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Review

The S phase checkpoint: When the crowd meets at the fork

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Abstract

Accumulation of unrepaired DNA lesions is the biggest threat to genomic stability. DNA damage checkpoints create windows of time that allow the cell to repair assaults on DNA in each phase of the cell cycle. When DNA lesions arise in S phase, however, the checkpoint machinery must work to coordinate DNA replication and repair processes. In fact some upstream components of the DNA damage checkpoint play parallel roles in maintaining the continuity of DNA replication and signaling to downstream components.
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Keywords: S phase checkpoint; DNA replication; DNA damage; ATM/ATR; Chk1/Chk2

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1. Introduction

DNA lesions arise from many different sources and can provoke a broad spectrum of repair intermediates. While damage to DNA can occur in all phases of the cell cycle, cells passing though S phase are the most susceptible to genotoxic stress [1]. Thus, in some cases cells have to repair DNA lesions in S phase before the DNA polymerase encounters the damage, while in others the checkpoint machinery may have to allow bypass of lesions and repair the damage later. This represents the most substantial difference between the S phase checkpoint and the G1 and G2 checkpoints. While in the latter the arrest of the cell cycle will allow DNA repair, in the former DNA replication will slow

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Table 1
Checkpoint homologs in different eukaryotes

Class of checkpoint protein	*S. cerevisiae*	*S. pombe*	Xenopus	Mammalian
Sensors	Rad24	Rad17	Rad17	Rad17
	Dcd1	Rad9	Rad9	Rad9
	Mec3	Hus1	Hus1	Hus1
	Rad17	Rad1	Rad1	Rad1
	Mec1	Rad3	ATR	ATR
	Dcd2/Pie1/Lcd1	Rad26	????	ATRIP
	Tel1	Tel1	ATM	ATM
Mediators	Rad9	Rhp1/Crb2	????	BRCA1, 53BP1
	Mrc1	Mrc1	Claspin	Claspin
Effectors	Rad53	Cds1	Cds1	Chk2
	Chk1	Chk1	Chk1	Chk1

down as a consequence of repair processes. Nevertheless, the S phase checkpoint shares components with the G1/S and G2/M checkpoints. Many sensors, mediators and effector kinases (summarized in Table 1) are common components of checkpoints in all phases of the cell cycle.

DNA damage-checkpoint proteins are generally well conserved and many of these proteins were initially identified in yeast. Some of these proteins (e.g. Rads) were well characterized before their cloning, thus the homonym of a certain Rad is not necessary its homologue. Melo and Toczyski [2] proposed a way to simplify nomenclature that we will follow with slight modifications: when homologs are referred to collectively we use the human name for a gene without prefix (e.g. ATR); when we refer to a specific homologue the name is preceded with a species-indicating prefix (h, human; sc, *S. cerevisiae*; sp, *S. pombe*; and x, Xenopus) and the human protein name for the same function will be given afterwards, in parentheses (e.g. scRad17 (Rad1)).

2. When do cells activate an S phase checkpoint?

The deleterious effects on DNA integrity in S phase of chemotherapeutic agents, UV light exposure, smoke and a plethora of mutagenic stimuli have been described [3]. Perhaps more relevantly, and unique to the S phase, not only external sources of mutagenic agents but reactive by-products of cellular metabolism can damage DNA. Indeed, perhaps the most dangerous type of DNA lesions, double strand breaks (DSBs), can arise during unperturbed DNA replication as forks pass though nicked DNA or through certain repair or recombination intermediates, or when forks stall at a site of DNA damage [4].

We can first ask what is the extent of DNA damage required to activate a checkpoint? It seems that the threshold for activation depends on both the number and type of lesions. While only a few DSBs can eventually activate a checkpoint [5–7], cells can handle a substantial accumulation of single stranded DNA (ssDNA) intermediates before the intra-S phase checkpoint response is initiated. This may relate to the fact that unperturbed replication accumulates ssDNA. For example, in *S. cerevisiae*, while around 300 bp of ssDNA accumulate during a normal S phase, 500 bp of ssDNA will activate a checkpoint [8]. During the S phase checkpoint the DNA repair machinery requires DNA replication to continue [9,10]. In fact the replication-related functions of DNA polymerase ε (Pol ε) and its interacting partners are required for efficient checkpoint activation in budding yeast [11]. DNA polymerase α (Pol α) primase activity is also required for the S phase checkpoint [12,13]. All of the above suggest that the main function of the S phase checkpoint is to maintain fork integrity. Cell viability results as an indirect effect of prevention of DNA replication fork catastrophe [14].

Another trigger of an S phase checkpoint is the direct inhibition of essential components of the replication machinery. Compounds such as hydroxyurea (HU) and aphidicolin (APH) trigger the replication checkpoint. HU is a ribonucleotide reductase inhibitor that leads to depletion of the small pool of cellular deoxyribonucleotide triphosphates while the latter directly inhibits the activity of Pol α. Treatment with these inhibitors leads to the accumulation of stalled replication forks. It is important to highlight that in this scenario the arrest is imposed on the cell and the main function of the checkpoint is to protect the replication fork from collapsing while DNA synthesis is stalled [15]. It is likely that DNA strand breaks resulting from stalled replication contribute to the activation of the S phase checkpoint. A more puzzling inducer of S phase checkpoint is hypoxia. The reasons for S phase accumulation when oxygen levels are low are not yet clearly understood. Intriguingly, hypoxia does not cause detectable DNA damage as measured by comet assays [16]. However, it has not yet been determined if there are any hypoxia-related DNA repair intermediates that could trigger the checkpoint response.

3. What is sensed by the DNA damage checkpoint?

DNA lesions induced by different agents trigger the recruitment of the repair and checkpoint machinery. Importantly, checkpoint pathways respond to a broad variety of DNA lesions with the result that different sets of repair protein bind preferentially to particular classes of lesions ([17,18] and references therein).

