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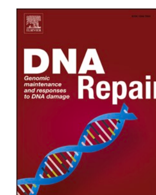

Checkpoint activation and recovery: regulation of the 9–1–1 axis by the PP2A phosphatase

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ABSTRACT

Genome integrity is continuously monitored by elaborate cellular networks, collectively referred to as the DNA damage response (DDR), which detect DNA lesions and transmit the information to downstream targets, thereby coordinating a broad range of biological processes. A crucial signal in this response is the generation of single-stranded DNA that, once coated by replication protein A (RPA), serves as a platform for recruiting the apical checkpoint kinase Mec1/ATR. Full activation of Mec1/ATR also requires the 9–1–1 complex, which provides a docking site for additional checkpoint mediators, such as Dpb11/TOPBP1 and Rad9/53BP1. These mediators are important for transducing the checkpoint signal from Mec1/ATR to the effector kinase Rad53/CHK2. The checkpoint signal transduction cascade is tightly regulated by phosphorylation events, which can be counteracted by phosphatases to ensure timely checkpoint inactivation once DNA repair is complete. In this review, we examine the mechanistic aspects of Mec1/ATR activation, with a particular focus on the 9–1–1 checkpoint axis in *Saccharomyces cerevisiae*. We discuss how phosphorylation and dephosphorylation dynamically regulate the checkpoint pathway, allowing cells to efficiently respond to genotoxic stress while ensuring a timely return to normal cell-cycle progression.

1. Introduction

Cells constantly face endogenous and exogenous threats that compromise DNA integrity, leading to genome instability. To safeguard the genetic information and ensure its faithful transmission across generations, cells have evolved the DNA damage response (DDR), a network of checkpoints and repair pathways that detect, signal, and counteract DNA lesions.

The DNA damage checkpoint is a signal transduction cascade that senses DNA damage and orchestrates a multifaceted cellular response [1,2]. In *Saccharomyces cerevisiae*, checkpoint activation follows a hierarchical kinase cascade, with Mec1 and Tel1 playing central roles [3]. These kinases belong to the phosphatidylinositol-3-kinase-related kinase (PIKK) family and are recruited to damaged DNA to initiate the checkpoint response. Tel1 is the yeast ortholog of human ATM, whose mutation causes the autosomal recessive disorder ataxia-telangiectasia [4–6], while Mec1 is the counterpart of human ATR, whose deficiency leads to Seckel syndrome [7]. Once activated, these kinases phosphorylate myriads of transducer proteins and downstream effectors [8,9], initiating a signaling cascade that controls cell-cycle progression, replisome stability, origin firing, DNA repair and deoxynucleotide triphosphate (dNTP) levels.

While phosphorylation-driven checkpoint activation is crucial for short-term survival, prolonged signaling can be detrimental. Once DNA damage is repaired, cells must dampen the checkpoint response to resume the cell cycle. Checkpoint inactivation is often mediated by phosphatases, which selectively remove phosphate groups from checkpoint components and effectors [10]. The balance between kinase-driven phosphorylation and phosphatase-mediated dephosphorylation ensures a timely and coordinated transition from an active checkpoint to recovery. In this review, we focus on the mechanisms governing Mec1/ATR activation, with particular emphasis on the 9–1–1 complex. This evolutionarily conserved clamp-like sensor plays a pivotal role in DNA damage recognition and checkpoint initiation. We discuss how its regulation through phosphorylation and dephosphorylation modulates checkpoint activation and timely recovery.

2. Mec1/ATR activation

Mec1/ATR is activated by a broad spectrum of DNA lesions,

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including those caused by dNTP depletion, ultraviolet radiation, topoisomerase poisons, DNA polymerase inhibitors, alkylating agents, and DNA crosslinkers. Despite their diversity, these lesions converge on a common DNA intermediate that serves as a key upstream signal for ATR activation, namely stretches of single-stranded DNA (ssDNA). Once formed, ssDNA is rapidly coated by the Replication Protein A (RPA) complex, which acts as a platform for recruiting the protein kinase Mec1/ATR [11–13]. ssDNA-RPA intermediates arise during DNA damage processing or replication fork stalling. Among the most severe threats to genome stability are double-strand breaks (DSBs), which undergo nucleolytic resection to generate 3'-ended ssDNA, an essential intermediate for homology search and strand invasion during homologous recombination (HR) [14,15]. The connection between ssDNA generation at the DSB ends and Mec1/ATR activation has been extensively studied using the HO endonuclease-induced DSB model in *S. cerevisiae* [16]. The HO enzyme allows mating-type switching by inducing a DSB at the *MAT* locus, which is subsequently repaired via HR. When repair of the HO-induced DSB is impaired due to the absence of the homologous donor loci *HML* and *HMR*, HO induction results in the formation of a single unrepairable DSB that is sufficient to induce a Mec1-dependent checkpoint, whose activation closely follows ssDNA formation that arises from 5' to 3' nucleolytic degradation of DSB ends [17].

