified ubiquitinated Hus1 and demonstrated the ubiquitination of Hus1 on lysine 136. A number of E3 ubiquitin ligases were identified as Hus1-interaction

partners that might be involved in the ubiquitination of the 9-1-1 complex. Although the obtained findings are reproducible we emphasize that this work is unfinished and therefore further research has to be undertaken to gain a better understanding of the underlying mechanisms of 9-1-1 ubiquitination. Nevertheless our preliminary results suggest that Hus1 is regulated in a manner that is dependent on E3 ligase EDD, possibly through ubiquitination. Importantly, depletion of EDD leads to increased sensitivity of cells to UV damage, a decrease in DNA damage-induced Chk1 phosphorylation and inefficient recruitment of the 9-1-1 complex to chromatin after genotoxic stress. In contrast to EDD, Rad18 seems to stabilize the 9-1-1 complex protein levels. Depletion of Rad18 decreases cell viability after UV damage and moreover it affects GFP-Rad9 focus formation and recruitment of the 9-1-1 complex to chromatin in response to DNA damage. The interesting results presented here create preliminary insight into the mechanism(s) of 9-1-1 ubiquitination and suggest that the E3 ubiquitin ligases EDD and Rad18 are implicated in the response of cells to UV damage.

MATERIALS AND METHODS

Plasmids and site-directed mutagenesis

The pcDNA3-myc-Rad1 and pcDNA3-myc-Hus1 were a kind gift from H.G. Wang (H. Lee Moffitt Cancer Center and Research Institute, Tampa, USA) and described previously [30, 36]. YFP-Rad18 by A. Inagaki [37] (Erasmus MC, Rotterdam, The Netherlands). eGFP-HA2-Rad9 (GFP-Rad9) was described before [24]. Flag-EDD was a gift from R.L. Sutherland (Garvan Institute of Medical Research, Sydney, Australia). Wild type and mutated (K48R and K63R) His-tagged ubiquitin expression plasmids were kindly provided by M. Benkirane (Institut de Génétique Humaine, Montpellier, France). Hus1 lysines 136, 141 and 144 were changed to arginine and EDD serine at position 2071 was changed to alanine using the QuickChange site-directed mutagenesis kit from Stratagene. To produce truncated Flag-EDD proteins we introduced a stop codon in the Flag-EDD cDNA at the positions 2446, 1369 or 577 using the indicated primers:

EDD D2446-2798 forward	agagcaagagtggatgaggcctaggaactcattattgcacatgg
EDD D2446-2798 reverse	ccatgtgcaataatgagttcctaggcctcatccactcttgctct
EDD D1369-2798 forward	catcttttgccagaagagcaataatacctcaatcagcaaagtgg
EDD D1369-2798 reverse	ccactttgctgattgaggtattattgctcttctggcaaaagatg
EDD D577-2798 forward	gacagctgtagatttcaactttgatctcctgaaagcttgaaaa
EDD D577-2798 reverse	ttttcaagctttcaggagatcaaagttgaaatctacagctgtc

GFP-Rad1 and Hus1-GFP cDNA was produced by PCR on pcDNA3-myc-Rad1 and pcDNA3-myc-Hus1 introducing a BamH1 and EcoR1 restriction-site at opposite sites of the amplified cDNA. The templates were ligated into pEGFP-C1 and N1 (Invitrogen) digested previously with BamH1 and EcoR1 restriction enzymes to generate C and N-terminally labeled eGFP versions of Rad1 and Hus1. Tagging of Hus1 at either side maintained interactions with endogenous Rad9 and Rad1, whereas C-terminal tagging of Rad1 disrupts the interaction with endogenous Rad9 and Hus1 (data not shown).

Cell culture and maintenance

U2OS and HEK 293T cells were grown using standard procedures. U2OS cells expressing

GFP-Rad9 and Hus1-GFP were grown in standard medium supplemented with 700 μg/ml of G418.
UV-light was administered using a 254 nm UV-C lamp (Philips) at 20 J/m^2 and cells harvested 1 hour post treatment. To stall replication forks, asynchronously growing cells were incubated with 5 μg/ml of aphidicolin (Sigma) for 20 hours, or 10 mM HU for 2 hours. IR (10 Gy) was induced using a ^{137}Cs source or a linear accelerator (LINAC) and cells were harvested 2 hours after treatment. MMS-treatment (0.02%) was performed for 1 hour.

Antibodies
Antibodies obtained from commercial sources were as follows: Orc2 (BD Pharmingen), Grb2 (BD Transduction), Rad9 (Novus), pS317 and pS345 Chk1 (Cell Signaling), Flag (Sigma), HA (Covance), Chk1, Rad18 and EDD (Abcam), and the following antibodies were obtained from Santa Cruz Biotechnology: Ku86 (C-20), Rad1 (N-18), Rad17 (H-300) and Chk1 (G-4).
Rabbit polyclonal anti-GFP, anti-TopBP1 and anti-Rad9 were described before [38-40]. Rabbit polyclonal Hus1 antibody was raised against recombinant Hus1 full length protein. Recombinant protein was obtained by cloning the cDNA corresponding in pET-28 (Novagen) expressed in E. coli and soluble protein was purified and used to immunize. A similar method was used to obtain antibodies against EDD, Rad18 and Rad1.

Transfection
Plasmid DNA was transfected into cells using the calcium phosphate transfection method.
siRNA oligonucleotides (Dharmacon Research) were transfected into cells using Oligofectamine (Invitrogen) according to the manufacturers instructions, and as previously described [16, 41, 42]. Cells were incubated for 48-72 hours after transfection prior to further analysis. Sequences of oligonucleotides were as follows:

Luc	CGUACGCGGAAUACUUCGAdTdT
Chk1	UCGUGAGCGUUUGUUGAACdTdT
EDD	GCAGUGUUCCUGCCUUCUUdTdT
HUWE1	GAGUUUGGAGUUUGUGAAGdTdT
Rad18	GGAAAGCCUCAGAAGUUCUUU
Ubc13	CAGAUUUGUGUUAAGAUAGUAUU
USP1	UCGGCAAUACUUGCUAUCUUAUU

Colony survival assays
Cells that were downregulated for either luciferase, Chk1, EDD, HUWE1 and Rad18 were counted and 1000 cells were seeded onto 60 mm diameter dishes and left for 12 hours to attach. Cells were treated with different doses of UV (5, 10, 15, 20 J/m^2) and incubated with fresh medium for 7-14 days, after which the colonies were fixed, stained and counted. All experiments were performed in triplicate.

Whole cell extracts and cell fractionation
For whole cell extracts, cells were washed in cold PBS, after which the cells were resuspended in Laemmli sample buffer (4% SDS, 20% glycerol, 120 mM Tris pH 6.8) and boiled for 5 min. Protein concentrations were determined using the Lowry protein assay. Chromatin fractionation was performed as previously described [16, 43].

Immunoprecipitations

HEK 293T-transfected cells were collected by trypsinization, washed with ice-cold PBS, and lysed in buffer (A) containing 50 mM HEPES [pH 7.5], 150 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 0.5% NP40, 10% glycerol and 1 mM DTT, supplemented with protease inhibitor cocktail (Roche), 1 mM NaF (Sigma), 1 mM Na3VO4 (Sigma) and 2 mM N-Etylmaleimide (Sigma) for 20 minutes on ice, in the presence of MG132 (Calbiochem) when indicated. Immunoprecipitation experiments were carried out using Flag M2-Agarose beads (Sigma), HA (F-7) beads (Santa Cruz), Protein A Sepharose beads (GE Healthcare) or Magnetic Ni-particles (Promega) and analyzed by Western blotting with the indicated antibodies.

For mass spectrometry, the purification of Flag tagged Hus1 and Rad9 was carried in HEK 293T cells transfected with His-ubiquitin and Flag-Hus1 or Flag-Rad9. Briefly, after lysis in buffer A for 1 hour the cellular extracts were spun down and the supernatant was incubated with Flag beads for 3 hours at 4ºC on a spinning wheel. Beads were washed 4 times with buffer A and Flag tagged proteins were eluted from the beads in four steps using 0.2 mg/ml Flag peptides (Sigma) under cold conditions. The elution fractions were pooled and incubated in buffer A with Magnetic Ni-particles (Promega) for 1 hour at 4ºC on a spinning wheel. After incubation the beads were washed 4 times in buffer A and the beads were collected using a magnet. Sample buffer (120 mM Tris [pH 6.8], 4% SDS, 20% Glycerol, 10% β-mercaptoethanol and 0.005% bromophenolblue) was than added to remove all proteins attached to the beads. Eluted protein extract were run on two SDS-PAGE gels, one would be analyzed by western blotting with the indicated antibodies and the other stained with the quick-CBB kit (Wako) for subsequent mass spectrometry analysis.

For mass spectrometric analysis ubiquitinated peptides were analyzed on an LTQ-Orbitrap (Thermo).

Immunofluorescence

For immunostaining, cells were fixed in 2% paraformaldehyde containing 0.2% Triton X-100 for 20 minutes at RT and then permeabilized with 0.1% Triton X-100 for 5 minutes. Samples were blocked in 1% FCS and immunostained with antibodies as indicated.

For detection of Hus1-GFP and GFP-Rad9 in U2OS, cells were fixed and permeabilized as above.

Cells containing >10 Hus1-GFP foci were scored as positive. Error bars on graphs represent the standard error of the mean of at least two independent experiments.

Images were made using a Cell Observer fluorescent microscope equipped with Axiovision software (Zeiss) or a Confocal Laser Scanning Microscope LSM 510 (Zeiss), equipped with a 488 nm Ar-laser and a 505-530 nm band pass filter for green fluorescence. Red fluorescence was detected using a 543 nm laser and 560 nm long pass filter.