It is also well established that the sensor kinases of the DNA damage-checkpoint pathway (ATM/ATR, discussed bellow) interact with DNA [4]. They likely recognize features of DNA such as topology or structure that are common to all DNA lesions such as a repair-intermediate. Single stranded DNA is one such a candidate for recognition and recruitment of checkpoint molecules and in fact ATR can interact with ssDNA [19,20]. Interestingly, different types of repair

processes such as nucleotide excision repair (NER) of UV induced lesions [21] and the high fidelity homologous recombination (HR) repair of double strand breaks [22] among others produce ssDNA intermediates. Stalled replication forks also expose extended regions of ssDNA [8]. Consistent with this, short patch base excision repair (BER) does not generate significant levels of ssDNA and does not activate the DNA damage checkpoint [23]. In cells ssDNA is always coated with the essential protein RPA. Interestingly, mutated RPA causes a defective checkpoint [6]. Furthermore, a link between RPA and the activation of ATR has been established while the ability of ATR to bind purified ssDNA is greatly reduced when the DNA is not coated with RPA [24]. Are there exceptions in which checkpoint kinases are activated independently of ssDNA/RPA intermediates? DSB could represent an exception since direct recognition of DSB by the checkpoint machinery has been reported [25]. However, the DSB activated sensor kinase, ataxia telangiectasia mutated (ATM) [26] is normally bound to the Mre11–Rad50–Nbs1 (MRN) nuclease complex [27]. In studies in which HR is inhibited, ssDNA accumulates as a consequence of 5′–3′ exonuclease activities [6] thus suggesting that the nuclease activity of MRN [28] plays a role in the generation of ssDNA during DSB repair. Finally, it has been proposed that DNA distortions can also activate ATM [7] and the apparently damage-free hypoxia induced S phase arrest could also represent a potential exception to this rule. However, it might be speculated that ssDNA could be exposed as a consequence of chromatin tension during those treatments.

4. What are the factors involved in S phase checkpoint?

Studies with yeast, invertebrates, frogs, and mammals have revealed sets of proteins that play roles in the signaling pathways involved in responding to extrinsic and intrinsic damage during S phase. To a significant extent the relevant gene products are conserved both structurally and functionally although differences and complexities exist. Following the same organization as in Table 1, we discuss below recent findings on the factors involved in sensing, mediating and effecting the S phase checkpoint.

4.1. The sensors

It is important to highlight that the S phase checkpoint in particular and the damage checkpoint in general cannot be visualized as a linear pathway. In fact, the simple “upstream-downstream” organization generally envisioned for many signaling mechanisms is not applicable to this intricate and complex network. For example, the phosphoinositol-3-phosphate kinases (PI3Ks) ATM and ATR cannot be considered exclusively as upstream components of the DNA damage-signaling pathway. Instead they can be visualized as a functional core that directly coordinates and controls the initiation, amplification and carrying through of the checkpoint through phosphorylation of many different targets. As an example, while ATR binding to the fork could fairly be considered a very “upstream” event of this pathway, its kinase activity is directly required for the activation of mediators, effector kinases and also molecules “downstream” of the effector such as CDC25 and p53. Although the main players of this network are introduced herein based on their presumed timing of loading onto chromatin, the concept of continuous and elaborate cross-talk between molecules must always be kept in mind for a better understanding of this process. The following are the main sensors of damage in S phase.

4.1.1. ATR/ATRIP

ATM and ATR are required for DNA damage responsive checkpoints in yeast and mammals. While hATM seems to play a key role in responding to DSBs after ionizing radiation (IR), hATR is activated after a wider variety of insults including UV light, HU-dependent replication inhibition and DNA methylation by methyl methane sulfonate (MMS) [26]. ATR, the primary S phase checkpoint kinase, plays roles in both damage sensing and DNA replication. This is in contrast to ATM which may only sense damaged DNA. In fact, the broader spectrum of ATR activating signals correlates with the much higher lethality of ATR loss [4]. ATR binds to ATRIP which works as a regulatory subunit [29–32]. Deletion of ATRIP renders the cell effectively ATR-null [30]. ATR can phosphorylate both ATRIP and RPA although the relevance of such phosphorylation is not known currently [33]. The ATR/ATRIP complex associates with RPA-coated DNA independently of any checkpoint proteins, which suggests that this complex directly recognizes damaged DNA. In fact, direct binding of the ATR/ATRIP complex to sites of damaged has been shown in yeast. Further, in mammals, colocalization of ATR and ATRIP into nuclear foci suggest their joint recruitment to sites of damage [30]. Although binding of isolated scLcd1/Ddc2/Pie1 (ATRIP) to DNA has been reported [25], this protein does not efficiently bind to DNA in the absence of scMec1 (ATR) suggesting that stable association between these molecules is required for DNA binding in vivo [34]. ATR not only binds to damaged DNA but has also been shown to interact with replicating DNA [35,36]. To associate with unperturbed DNA replication forks, the ATR/ATRIP complex requires the previous loading of RPA on replicating ssDNA [33]. This event does not require Pol α suggesting that ATR appearance in replicating forks takes place even before Pol α recruitment (Fig. 1A). The early association of ATR with replication forks is consistent with its newly identified role in the modulation of the timing of origin firing during unperturbed replication [37]. Other ATR functions in the absence of stress could include scanning for changes in the speed and processivity of polymerases or in the extent of ssDNA accumulation at the fork. In all cases, full activation of ATR/ATRIP requires the independent loading of a second complex, Rad17/9-1-1, onto DNA.