In both yeast and mammals, DSB resection occurs in two steps, mediated by distinct short-range and long-range nucleases (Fig. 1) [14,15]. The initial short-range resection is carried out by the evolutionarily conserved Mre11-Rad50-Xrs2/NBS1 (MRX/MRN) complex, aided by Sae2 (CtIP in mammals), which cleaves the 5'-terminated strands at the DSB ends. Subsequently, the Mre11 exonuclease trims DNA back toward the break site, generating 100–300 nucleotide-long 3'-terminated ssDNA overhangs [18–22]. The ability of Sae2 to promote Mre11 endonuclease activity requires CDK-mediated Sae2 phosphorylation [23,24], and this control allows DSB resection to take place only during the S and G2 phases of the cell cycle. In the long-range resection, either Exo1 or Dna2, in conjunction with the Sgs1 helicase, extends the ssDNA in the 5'-3' direction [19,20,25–29]. Inactivation of Exo1 or Dna2/Sgs1 alone results in a minor resection defect, whereas DSB resection is severely impaired when both pathways are disrupted simultaneously [19,20]. Exo1 can degrade 5′-terminated DNA strands within a duplex DNA molecule [30], whereas Dna2 is recruited to ssDNA ends and processes them in an endonucleolytic manner [31]. The resection activity of Dna2 depends on a helicase, provided by Sgs1 in yeast and by either BLM or WRN in human cells [20,32,33].

Similar to ATR, Mec1 functions as a stable dimeric complex with Ddc2 (ATRIP in humans), which allows Mec1 recruitment to RPA-coated ssDNA through a direct interaction with RPA [13,34,35]. Upon activation, Mec1/ATR transduces checkpoint signals to the downstream checkpoint kinases Rad53 (CHK2 in humans) and Chk1 (CHK1 in humans), which initiate two parallel checkpoint branches [36]. Rad53 activation relies on mediator proteins such as Rad9 (53BP1 in humans)

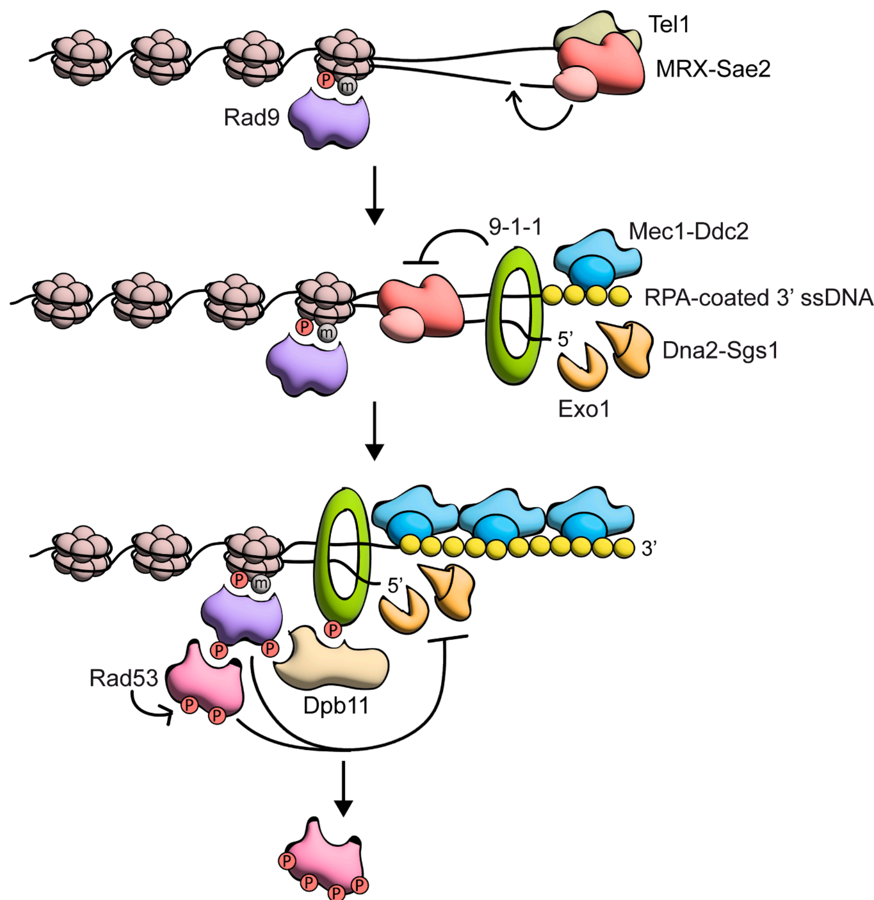

Fig. 1. Model for Mec1 activation in response to DNA DSBs. The MRX-Sae2 complex is rapidly recruited to DNA ends. Rad9 is pre-bound to chromatin via interactions with methylated histone H3 (gray dots). MRX recruits Tel1, which phosphorylates histone H2A on S129 (red dots), further enriching Rad9 at DSBs. MRX-Sae2 initiates DSB resection by nicking the 5'-terminated strands. Exo1 and Dna2-Sgs1 nucleases generate ssDNA, which is coated by RPA, thus allowing Mec1-Ddc2 recruitment. Initiation of DSB resection also promotes the association of the 9–1–1 complex at the 5' recessed end of the ssDNA-dsDNA junction, where it inhibits MRX-mediated cleavage events. Mec1, in turn, phosphorylates Ddc1 at T602 (red dots), creating a docking site for Dpb11. Cdk1-phosphorylated Rad9 binds Dpb11 that acts as a scaffold to promote Rad9-Mec1 interaction and therefore Rad9 phosphorylation by Mec1. Phosphorylated Rad9 recruits Rad53 molecules to allow Rad53 trans-autophosphorylation. Fully activated Rad53 is then released. Rad9 and Rad53 inhibit Exo1 and Dna2-Sgs1 nucleases.