RESULTS

Hus1 is ubiquitinated at lysine 136

To investigate if the 9-1-1 complex is modified through ubiquitination we set up an assay using transiently expressed Flag- or GFP-tagged Hus1, Rad1 or Rad9 together with epitope-labeled ubiquitin in human HEK 293T cells. Since a previous report suggested that human Hus1 is ubiquitinated we concentrated most of our investigation on the Hus1 subunit of the complex [30]. Cell extracts containing Flag-Hus1 were lysed and incubated with beads

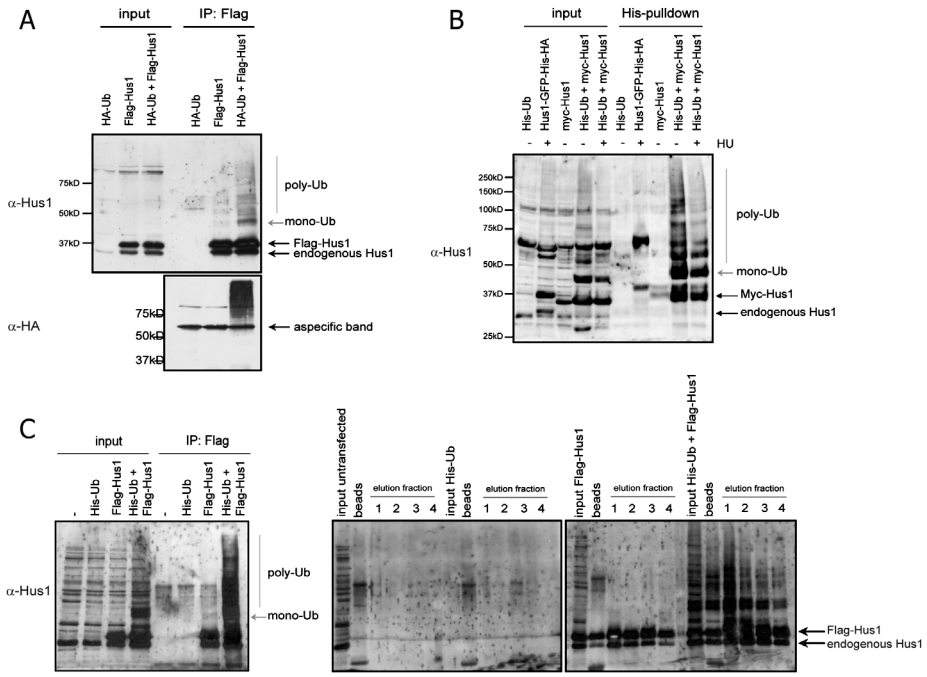

Figure 1: Hus1 ubiquitination and purification. (A) HEK 293T cells were transfected with HA-ubiquitin and Flag-Hus1, cell extracts with Flag beads to perform immunoprecipitation as described in materials and methods. Protein extracts were analyzed by western blotting with the indicated antibodies. (B) As in (A) but now incubated with His-beads. (C) Tandem tag purification of ubiquitinated Hus1 in HEK 293T cells as described in materials and methods.

containing covalently linked Flag antibodies. Immunoprecipitated Flag-Hus1 was eluted from the beads in denaturing conditions. Upon overexpression of ubiquitin, Hus1 was mono- and poly-ubiquitinated, seen as Hus1 running with slower mobility (Figure 1A). Similar results were obtained when a pulldown for His tagged ubiquitin in the presence of Hus1 was performed (Figure 1B). Treatment with the replication inhibitor HU did not influence Hus1 ubiquitination in this assay (data not shown). Besides Hus1, GFP-Rad9 also became mono- and poly-ubiquitinated and interestingly GFP-Rad1 was mono-ubiquitinated in our experimental setup (Figure 7A and B).

To identify the lysine(s) in Hus1 that is ubiquitinated and additionally reveal potential proteins involved in Hus1 ubiquitination, we performed a tandem tag purification for ubiquitinated Hus1 followed by mass spectrometry analysis of (co-)immunoprecipitated proteins [44, 45]. Flag-Hus1 was immunoprecipitated in the presence of His-tagged ubiquitin (Figure 1C). Using Flag peptides Flag-Hus1 was specifically eluted from the beads. The elution fractions were pooled and incubated with magnetic His-beads to enrich for ubiquitinated Hus1. Protein samples were run on SDS-PAGE, stained with Coomassie and the complete lanes were analyzed by mass spectrometry (Figure 2A).

Mass spectrometry results identified lysine 136 of Hus1 as being ubiquitinated. To validate and address the function of this modification we mutated lysine 136 to arginine disrupting its potential ubiquitination. We also mutated the two closely located lysines 141 and

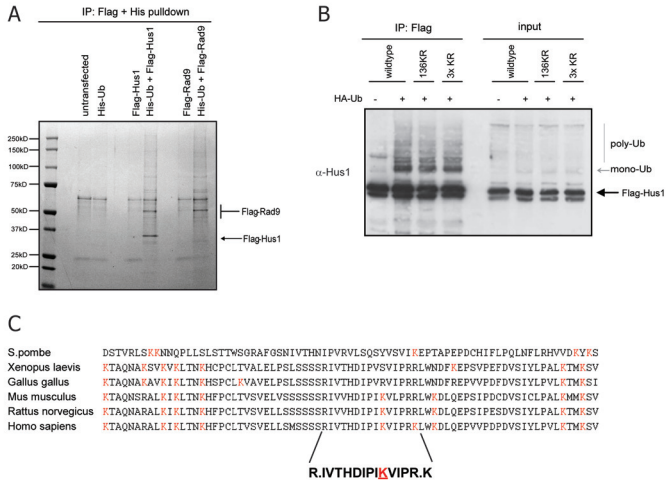

Figure 2: Identification of ubiquitinated lysine(s) in Hus1. (A) Coomassie stained SDS-PAGE gel for mass spectrometry containing the purified Hus1 and Rad9 protein extracts. (B) HEK 293T cells were transfected with HA-ubiquitin together with Flag-Hus1 wild type, K136R or K136R, K141R, K144R triple mutant. After lysis extracts were used for anti-Flag immunoprecipitation and protein extracts were analyzed by western blotting. (C) Hus1 amino acid sequence comparison between different species, shown are the 77 amino acids in between position 92 and 196, in total Hus1 contains 280 amino acids. Depicted is the ubiquitinated Hus1 peptide (IVTHDIPIKVIPR) that was identified through mass spectrometry.

144 to exclude the possibility that these neighbouring lysines would be ubiquitinated instead. Expression of the K136R and triple K136R/K141R/K144R mutant only slightly influenced Hus1 ubiquitination in our ubiquitination assay (Figure 2B). Although lysine 136 is situated in a well conserved part of the Hus1 protein the lysine itself is not conserved throughout different species (Figure 2C). These results suggest that additional lysine(s) in Hus1 are ubiquitinated, but those were not identified by the mass spectrometry analysis. Alternatively the ubiquitination of Hus1 might be influenced by overexpressing of ubiquitin in our assay, since we did not observe ubiquitination of Hus1 in cell extracts without transient overexpression of ubiquitin (Figure 1). We were also unable to immunoprecipitate endogenous Hus1 with or without overexpression of ubiquitin (data not shown). To minimize potential adverse effects of transiently expressed ubiquitin we titrated down the amount of ubiquitin necessary to detect Hus1 ubiquitination on western blotting and used this low amount to test ubiquitination of the Hus1-K136R mutant and even under those conditions the Hus1 mutant became ubiquitinated (data not shown). Nonetheless we cannot be absolutely certain that we are able to address the loss of ubiquitination in the K136R mutant under these experimental conditions. Further investigations will be necessary to address the relevance of Hus1 ubiquitination on lysine 136.

EDD, the candidate E3 ligase for Hus1 ubiquitination

Mass spectrometry analysis of purified (ubiquitinated) Hus1 also identified potential interaction partners of Hus1, which are depicted in Table 1. Selections of the results are shown in three categories, namely proteins from the ATR-Chk1 pathway (including known Hus1 interaction partners), DNA repair proteins and potential regulators involved