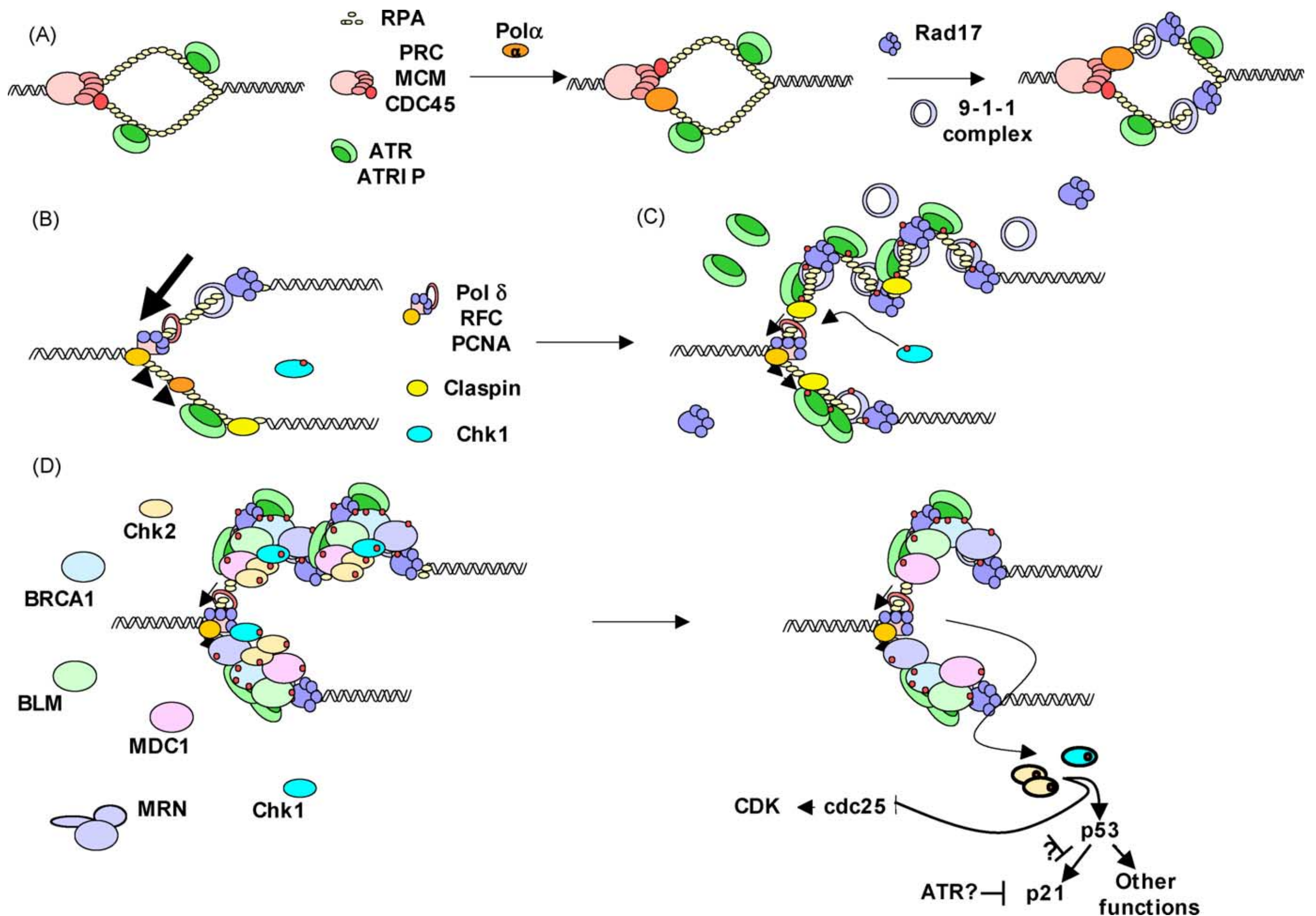

Fig. 1. Signal propagation at the stalled fork. (A) During the initiation phase of DNA replication the pre-RC MCM complex and CDC45 load onto origins of replication. RPA binds to regions of single stranded DNA. ATR/ATRIP complexes are then loaded onto chromatin by direct binding to RPA. After Pol α primase loading, Rad17/9-1-1 complexes are recruited to the newly unwound fork. It is not clear if the ATR/ATRIP complexes are actively signaling at this stage. (B) During the elongation phase of DNA replication the RFC/PCNA clamp loading complex is loaded onto DNA. The DNA Pol δ complex extends the leading strand and Pol α synthesizes the lagging strand. Claspin is bound to the fork and Chk1 has a role in the maintenance of fork stability. (C) As a consequence of blocked replication or repair of lesions on DNA, the S phase checkpoint is activated and the signal finally arrives to the replicating fork (see text) resulting in slowing down of DNA replication. Fork asymmetry results from different rates of synthesis in the leading and lagging strands. Here Rad17/9-1-1 complexes are more efficiently phosphorylated by ATR/ATRIP complexes. Phosphorylation is indicated by red dots. (D) Mediators and Chk kinases 1 and 2 are recruited to sites of damage and are phosphorylated within nuclear foci. While mediators can contribute to the effectiveness of the S phase checkpoint in an effector kinase-independent manner, activated Chks are released from foci and phosphorylate their targets CDC25 and perhaps p53. p53 transcriptional activity might be modulated to ensure the reversibility of the arrest.

4.1.2. *Rad17/9-1-1*

A second complex required for effective checkpoint activation in yeast and humans is the replication factor C (RFC)/PCNA-like 9-1-1 complex. The 9-1-1 complex is formed by three proteins: Rad9, Rad1 and Hus1 which create a ring-shaped structure and thereby share structural and functional similarity with the DNA polymerase processivity clamp PCNA [38–40]. Rad17 is similar in sequence to the large subunit of replicating factor C (RFC) which opens the PCNA clamp complex and loads it onto DNA and in fact Rad17 forms a complex with the four small subunits of RFC. Further, Rad17 binds to and is required for the localization of 9-1-1 clamp-like complex on DNA [34,41,42]. Accordingly, siRNA-mediated downregulation of hRad17 results in a strong reduction in hRad9 loading onto damaged chromatin [43]. Similarly to ATR/ATRIP, the Rad17/9-1-1 complex associates with chromatin in a manner that requires Rad17 interaction with RPA [43]. Rad17/9-1-1 also binds to DNA during unperturbed replication although its recruitment to the replication fork takes place later than the arrival of ATR/ATRIP. This has been shown in extracts from *Xenopus laevis* where xATR (ATR) associates with chromatin before DNA polymerase α while xHus1 (Hus1) association with DNA requires previous recruitment of DNA polymerase α [44,45] (see Fig. 1A). Although binding of Rad17/9-1-1 and ATR/ATRIP to DNA are independent processes [43,46] both complexes are required for a successful activation of the checkpoint when DNA damage arises [43,47]. Both ATR and ATM can phosphorylate Rad17 and Rad9 ([26] and references therein). Rad17 phosphorylation by ATR requires

