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REVIEW

Eukaryotic DNA damage checkpoint activation in response to double-strand breaks

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Abstract Double-strand breaks (DSBs) are the most detrimental form of DNA damage. Failure to repair these cytotoxic lesions can result in genome rearrangements conducive to the development of many diseases, including cancer. The DNA damage response (DDR) ensures the rapid detection and repair of DSBs in order to maintain genome integrity. Central to the DDR are the DNA damage checkpoints. When activated by DNA damage, these sophisticated surveillance mechanisms induce transient cell cycle arrests, allowing sufficient time for DNA repair. Since the term "checkpoint" was coined over 20 years ago, our understanding of the molecular mechanisms governing the DNA damage checkpoint has advanced significantly. These pathways are highly conserved from yeast to humans. Thus, significant findings in yeast may be extrapolated to vertebrates, greatly facilitating the molecular dissection of these complex regulatory networks. This review focuses on the cellular response to DSBs in *Saccharomyces cerevisiae*, providing a comprehensive overview of how these signalling pathways function to orchestrate the cellular response to DNA damage and preserve genome stability in eukaryotic cells.

Keywords Cancer · Checkpoint · DNA damage · Double-strand break · Yeast · Genome instability

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Abbreviations

ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
ATRIP	ATR interacting protein
BRCT	BRCA1 carboxyl terminal
CAD	Chk1 activation domain
CDK	Cyclin-dependent kinase
DDK	Dbf4-dependent kinase
DDR	DNA damage response
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	Double-strand break
GCRs	Gross chromosomal rearrangements
G1	Gap phase 1
G2	Gap phase 2
HR	Homologous recombination
IR	Ionising radiation
M	Mitosis
MEN	Mitotic exit network
MMS	Methyl methanesulfonate
MRX/MRN	Mre11-Rad50-Xrs2/MRE11-RAD50-NBS1
NHEJ	Non-homologous end joining
PIKK	Phosphoinositide 3-kinase related kinase
PTM	Post-translational modification
RNR	Ribonucleotide reductase
RPA	Replication protein A
SCD	SQ/TQ cluster domain
UV	Ultraviolet

Introduction

The genomic integrity of an organism is constantly being challenged by DNA insults arising from exogenous sources, including ionising radiation (IR), ultraviolet (UV) radiation and carcinogenic agents, as well as endogenous

stresses resulting from DNA replication errors and by-products of cellular metabolism, such as reactive oxygen species [1]. The capacity to deal effectively with spontaneous- or environmentally-induced DNA damage is crucial for cellular survival and the maintenance of genomic stability, as inaccuracies in these processes can lead to chromosomal aberrations and cancer [2].

Consequently, cells have evolved sophisticated surveillance mechanisms termed DNA damage checkpoints [3, 4] that monitor the successful completion of cell cycle events and initiate a coordinated cellular response when DNA damage is detected [5–7]. Activation of the DNA damage checkpoint results in cell cycle arrest, activation of transcriptional programmes, initiation of DNA repair or, if the damage is too severe, cellular senescence or programmed cell death [8]. These mechanisms act in concert to preserve genomic integrity and thus the fidelity of cell propagation. Once repair is completed, the DNA damage checkpoint response is down-regulated and cells re-enter the cell cycle in a process known as recovery. Alternatively, if the lesion is irreparable, cells may undergo adaptation and eventually re-enter the cell cycle in the continued presence of DNA damage [9].

DNA double-strand breaks (DSBs) constitute one of the most cytotoxic forms of DNA damage and pose a significant threat to cell viability, survival and homeostasis. DSBs have the potential to promote gross chromosomal rearrangements (GCRs) and potentially deleterious mutations that promote tumourigenesis [10]. Indeed, defects in many proteins with known roles in the DNA damage response (DDR) have been implicated not only in cancer development but also in neurodegenerative disorders, developmental defects, immune deficiency disorders and sterility [11]. Therefore, understanding the molecular mechanisms governing the cellular response to DSBs will not only serve to further our knowledge of the complex biology underpinning these intricate signalling networks but it may also identify exciting avenues for the development of novel and targeted mechanism-based therapeutics with the potential to significantly impact human health. This review focuses on DNA damage checkpoint activation in response to DSBs with particular focus on the budding yeast *Saccharomyces cerevisiae*. We describe the currently proposed models for how these lesions are detected and discuss the intricate signal transduction mechanisms underlying the DDR. These DNA damage checkpoints are evolutionarily conserved, and therefore reported similarities between *S. cerevisiae* and vertebrates will also be discussed in this context.