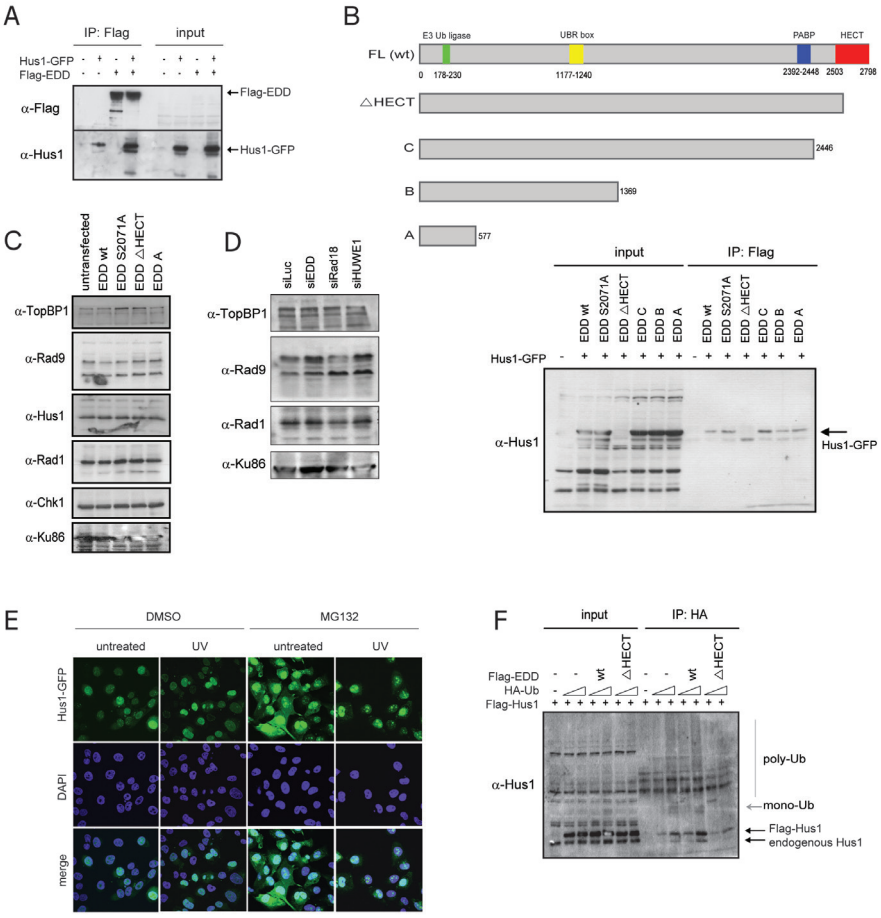

Figure 3: Functional relationship between Hus1 and the EDD ubiquitin ligase. (A) Co-immunoprecipitation experiment in HEK 293T cells transfected with Hus1-GFP and Flag-EDD using Flag beads. Protein extracts were analyzed by western blotting with the indicated antibodies. (B) Diagram describing the different EDD domain mutants. HEK 293T cells were transfected with the indicated Flag-EDD mutants together with Hus1-GFP. Flag immunoprecipitation was performed and analyzed as previously discussed (C) Different EDD mutant constructs were expressed in U2OS cells, after lysis the extracts were analyzed by western blotting using the indicated antibodies. (D) U2OS cells were transfected with siRNA oligos as described in materials and methods targeting luciferase, EDD, Rad18 and HUWE1. After 48 hours the cells were lysed and run on SDS-PAGE and analyzed by western blotting with the indicated antibodies. (E) Hus1-GFP cells were untreated or treated with MG132 (10 μM) or UV-irradiation (20 J/m^2) or a combination of both. MG132 was added 30 minutes before administrating UV light, cells were fixed 1,5 hours later. (F) HEK 293T cells were transfected with Flag-Hus1 and increasing amounts of HA-ubiquitin in the presence of either wild type or a HECT domain mutant of EDD. Immunoprecipitation was performed using HA-beads in the presence of MG132 (10 μM). The extracts were loaded on SDS-PAGE and analyzed by western blotting with the indicated antibody.

in ubiquitination. The listed proteins were not present in the control samples without expression of Flag-Hus1. The mascot scores indicate the probability of the described protein as a potential Hus1 interacting protein (we take scores of 45 or higher as indicative of a bone fide interaction). Importantly, the results in Table 1 show that, in the presence of ubiquitin, Hus1 interacts with a number of E3 ubiquitin ligases, namely EDD (UBR5), HUWE1 and Rad18. In parallel we performed mass spectrometry analysis on the lanes containing purified ubiquitinated Rad9, as shown in Figure 2A. The results from this analysis are summarized in Table 2 and also identified EDD (UBR5), HUWE1 and Rad18 as potential Rad9 interacting proteins. Mass spectrometry did not identify any ubiquitinated lysines in Rad9, which was surprising since the immunoprecipitation of GFP-Rad9 in the presence of His-tagged ubiquitin resulted in slower migrating forms of Rad9 as seen in Figure 7A. Possibly the abundance of ubiquitinated Rad9 was not high enough to be identified by the mass spectrometry analysis.

EDD and HUWE1, HECT domain-containing E3 ligases, were identified as interactors of the 9-1-1 complex. Both proteins were previously implicated in the DNA damage response such as the ubiquitination of TopBP1 [27, 28, 46-50] and activation of Chk2 and the checkpoint arrest upon DSB induction [51, 52]. This makes them interesting candidates for regulating the 9-1-1 complex.

To investigate the functional relationship between EDD and Hus1 we started by confirming the interaction between EDD and Hus1 through co-immunoprecipitation. After overexpression of Flag-labelled EDD together with GFP-tagged Hus1 and immunoprecipitation of Flag-EDD, Hus1-GFP was identified, thereby confirming that EDD and Hus1 interact (Figure 3A). Damage induction by UV light did not change this binding (data not shown). To identify the region of EDD important for the interaction with Hus1, we performed a Hus1 binding analysis using different Flag-tagged EDD deletion mutants. Additionally we mutated EDD serine 2071, a site that is phosphorylated in response to DNA damage in an ATM/ATR-dependent manner, to determine if phosphorylation of this serine is required for the binding of EDD to Hus1 [49]. Flag immunoprecipitations indicated that Hus1 binds to the N-terminal region of EDD, as it is still able to bind to small EDD fragment A, containing the E3 ubiquitin ligase domain (Figure 3B). Mutation of serine 2071 did not abrogate the Hus1-EDD interaction, demonstrating that the ATM/ATR-dependent phosphorylation of EDD is not necessary for the interaction with Hus1.

To investigate if EDD regulates Hus1 protein levels we overexpressed EDD in U2OS cells. Expression of full length EDD or the EDD-S2071A mutant resulted in a small but significant decrease of Rad9 protein levels as judged by western blotting (Figure 3C). Deletion of the HECT domain resulted in an increase of Rad9 protein back to wild type levels suggesting that the HECT domain might be necessary for Rad9 degradation (Figure 3C). Next we downregulated EDD protein levels in U2OS by siRNA. In the same experiment we performed siRNA-mediated downregulation of HUWE1 and Rad18. Depletion of EDD and HUWE1 resulted in slightly higher Rad9 and Rad1 proteins levels. In contrast, depletion of Rad18 resulted in a decrease of Rad9 and Rad1 protein levels (Figure 3D). These results suggest that EDD and HUWE1 might regulate the 9-1-1 complex by targeting the proteins for ubiquitin-dependent, proteosomal-mediated degradation. To investigate this hypothesis we treated Hus1-GFP expressing cells with proteosome-inhibitor (MG132) and UV light and thereafter analyzed them under the microscope. In the presence of MG132, Hus1-GFP protein levels rise substantially both in the nucleus and the cytoplasm, suggesting inhibition of proteasome-dependent degradation (Figure 3E). In the presence of UV damage, Hus1-

GFP localization into foci is abrogated in the presence of MG132. Our results collectively suggest that Hus1 localization and protein levels are regulated through ubiquitination.
To address if EDD is involved in the ubiquitination of Hus1, a HECT domain mutant or the full length version of EDD were expressed and the amount of Hus1 ubiquitination after immunoprecipitation using HA-tagged ubiquitin was determined. Western blotting shows that expression of full length EDD resulted in more ubiquitinated Hus1, whereas the expression of the EDD-HECT domain mutant resulted in less Hus1 ubiquitination (Figure 3F). These data suggest that EDD is an attractive candidate E3 ligase involved in Hus1 ubiquitination mediated by its HECT domain.

EDD is involved in the ATR-mediated DNA damage response
Since EDD was described to be involved in the DNA damage-induced checkpoint activation after ionizing radiation (IR) and the results described above suggest that EDD regulates Hus1 ubiquitination [51, 52], we wondered if EDD also plays a role in the ATR-mediated checkpoint response in general. We set out by determining the UV-sensitivity of U2OS cells after siRNA-mediated downregulation of EDD. A similar experiment was performed after depletion of HUWE1. Downregulation of either EDD or HUWE1 increased the sensitivity of cells for UV damage, although to a lesser extent as knock down of Chk1 (Figure 4A).
Next we determined if EDD is required for Chk1 phosphorylation by depleting EDD and investigating the phosphorylation status of Chk1 in response to UV or HU. Downregulation of EDD caused a defect in UV-induced phosphorylation of Chk1 on serine 317 and serine 345 but the effect was less obvious after HU treatment (Figure 4B). Phosphorylation of Chk1 was also studied by immunofluorescence, combined with Cyclin B staining. During S phase Cyclin B levels start to increase and Cyclin B localizes in the cytoplasm until the G2/M transition [53]. Figure 4C shows a decrease in UV-induced Chk1 phosphorylation in cells depleted for EDD. Downregulation of EDD also results in less Cyclin B staining suggesting that EDD depletion affects cell cycle progression. Depletion of EDD for 72 hours prevented cells from entering S-phase resulting in an increased population of cells at the G1/S border (data not shown). The low amount of S phase cells might therefore be an explanation for the decreased levels of UV-induced Chk1 phosphorylation that were observed in Figures 4B and 4C.
In response to DNA damage the 9-1-1 complex is loaded onto the DNA by Rad17-RFC, seen by the accumulation of these proteins onto damaged chromatin [11]. To address the efficiency of 9-1-1 recruitment to chromatin after DNA damage in EDD-depleted cells, chromatin fractionation was performed. Whereas the 9-1-1 complex proteins were recruited to chromatin upon UV and HU treatment in control downregulated cells, EDD depletion resulted in less 9-1-1 proteins in the chromatin fraction (Figure 4D). These results suggest a role for EDD in the regulation of 9-1-1 complex localization in response to DNA damage, possibly through the ubiquitination of Hus1.