(Fig. 1). In response to DNA damage, Mec1 phosphorylates Rad9 at multiple sites, inducing structural changes that promote Rad9 multimerization via its BRCT domains [37–40]. Phosphorylated Rad9 first acts as an adaptor, which facilitates Mec1-Rad53 interaction and Mec1-dependent Rad53 phosphorylation. Subsequently, Rad9 functions as a scaffold, bringing multiple Rad53 molecules into close proximity, to allow Rad53 trans-autophosphorylation and activation [41–43]. Once phosphorylated, Rad53 molecules dissociate from Rad9, propagating the checkpoint signal.

3. The 9–1–1 complex

In both yeast and mammals, the recruitment of Mec1/ATR-Ddc2/ATRIP to RPA-coated ssDNA alone is not sufficient to activate Mec1/ATR. Studies in *Xenopus laevis* egg extracts have shown that Mec1/ATR activation requires an adjacent double-stranded DNA (dsDNA) region, forming a ssDNA-dsDNA junction, which is specifically recognized by the 9–1–1 complex [44]. The 9–1–1 complex is a ring-shaped heterotrimeric clamp, composed of RAD9-RAD1-HUS1 in humans and Ddc1-Rad17-Mec3 in yeast. Structurally, it resembles the processivity clamp PCNA, sharing a similar ring-like architecture [45–47]. Like PCNA, the 9–1–1 complex requires a specialized ATP-dependent clamp loader for its loading onto DNA. However, while PCNA is loaded by the canonical replication factor C (RFC) clamp loader, the 9–1–1 complex relies on an alternative clamp loader, Rad24-Rfc2–5 in yeast and RAD17-RFC2–5 in humans. This loader shares four subunits (Rfc2–5) with RFC but possesses a distinct large subunit (Rad24/RAD17), which confers specificity [48–50]. The specificity of Rad24-RFC for the 9–1–1 complex arises from direct interactions between Rad24 and the 9–1–1 subunits Ddc1 and Mec3 [51].

Structurally, Rad24-RFC and the 9–1–1 complex associate as two planar rings [52], whereas the RFC clamp loader binds the PCNA ring in a spiral conformation [53]. Rad24 further dictates the selectivity of the Rad24-RFC clamp loader for a 5’ recessed DNA end, as it possesses structural elements that enable high-affinity interactions of the free 5’ end and the 3’ overhang in a DNA binding cleft on its surface, leading to the perfect positioning of the ssDNA inside the 9–1–1 clamp (Fig. 2A and B).

Unlike the RFC-PCNA complex, which can preassemble in the absence of DNA, the 9–1–1 clamp is loaded onto DNA only after binding to Rad24-RFC in an open conformation. Upon interaction with the Rad24-RFC-DNA complex, the 9–1–1 ring opens up due to a rotation of the Mec3 subunit, stabilized by the interaction of Rad24 with both Ddc1 and Mec3 itself. The 9–1–1 clamp exhibits a large opening (27 Å), significantly wider than what would be necessary for ssDNA to pass through, though whether this feature is essential for its function remains unclear. Once the 9–1–1 complex is loaded, the hydrolysis of the ATP cofactors, bound by the RFC subunits, induces conformational changes that release the 9–1–1 complex that clamps on ssDNA (Fig. 2C and D).

4. The 9–1–1 axis in checkpoint activation

Mec1-Ddc2 and the 9–1–1 complexes localize independently to sites of DNA damage [54–56], suggesting that the DNA damage site itself