Overview of the DNA damage response in *S. cerevisiae*

The unicellular eukaryotic budding yeast, *S. cerevisiae*, is a well-characterised and versatile model organism and has been instrumental in initial discoveries related to cell cycle control and the DDR [4, 12–14]. The DDR in *S. cerevisiae* relies on several classes of checkpoint and repair genes which are evolutionarily conserved in vertebrates [15]. The principal proteins in *S. cerevisiae* involved in the early steps of DSB checkpoint activation are listed in Table 1 along with their *Schizosaccharomyces pombe* and human homologues.

Faithful replication and propagation of genetic information is paramount for cell viability and genomic stability. Hence, cells have evolved a complex regulatory network termed the cell cycle control system which coordinates the order and timing of cell cycle events [16]. The *S. cerevisiae* mitotic cell cycle (Fig. 1), like all eukaryotes, consists of four phases [17]. The first phase, Gap phase 1 (G1), is the main phase of growth and activation of transcriptional pathways required for the subsequent S phase [18]. In late G1, once cells have reached the critical size and sufficient nutrients are available, they pass through START (termed the restriction point in vertebrates) and enter S phase where the genome is replicated. Following S phase, cells enter Gap phase 2 (G2) and prepare for entry into mitosis (M), the phase in which the duplicated chromosomes are segregated and the cell divides in two. The major processes of the cell cycle occur in S phase where chromosomes must be accurately duplicated and in M phase where they must be properly segregated. It is vital that cells exit mitosis and proceed through cytokinesis only after chromosome segregation is complete. Thus, there are two main transition points in the cell cycle: one at the G1/S transition and the other at the G2/M phase boundaries [16]. It is important to note that, unlike *S. pombe* and vertebrates, the G2/M transition is not well defined in *S. cerevisiae* and events traditionally considered as mitotic, such as spindle pole body duplication and mitotic spindle formation, are initiated during S phase in order to facilitate bud formation and nuclear migration [19, 20]. Consequently, it is the metaphase to anaphase transition rather than the G2 to M transition that is regulated by the G2/M DNA damage checkpoint in *S. cerevisiae* [21]. The key regulators of the cell cycle are a family of serine/threonine kinases known as cyclin-dependent kinases (CDKs) which phosphorylate numerous substrates [22, 23] that play pivotal roles in major cell cycle events, including DNA replication and mitosis [24]. In *S. cerevisiae*, all cell cycle events are controlled by a single essential CDK known as Cdk1/Cdc28 [14, 25, 26].

In *S. cerevisiae*, DNA damage checkpoints operate at three distinct stages in the cell cycle (Fig. 1). The G1 checkpoint arrests cells at the G1/S transition prior to START [27–29] before cells irreversibly commit to the next cell cycle. This transient arrest delays bud emergence, spindle pole body duplication and S phase entry, allowing

Table 1 Homologues of the central components of the DNA damage checkpoint in eukaryotes. DNA damage checkpoint proteins and protein complexes involved in the early steps of double-strand break DNA damage checkpoint activation in *S. cerevisiae*, and their structural or functional homologues in *S. pombe* and *H. sapiens*

Class of protein	*S. cerevisiae*	Proposed function(s)	*S. pombe*	*H. sapiens*
Sensors	Mec1-Ddc2	Principal PIKK complex involved in sensing DNA damage and transducing the checkpoint signal. Mec1 binds Ddc2, which mediates its recruitment to sites of damage	Rad3-Rad26	ATR-ATRIP
	Tel1	PIKK primarily involved in telomere maintenance. Partial overlap with Mec1 in the DNA damage response.	Tel1	ATM
	Mre11-Rad50-Xrs2	Initial sensing and signalling of DSBs. Involved in DSB repair via HR and NHEJ.	Rad32-Rad50-Nbs1	MRE11-RAD50-NBS1
	Rad24-Rfc2-5 (RFC-like complex)	Loading of Ddc1-Rad17-Mec3 (9-1-1) complex onto DNA. Role in DNA damage signalling.	Rad17-Rfc2-5	RAD17-RFC2-5
	Ddc1-Rad17-Mec3 (PCNA-like complex)	Heterotrimeric complex, structurally related to PCNA, also called 9-1-1. DNA damage signal transduction and recruitment of other checkpoint proteins.	Rad9-Rad1-Hus1	RAD9-RAD1-HUS1
	Dpb11	Replication initiation protein and checkpoint sensor recruited to sites of damage by 9-1-1 complex, where it activates Mec1	Cut5	TopBP1
DSB processing	Sae2	Endonuclease that functions with MRX complex in the first step of DNA DSB resection.	Ctp1	CtIP
	Exo1	5′–3′ exonuclease involved in recombination and DNA repair.	Exo1	EXO1
	Sgs1	RecQ-like helicase that functions with Dna2 to process DSBs.	Rqh1	BLM
	Dna2	5′ flap endonuclease/helicase that functions with Sgs1 to resect DSBs.	Dna2	DNA2
Adaptors/ mediators	Rad9	Molecular adaptor/mediator required for DNA damage signal transduction and DSB repair. Required for Rad53 and Chk1 activation.	Crb2	53BP1; BRCA1; MDC1
	Mrc1	Molecular adaptor required for S phase checkpoint activation. Required for Rad53 activation in response to replication stress.	Mrc1	Claspin
Effectors	Rad53	Effector kinase in replication and DNA damage checkpoints.	Cds1	CHK2
	Chk1	Effector kinase involved in signal transduction.	Chk1	CHK1