Rad18-dependent regulation of the 9-1-1 complex
Although the mass spectrometry results indicate a relatively low mascot score for the Hus1-Rad18 interaction (Table 1), Rad18 is an interesting candidate for regulating 9-1-1 because of its involvement in the ubiquitination of the Rad1 homologue in *S. cerevisiae* and its requirement for the ubiquitination of the PCNA trimer in human cells [32, 54]. Therefore we were interested in addressing its possible function in regulation of human 9-1-1. First, we confirmed the interaction between Hus1 and Rad18 by co-immunoprecipitation, which

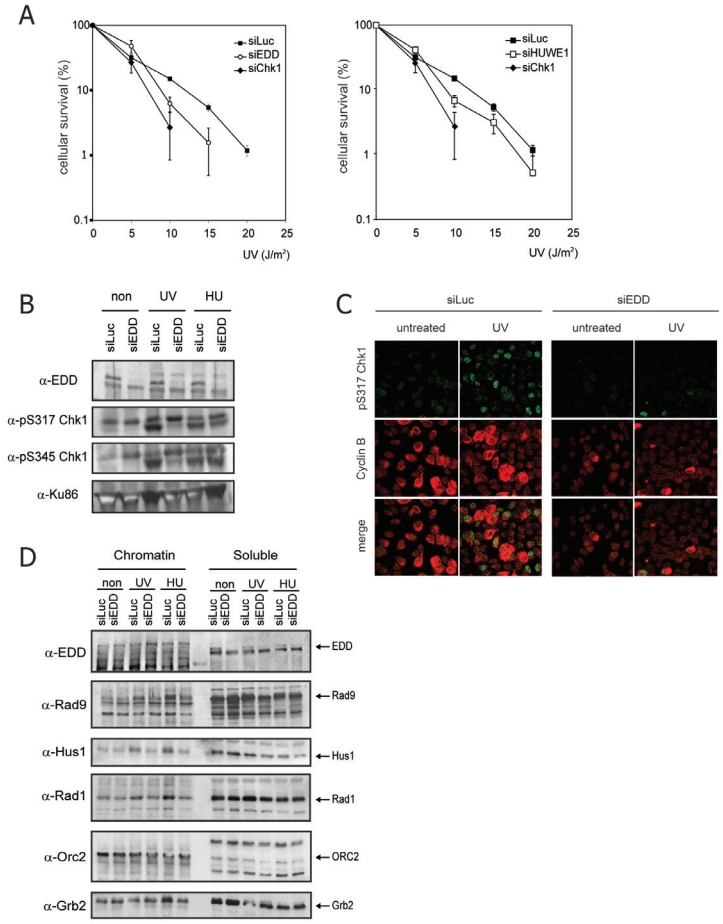

Figure 4: EDD involved in the DNA damage response through regulation of Hus1. (A) Cellular survival assay after different doses of UV using U2OS cells depleted for the indicated proteins. (B) U2OS cells were transfected with siRNA oligos targeting luciferase or EDD for 72 hours and thereafter untreated or treated with HU (10 μM) for 3 hours or UV (20 J/m^2) and fixed after 1 hour. Cell extracts were analyzed by western blotting with the indicated antibodies. (C) U2OS cells were depleted for 72 hours with oligos directed against luciferase or EDD, than untreated or UV-irradiated (20 J/m^2) and fixed 1 hour later. Immunofluorescence was performed with antibodies directed against the indicated proteins. (D) U2OS cells were transfected with siRNA oligos against luciferase and EDD. 48 hrs later cells were left untreated, incubated with 10 μM HU for 3 hours or UV-irradiated (20 J/m^2) and lysed 1 hour later. Chromatin fractionation was performed as described in materials and methods.

shows that YFP-Rad18 binds immunoprecipitated Flag-Hus1 (Figure 5A).

Upon replication stress induced by aphidicolin, GFP-Rad9 localizes into discrete nuclear foci as shown in Figure 5B [40]. Staining of these cells with antibodies recognizing Rad18 resulted in aphidicolin-induced Rad18 foci, which partially colocalized with GFP-Rad9 (Figure 5B), suggesting that Rad9 and Rad18 are involved in the cellular response to similar types of replication stress-induced DNA lesions.

To investigate the relationship between the 9-1-1 complex proteins and Rad18 after DNA

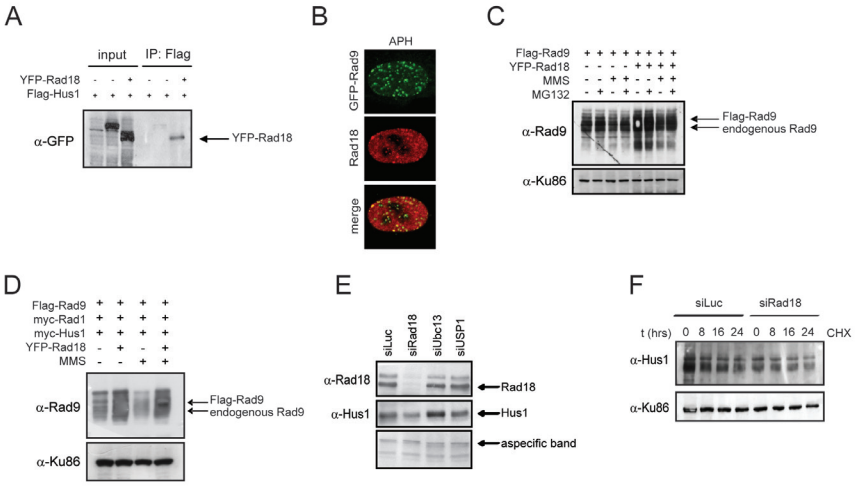

Figure 5: Rad18 involved in the regulation of the 9-1-1 complex. (A) HEK 293T cells were transfected with Flag-Hus1 and YFP-Rad18 plasmid DNA. 48 hours later cell extracts were prepared and used for immunoprecipitation using Flag beads. Protein extracts were analyzed by western blotting with the indicated antibody. (B) GFP-Rad9 cells were treated with 5 μg/ml aphidicolin for 16 hours, cells were fixed and stained with antibodies against Rad18 by immunofluorescence. (C) HEK 293T cells expressing Flag-Rad9 and YFP-Rad18 were treated with 0.02% MMS for 1 hour. MG132 (10 μM) was added 30 minutes before treatment with MMS. Cell extracts were analyzed by western blotting with the indicated antibodies. (D) Similar as in (C) however cells were transfected with different plasmids as indicated. (E) U2OS and Hus1-GFP cells were depleted for luciferase, Rad18, Ubc13 and USP1. 48 hrs after transfection cells were lysed and analyzed by western blotting with the indicated antibodies. (F) 48 hours after transfection with siRNA oligos against luciferase or Rad18 U2OS cells exposed to CHX (Sigma) and fixed at the indicated timepoints.

damage, cells were transfected with Flag-Rad9 and YFP-Rad18 and treated with the DNA damaging agent methyl methanesulfonate (MMS) and proteosome inhibitor MG132. Combined expression of Rad9 and Rad18 resulted in significantly higher Rad9 protein levels compared to the expression of Rad9 without Rad18 (Figure 5C). This was reduced after treatment with MMS and moreover the expression of Rad9 in the presence of MMS without Rad18 resulted in a reduction of Rad9 protein levels. Inhibition of proteosomal-mediated degradation by MG132 only mildly affected Rad9 proteins levels (Figure 5C).

To rule out any possible bias due to the transient expression of one single 9-1-1 subunit we overexpressed each of the components of the 9-1-1 complex and treated the cells with MMS. As was observed after the expression of only Rad9, MMS-treatment resulted in lower protein levels of Rad9. Expression of Rad18 greatly enhanced Rad9 protein levels as seen before therefore we conclude that the expression of one single 9-1-1 complex subunit did not influence the outcome of the experiments (Figure 5D). All together, these results suggest that Rad18 might stabilize 9-1-1 complex protein levels.

As mentioned before, Rad18 is responsible for the ubiquitination of the PCNA complex [54]. Ubc13 acts as the E2 ubiquitin conjugating enzyme in that reaction and USP1 is the responsible deubiquitinating enzyme [55, 56]. To investigate the relationship between the 9-1-1 complex and Rad18, Ubc13 and USP1, these proteins were depleted in U2OS cells. Downregulation of Rad18 resulted in a small decrease in Hus1 protein levels (Figure 5E). In contrast, depletion of Ubc13 resulted in higher Hus1 protein levels, whereas knock down of

USP1 did not have an effect (Figure 5E). Although preliminary, these results may point out that Ubc13 may also be involved in the regulation of Hus1.
Although the depletion of Rad18 led to a decrease in Hus1 levels, we investigated if the loss of Rad18 would influence Hus1 protein stability over time. Rad18 was downregulated in U2OS cells and cycloheximide was added to inhibit protein synthesis. Depletion of Rad18 did not influence Hus1 turnover, but it did reduce the initial Hus1 protein levels suggesting that Rad18 is not involved in the regulation of Hus1 protein levels through degradation (Figure 5F).
To obtain more evidence for the involvement of Rad18 in regulating the ATR-mediated checkpoint response we tested the UV sensitivity of cells depleted of Rad18. Cells lacking Rad18 become more sensitive for UV damage than control cells, indicating that Rad18 plays a role in the UV-induced DNA damage response (Figure 6A).
The results above indicate that Rad18 regulates 9-1-1 protein levels. To address the function of Rad18 in Hus1-GFP localization we performed immunofluorescence of Hus1-GFP cells after depletion of Rad18. In response to UV-irradiation Hus1-GFP, like GFP-Rad9, localizes into nuclear foci [24]. siRNA-mediated downregulation of Rad18 resulted in lower Hus1-GFP protein levels, which are comparable to the results in Figure 5E (Figure 6B left panel). Depletion of Rad18 resulted in 20-30% reduction of cells with UV-induced Hus1-GFP foci (Figure 6B). In response to UV or HU, Hus1-GFP, endogenous Rad9 and Rad1 present in the chromatin fractions increased. Downregulation of Rad18 resulted in a less efficient recruitment of the 9-1-1 complex proteins to damaged chromatin (Figure 6C). Taken together, these results suggest that Rad18 might stabilize the 9-1-1 complex protein levels and thereby may facilitate the localization of the 9-1-1 complex to sites of DNA lesions.

