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Mechanisms of ATR-mediated checkpoint signalling

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1. 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 localisation 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.

2. 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 relocalises 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, stabilisation 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 relocalisation 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.

3. 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-stabilisation 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.

4. TRIGGERING ATR SIGNALLING

4.1. 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

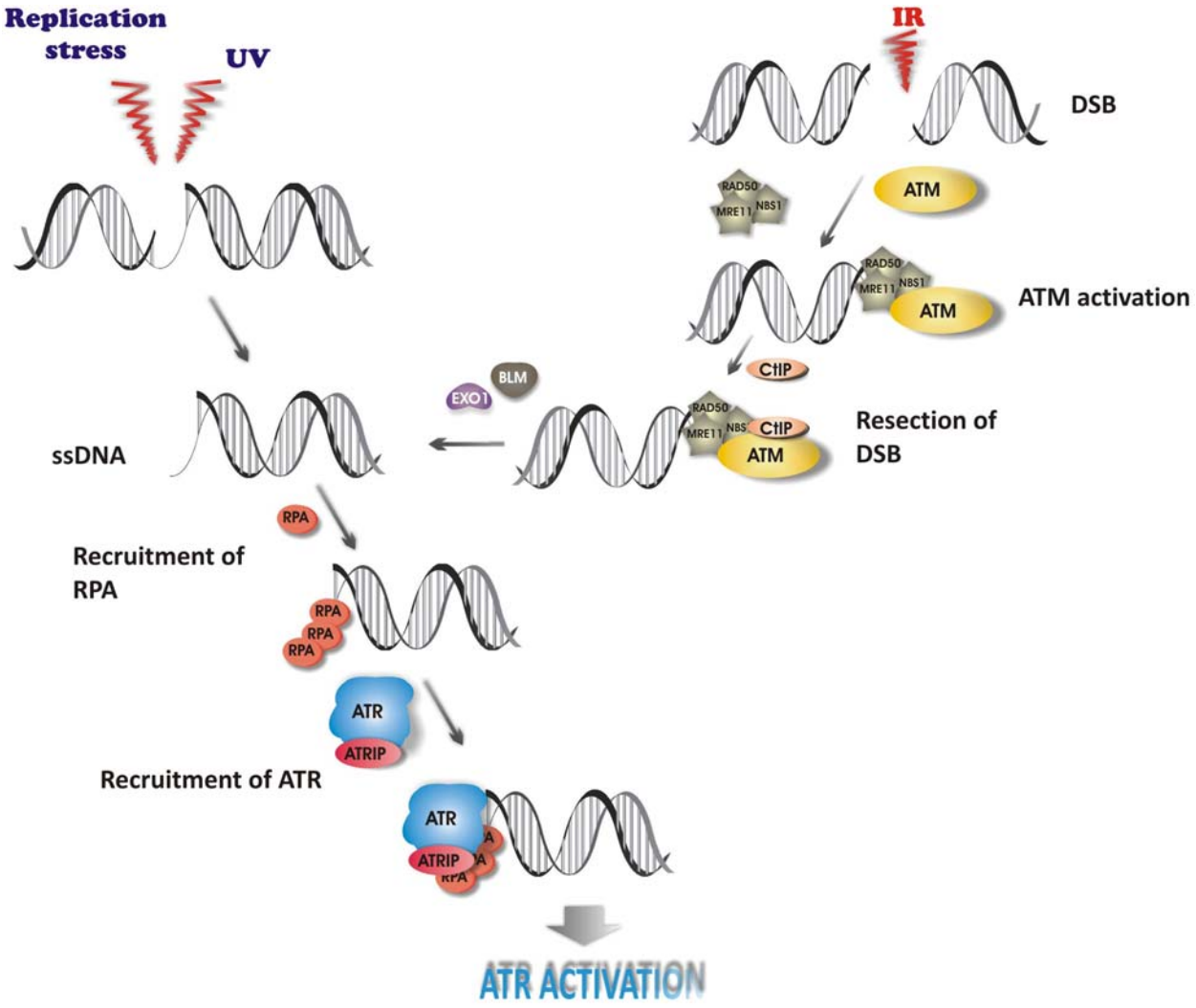

Figure 1. ATR can be activated by different types of DNA lesions. See text for details.

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) (Figure 1).

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).

4.2. 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 localisation 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).