both Rad17 loading onto chromatin and Rad17/Hus1 interaction which can take place during unperturbed DNA synthesis [43]. After damage, the phosphorylation of Rad17 and Rad9 by ATR is significantly enhanced as a consequence of increased interaction between the 9-1-1 and DNA after damage [43,48–50] (Fig. 1C). hRad17 and hRad9 phosphorylation by ATM has also been documented (see [26] and references therein). Disruption of hRad1 primarily affects hATR-dependent activation of the effector kinase hChk1, but does not affect hATM-dependent activation of the second effector kinase hChk2 [51], thus providing further evidence for a tighter functional interaction between Rad17/9-1-1 and ATR. Relevantly, during unstressed replication Rad9 participates in ATR signal to Chk1 [52]. Although Rad17/9-1-1 association with DNA is essential for the activation of the checkpoint, this complex is not required for the phosphorylation of all ATR substrates (see below). For example, ATRIP is phosphorylated by ATR in the absence of the 9-1-1 complex [29,31] and phosphorylation of the core histone H2AX (see details in sections below) was observed in the absence of this complex [53].

4.1.3. ATM and the MRN complex

As mentioned previously, DSBs can take place during unperturbed DNA replication as a consequence of stalled forks or oxidative stress. Moreover, DSBs are necessary in cellular processes such as VDJ recombination and meiosis. ATM has a major role in sensing this particular type of damage [26] and a complex of three proteins Mre11, Rad50 and Nbs1 (Xrs2 in yeast) termed the MRN complex contributes to recruitment of active ATM to sites of DSBs [54,55]. Mre11 has an exonuclease activity [28] while Rad50 and Nbs1 stimulate Mre11 enzymatic activity. Nbs1 has also a BRCA1 C-terminal domain (BRCT) that is responsible for protein–protein interactions between checkpoint-related molecules (see also the mediators section of this review). In agreement with a central role of the MRN complex in the processing of DSBs, the lack of each component of this complex results in cancer prone diseases and their disruption in mice results in embryonic lethality ([54] and references therein). In undamaged cells, ATM kinases are kept together as soluble inactive dimers [7]. The exact mechanism of ATM activation by DSBs has not been revealed but it involves ATM auto-phosphorylation and dissociation of ATM multimers [7] (reviewed in [56]). Activated ATM monomers phosphorylate soluble substrates (see their section bellow) or are recruited to chromatin where they phosphorylate the MRN components which are already localized at the site of damage [57]. Reciprocally, the key function of the MRN complex is likely ATM recruitment to DSBs [26,58]. In keeping with a central role of the MRN complex in ATM signaling, downregulation of both the ATM target effector kinase Chk2 and MRN are necessary for complete recovery of radioresistant DNA synthesis (RDS), thus suggesting that two parallel pathways (ATM-Chk2 and ATM-MRN) cooperate during the intra-S checkpoint [46]. Furthermore, this heterotrimeric complex might also coordinate the arrival of the checkpoint and repair machinery to DNA breaks since the MRN foci strikingly disassembles at the time of recruitment of the HR machinery to damaged DNA [55]. MRN might also participate in the surveillance of unperturbed S phase since it is retained in an insoluble chromatin bound fraction during S phase independently of ATM or ATR [57].

While strong evidence demonstrates that ATR and ATM are activated by different type of DNA damage they might coordinately contribute to the resolution of the same DNA lesions. In fact, the initial response to DSBs is strictly ATM-dependent, but the ssDNA resulting from the processing of DSBs promotes a slower activation of ATR which cooperates with ATM in the maintenance of the intra-S phase checkpoint and in the inhibition of late origin firing [37,59]. Confirming the above, temporal analysis performed in living cells has shown the initial recruitment of scMre11 and scTel1 (ATM) to DSBs followed by the association of RPA with processed ssDNA several minutes after Mre11 [55]. Reciprocally, ATM and MRN also cooperate with ATR in unperturbed cell cycle progression. ATM is transiently activated during cell cycle progression probably as a consequence of sporadic formation of DSBs. A related finding is that xATM and xATR inhibition by caffeine accelerates the initiation of DNA replication in Xenopus probably by promoting the firing of adjacent origins by S phase promoting kinase [37].

4.2. The mediators

After ATM/ATR activation and phosphorylation of the above-mentioned sensors, a number of proteins are recruited to the damaged DNA. Once activated, many such proteins remain at the site of damage while others (Chk1 and Chk2) are released to activate soluble targets [60]. Many of them are responsible for the activation of self-amplifying rounds of signals that ensure a sustained Chk1 and Chk2 response during DNA damage and depletion of these proteins by siRNA results in RDS [61–64]. The following summarizes what is known about their participation in signal propagation during the S checkpoint. As with other S phase checkpoint factors, mutations in genes encoding many of these proteins result in genetic disorders associated with predisposition to many types of cancers (see [26] and references therein).