DISCUSSION

The preliminary results that are presented in this chapter imply that the 9-1-1 complex is modified by ubiquitination. To detect this post-translational modification we setup an experimental assay to detect and purify ubiquitinated 9-1-1. We were able to demonstrate ubiquitination of the 9-1-1 complex members upon overexpression only and in the presence of exogenous ubiquitin. It will therefore be challenging but necessary to demonstrate the ubiquitination of the endogenous 9-1-1 complex components without the expression of ubiquitin in future research.
The recently published crystal structure of the 9-1-1 complex indicates that Hus1 lysine 136 is an accessible amino acid, supporting our mass spectrometry results showing that this lysine is ubiquitinated [57]. However, we failed to detect a major decrease in ubiquitination of Hus1 in the K136R mutant, which might have different explanations. First, additional lysines in Hus1 are ubiquitinated under normal conditions. Second, the overexpression of ubiquitin could trigger the ubiquitination of other lysines. Lysines that normally do not get ubiquitinated are more prone for ubiquitination in the presence of large quantities of ubiquitin. Third, the results might be influenced by the presence of endogenous Hus1. Immunoprecipitation of epitope tagged-Hus1 resulted in binding of endogenous ubiquitinated Hus1, thereby possibly influencing the results obtained with the Hus1 K136R mutant (Figure 1A, 1C, 2B). Fourth, the mutation of an ubiquitinated lysine residue results in the ubiquitination of adjacent (accessible) lysine(s). Lysines residues that are in close proximity to the E3 ubiquitin binding site may adversely become ubiquitinated due to the K to R mutation. However, additional mutation of the 2 lysines close to lysine 136 (141 and

144) also did not abrogate ubiquitination of Hus1 (Figure 2B). Expression of Hus1 K136R in cells lacking Hus1 may avoid some of these discussed problems, additionally the DNA damage-induced focus formation of Hus1 K136R could also be addressed in this manner. Mouse cells lacking Hus1 exist and furthermore lysine 136 is conserved in mice, making this a suitable system [18].

The 9-1-1 complex shows structural similarities to the PCNA trimer, the processivity factor for replicative DNA polymerases [57]. PCNA is mono- and poly-ubiquitinated on lysine 164 in an Rad18/Rad6-dependent manner and is also sumoylated at this residue [54]. Whereas lysine 48 polyubiquitination results in degradation of the target protein via the proteasome, lysine 63 polyubiquitination leads to modification of protein function. PCNA can be polyubiquitinated in both ways [58] and these different types of ubiquitination are likely to also play a role in the regulation of the 9-1-1 checkpoint clamp. Our preliminary results show that the 9-1-1 complex can be regulated in different ways, possibly through both monoubiquitination and lysine 48- and lysine 63-linked polyubiquitination.

Mass spectrometry experiments identified a number of enzymes involved in ubiquitination, as Hus1-interactors. Among these enzymes were the E3 ligases EDD, HUWE1 and Rad18, the E2 Ubc13 and deubiquitin enzyme (DUB) USP1. Experiments overexpressing or depleting EDD suggest that it regulates 9-1-1 complex levels through ubiquitin-mediated degradation. Protein levels were nonetheless only slightly affected and in particular those of Rad9, which might suggest that other E3 ubiquitin ligases like HUWE1 may also be involved or act redundantly upon EDD depletion. It will therefore be interesting to see how simultaneous overexpression or depletion of EDD and HUWE1 (or UBR2 and UBR4) affects 9-1-1 protein levels. Depletion of EDD also affected 9-1-1 localization and subsequent Chk1 phosphorylation suggesting that 9-1-1 polyubiquitination is necessary for checkpoint function and therefore does not target the complex for degradation but modifies its activity. Although we cannot rule our either hypothesis at this point, we favour this explanation since the 9-1-1 complex levels only seemed to be mildly affected in the absence of EDD, whereas the checkpoint defects were much more obvious.

Due to the structural similarity between 9-1-1 and PCNA, E3 ligase Rad18 is an interesting candidate to be involved in Hus1 ubiquitination. However, Rad18 does not seem to be involved in the ubiquitin-mediated degradation of the 9-1-1 complex since Rad18 depletion does not change Hus1 protein stability, as shown in Figure 5F. In addition, overexpression of Rad18 resulted in an increase in Rad9 levels, therefore Rad18 might stabilize 9-1-1 protein through an ubiquitin-modification (mono- or lysine 63-linked ubiquitination) resulting in a more stable version of the 9-1-1 complex, which is required for the localization to sites of DNA damage. Alternatively, Rad18 might regulate 9-1-1 in an indirect manner, possibly by regulating an E3 ligase involved in 9-1-1 ubiquitination like EDD.

Our analysis also identified Ubc13, an E2 conjugating enzyme as Hus1-interacting protein. Depletion of an E2 conjugating enzyme involved in the proteosome-mediated ubiquitination of the 9-1-1 complex would indeed result in higher complex protein levels. Depletion of Ubc13 resulted in higher Hus1 protein levels, suggesting that Ubc13 might also regulate the 9-1-1 complex (Figure 5E). The DUB USP1 is involved in deubiquitinating PCNA and depletion of this enzyme could result in higher protein levels, however only if it would remove ubiquitin moieties that are responsible for ubiquitin-mediated degradation [56, 59]. Further research is required to clarify these preliminary observations regarding regulatory enzymes of ubiquitination of the 9-1-1 complex.

The *S. cerevisiae* homologue of Rad1 is mono-ubiquitinated on lysine 197 and this modification is required for efficient phosphorylation of scRad53 (ref). It will therefore be

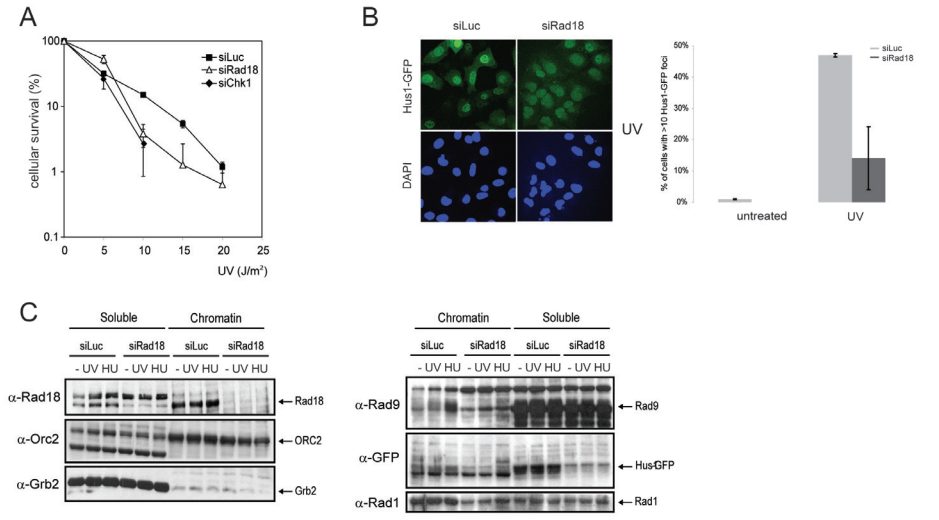

Figure 6: Rad18 involved in the UV-induced DNA damage response. (A) Cellular survival of U2OS cells depleted for the indicated proteins using different doses of UV light. (B) Hus1-GFP cells were transfected with siRNA oligos against luciferase or Rad18 and untreated or treated with UV (20 J/m^2) and fixed after 1 hour. The number of cells containing (more than 10) Hus1-GFP foci was counted. Error bars represent the SEM of two independent experiments. (C) U2OS cells were depleted with siRNA oligos against luciferase or Rad18 for 48 hours and then untreated or treated with HU (10 μM) or UV (20 J/m^2) and lysed after 1 hour. Chromatin fractionation was performed as described in materials and methods.

interesting to address the phenotype of this modification in mammalian cells [32]. As the *S. cerevisiae* and mammalian 9-1-1 amino acid sequences vary greatly, direct comparison between the two proteins cannot be made. But as mentioned before, the 9-1-1 complex shares structural resemblance to the PCNA sliding clamp and the crystal structure of the human 9-1-1 complex reveals that Rad1 lysine 185 is the structural equivalent of the well known ubiquitinated lysine 164 in PCNA. It will be interesting to determine if Rad1 lysine 185 becomes ubiquitinated and what the function of this modification in human cells is [33, 57, 60, 61]. A similar experimental setup as described in this chapter can be used to purify and identify any ubiquitinated lysines in Rad1.

Besides a function for 9-1-1 ubiquitination in the regulation of the DNA damage checkpoint, we speculate that it could also be important in other cellular processes. In response to DNA damage and the subsequent formation of RPA-coated ssDNA, the 9-1-1 complex is placed at the 3'ssDNA/dsDNA junction, opposite from PCNA at the 5'ssDNA/dsDNA junction [10]. In yeast, the 9-1-1 clamp can physically interact with translesion synthesis (TLS) polymerases [62, 63]. Possibly mediated through ubiquitination, the 9-1-1 complex may facilitate TLS by loading low fidelity DNA polymerases onto DNA. This might be in cooperation with PCNA, which was demonstrated to be involved in TLS [57, 64].

In conclusion, we have shown that the human Hus1 subunit of the 9-1-1 complex is ubiquitinated in our experimental conditions. In addition, different E3 ubiquitin ligases were implicated in the regulation of 9-1-1, possibly through ubiquitination. These results suggest that similar to PCNA, different proteins are responsible for modifying the 9-1-1 through ubiquitination.

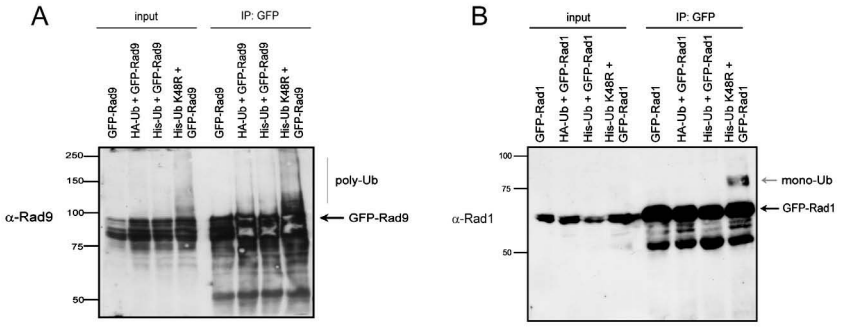

Figure 7: Ubiquitination of Rad9 and Rad1. (A) HEK 293T cells were transfected with GFP-Rad9 and HA-ubiquitin, His-ubiquitin or His-ubiquitin K48R. Cell extracts were immunoprecipitated using GFP antibodies and Protein A Sepharose beads as described in materials and methods. Protein extracts were analyzed by western blotting. (B) Similar as in (A) but now with GFP-Rad1.