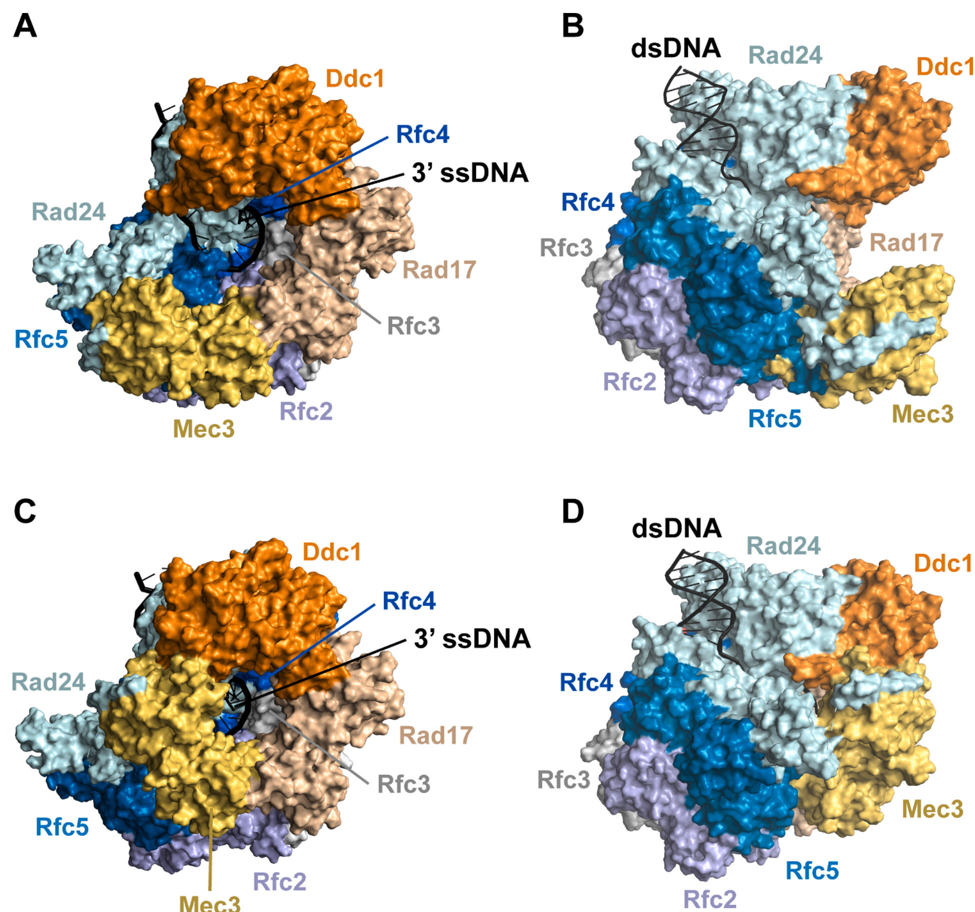

Fig. 2. Structure of the *S. cerevisiae* Rad24-RFC-9–1–1 DNA ternary complex. The Rad24-RFC-9–1–1 clamp complexed with DNA is shown in the open state (A and B, rotated of 90° along the Y axis) immediately after the complex formation, and in the closed state (C and D, rotated of 90° along the Y axis) following ATP hydrolysis on RFC subunits and rotation of Mec3 to reconstitute the 9–1–1 ring on a 3’ ssDNA overhang. The subunits are indicated with a color code. The disordered regions, such as the Rad24 C-terminal coiled coil or the long C-terminal tails of Ddc1 and Rad17, are not represented.

serves as a recruitment platform that concentrates both Mec1-Ddc2 and 9–1–1 in the same region to enhance checkpoint activation. Supporting this model, artificially tethering Mec1-Ddc2 and the 9–1–1 complex to a chromosomal array is sufficient to trigger checkpoint activation even in the absence of DNA damage [57]. Moreover, the extent of Rad53 phosphorylation correlates with the number of colocalized checkpoint molecules, highlighting how Mec1 and 9–1–1 cooperatively amplify the DNA damage response [57].

Once loaded at the ssDNA-dsDNA junction, the 9–1–1 complex promotes Mec1 activation by interacting with Dpb11 (TOPBP1 in humans), which, in turn, recruits Rad9 to sites of DNA damage, forming the so-called 9–1–1 axis (Fig. 1) [58–61]. By facilitating Rad9 recruitment, the 9–1–1 complex enables Mec1 to phosphorylate Rad9, generating phospho-docking sites essential for Rad53 recruitment and activation (Fig. 1). In budding yeast, the interaction between 9–1–1 and Dpb11 depends on phosphorylation of the Ddc1 subunit at threonine 602, which is specifically recognized by the BRCT3/4 domains of Dpb11 [60,62]. According to Alphafold3 structural predictions, phosphothreonine 602 of Ddc1 is recognized by BRCT4 of Dpb11, with additional involvement of the structured N-terminal domain of Ddc1 (Fig. 3), although no experimental data are currently available to confirm this interaction. Similarly, Rad9 binding to Dpb11 requires phosphorylation at serine 462 and threonine 474, which are recognized by the BRCT1/2 domains of Dpb11 [61]. These phosphorylation-dependent interactions facilitate the assembly of a core checkpoint complex, in which Dpb11 bridges the 9–1–1 complex to Rad9, ensuring robust checkpoint activation. This regulatory mechanism is conserved in *Schizosaccharomyces pombe* and mammals [63–66]. In addition to Dpb11, Mec1 activation is also stimulated by Ddc1 alone and by the Dna2 nuclease/helicase during S phase [67], although these activation mechanisms are likely not conserved across eukaryotes.

Rad9 association with DNA is not solely dependent on Dpb11 (Fig. 1). In undamaged cells, Rad9 is already bound to chromatin via its Tudor domain, which interacts with histone H3 methylated at lysine 79 (H3K79me), a constitutive euchromatin mark catalyzed by the methyltransferase Dot1 [68–72]. Upon DNA damage, Rad9 is further recruited to DNA lesions via its BRCT domains, which specifically recognize phosphorylated histone H2A (γH2A in yeast, γH2AX in mammals), a DNA damage-induced chromatin modification catalyzed by Mec1 and Tel1 [73–75]. While H3K79 methylation is a constitutive chromatin mark [76], γH2A spreads across kilobases around a DSB, creating a broad damage-associated chromatin domain [74]. In contrast, Dpb11 localization is more restricted, as it is specifically recruited to damage sites through its interaction with the Ddc1 subunit of the 9–1–1 complex. This hierarchical recruitment ensures that Rad9 binding is highly regulated, fine-tuning the checkpoint response according to the nature and extent of the DNA lesion.

Interestingly, γH2A phosphorylation responds to low levels of ssDNA but does not increase further with additional ssDNA accumulation [77]. In contrast, hyperactivation of the 9–1–1 axis leads to excessive Rad53 phosphorylation, even when Mec1-Ddc2 association is reduced [77]. This suggests that the extent of 9–1–1 binding at a DSB quantitatively influences Rad53 activation. Given that Rad53 phosphorylation leads to checkpoint-mediated cell-cycle arrest, these findings underscore the importance of regulating Mec1/ATR activation, preventing unnecessary checkpoint responses unless ssDNA accumulation exceeds a critical threshold.