4.2.1. Claspin

The function of this highly conserved mediator relates to the activation of Chk kinases in S phase [65–67]. In yeasts, scMrc1 (Claspin) and spMrc1 (Claspin) are required for scRad53 (Chk2) and spCds1 (Chk2) activation respectively during S phase [66,67]. In Xenopus, xClaspin is essential for the xATR-dependent activation of xChk1 after UV irradiation or aphidicolin [68]. In mammals, Claspin phosphorylation in response to DNA damage and replication stress results in recruitment and phosphorylation of BRCA1 (see below) and subsequent activation of Chk1 [69]. Intriguingly, while xRPA is required for xRad17 and xATR recruitment to chromatin, xClaspin loading onto DNA is independent of xRPA. This

suggests that Claspin loads onto DNA just after its initial unwinding [70] and is in keeping with its recently reported ring shaped structure and high affinity for branched DNA [71]. In fact, Claspin participates in unperturbed DNA replication in Xenopus and mammals [69,70]. Downregulation of Claspin results in both slower cell proliferation [69] and CDC25A upregulation [52]. Surprisingly, overexpression of Claspin also leads to increased cell proliferation which may suggest a dual role both as a tumor suppressor and as an oncogene [69]. Claspin also participates in adaptation processes during long-term replication blockage. In fact, experiments performed in Xenopus egg extracts have shown that during long exposure to aphidicolin Claspin gets phosphorylated by the Xenopus-Polo-like kinase (PLX1) which promotes both Claspin removal from DNA, Chk1 inactivation and termination of the checkpoint [72]. Thus, through the modulation of Chk1 activity, Claspin plays an essential role in DNA replication as well as the initiation and the termination of the checkpoint response to damaged DNA.

4.2.2. Foci formation during the S phase checkpoint

A second group of mediators shares the capacity to create multimeric complex (foci) at sites of damage [73]. These large proteins contain motifs such as BRCA1 carboxiterminal and forkhead associated (FHA) that facilitate the formation of large complexes containing sensor kinases, mediators and effector kinases. They are all targets of ATM/ATR and can participate in damage recognition and/or the transmission of signals to Chk1 and Chk2 (see Fig. 1D). This cluster of proteins along with histone H2AX forms a network that apparently promotes the local concentration of ATM/ATR and their targets in what has been defined as the “upstream and downstream mingle” [26]. Although current microscopy techniques do not allow distinguishing which protein is recruited first to these damage-induced foci it is clear that they participate together in checkpoint, replication and repair pathways. Importantly, many of them can inhibit RDS and promote cell survival after DNA damage independently of Chk1 activation [46,74]. Thus, some of these mediators during the S phase checkpoint might work in a parallel and separate pathway branch, which functions independently of Chk kinases. Here too defective expression of many of them results in genetic disorders with predisposition to many types of cancers (see [26] and references therein).

4.2.2.1. H2AX. Histone H2AX cannot be defined as a mediator protein but participates in the early steps of foci formation around damaged DNA. A critical function of H2AX is related to holding broken chromosomal ends in proximity [75]. While H2AX does not have a role in DNA lesion recognition and is apparently randomly incorporated into nucleosomes [76], formation of multiprotein structures around DNA lesions has often been monitored by determining the localization of the phosphorylated fraction of H2AX. H2AX phosphorylation by ATM/ATR and takes place rapidly after DNA damage or replicative stress [77,78]. Phosphorylated H2AX is not required for the initial recruitment of mediators containing BRCT domains to sites of damage [76] but it is essential for the formation of IR-induced repair foci [79–82]. Accordingly, BRCT-containing proteins had been shown to bind specifically to the phosphorylated motif of H2AX [81,83,84] which might directly influence the rate of exchange of mediators proteins onto DNA thus promoting the maintenance of foci structures. Supporting its role in the checkpoint response, impaired H2AX contributes to genomic instability both in humans [75] and in mice [82].

4.2.2.2. BRCA1. The BRCA1 tumor suppressor is a mediator protein that has been group together with scRad9 (BRCT) and spCrb2 (BRCT) based on their possible functional similarities. While lacking significant over-all homology, each possesses BRCT repeats that may account for their ability to organize into foci. This clustering activity or BRCA1 seems to play an essential role in checkpoint activation. In yeasts, scRad9 (BRCT) participates in scTel1 (ATR) dependent activation of the effector kinase scRad53(Chk2) during the S phase checkpoint [85]. In this case, oligomerization though BRCT domains follows the initial phosphorylation of scRad9 (BRCT) by scMec1 (ATM) or scTel1 (ATR) [86–88]. Intriguingly, chaperone functions can also modulate phosphorylation of scRad9 (BRCT) and scRad53 (Chk2) suggesting that there are some yet unexplored levels of regulation of BRCT proteins during checkpoints [89]. After initial ATM/ATR phosphorylation scRad9 (BRCT)-bound-scRad53 (Chk2) is fully activated by auto-phosphorylation and it is then released from scRad9 multiprotein complexes [90–92]. In mammals it seems that a rather similar process occurs involving a crucial role of BRCA1 [93]. Repair foci termed BRCA1-associated genome surveillance complex (BASC) containing the tumor suppressor BRCA1 and other repair proteins have been reported [73]. BRCA1 is phosphorylated by ATM or ATR [94,95]. ATR not only phosphorylates but also colocalizes with BRCA1 in nuclear foci during stalled replication [95]. BRCA1 facilitates the ability of ATM or ATR to phosphorylate some downstream targets including Chk2, the tumor suppressor p53 and Nbs1 but does not affect ATM/ATR-dependent phosphorylation of Claspin, Rad9, Hus1 and Rad17 [69,96]. ATM positively regulates BRCA1 in at least two ways: direct phosphorylation of BRCA1 at S1387 and phosphorylation of the BRCA1 inhibitor CtBP interacting protein (CtIP), thus resulting in stimulation of BRCA1 [97]. The interaction of BRCA1 with ATM/ATR targets such as Chk2 may require remodeling of BRCA1 by chaperone activities as demonstrated for scRad9 (BRCA1) [98]. BRCA1/Chk2 interaction is transient [60] and complex dissociation is promoted by Chk2-dependent phosphorylation of BRCA1 at sites different than those modified by sensor kinases [99]. BRCA1 is also required for the activation of the second effector kinase, Chk1, and for the intra-S phase checkpoint when DNA is damaged [100].

Other activities of BRCA1, such as ubiquitin conjugation are increased in BRCA1 foci during S phase and in response

to replication stress and DNA damage although the contribution of this BRCA1 activity to the checkpoint awaits to be tested [101]. It is also interesting to highlight that defects in BRCA2, a tumor suppressor closely related to BRCA1, reveal that this protein participates in the maintenance of the Y-shaped structure of stalled replication forks, and thus protects the genome from double strand breaks [102]. Taken together, these data indicate a clear role of these mediators in the maintenance of genomic stability during unperturbed or stressed S phase progression.