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REFERENCES

1. Kanaar, R., C. Wyman, and R. Rothstein, *Quality control of DNA break metabolism: in the 'end', it's a good thing.* Embo J, 2008. **27**(4): p. 581-8.
2. Hoeijmakers, J.H., *Genome maintenance mechanisms for preventing cancer.* Nature, 2001. **411**(6835): p. 366-74.
3. Sancar, A., et al., *Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints.* Annu Rev Biochem, 2004. **73**: p. 39-85.
4. O'Driscoll, M. and P.A. Jeggo, *The role of double-strand break repair - insights from human genetics.* Nat Rev Genet, 2006. **7**(1): p. 45-54.
5. Shiloh, Y., *ATM and related protein kinases: safeguarding genome integrity.* Nat Rev Cancer, 2003. **3**(3): p. 155-68.
6. Harper, J.W. and S.J. Elledge, *The DNA damage response: ten years after.* Mol Cell, 2007. **28**(5): p. 739-45.
7. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes Dev, 2001. **15**(17): p. 2177-96.
8. Zou, L. and S.J. Elledge, *Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.* Science, 2003. **300**(5625): p. 1542-8.
9. Zou, L., D. Liu, and S.J. Elledge, *Replication protein A-mediated recruitment and activation of Rad17 complexes.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 13827-32.

10. Majka, J., et al., *Replication protein A directs loading of the DNA damage checkpoint clamp to 5'-DNA junctions.* J Biol Chem, 2006. **281**(38): p. 27855-61.
11. Zou, L., D. Cortez, and S.J. Elledge, *Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin.* Genes Dev, 2002. **16**(2): p. 198-208.
12. Delacroix, S., et al., *The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1.* Genes Dev, 2007. **21**(12): p. 1472-7.
13. Cimprich, K.A. and D. Cortez, *ATR: an essential regulator of genome integrity.* Nat Rev Mol Cell Biol, 2008. **9**(8): p. 616-27.
14. Kumagai, A., et al., *TopBP1 activates the ATR-ATRIP complex.* Cell, 2006. **124**(5): p. 943-55.
15. Lee, J., A. Kumagai, and W.G. Dunphy, *The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR.* J Biol Chem, 2007. **282**(38): p. 28036-44.
16. Smits, V.A., P.M. Reaper, and S.P. Jackson, *Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response.* Curr Biol, 2006. **16**(2): p. 150-9.
17. Weiss, R.S., et al., *Hus1 acts upstream of chk1 in a mammalian DNA damage response pathway.* Curr Biol, 2002. **12**(1): p. 73-7.
18. Weiss, R.S., T. Enoch, and P. Leder, *Inactivation of mouse Hus1 results in genomic instability and impaired responses to genotoxic stress.* Genes Dev, 2000. **14**(15): p. 1886-98.
19. Hopkins, K.M., et al., *Deletion of mouse rad9 causes abnormal cellular responses to DNA damage, genomic instability, and embryonic lethality.* Mol Cell Biol, 2004. **24**(16): p. 7235-48.
20. Kobayashi, M., et al., *Critical role for chicken Rad17 and Rad9 in the cellular response to DNA damage and stalled DNA replication.* Genes Cells, 2004. **9**(4): p. 291-303.
21. Parrilla-Castellar, E.R., S.J. Arlander, and L. Karnitz, *Dial 9-1-1 for DNA damage: the Rad9-Hus1-Rad1 (9-1-1) clamp complex.* DNA Repair (Amst), 2004. **3**(8-9): p. 1009-14.
22. Bao, S., et al., *ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses.* Nature, 2001. **411**(6840): p. 969-74.
23. Wang, X., et al., *Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress.* Mol Cell, 2006. **23**(3): p. 331-41.
24. Medhurst, A.L., et al., *ATR and Rad17 collaborate in modulating Rad9 localisation at sites of DNA damage.* J Cell Sci, 2008. **121**(Pt 23): p. 3933-40.
25. Roos-Mattjus, P., et al., *Phosphorylation of human Rad9 is required for genotoxin-activated checkpoint signaling.* J Biol Chem, 2003. **278**(27): p. 24428-37.
26. Mamely, I., et al., *Polo-like kinase-1 controls proteasome-dependent degradation of Claspin during checkpoint recovery.* Curr Biol, 2006. **16**(19): p. 1950-5.
27. Herold, S., et al., *Miz1 and HectH9 regulate the stability of the checkpoint protein, TopBP1.* Embo J, 2008. **27**(21): p. 2851-61.
28. Honda, Y., et al., *Cooperation of HECT-domain ubiquitin ligase hHYD and DNA topoisomerase II-binding protein for DNA damage response.* J Biol Chem, 2002. **277**(5): p. 3599-605.
29. Tomida, J., et al., *DNA damage-induced ubiquitylation of RFC2 subunit of replication factor C complex.* J Biol Chem, 2008. **283**(14): p. 9071-9.
30. Hirai, I., T. Sasaki, and H.G. Wang, *Human hRad1 but not hRad9 protects hHus1 from ubiquitin-proteasomal degradation.* Oncogene, 2004. **23**(30): p. 5124-30.

31. Huang, J., et al., *Jab1 mediates protein degradation of the Rad9-Rad1-Hus1 checkpoint complex.* J Mol Biol, 2007. **371**(2): p. 514-27.
32. Fu, Y., et al., *Rad6-Rad18 mediates a eukaryotic SOS response by ubiquitinating the 9-1-1 checkpoint clamp.* Cell, 2008. **133**(4): p. 601-11.
33. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.
34. Davies, A.A., et al., *Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a.* Mol Cell, 2008. **29**(5): p. 625-36.
35. Huttner, D. and H.D. Ulrich, *Cooperation of replication protein A with the ubiquitin ligase Rad18 in DNA damage bypass.* Cell Cycle, 2008. **7**(23): p. 3629-33.
36. Hirai, I. and H.G. Wang, *A role of the C-terminal region of human Rad9 (hRad9) in nuclear transport of the hRad9 checkpoint complex.* J Biol Chem, 2002. **277**(28): p. 25722-7.
37. Inagaki, A., et al., *Dynamic localization of human RAD18 during the cell cycle and a functional connection with DNA double-strand break repair.* DNA Repair (Amst), 2009. **8**(2): p. 190-201.
38. Toueille, M., et al., *The human Rad9/Rad1/Hus1 damage sensor clamp interacts with DNA polymerase beta and increases its DNA substrate utilisation efficiency: implications for DNA repair.* Nucleic Acids Res, 2004. **32**(11): p. 3316-24.
39. Danielsen, J.M., et al., *HCLK2 Is Required for Activity of the DNA Damage Response Kinase ATR.* J Biol Chem, 2009. **284**(7): p. 4140-4147.
40. Warmerdam, D.O., et al., *Cell cycle-dependent processing of DNA lesions controls localization of Rad9 to sites of genotoxic stress.* Cell Cycle, 2009. **8**(11).
41. Adams, K.E., et al., *Recruitment of ATR to sites of ionising radiation-induced DNA damage requires ATM and components of the MRN protein complex.* Oncogene, 2006. **25**(28): p. 3894-904.
42. Dart, D.A., et al., *Recruitment of the cell cycle checkpoint kinase ATR to chromatin during S-phase.* J Biol Chem, 2004. **279**(16): p. 16433-40.
43. Mendez, J. and B. Stillman, *Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis.* Mol Cell Biol, 2000. **20**(22): p. 8602-12.
44. Kaiser, P. and J. Wohlschlegel, *Identification of ubiquitination sites and determination of ubiquitin-chain architectures by mass spectrometry.* Methods Enzymol, 2005. **399**: p. 266-77.
45. Komander, D., *The emerging complexity of protein ubiquitination.* Biochem Soc Trans, 2009. **37**(Pt 5): p. 937-53.
46. Parsons, J.L., et al., *Ubiquitin ligase ARF-BP1/Mule modulates base excision repair.* Embo J, 2009. **28**(20): p. 3207-15.
47. Hall, J.R., et al., *Cdc6 stability is regulated by the Huwe1 ubiquitin ligase after DNA damage.* Mol Biol Cell, 2007. **18**(9): p. 3340-50.
48. Hall, J.R., et al., *Cdt1 and Cdc6 are destabilized by rereplication-induced DNA damage.* J Biol Chem, 2008. **283**(37): p. 25356-63.
49. Matsuoka, S., et al., *ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage.* Science, 2007. **316**(5828): p. 1160-6.
50. Henderson, M.J., et al., *EDD, the human hyperplastic discs protein, has a role in progesterone receptor coactivation and potential involvement in DNA damage response.* J Biol Chem, 2002. **277**(29): p. 26468-78.
51. Henderson, M.J., et al., *EDD mediates DNA damage-induced activation of CHK2.* J Biol

Chem, 2006. **281**(52): p. 39990-40000.
52. Munoz, M.A., et al., *The E3 ubiquitin ligase EDD regulates S-phase and G(2)/M DNA damage checkpoints.* Cell Cycle, 2007. **6**(24): p. 3070-7.
53. Jackman, M., M. Firth, and J. Pines, *Human cyclins B1 and B2 are localized to strikingly different structures: B1 to microtubules, B2 primarily to the Golgi apparatus.* Embo J, 1995. **14**(8): p. 1646-54.
54. Ulrich, H.D., *Regulating post-translational modifications of the eukaryotic replication clamp PCNA.* DNA Repair (Amst), 2009. **8**(4): p. 461-9.
55. Ulrich, H.D. and S. Jentsch, *Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair.* Embo J, 2000. **19**(13): p. 3388-97.
56. Nijman, S.M., et al., *The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway.* Mol Cell, 2005. **17**(3): p. 331-9.
57. Dore, A.S., et al., *Crystal structure of the rad9-rad1-hus1 DNA damage checkpoint complex--implications for clamp loading and regulation.* Mol Cell, 2009. **34**(6): p. 735-45.
58. Brun, J., et al., *Regulation of PCNA polyubiquitination in human cells.* BMC Res Notes. **3**(1): p. 85.
59. Huang, T.T., et al., *Regulation of monoubiquitinated PCNA by DUB autocleavage.* Nat Cell Biol, 2006. **8**(4): p. 339-47.
60. Stelter, P. and H.D. Ulrich, *Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation.* Nature, 2003. **425**(6954): p. 188-91.
61. Ulrich, H.D., *Conservation of DNA damage tolerance pathways from yeast to humans.* Biochem Soc Trans, 2007. **35**(Pt 5): p. 1334-7.
62. Sabbioneda, S., et al., *The 9-1-1 checkpoint clamp physically interacts with polzeta and is partially required for spontaneous polzeta-dependent mutagenesis in Saccharomyces cerevisiae.* J Biol Chem, 2005. **280**(46): p. 38657-65.
63. Kai, M., et al., *Rad3-dependent phosphorylation of the checkpoint clamp regulates repair-pathway choice.* Nat Cell Biol, 2007. **9**(6): p. 691-7.
64. Jansen, J.G., M.I. Fousteri, and N. de Wind, *Send in the clamps: control of DNA translesion synthesis in eukaryotes.* Mol Cell, 2007. **28**(4): p. 522-9.