Interestingly, the 9–1–1 checkpoint axis is particularly active in response to DNA-damaging agents when long-range resection is impaired, such as in *exo1*Δ *sgs1*Δ mutant cells [78]. This enhanced checkpoint activation contributes to the increased sensitivity of these cells to DNA damage, as failure of 9–1–1 to recruit Dpb11 and Rad9 partially suppresses the DNA damage sensitivity of *exo1*Δ *sgs1*Δ cells [78]. In the absence of both Exo1 and Sgs1, ssDNA formation still occurs, but it is primarily mediated by Mre11 nuclease activity, which generates recessed 5'-end structures of approximately 100–300 nucleotides [19,20,78,79]. Since the 9–1–1 complex preferentially binds ssDNA-dsDNA junctions [48,50,80], accumulation of these 5' recessed structures in *exo1*Δ *sgs1*Δ cells enhances 9–1–1 recruitment. Consequently, this strengthens Dpb11 and Rad9 association, leading to prolonged Rad53-mediated cell-cycle arrest and exacerbating DNA damage sensitivity of these mutant cells.

5. The 9–1–1 axis in DSB resection

The 9–1–1 complex regulates DSB resection by mediating the recruitment of Rad9, which inhibits long-range resection by limiting the activity of Exo1 and the Dna2-Sgs1 nucleases. This inhibition occurs either directly [81–85] or indirectly via Rad53 activation, which phosphorylates and inhibits Exo1 [86] (Fig. 1). However, in the absence of Rad9, the 9–1–1 complex has been shown to stimulate resection by facilitating Exo1 and Sgs1-Dna2 recruitment to DSBs [87]. Since the interaction of 9–1–1 with Dpb11 depends on Mec1-dependent phosphorylation of the Ddc1 subunit of the 9–1–1 complex [60,62], this

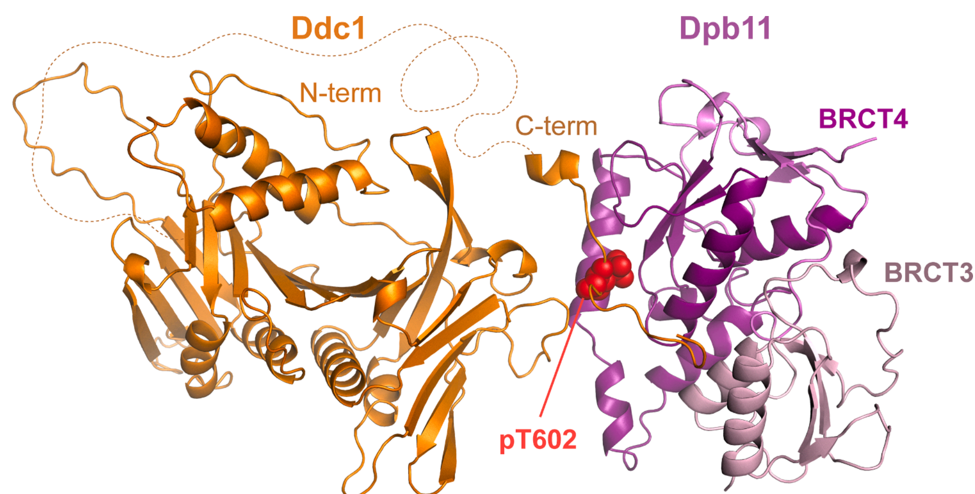

Fig. 3. Alphafold3 prediction of the complex of Ddc1 with Dpb11. The complete sequence of *S. cerevisiae* Ddc1 with phosphorylated T602 residue and Dpb11 proteins were modeled using the structure predictor Alphafold3. The substructure with consistent predictions from five generated models is represented as a cartoon. The long-disordered tail of Ddc1 is not represented (dashed line), except for the last 20 amino acids. Phosphothreonine 602 (pT602) of Ddc1 is represented as red spheres.

suggests that Mec1 activation may function as a regulatory switch, shifting the role of 9–1–1 from promoting resection to inhibiting it. Given that Rad9 transduces checkpoint signals from Mec1 to Rad53, this switch likely serves to prevent excessive accumulation of ssDNA, which could otherwise interfere with checkpoint inactivation and contribute to genomic instability.

The inhibitory effect of Rad9 on DSB resection is counteracted by Fun30, a Swr1-like ATP-dependent chromatin remodeler that forms a ternary complex with 9–1–1 and Dpb11 in a phosphorylation-dependent manner [88–90]. The Fun30 human ortholog, SMARCAD1, also antagonizes 53BP1 and promotes DNA end resection, suggesting evolutionary conservation [88]. Loss of Fun30 increases Rad9 association with DSBs, whereas the DSB resection defect of *fun30* mutants can be suppressed by *RAD9* deletion [88–90]. Interestingly, Rad9 and Fun30 appear to share the binding site for Dpb11 [90], suggesting that their antagonism depends on a competition between Fun30 and Rad9 for Dpb11 interaction. However, as Rad9 recognizes specific histone modifications and Fun30 can slide nucleosomes and mediate histone dimer exchange [91,92], Fun30 may also promote the direct removal of Rad9 from nucleosomes or eliminate Rad9 binding sites by catalyzing histone dimer exchange [93].

Beyond its role in long-range resection, the 9–1–1 complex also limits MRX-mediated short-range resection. In *exo1*Δ *sgs1*Δ cells, the absence of 9–1–1 leads to an increase in resection length, from approximately 350 nucleotides to over 1.7 kb [78]. This extended resection is dependent on Mre11 nuclease activity but does not require the function of 9–1–1 in recruiting Dpb11 and Rad9 [78]. A reconstituted short-range resection system in yeast has shown that the MRX complex, together with phosphorylated Sae2, proceeds by stepwise endonucleolytic incisions [94]. Given that the 9–1–1 complex preferentially binds to 5’ recessed ssDNA-dsDNA junctions, which mark the leading edge of resection, and is capable to slide along dsDNA [50,80], its mobility may allow it to move ahead of the resection machinery, physically blocking MRX from accessing the flanking dsDNA and thereby exerting further DNA cleavage events.