4.2.2.3. MDC1. Mediator of DNA damage-checkpoint protein 1 (MDC1) is another BRCT containing protein with a central role in the S phase checkpoint. MDC1 binds to phosphorylated H2AX [103] and is required for the recruitment of NSB1, one of the components of the MRN complex to broken DNA [28,104]. Consistent with this, downregulation of MDC1 results in defective ATM activation [105]. MDC1 also promotes the assembly of 53BP1, BRCA1 into foci and facilitates the phosphorylation of SMC1 (see bellow) by ATR [63,74,81,106,107]. Although downregulation of MDC1 with siRNA does not completely abolish Nbs1, Chk2, or other checkpoint responses, it causes RDS [61].

4.2.2.4. 53BP1. p53 binding protein 1 (53BP1) is a BRCT containing protein important both for the intra-S and G2 checkpoint after IR [62,82,108]. 53BP1 regulates the phosphorylation of Chk2, BRCA1 and SMC1 (see below) at sites of damage after IR [62,82,108,109]. Moreover, defective localization of BRCA1 to foci has been observed in 53BP1 mutant cells [62] suggesting that there is sequential recruitment of MDC1, 53BP1 and BRCA1 to foci.

4.2.2.5. SMC1. Structural maintenance of chromosomes 1 (SMC1) is a component of the cohesin complex that is required for sister chromatid cohesion during S phase. SMC1 is phosphorylated by ATM in an Nbs1 [63,74] and BRCA1 [106] dependent manner. In line with its reported phosphorylation after exposure to a broad range of stimuli including IR, HU and UV light [63], SMC1 is also phosphorylated by ATR. Supporting the importance of the clustering activities of Nbs1, SMC1 phosphorylation does not require previous phosphorylation of Nbs1 [110]. Downregulation of 53BP1 has also been shown to impair phosphorylation of SMC1 by ATM [108] and interference with SMC1 phosphorylation by ATM abrogates the S phase checkpoint [63,74]. Lack of SMC1 phosphorylation also results increased chromosome aberrations after DNA damage [106] but the mechanism by which phosphorylated SMC1 prevents RDS is currently unknown. Recent evidence also suggests that both the upstream signaling pathway required for SMC1 phosphorylation and events downstream of SMC1 are not totally overlapping with the Chk1 pathway [111]. Consistently, while Chk1 activation is mandatory for checkpoint activation, phosphorylation of SMC1 is required for cell survival, thus suggesting that SMC1 and Chk1 regulate two branches of the DNA damage response [111].

4.2.2.6. FANCD2 (Fanconi Anemia complementation group D2). Eight Fanconi Anemia proteins are essential for sensing interstrand crosslinks in DNA. Several of these proteins including FANCA, FANCC, FANCE, FANCF and FANCG form a multimeric complex that promote the mono-ubiquitination of FANCD2 after DNA damage ([112] and references therein). FANCD2 is also a recently identified target of ATM [113,114]. These two post-translational modifications, phosphorylation and mono-ubiquitination, modulate different biological functions of FANDC2. While the ATM-dependent phosphorylation of FANCD2 is required for S phase arrest and thus might function as an amplifier of the checkpoint signal [112], the ATM-independent mono-ubiquitination of FANCD2 is ATM independent, is required for localization of FANCD2 into BRCA1 foci which is relevant for the crosslink repair functions of FANC2 [115]. While foci recruitment of FANCD2 depends on BRCA1, FANCDL, a novel E3 ligase component of the Fanconi Anemia complex is responsible for FANCD2 ubiquitination [116]. Ubiquitinated FANCD2 promotes recruitment of FANCD1 (BRCA2) to chromatin, an event that appears to be necessary for HR [117]. As with other mediators, FANCD2 interacts with MRN, and this interaction contributes to the checkpoint in a pathway that does not apparently involve the activation of the effector kinases [114].

4.2.2.7. BLM and WRN helicases. The formation and migration of reversed forks might be also required during S phase checkpoint for successful repair of some DNA breaks [4]. Interestingly, the Bloom's syndrome helicase (BLM) and the Werner's syndrome helicase (WRN) which have the ability to catalyze the resolution of Holliday junctions in vitro [118,119] are phosphorylated during replication blockage [120]. Phosphorylation of xBLM by xATR is also required during unperturbed DNA replication and it depends on xRad17 but not xClaspin [121]. In line with this, Bloom's syndrome (BS) cells display abnormalities in the timing of normal replication and are hypersensitive to HU [122]. BLM associates with and is phosphorylated by ATM [123] and ATR [124], colocalizes with H2AX, BRCA1 and Nbs1 [123,125] and is required for correct relocalization of the MRN complex after replication blockage or IR [120]. Defective phosphorylation of BLM by ATR impairs recovery from blocked S phase suggesting indeed that the accumulation of damaged DNA is dependent on fork instability [125,121]. Moreover, BRCA1 and Nbs1 foci formation is defective in cells from Blooms syndrome patients [124].

A role of WRN in the S phase checkpoint has also been demonstrated. In fact, alterations of the normal topology of forks have been reported in Werner's syndrome (WS) cells [126]. WRN is phosphorylated in an ATR/ATM-dependent manner during S phase and DNA damage checkpoints [127] and relocates into nuclear foci in response to DNA damag-

ing agents and colocalizes with RPA and Rad51 [128,129]. The recruitment of BLM to DNA damage-induced foci depends on a functional interaction with 53BP1 [130]. WRN also interacts with Pol δ and may facilitate Pol δ-dependent replication and/or repair [131]. Importantly, WRN may also be required for re-start of DNA replication and it could be involved in the correct resolution of recombination intermediates that arise from replication arrest due to either DNA damage or replication fork collapse [127].