Flag-Hus1

Gene name	Description/function	Mascot score
ATR-Chk1 pathway		
Rad1	Cell cycle checkpoint protein Rad1	556
Hus1	Cell cycle checkpoint protein Hus1	446
ATR	Serine/threonine-protein kinase ATR	54
RFC5	Replication factor C subunit 5	45
Claspin	Isoform 1 of Claspin	37
DNA repair		
TRIM28 (KAP1)	Transcription intermediary factor 1-beta	642
PCNA	Proliferating cell nuclear antigen	208
FEN1	Flap endonuclease 1	87
XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6	83
Ubiquitination		
UBR3	E3 ubiquitin-protein ligase UBR3	45

Flag-Hus1 + His-ubiquitin

Gene name	Description/function	Mascot score
ATR-Chk1 pathway		
Hus1	Cell cycle checkpoint protein Hus1	1060
Rad1	Cell cycle checkpoint protein Rad1	556
RFC4	Replication factor C subunit 4	534
RFC5	Replication factor C subunit 5	516
RFC2	Replication factor C subunit 2	433
RFC1	Replication factor C subunit 1	202
RPA70	Replication protein A 70 kDa DNA-binding subunit	178
TopBP1	DNA topoisomerase 2-binding protein 1	144
Rad17	Cell cycle checkpoint protein Rad17	114
RFC3	Replication factor C subunit 3	112
RPA32	Replication protein A 32 kDa DNA-binding subunit	61
ATR	Serine/threonine-protein kinase ATR	42
DNA repair		
TRIM28 (KAP1)	Transcription intermediary factor 1-beta	1494
PCNA	Proliferating cell nuclear antigen	650
FEN1	Flap endonuclease 1	416
Rad50	DNA repair protein Rad50	348
MSH2	DNA mismatch repair protein Msh2	174
Mre11	Double-strand break repair protein MRE11A	144
MSH3	DNA mismatch repair protein Msh3	89
XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6	66
MSH6	DNA mismatch repair protein Msh6	47
BLM	Bloom syndrome protein	45
Ubiquitination		
UBR5 (EDD)	E3 ubiquitin-protein ligase UBR5	790
HUWE1	E3 ubiquitin-protein ligase HUWE1	854
USP5	Ubiquitin carboxyl-terminal hydrolase 5	461
UBR4	E3 ubiquitin-protein ligase UBR4	421
USP9	Ubiquitin specific protease 9	304
RNF40	E3 ubiquitin-protein ligase BRE1B	274
RNF20	E3 ubiquitin-protein ligase BRE1A	112
UBE2E1	Ubiquitin-conjugating enzyme E2 E1	79
TRIM33	E3 ubiquitin-protein ligase TRIM33	76
USP48	USP48 protein	66
UBR2	E3 ubiquitin-protein ligase UBR2	45
Rad18	E3 ubiquitin-protein ligase Rad18	45

Table 1: Hus1 interaction partners identified by mass spectrometry analysis of Flag-Hus1 immono-precipitations in presence or absence of His-ubiquitin.

Flag-Rad9

Gene name	Description/function	Mascot score
ATR-Chk1 pathway		
Rad9	Cell cycle checkpoint control protein Rad9A	704
Rad1	Cell cycle checkpoint protein Rad1	175
Hus1	Cell cycle checkpoint protein Hus1	86
ATR	Serine/threonine-protein kinase ATR	72
TopBP1	DNA topoisomerase 2-binding protein 1	39
DNA repair		
TRIM28 (KAP1)	Transcription intermediary factor 1-beta	706
PCNA	Proliferating cell nuclear antigen	381
FEN1	Flap endonuclease 1	117
XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6	46
Rad50	DNA repair protein Rad50	45
WRN	Werner syndrome ATP-dependent helicase	45

Flag-Rad9 + His-ubiquitin

Gene name	Description/function	Mascot score
ATR-Chk1 pathway		
TopBP1	DNA topoisomerase 2-binding protein 1	1257
Rad9	Cell cycle checkpoint control protein Rad9A	808
RFC5	Replication factor C subunit 5	432
RPA70	Replication protein A 70 kDa DNA-binding subunit	415
RFC4	Replication factor C subunit 4	326
RFC2	Replication factor C subunit 2	325
RFC1	Replication factor C subunit 1	275
RFC3	Replication factor C subunit 3	184
RPA32	Replication protein A 32 kDa DNA-binding subunit	180
Hus1	Cell cycle checkpoint protein Hus1	107
Rad1	Cell cycle checkpoint protein Rad1	66
Claspin	Isoform 1 of Claspin	54
RPA14	Replication protein A 14 kDa DNA-binding subunit	46
DNA repair		
TRIM28 (KAP1)	Transcription intermediary factor 1-beta	1392
Rad50	DNA repair protein Rad50	588
PCNA	Proliferating cell nuclear antigen	584
FEN1	Flap endonuclease 1	422
MSH2	DNA mismatch repair protein Msh2	196
Mre11	Double-strand break repair protein MRE11A	162
MSH6	DNA mismatch repair protein Msh6	69
XRCC6	X-ray repair complementing defective repair in Chinese hamster cells 6	63
Ubiquitination		
HUWE1	E3 ubiquitin-protein ligase HUWE1	1158
RNF40	E3 ubiquitin-protein ligase BRE1B	929
UBR5 (EDD)	E3 ubiquitin-protein ligase UBR5	632
TRM33	E3 ubiquitin-protein ligase TRIM33	202
UBR2	E3 ubiquitin-protein ligase UBR2	182
UBE2E1	Ubiquitin-conjugating enzyme E2 E1	143
RLIM	E3 ubiquitin-protein ligase RNF12	122
RNF20	E3 ubiquitin-protein ligase BRE1A	117
HECTD3	E3 ubiquitin-protein ligase HECTD3	95
RNF2	E3 ubiquitin-protein ligase RING2	87
UBR4	E3 ubiquitin-protein ligase UBR4	84
Rad18	E3 ubiquitin-protein ligase Rad18	66
HERC1	E3 ubiquitin-protein ligase HERC1	59

Table 2: Rad9 interaction partners identified by mass spectrometry analysis of Flag-Rad9 immono-precipitations in presence or absence of His-ubiquitin.

Abbreviations

AA	amino acid
AT	ataxia telangiectasia
ATM	ataxia telangiectasia mutated
ATR	ATM- and Rad3-related
ATRIP	ATR-interacting protein
BrdU	bromodeoxyuridine
cDNA	complementary DNA
CPD	cyclobutane pyrimidine dimer
DNA	deoxyribonucleic acid
DSB	double-stranded break
FACS	fluorescence-activated cell sorting
FRAP	fluorescence recovery after photobleaching
FLIP	fluorescence loss in photobleaching
γH2AX	phosphorylated H2AX
GFP	green fluorescent protein
Gy	Gray
HR	homologous recombination
HU	hydroxyurea
IR	ionizing radiation
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MMS	methyl methanesulfonate
Nbs1	Nijmegen breakage syndrome protein 1
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
PCNA	proliferating cell nuclear antigen
PI	propidium iodide
PIKK	phosphoinositide 3-kinase-like protein kinase
RFC	replication factor C
RPA	replication protein A
S.cerevisiae	Saccharomyces cerevisiae
S.pombe	Schizosaccharomyces pombe
ssDNA	single-stranded DNA
TLS	DNA translesion synthesis
TopBP1	topoisomerase IIβ binding protein
UV	ultraviolet
6-4 PP	pyrimidine (6-4) pyrimidone photoproduct

Nederlandse samenvatting

DNA is de drager van erfelijke eigenschappen van cellen, de bouwstenen van het lichaam. Beschadigingen in het DNA, veroorzaakt door bijvoorbeeld ioniserende en ultraviolette (UV) straling in zonlicht, moeten zo goed mogelijk hersteld worden omdat fouten in het DNA kunnen leiden tot het ontstaan van kanker.

Tijdens de deling doorloopt de cel de celcyclus, waarin het erfelijke materiaal verdubbeld wordt en dit vervolgens verdeeld wordt over de twee dochtercellen. Om te voorkomen dat beschadigingen in het DNA doorgegeven worden aan de dochtercellen bestaan er controlemechanismen die continue het DNA afspeuren op beschadigingen. Bij de detectie van DNA schade worden deze controlemechanismen geactiveerd, zodat de celdeling wordt geblokkeerd om de cel tijd te geven het beschadigde DNA te herstellen. Wanneer cellen niet efficiënt reageren op DNA schade kan dit ten eerste lijden tot een versnelde celdeling. Ten tweede, door het niet correct repareren van DNA, kunnen er mutaties en breuken ontstaan. Beide zorgen voor een abnormaal functioneren van de cel met op een lange termijn tumorvorming tot gevolg. Een juiste reactie op DNA schade is daarom essentieel voor het voortbestaan van cellen en het organisme. Mijn proefschrift beschrijft de initiële respons van cellen op DNA schade. Door het analyseren van eiwitten die betrokken zijn bij de reactie op DNA schade in levende cellen met behulp van de microscoop zijn wij meer te weten gekomen over de regulatie van dit belangrijke proces.