6. Recovery from checkpoint activation

Once DNA lesions are repaired, cells must turn off the checkpoint cascade to resume cell-cycle progression in a process known as checkpoint recovery. This transition is marked by the reappearance of unphosphorylated Rad53 [95]. The loss of Rad53 phosphorylation does not require new protein synthesis [96], suggesting that Rad53 is dephosphorylated rather than degraded. Checkpoint deactivation involves various phosphatases, which likely remove activating phosphorylation events from key checkpoint components.

Protein phosphatases are broadly classified based on their sequence, structure, and catalytic mechanism into three major families: phosphoprotein phosphatases (PPP), including PP1, PP2A, PP2B, and PP4, metal-dependent protein phosphatases (PPM), and phospho-tyrosine phosphatases (PTM) [10]. In budding yeast, Rad53 deactivation after DNA damage relies on the PPM family phosphatases, Ptc2 and Ptc3 (Fig. 4). In the absence of both Ptc2 and Ptc3, phosphorylated Rad53 persists even after DSB repair is completed [97,98]. Furthermore, Ptc2 directly dephosphorylates Rad53 in vitro, confirming its role in checkpoint recovery [97]. However, Ptc2 and Ptc3 are dispensable for Rad53 deactivation in response to DNA methylation or replication stress [99]. Instead, Rad53 dephosphorylation and cell-cycle recovery after treatment with methyl methanesulfonate rely on PP4, whose catalytic subunit Pph3 directly binds Rad53 [100,101] (Fig. 4). Pph3-mediated Rad53 dephosphorylation enhances DNA resection by counteracting the inhibitory effect exerted by Rad9 on the Dna2-Sgs1 complex [102]. Pph3, Ptc2, and Ptc3 exhibit partial functional redundancy. Cells lacking all three phosphatases (*pph3*Δ *ptc2*Δ *ptc3*Δ) show severe defects in Rad53 deactivation following DNA methylation damage, suggesting that Ptc2 and Ptc3 can compensate for Pph3 loss and vice versa [99].

Beyond its role in Rad53 deactivation, Pph3 has been involved in dephosphorylating γH2AX (Fig. 4) [103]. The persistence of γH2A phosphorylation in *pph3*Δ cells impairs checkpoint inactivation and re-entry into the cell cycle after DNA repair. In fact, abolishing phosphorylation of H2A Ser129 restores checkpoint recovery in *pph3*Δ cells [103]. Interestingly, the specificity of Pph3 for dephosphorylating Rad53 and γH2A is regulated by distinct binding partners. In fact, while Pph3 activity in mediating Rad53 dephosphorylation after treatment

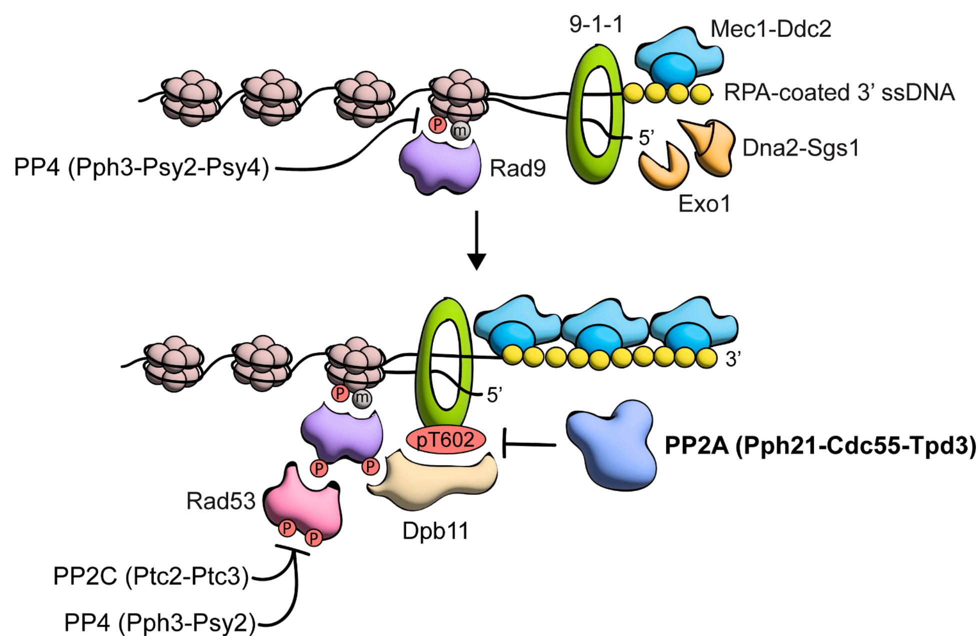

Fig. 4. Checkpoint deactivation by phosphatases. PP4 (Pph3-Psy2-Psy4) is involved in γH2A dephosphorylation, whereas both PP2C (Ptc2-Ptc3) and PP4 (Pph3-Psy2) bind Rad53 and mediate its dephosphorylation. PP2A negatively regulates checkpoint signaling by the 9–1–1 complex by interacting with Ddc1 and counteracting Ddc1-Dpb11 complex formation. This occurs at the level of Dpb11 recognition of phosphorylated Ddc1 T602.

with methyl methanesulfonate requires Psy2, but not Psy4 [100], both Psy2 and Psy4 are involved in γH2A dephosphorylation [103], suggesting that changing the composition of the PP4 complex can modulate its substrate specificity. Supporting a broader role of Pph3 in DNA damage signaling, mutations in *PPH3* and *PSY2* were identified as suppressors of the sensitivity of *mec1–100* cells to the DNA synthesis inhibitor hydroxyurea [104], suggesting that PP4 regulates Mec1 targets during replication stress. In agreement with this, Pph3-Psy2 interacts with Mec1-Ddc2 and promotes the dephosphorylation of Mec1 substrates, including Mec1 itself at Ser1991 [104].