time for DNA lesions to be repaired before the onset of DNA replication [27, 28, 30]. However, certain DNA aberrations such as alkylated DNA do not activate the G1 checkpoint and instead cells pass through START. Essentially, these lesions elicit a checkpoint response during S phase since they need to be converted to secondary lesions during DNA replication before being recognised by the checkpoint machinery [31]. The intra-S phase checkpoint slows the rate of replication in response to DNA damage [32], coordinating fork repair mechanisms and cell cycle progression to ensure the fidelity and completion of replication before cells enter mitosis. As discussed above, the G2/M checkpoint arrests cells at the metaphase to anaphase transition, preventing cells from progressing through mitosis in the presence of DNA damage [4].

If DNA damage can be mended efficiently, the lesion is rapidly repaired without induction of cell cycle arrest [33]. However, when the damage cannot be repaired quickly or persists, the DNA damage checkpoint is activated [34]. The apical protein kinases in this evolutionarily conserved signal transduction cascade are members of a family of phosphoinositide 3-kinase-related kinases (PIKKs) which include *S. cerevisiae* Tel1 and Mec1, *S. pombe* Tel1 and Rad3, and mammalian ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) [6, 35].

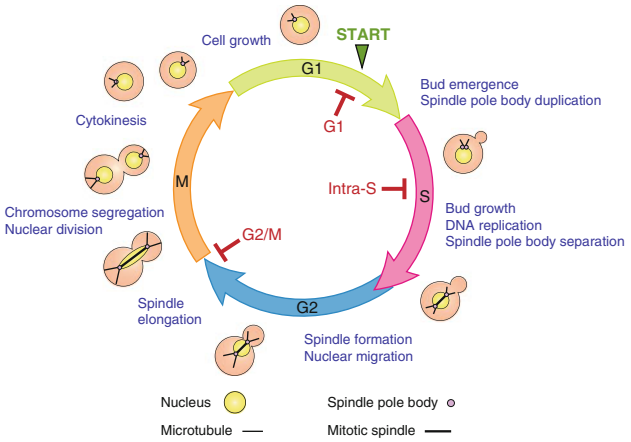

Fig. 1 *Saccharomyces cerevisiae* cell cycle and the DNA damage checkpoints. The budding yeast cell cycle, illustrating the specific cellular morphologies at each cell cycle stage. Also depicted are the three DNA damage checkpoints; the G1 checkpoint which arrests cells prior to START, the intra-S phase checkpoint which slows the rate of DNA replication and the G2/M checkpoint which arrests cells at the metaphase/anaphase transition. Once damage is repaired, the checkpoint is down-regulated and cells resume cycling

A homologue of DNA-PKcs has not been identified in *S. cerevisiae* or *S. pombe* [36]. In *S. cerevisiae*, Mec1 is often considered to be the principal PIKK since *mec1* mutants are severely sensitive to DNA damage [34] and Tel1 function is mostly apparent when several DSBs are generated [80]. In reality, both Tel1 and Mec1 have important roles in DSB signalling, mostly similar to their vertebrate homologues: ATM (*sc*Tel1) functions in response to DSBs [37], while ATR (*sc*Mec1) is activated via its recruitment to replication protein A (RPA)-coated ssDNA [38, 39] which occurs at stalled replication forks and also at DSBs which have undergone resection, a process which is dependent upon prior activation of ATM [40–42]. These PIKKs promote the activation of downstream effector kinases (*sc*Chk1 and Rad53; *sp*Chk1 and Cds1; human CHK1 and CHK2), which function to target downstream components of the DDR as well as amplifying the initial DDR signal [43]. In *S. cerevisiae*, Mec1 activates both Rad53 and Chk1 [44] while in vertebrate cells ATM primarily activates CHK2 (*sc*Rad53) and ATR activates CHK1 (*sc*Chk1) [43]. PIKK-dependent activation of the effector kinases is regulated by mediator proteins, which include the prototypical mediator protein *sc*Rad9 (equivalent to *sp*Crb2; human 53BP1, BRCA1, MDC1) that function as molecular adaptors to recruit proteins to sites of damage and mediate the PIKK-dependent phosphorylation of downstream substrates [8]. Activation of downstream targets is often mediated by phosphorylation events which modulate the transcription level of repair genes and regulate cell cycle transitions by influencing the stability and/or localisation of proteins involved in cell cycle progression or checkpoint maintenance [45]. A major surprise in recent years has been the discovery that protein kinases other than the classical PIKK and CHK kinases also regulate DDR protein function [46]. Additionally, it is becoming clearer that other post-translational modifications (PTMs) are important for DNA damage checkpoint regulation, particularly those linked to chromatin modulation [47]. Mapping the complex biochemical interplay between these post-translational regulatory mechanisms represents a challenge of significant importance in order to gain a more comprehensive understanding of the DDR. Here, we present the known and most current mechanisms postulated to regulate the DDR.