Onderzoek in de laatste decennia heeft aangetoond dat het ATR-Chk1 controlemechanisme (*checkpoint*) een belangrijke sleutel is in de reactie van cellen op DNA schade. Een veelvoud aan verschillende eiwitten zijn betrokken in deze eiwitcascade ofwel 'pathway'. In **Hoofdstuk 1** worden de verschillende componenten van de ATR-Chk1 pathway geïntroduceerd en hun functie besproken. Hoe het ATR-Chk1 en andere signaaltransductieroutes samenwerken met DNA reparatie mechanismen wordt beschouwd in **Hoofdstuk 2.**

Tot op heden waren de eiwitten die betrokken zijn bij de ATR-Chk1 pathway vooral door middel van biochemische technieken onderzocht en er was nog maar weinig bekend over hoe deze eiwitten zich gedragen in levende cellen. Om dit te onderzoeken hebben wij verschillende eiwitten in de ATR-Chk1 pathway gelabeld met een fluorescerend eiwit genaamd GFP (groen fluorescerend eiwit). In **Hoofdstuk 3** beschrijven wij de methode die is opgezet om dit te doen en om cellen te maken die deze eiwitten stabiel tot expressie brengen om ze zodoende geschikt te maken voor levende cel analyse onder de confocale fluorescentie microscoop. Door middel van deze stabiele cellijnen, het bleken van de fluorescentie en videomicroscopie (mobiliteitstudies) laten wij zien dat het ATRIP-ATR eiwit complex zich anders gedraagt na UV straling dan het Rad9-Rad1-Hus1 (9-1-1) complex. ATRIP lijkt zich sneller naar het beschadigde DNA te bewegen dan de eiwitten van het 9-1-1 complex. Beide complexen zijn betrokken bij het detecteren van DNA schade en het activeren van het controlemechanisme dat de celdeling tijdelijk blokkeert.

De eiwitten die betrokken zijn bij DNA schade detectie hopen zich na het geven van schade

op in zogenaamde *foci* in de kern van de cel. Door middel van deze observatie bestuderen wij in **Hoofdstuk 4** de relatie tussen celdeling en DNA reparatie mechanismen. De lokalisatie van Rad9 in de cel blijkt afhankelijk te zijn van het type DNA schade en de celcyclus fase waarin deze schade wordt veroorzaakt. In **Hoofdstuk 4** gaan wij hier verder op in en laten zien dat na UV straling, in tegenstelling tot ioniserende straling, de ATR-Chk1 pathway niet wordt geactiveerd in de G2 fase van de celcyclus. De p38 signaaltransductieroute speelt hierbij een veel belangrijkere rol, wat laat zien dat verschillende controlemechanismen tijdens de celcyclus differentieel gereguleerd worden.

In reactie op DNA schade is de ATR kinase verantwoordelijk voor de modificatie van Chk1. Dit proces is afhankelijk van het 9-1-1 eiwit complex. De modificatie van Chk1 zorgt ervoor dat de celcyclus wordt stopgezet om het DNA te repareren. In **Hoofdstuk 5** onderzoeken wij deze gebeurtenis in detail door middel van mobiliteitstudies en laten zien dat de fosforylering van Rad17 door ATR nodig is voor een stabiele associatie van het 9-1-1 complex op beschadigd DNA, wat implicaties heeft voor de werking van het ATR-Chk1 controlemechanisme. Wij laten hiermee ook zien dat mobiliteitstudies een goede aanvulling zijn op bestaande biochemische technieken doordat zij het effect van kortdurende eiwit-eiwit interacties en eiwit modificaties op het gedrag van eiwitten laten zien. In **Hoofdstuk 6** laten wij zien dat het 9-1-1 complex wordt gemodificeerd door ubiquitinering, en dan met name de Hus1 component van het 9-1-1 complex. Met behulp van massa-spectrometrie hebben wij het gemodificeerde aminozuur in Hus1 geïdentificeerd en ten slotte speculeren wij over welke eiwitten verantwoordelijk zouden kunnen zijn voor deze modificatie.

Curriculum Vitae

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Research experience:

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Research skills:
Confocal microscopy course
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In-depth courses: (e.g. Research school, Medical training)
Molecular and Cell Biology
Reading and discussing literature
Cellular Response to DNA Damage and Cancer Risk

International conferences:
DNA Damage Response and Repair Mechanisms 2009, Crete, Greece
Ataxia-Telangiectasia (AT) workshop 2008, Otsu, Japan

Seminars and workshops:
The 4th International Summer School "DNA and Chromosomes: Physical and Biological Approaches" 2006, France
Annual MGC graduate student workshop 2006-2009

Presentations:
Ataxia-Telangiectasia (AT) workshop 2008, Otsu, Japan
Integrated project DNA repair, 3rd annual review meeting 2008, Rotterdam, the Netherlands
NWO-CW study meeting Nucleic acids 2008, Lunteren, the Netherlands
MGC graduate student workshop 2007, Maastricht, the Netherlands

Awards:
Young investigator award Ataxia-Telangiectasia workshop 2008, Otsu, Japan

Organizing skills:
MGC graduate student workshop 2007, Maastricht, the Netherlands

Didactic skills:
Supervisor Master Student 2009-2010
Teaching practical skills *in vivo* imaging course 2007-2009

List of publications

Warmerdam DO, Kanaar R, Smits VA.
Differential dynamics of ATR-mediated checkpoint regulators
J Nucl Acids, *in press*

Smits VA, **Warmerdam DO**, Martin Y, Freire R.
Maintenance of genome stability by ATR-mediated checkpoint signalling
Front Biosci. 2010 June 1;15, 840-853

Luijsterburg MS, von Bornstaedt G, Gourdin AM, Politi AZ, Moné MJ, **Warmerdam DO**, Goedhart J, Vermeulen W, van Driel R, Höfer T.
Stochastic and reversible assembly of a multiprotein DNA repair complex ensures accurate target site recognition and efficient repair
J Cell Biol. 2010 May 3;189(3):445-63

Warmerdam DO, Kanaar R.
Dealing with DNA damage: relationships between checkpoint and repair pathways
Mutat Res. 2010 April - June;704(1-3):2-11

Luijsterburg MS, Dinant C, Lans H, Stap J, Wiernasz E, Lagerwerf S, **Warmerdam DO**, Lindh M, Brink MC, Dobrucki JW, Aten JA, Fousteri MI, Jansen G, Dantuma NP, Vermeulen W, Mullenders LH, Houtsmuller AB, Verschure PJ, van Driel R.
Heterochromatin protein 1 is recruited to various types of DNA damage
J Cell Biol. 2009, 185(4):577-86

Warmerdam DO, Freire R, Kanaar R, Smits VA.
Cell cycle-dependent processing of DNA lesions controls localization of Rad9 to sites of genotoxic stress
Cell Cycle. 2009, 8(11):1765-74

Medhurst AL*, **Warmerdam DO***, Akerman I, Verwayen EH, Kanaar R, Smits VA, Lakin ND.
*These authors contributed equally to this work.
ATR and Rad17 collaborate in modulating Rad9 localization at sites of DNA damage
J Cell Sci. 2008, 121(23):3933-40

Wyman C, **Warmerdam DO**, Kanaar R.
From DNA end chemistry to cell-cycle response: the importance of structure, even when it's broken
Mol Cell. 2008, 30(1):5-6

Zotter A, Luijsterburg MS, **Warmerdam DO**, Ibrahim S, Nigg A, van Cappellen WA, Hoeijmakers JH, van Driel R, Vermeulen W, Houtsmuller AB.
Recruitment of the nucleotide excision repair endonuclease XPG to sites of UV-induced DNA damage depends on functional TFIIH
Mol Cell Biol. 2006, 26(23):8868-79

Dankwoord

The last couple of pages in this thesis,... finally! Curious if you might be in there? Or wondering how many times the word thank you (*bedankt* in Dutch) is written? Yes,.. read on quickly! No,.. read on quickly as well!

The lab is almost empty on a sunny late Saturday afternoon somewhere in California. It has been only a few weeks since I have started working in this new environment. A new lab and a lot of distance between here and NL, a perfect setting to look back on the years as a PhD student, years that went by quickly….
De afgelopen vier en een half jaar waren een leuke en interessante tijd voor me. Ik heb heel veel nieuwe dingen geleerd, in een nieuwe stad gewoond, baanbrekende experimenten gedaan ☺ en een heleboel leuke en toffe mensen ontmoet.

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Martijn, bedankt voor de samenwerkingen en succes met het afronden van je postdoc in Stockholm. Stavros thanks for the pleasant collaboration and I hope things work out with the remaining experiments. I would also like to acknowledge Nick Lakin and Annette Medhurst for our successful collaboration and thank Nick for being a member in my defence committee. *Muchas gracias* to all the members of the Freire lab for their help, nice atmosphere and *tenderete*! Jeroen, bedankt voor de mass-spec analyzes en samenwerking. Veel van mijn experimenten zijn uitgevoerd met de confocale microscoop en daarbij heb ik ondersteuning gehad van Gert, Alex, Adriaan, Chris en Martin. Heel erg bedankt daarvoor! Jasperina en Marieke bedankt voor alle hulp en het oplossen van al die verschillende regeldingetjes.
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Lieve Eva, ik heb zo ontzettend veel zin in al onze nieuwe avonturen. Bedankt voor je onvoorwaardelijke steun en liefde. Ik hou van jou voor altijd!

While I'm typing these last words, the sun is slowly disappearing behind one of the buildings, the radio is playing:

Hold your own, know your name and go your own way ..... and everything will be fine!

(Jason Mraz feat. James Morrison - Details in the Fabric)