The PP2A phosphatase has also been implicated in checkpoint attenuation during replication stress. In yeast, PP2A has been shown to limit Mec1 signaling, preventing excessive checkpoint activation under replication stress conditions [105]. In mammalian cells, PP2A constitutively interacts with ATM, even in the absence of DNA damage [106]. This suggests that PP2A acts as a negative regulator, keeping ATM in check under normal conditions and preventing unnecessary checkpoint activation. Consistently, inhibition of PP2A in undamaged cells leads to spontaneous ATM autophosphorylation and activation, further supporting its role as a checkpoint suppressor [106].

In addition to phosphatases, Rad53 activation in response to replication stress or to DNA DSBs is downregulated by the scaffold proteins Slx4 and Rtt107. Slx4 directly interacts with Dpb11, while Rtt107 was shown to be important in stabilizing the interaction [107]. The Slx4-Dpb11 interaction counteracts Dpb11-Rad9 binding and therefore Rad9 engagement at DNA lesions [107–110], suggesting that Slx4 downregulates Rad53 signaling via a competition-based mechanism. Cells lacking both *SLX4* and the PP4-subunit *PPH3* display a synergistic increase in Rad53 signaling [111]. As Slx4-mediated dampening is thought to counteract de novo Rad53 activation, but not de-activate already activated Rad53, Slx4 might act locally to downregulate Rad53 activation, while PP4 acts on pools of active Rad53 that have diffused from the site of lesions. Since Rad9 acts as an inhibitor of DSB resection, Slx4 also promotes the nucleolytic processing of DSBs [112]. In agreement with the notion that Slx4-Rtt107 competes with Rad9 for recruitment to DNA lesions, decreased resection observed in cells lacking Slx4 or Rtt107 correlates with increased binding of Rad9 to DSBs [112].

7. Regulation of the 9–1–1 axis by PP2A

In *S. cerevisiae*, the interaction between the 9–1–1 complex and Dpb11 depends on the phosphorylation of Ddc1 at threonine 602 [60,62], while Dpb11 binds to Rad9 phosphorylated at serine 462 and threonine 474 [61]. Given the central role of phosphorylation in assembling the 9–1–1 axis, phosphatase-mediated dephosphorylation events could, conceivably, counteract its formation and regulate checkpoint activation.

The PP2A phosphatase complex comprises a scaffold A subunit (Tpd3), a regulatory B subunit (Cdc55, Rts1, or Rts3), and two interchangeable catalytic C subunits (Pph21 and Pph22) [113]. The B subunit plays a crucial role in substrate specificity and PP2A localization. Notably, gain-of-function mutations in Cdc55 (*cdc55-Q335E*) and Tpd3 (*tpd3-I384N*) have been identified as suppressors of the DNA damage sensitivity of cells lacking both Exo1 and Sgs1, which exhibit defects in long-range resection [78]. The hypersensitivity of *exo1*Δ *sgs1*Δ cells to genotoxic agents is due to persistent activation of the 9–1–1 checkpoint axis, as demonstrated by the fact that abolishing Ddc1 phosphorylation at T602 suppresses this sensitivity [78]. The Cdc55^{Q335E} and Tpd3^{I384N} variants restore DNA damage resistance in *exo1*Δ *sgs1*Δ cells by downregulating the 9–1–1 checkpoint axis [114]. This downregulation depends on Cdc55 ability to interact with Ddc1 and counteract Ddc1-Dpb11 complex formation. Specifically, it affects the step in which Dpb11 recognizes Ddc1 phosphorylated at Thr602, a critical event for the assembly of the Dpb11-Ddc1 complex (Fig. 4) [60,62]. In fact, the checkpoint downregulation mediated by Cdc55^{Q335E} and Tpd3^{I384N} is abolished when T602 is replaced with glutamic acid, mimicking constitutive phosphorylation. Conversely, the absence of Cdc55 or Tpd3 leads to the opposite effect, resulting in sustained checkpoint activation, a phenotype that is rescued when T602 is substituted with a non-phosphorylatable alanine residue.

Neither the Cdc55^{Q335E} nor Tpd3^{I384N} mutations affect regions that interact with the PP2A catalytic subunit (Fig. 5), suggesting that these mutations alter substrate recognition rather than the catalytic function of the phosphatase. Consistently, the Cdc55^{Q335E} mutant exhibits an enhanced interaction with Ddc1, a largely disordered protein whose structure cannot be modeled. The observation that Ddc1 phosphorylation increases with similar kinetics in both wild-type and *cdc55-Q335E* cells after PP2A catalytic inhibition suggests that the stronger Ddc1-Cdc55^{Q335E} interaction makes Thr602 a more favourable substrate for dephosphorylation rather than shielding it from Mec1 phosphorylation. It remains unclear whether the *cdc55-Q335E* and *tpd3-I384N* mutations represent gain-of-function alterations specific to Ddc1 or whether they enhance dephosphorylation of other substrates as well. Given the limited understanding of PP2A substrate recognition, elucidating the molecular mechanisms affected by these mutations could provide important insights into how specific phosphorylation sites are selectively targeted for dephosphorylation. A recent model suggested the presence of conserved motifs responsible for substrate recognition by human B55α [115], which are fully conserved in the yeast ortholog Cdc55. An AlphaFold3-based model of the interaction between Ddc1 and yeast PP2A supports the conservation of this substrate recruitment module in yeast (Fig. 5). The Cdc55^{Q335E} mutation may influence the flexibility of the substrate-binding pocket, potentially affecting the affinity for different substrates.