4.3. Rad17 and Rad9-Rad1-Hus1

The localisation 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

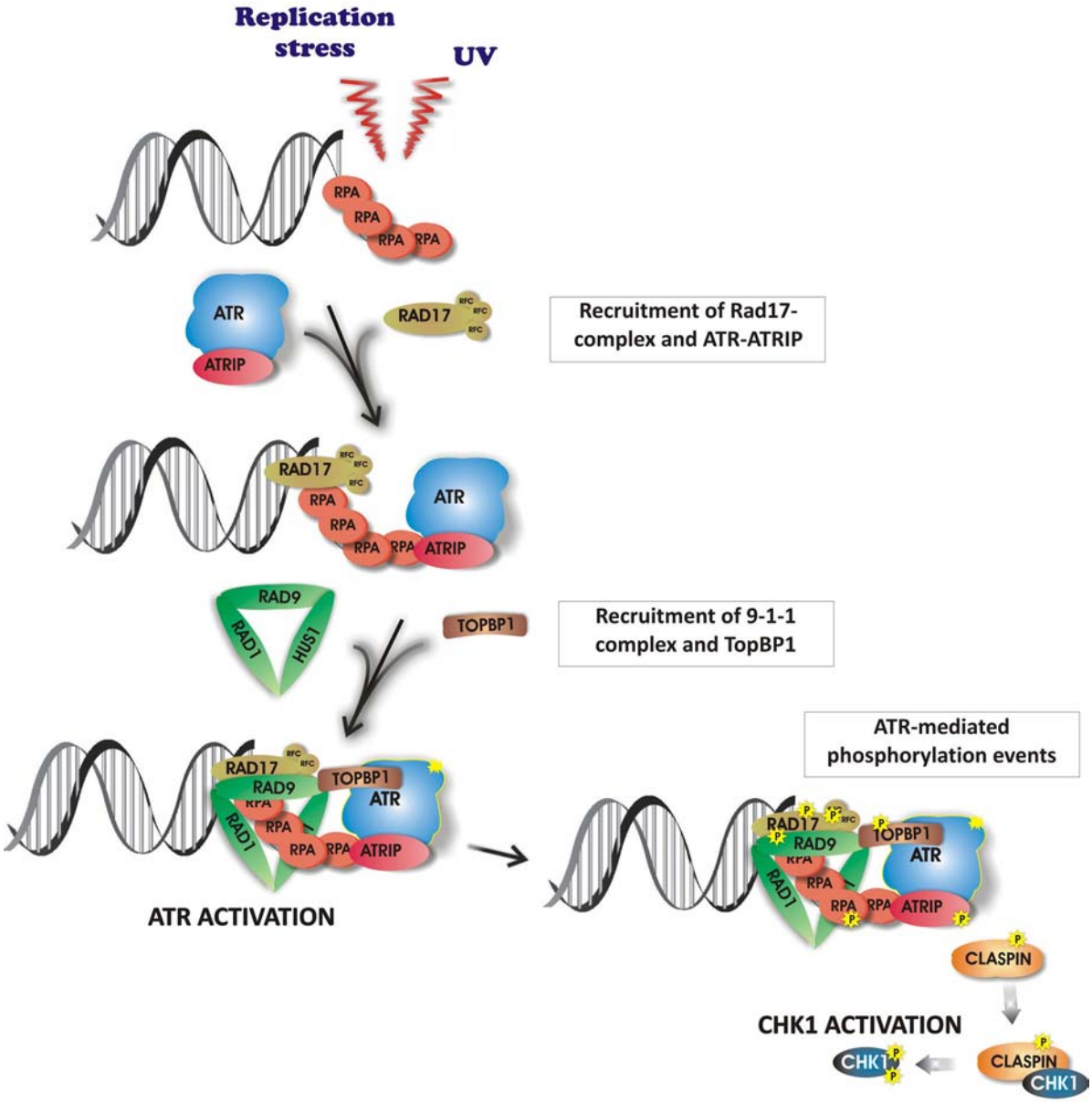

Figure 2. Factors involved in the ATR pathway, resulting in activation of effector kinase Chk1. See text for details.

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 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.

4.4. 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.

5. 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 localisation 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).

6. 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 relocalise 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 colocalisation of these fusions resulted in phosphorylation of downstream effector kinase scRad53 and a cell cycle arrest. Interestingly, artificial localisation of scDdc1 bypasses the requirement of the *S. cerevisiae* Rad17 (scRad24) clamp loader. From these data can be concluded that colocalisation 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) (D. Warmerdam and V. Smits, unpublished). The rapid disassembly of the activated proteins was hypothesised 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 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.

7. 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.

7.1. 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.

7.2. 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.

8. 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 hypothesised 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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Abbreviations: ATM: ataxia telangiectasia mutated; ATR: ATM- and Rad3-related; ATRIP: ATR-interacting protein; DSB: double-stranded break; HU: hydroxyurea; IR: ionizing radiation; MEF: mouse embryonic fibroblasts; NER: nucleotide excision repair; PIKK: phosphoinositide 3-kinase-like protein kinase; RPA: replication protein A; sc: *S. cerevisiae*; ssDNA: single-stranded DNA; TLS: translesion synthesis; UV: ultraviolet.

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