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Review

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.

Contents

1. 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

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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 stability. 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 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–CDK1/2 is responsible for the entry into mitosis. The activities of the Cylin–CDK complex are negatively controlled by post-translational 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 proteosomal 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.

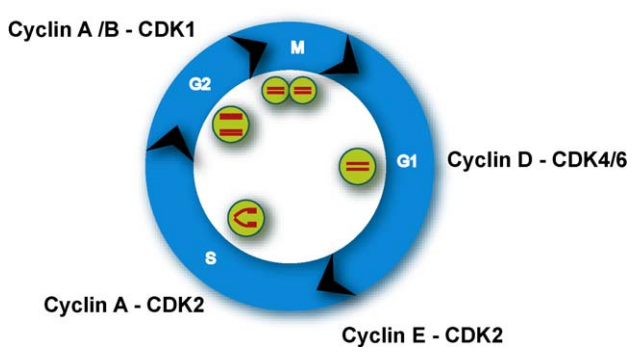

Fig. 1. Cyclin–CDK regulation throughout the different phases of the cell cycle. During the G1 phase, the Cyclin D–CDK4/6 complex is responsible for cell cycle progression after which the Cyclin E–CDK2 complex come up, leading to further progression into S-phase and duplication of the DNA. Cyclin A–CDK2 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.

2. 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.

2.1. ATM signaling

In response to DSBs, ATM activation is necessary for checkpoint activation [15,16]. The full activation of ATM is dependent on 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 at Ser1981, which coincides with ATM forming active monomers [18–20]. Besides Ser1981, ATM is also autophosphorylated at Ser367 and Ser1893. Cells harboring mutations in these sites are more sensitive to DSBs and show defective checkpoint signaling [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 a mutated form of ATM Ser1987 (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 phosphorylated 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 signaling 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 signaling [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 3.1), 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].

2.2. ATR signaling

After DNA damage infliction, ATR signaling 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].

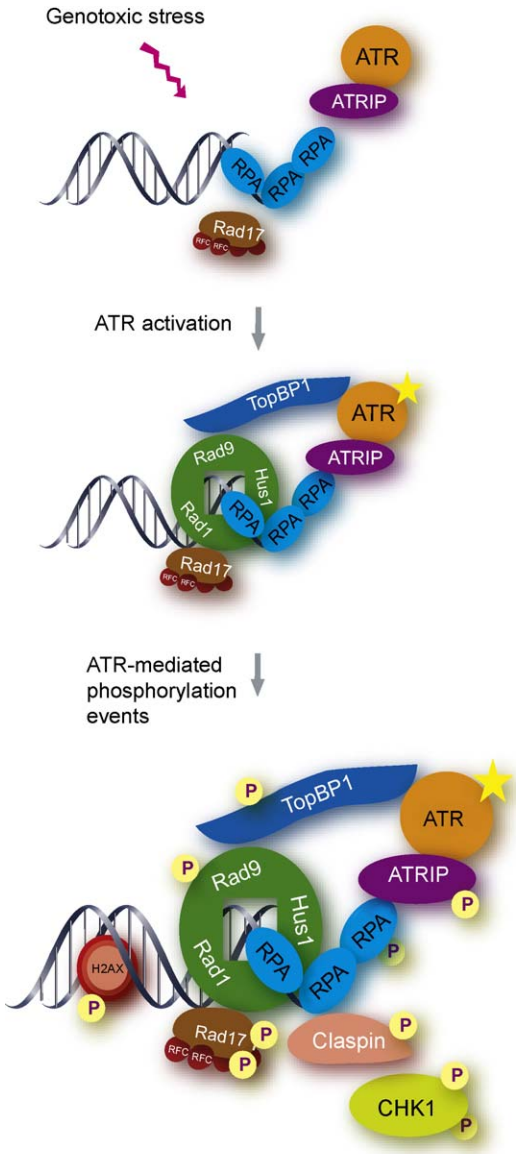

Fig. 2. Genotoxic stress leads to many ATR-mediated phosphorylation events and subsequent checkpoint activation, amplification and maintenance. RPA coated ssDNA functions as the initial signal for ATR-mediated checkpoint activation, through the independent recruitment of the ATR–ATRIP and Rad17–RFC(2–5) 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, Rad17, RPA, TopBP1, ATRIP and H2AX by ATR are all suggested to play a role in checkpoint regulation, indicating the importance of signaling through phosphorylation in checkpoint control.

The PCNA and 9-1-1 sliding clamps, preferably load onto different DNA substrates. In the case of a DNA damage-induced ssDNA gap 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 signaling [58].