Mechanisms of DNA damage checkpoint activation in response to double-strand breaks

Of the diverse types of DNA lesion, DSBs are the most cytotoxic form of DNA damage and pose a serious threat to cell viability. In response to even a single DSB, cells must trigger a series of events to promote repair of the lesion in order to survive and restore chromosomal integrity [48]. Ionising radiation, chemical agents, oxidative stress, and collapsed replication forks are thought to all lead to the formation of DSBs. Moreover, DSBs are required for normal cellular processes such as chromosome segregation during meiosis, V(D)J recombination in immunoglobulin genes, which occurs specifically in vertebrates, and mating-type switching in yeast [11, 49]. Much work has elucidated the cellular response to DSBs and identified the regulated recruitment and activation of the checkpoint machinery at sites of damage [15].

Sensing DNA double-strand breaks

A first step in the activation of the DNA damage checkpoint is sensing of the damaged DNA. Initially thought to be the role of checkpoint proteins, the DSB repair proteins have been found to be important sensors of DNA damage. In *S. cerevisiae*, DSBs are specifically and independently recognised by both the Ku (yKu70/yKu80) and MRX (Mre11-Rad50-Xrs2) repair complexes (Fig. 2a), which compete for binding to unprocessed DSB ends [50–55]. The Ku complex, normally localised to telomeres, relocates to the site of the DSB and wraps around the dsDNA ends in a sequence-independent manner holding them together to ensure they are properly aligned for rejoining [51, 56, 57]. In vertebrates, the Ku heterodimer recruits DNA-PKcs, forming the DNA-PK holoenzyme, which functions as a DNA end-bridging factor [58–60]. Together with the MRN (MRE11-RAD50-NBS1) complex, they function in tethering the DNA ends together [61]. Although DNA-PKcs is