4.3. The effectors

Two key effector kinases Chk1 and Chk2 play critical roles in causing cell cycle arrest resulting from phosphorylation of their substrates. Although these kinases are very different in terms of their domain organization, upon activation after the checkpoint initiation, they phosphorylate a number of common targets [132] and have similar consensus phosphorylation sequences. While scRad53 (Chk2) is central for the S phase checkpoint in *S. cerevisiae*, in higher eukaryotes Chk1 is activated by ATR after a broad range of stimuli while Chk2 is activated mainly by ATM [2]. Activation of ATR/ATM and recruitment of BRCT proteins to DNA triggers the subsequent re-localization of Chk1 and Chk2 proteins to those multiprotein structures described above. After IR, phosphorylation of Chk2 by ATM apparently takes place within these large complexes [60,132]. This event leads to phosphorylation, dimerization and final activation of Chk2 by trans-autophosphorylation and spreading of Chk2 throughout the nucleoplasm (Fig. 1D). Active Chk2 dimers can then phosphorylate soluble substrates such as CDC25 proteins and perhaps p53 although once activated Chk2 monomers have also been shown to have activity [133]. Forced retention of Chk2 at sites of damage reduces the activation of one of its targets, the tumor suppressor p53 [60]. Other factors involved in Chk2 activation are 53BP1 [62], MDC1 [61,134], and the nuclease MRN complex [27].

The other effector kinase, Chk1, is targeted by both ATM and ATR after a broad range of stimuli such as UV light, stalled replication and some other drugs. In contrast to Chk2, Chk1 does not require dimerization or transphosphorylation for full activation [132]. However, optimal phosphorylation of Chk1 requires interaction with BRCA1, Claspin and Rad17/9-1-1 complex formation [43,47,100,135].

Important differences in cell cycle-dependent expression of Chk1 and Chk2 have been reported. While Chk1 is present in significant quantities only in S and G2 phases of the cell cycle and is expressed at very low levels in quiescent and differentiated cells [136], Chk2 is present throughout the whole cell cycle [137]. Similarly, mice which are null for both ATR and Chk1 are not viable and fibroblasts derived from their embryos die in culture in a manner resembling mitotic catastrophe [138,139]. More recently, Chk1 deficient tumor cell lines have been identified but, in line with a central role of Chk1 during replication and stress, they exhibit multiple checkpoint and survival defects [140]. Furthermore, although Chk1 siRNA does not alter the cell cycle profile or induce apoptosis in human cancer cell lines [141], dominant negative Chk1 has been reported to affect genomic stability under certain conditions [142,143]. Remarkably, an important role of Chk1 in the maintenance of fork integrity has been revealed. Both scMEC1 (ATR) and scRad53 (Chk1) mutants cannot complete replication after release from replication block [144,145]. scRad53 (Chk1) is required to complete replication after HU treatment by preventing collapse of the fork when replication is paused [15]. Electron microscopy revealed that a scRad53 (Chk1) mutant accumulates longer ssDNA and Holliday junctions caused by fork reversal [8]. Importantly, while scRad53 (Chk1) promotes the maintenance of replication "competence", replication slowdown takes place both in the presence and in the absence of scMec1 (ATR) or scRad53 (Chk1). In the latter case however, replication will stop before completion. Thus, Chk1 "adapts" the replication machinery to a slower rate of processivity preventing in that way its dissociation from DNA. This suggests a central role of Chk1 in the re-start of DNA synthesis [4]. Moreover, the Chk1 inhibitor, UCN-01, disturbs both origin firing and fork viability when DNA synthesis is stalled [146]. Based on these observations it is thus surprising that ATR knockout mouse cell lines generated using the cre-lox system reveal that the replication checkpoint is intact in the absence of both ATR and activated Chk1. The recent identification of MAPKAP kinase-2 as a "Chk3" component of the S phase checkpoint after UV irradiation supports the likelihood of further complexity that might explain these observations [147]. Further, similar results were obtained in an ATR−/−ATM−/− background thus supporting the existence of an ATR/ATM-independent mechanism that prevents mitotic entry [148] that could possibly depend on diffusible inhibitors described elsewhere [149]. Importantly, despite the active ATR/Chk1-independent checkpoint, ATR knockout cells enter mitosis with chromosome breaks which suggests that the DNA stabilizing functions of ATR/Chk1 pathway are not dispensable.

Despite their similar biochemical functions, the contributions of Chk1 and Chk2 to development and survival are strikingly different [139,150]. Biochemical data supports a narrower role of Chk2 that is time and species dependent and is more limited to DSB-induced checkpoint signals [46,151]. Importantly, human cells expressing functionally impaired Chk2 manifest RDS thus suggesting a central role of Chk2 in the intra-S phase checkpoint [151]. However, this is not the case for Chk2 deficient mouse cells in which Chk1 could have compensated for that function [152]. In fact, Chk1 is now viewed as the "workhorse" kinase while Chk2 serves as an important amplifier whose contribution is particularly important for response to DSBs [132]. For example, the IR-dependent intra-S phase arrest requires *both* basal Chk1 phosphorylating activity and the further enhancement of substrate phosphorylation by DSB-activated Chk2 kinase activity [153]. Consistent with a central role of Chk1 in the S phase checkpoint, its inactivation might be crucial for check-

point reversibility. In fact, Claspin inactivation by Polo-like kinase [72] and Chk1 dephosphorylation by PP1 ([154] and references therein) are required for checkpoint recovery.

Finally, it is important to highlight that the original delineation of ATM-Chk2 and ATR-Chk1 as parallel-independent signals has been recently complicated somewhat by the identification of cross talk between these pathways. In one case ATM-independent activation of Chk2 has been reported [150]. ATM-dependent activation of Chk1 has been documented as well after IR [153,155] and ATR regulates a late IR response in mouse cells [148].

The most well studied targets of Chk1 and Chk2 are CDC25 and p53 and the biological relevance of their phosphorylation for the S phase checkpoint is discussed extensively in the next section. Other ATM-Chk2 targets identified recently are TLK, which links chromatin remodeling to the DNA damage checkpoint [156], PML [157], PLK3 [158] and E2F [159]. The implication of modulations in these proteins' activity by the DNA damage checkpoint during S phase waits to be unraveled.