Artificial localization of the Mec1 and Ddc1, the *S. cerevisiae* equivalents of ATR and Rad9 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].

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 harboring 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].

3. 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.

3.1. 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 CtIP, the MRN complex 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].

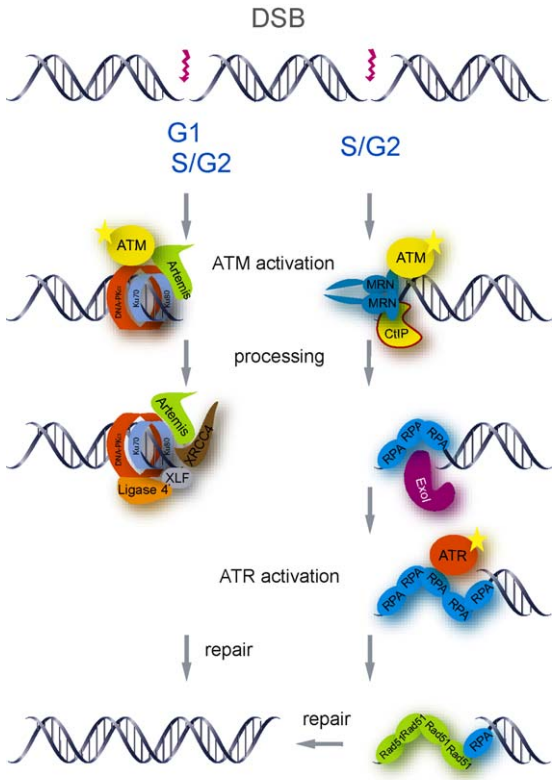

Fig. 3. Cell cycle-dependent processing of DSBs and subsequent checkpoint activation. DSBs generated in G1 and S/G2 can be repaired by NHEJ. The Ku70/80 complex binds the broken DNA ends first. ATM becomes activated, inducing a checkpoint response. The Artemis nuclease trims down the DNA end creating 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.

3.2. 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 behavior. Combining these data with the use of computer modeling will gain further insight into how the NER pathway as a whole behaves in real time in the context of the nucleus [110,111].

4. Processing of DNA lesions

In response to DNA damage, the checkpoint is activated only when the DNA alterations are processed into intermediate structures. Consequently the lesions must be recognized and processed first, before the DNA damage checkpoint becomes fully activated. Recently 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 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 coworkers 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/EmeI [120]. Mus81/EmeI 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/EmeI and SLX1 [122–126]. SLX4 in combination with Mus81/EmeI 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.

5. 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.

5.1. 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 fulfill their job due to the need for an identical template? Or is the homologous recombination machinery actively being shut-down 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 favorable [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 favor 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.

5.2. 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 signaling 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 signaling also outside of S-phase [149–151]. UV-induced lesions outside of S-phase will also be recognized by NER. During NER-dependent

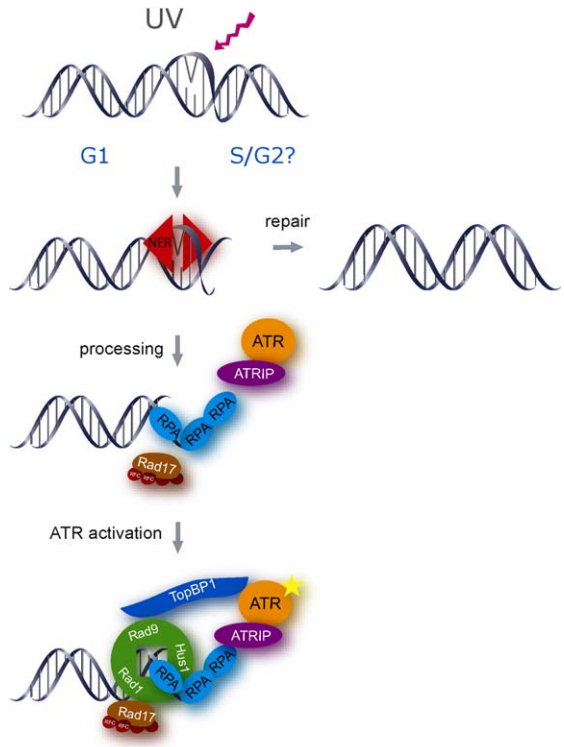

Fig. 4. NER-dependent checkpoint activation after UV-irradiation. UV lesions are recognized by NER and thereafter either directly repaired or processed further to create ssDNA gaps. RPA quickly accumulates at the ssDNA gap after which the ATR–ATRIP and Rad17–RFC(2–5) complexes independently localize 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.

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.

5.3. 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].

6. 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 favor 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 signaling 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.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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