Since the 9–1–1 complex recruits Rad9 to DSBs via Dpb11, where Rad9 inhibits long-range resection by antagonizing Exo1 and the Dna2-Sgs1 complex [81–84,116], the presence of the Cdc55^{Q335E} mutant enhances DSB resection by reducing Rad9 recruitment at DSBs. Conversely, the loss of Cdc55 or Tpd3 impairs long-range resection due to increased Rad9 association with DSBs. These findings indicate that PP2A-dependent dephosphorylation of Ddc1 plays a key role in balancing checkpoint activation and DSB resection, ensuring that

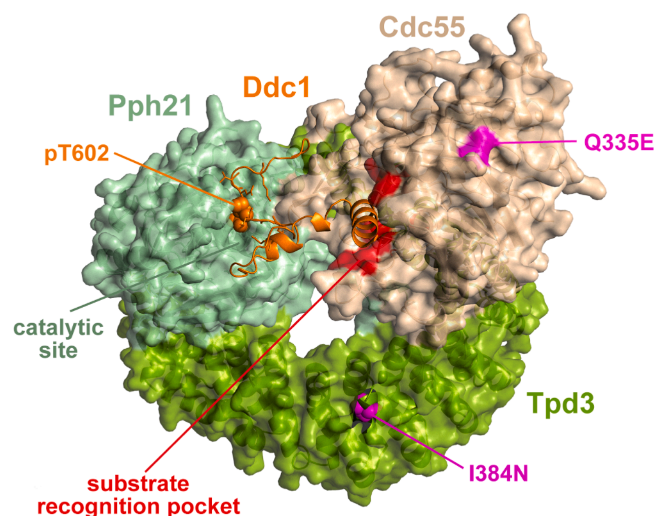

Fig. 5. Alphafold3 prediction of the complex of Ddc1 with the PP2A subunits Pph21, Cdc55, and Tpd3. The structure of PP2A with the Tpd3 scaffold subunit, the Cdc55 regulatory subunit, and the Pph21 catalytic subunit, together with Ddc1 phosphorylated on T602, was modeled with Alphafold3. The phosphatase subunits are represented as a cartoon with a semitransparent surface. The red surface marks the substrate recognition site of Cdc55. The Ddc1 562–611 aa tail is represented as a cartoon, while phosphorylated T602 (pT602) is represented as spheres. The purple spheres represent the residues affected by the *cdc55-Q335E* and *tpd3-I384N* mutations, which are located far from the catalytic site.

checkpoint signaling does not persist longer than necessary and allowing proper DNA end processing.

8. Concluding remarks

The DNA damage checkpoint plays a fundamental role in maintaining genome stability by detecting DNA lesions and initiating a cascade of phosphorylation events that halt cell-cycle progression and enable repair. Phosphorylation is a key regulatory mechanism in checkpoint signaling, as it creates binding surfaces for protein-protein interactions. However, once the damage is resolved, checkpoint deactivation is equally critical to allow cells to resume normal division. This transition is finely tuned through the opposing actions of protein kinases and phosphatases. Among phosphatases, PP2A plays a crucial role in modulating the 9–1–1 axis, particularly by dephosphorylating Ddc1, which influences Rad9 recruitment and checkpoint persistence. This regulatory function ensures a timely shift from DNA damage signaling to recovery, balancing checkpoint activity to prevent unnecessary cell-cycle arrest. Through these actions, phosphatases not only ensure timely checkpoint inactivation but also influence DNA end resection, promoting genome integrity.

PP2A typically functions as a tumor suppressor in cancer [117]. It has been found to be inactivated in various malignancies such as colorectal, breast, oral squamous cell carcinomas, and acute myeloid leukemia. Moreover, the PP2A inhibitor okadaic acid has been shown to induce tumor formation in mice, suggesting that loss of PP2A activity can contribute to cancer development [118,119]. Inactivation of PP2A in cancer is typically caused by mutations, post-translational modifications, or increased expression of endogenous PP2A inhibitors [120,121]. Major signaling pathways affected by PP2A dysfunction include PI3K-AKT, Wnt, mTOR, and MAPK, all of which are critical in cancer progression [120,121]. Therefore, therapeutic strategies aimed at reactivating PP2A could restore its tumor-suppressive function by re-establishing proper regulation of these pathways.

However, the finding that PP2A can promote the inactivation of the DNA damage checkpoint response, which normally acts as a barrier to tumorigenesis, raises the concern that PP2A reactivation in cells with unrepaired DNA damage might lead to cell-cycle progression, thereby potentially contributing to tumor development. Consequently, therapeutic approaches that achieve substrate specificity by selectively targeting PP2A regulatory complexes involved in oncogenic signaling may enhance the effectiveness of cancer therapies while minimizing adverse effects on genome stability.

CRediT authorship contribution statement

Renata Tisi: Writing – review & editing, Writing – original draft, Conceptualization. **Erika Casari:** Writing – review & editing, Writing – original draft, Conceptualization. **Maria Pia Longhese:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

No data was used for the research described in the article.

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