5. Signaling to CDC25 and p53 during the S phase checkpoint

The current view of the S phase checkpoint envisions at least two parallel pathways. The first involves foci formation by BRCT domain-containing proteins and the second involves Chk kinase-dependent CDC25 degradation. CDC25 phosphatases promote DNA synthesis by dephosphorylation and activation of CDK2 and CDK1 [160]. CDC25 phosphatases are substrates of both Chk1 and Chk2 in all phases of the cell cycle. Chk1 and Chk2 phosphorylate CDC25A promoting its ubiquitination and degradation (reviewed in [132]). Chk2-dependent destruction of CDC25C might be a key determinant for G2/M arrest. Degradation of CDC25A is required for the intra-S phase checkpoint [161]. As mentioned before, the housekeeping activity of Chk1 and the damage-dependent activation of Chk2 might have cooperative effects on cell cycle arrest [153]. The ATM/ATR-Chk1/Chk2-CDC25 signaling pathway represents a reversible, fast response to DNA damage [162] (Fig. 1E). The irreversible slower response to DNA damage requires p53 stabilization [162].

The ATM and ATR kinases can phosphorylate p53 and its E3 ligase, MDM2, at key sites that should attenuate their interaction and promote p53 stabilization [26]. However, although p53 stabilization has been observed after a plethora of genotoxic or oncogenic stress [163], the mechanisms of p53 upregulation after DNA damage is still not well understood. Although ATM/ATR can phosphorylate both p53 and MDM2, Ser20 and Thr18 phosphorylation are required to prevent the p53/MDM2 interaction [26]. The role of Chk2 in Ser20 phosphorylation, while supported by a large body of evidence (reviewed in [132]), has been recently challenged by reports showing that p53 is stabilized after irradiation and phosphorylated at S20 after Chk2 elimination by gene knockout [164] and is also stabilized and active after DNA damage after Chk1 and Chk2 siRNA interference [165]. However, an interaction between p53 and Chk2 and resulting allosteric activation of Chk2 has been proposed [166]. While this could explain the lack of a Chk kinase consensus sequence in p53 [165,167], Chks-independent stabilization of p53 after damage cannot be ruled out. Perhaps the ATR/ATM/Chk1-independent DNA damage-signaling pathways described above may provide redundancy in signaling to p53 [148]. Regardless of the source of upregulating signals, p53 does accumulate after both replication blockage and damage-induced intra-S phase checkpoint. Moreover, p53 is stabilized after hypoxia [168] and hyperoxia [169] induced S phase arrest. Importantly, however, p53 transcriptional activity is selectively impaired in at least some cell lines with particularly defective accumulation of the cyclin kinase inhibitor p21 [170–172]. Intriguingly as well, after initiation or the hypoxic S phase checkpoint, p53 fails to upregulate some of its targets including p21 [168]. After IR, although cells arrested in every phase of the cell cycle show elevated levels of p21, p21 accumulation is not observed in S phase arrested cells [173]. Moreover, a p53-dependent arrest in S phase that results from starvation of pyrimidine nucleotides by treatment with the inhibitor PALA does not result in and does not require p21 upregulation [174]. Importantly, p21 levels were observed to increase after HU removal correlating with G2 entrance [175] which supports the notion that the S phase checkpoint is dependent on p21 downregulation. It is relevant that p21 degradation is enhanced after both HU treatment [171] and UV light [176], suggesting the existence of converging signals that maintain p21 levels low during S phase arrest, probably ensuring the reversibility of the arrest. In Xenopus, p21 degradation takes place on replicating DNA [177,178] which could imply the intriguing possibility of a direct interaction between p21 and the checkpoint machinery. Interesting as well is the activation of a p21 degradation pathway that depends on ATR after UV treatment [176]. p21 functions that could be incompatible with S phase arrest could include inhibition of PCNA-dependent DNA replicative elongation [171] and DNA repair [176], downregulation of Chk1 [172], endoreduplication [179] and possibly as well, inhibition of the newly reported E2F repair-related activities ([180] and references therein). We hypothesize that inhibition of p53 cell cycle arrest activities during S phase block will allow DNA synthesis to re-start when the damage is repaired or the replication inhibitor removed. In fact, prolonged arrest in S phase results in cellular stasis that correlates with irreversible stops in origin firing [181]. Downregulation of p21 mRNA transcription is not evidence of blanket suppression of p53 transcriptional activity during S phase arrest since induction of some p53 apoptotic targets or p53 transcriptional repression is not impaired under these conditions [168,171,172]. In fact, p53-dependent reduction of cyclin A and cyclin B levels has been reported in cells damaged in S phase

[182]. Moreover, p53 has been reported to prevent mitotic entry when DNA synthesis is blocked [183]. The selective regulation of p53 downstream signaling may serve to ensure the effectiveness of the arrest-free checkpoint (Fig. 1E).

6. Concluding remarks

Of cellular checkpoints, the S phase checkpoint is the most complex and elaborate. This is because it needs to respond to endogenous and exogenous signals in a manner that ensures stability and fidelity of replication. It is clear that the viability of organisms is highly unlikely in the absence of many components of the S phase damage-checkpoint pathway. Indeed increasing evidence demonstrates that the tasks of these "DNA sentinels" are not relegated only to situations of damage. They also participate in normal replication by protecting the stability of replicating forks and the timing the firing of origins of replication [37]. Furthermore, in some scenarios a group of checkpoint proteins is mainly in charge of survival while in others they are primarily in charge of reduction in the rate of DNA synthesis (e.g. [111]). While substantial progress in the understanding of this pathway has been achieved, some interesting topics require further exploration. There is still a great deal to learn about the roles of some of the factors, in particular how RPA coordinates checkpoints, the relationship between the ATM and ATR sensors, the timing of both initiation and resolution of checkpoint activities, how checkpoints affect chromatin remodeling and transcription, the factors involved in recovery from the checkpoint and many other features. In the coming years, new findings that provide insight into this essential component of the cell will undoubtedly emerge.

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