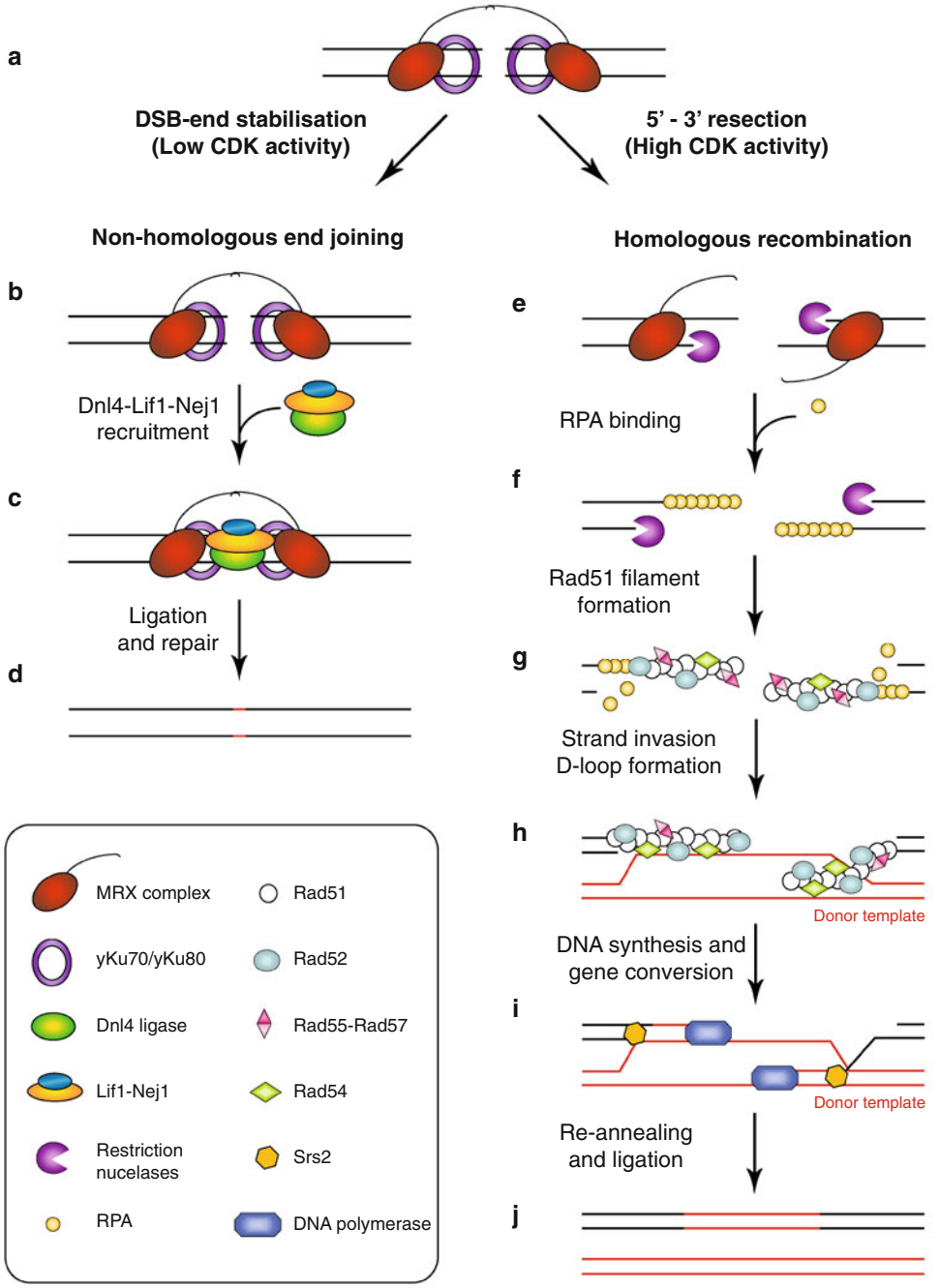

Fig. 2 Model of DSB repair by NHEJ and HR pathways in *S. cerevisiae*. Following DSB creation, the DNA ends are tethered by the MRX and Ku complexes (**a**). During G1 phase, when CDK activity is low, DSBs are primarily repaired via NHEJ. In NHEJ, DSB ends are further stabilised by MRX and yKu70/yKu80 (**b**). The Dnl4-Lif1-Nej1 ligase complex is then recruited (**c**), the broken DNA ends are ligated, and the lesion repaired (**d**). In S/G2 phases, when CDK activity is high, DSBs are repaired by HR. Firstly, DNA-end resection is initiated by Sae2 and the MRX complex. This is followed by a more processive resection catalysed by Exo1 and/or Dna2 in collaboration with the Sgs1 helicase (**e**). RPA binds to ssDNA (**f**). RPA-coated ssDNA is a substrate for Rad51 filament formation, involving Rad52, Rad55-Rad57 and Rad54 (**g**). Rad51 filament homology search and strand invasion lead to the formation of a D-loop (**h**). This is followed by disassembly of the Rad51 filament mediated by the Srs2 helicase, and subsequent DNA synthesis (**i**). Resolution of the Holliday junction is followed by re-annealing and ligation of the newly synthesised DNA (**j**)

not conserved in *S. cerevisiae* [62], the MRX complex seems to carry out this function alone as it has robust DNA end-bridging activity [63] and facilitates the bridging of DSB ends via the zinc-hook motifs located in the coiled-coil region of the Rad50 subunit, similar to MRN in vertebrate cells [64–66]. Non-homologous end joining (NHEJ) is the primary DSB repair pathway in G1 phase. In G1, processive resection is significantly reduced due to low CDK activity (Fig. 2b–d) and inhibition by the NHEJ machinery; binding of the Ku complex to DSB ends delays the onset of resection and provides a window of opportunity for DSB repair via NHEJ [53, 67–70]. Conversely, DSB repair via homologous recombination (HR) is restricted to S/G2 phases when CDK activity is high (Fig. 2e–j) and a suitable repair template for HR is available (i.e. the sister chromatid, which is used preferentially as a recombination template over a homolog in G2 diploid cells) [69–72]. Detailed molecular mechanisms for both NHEJ and HR have been recently described in the literature [73, 74].

In *S. cerevisiae*, the MRX complex is the principal sensor required for DSB-induced checkpoint activation

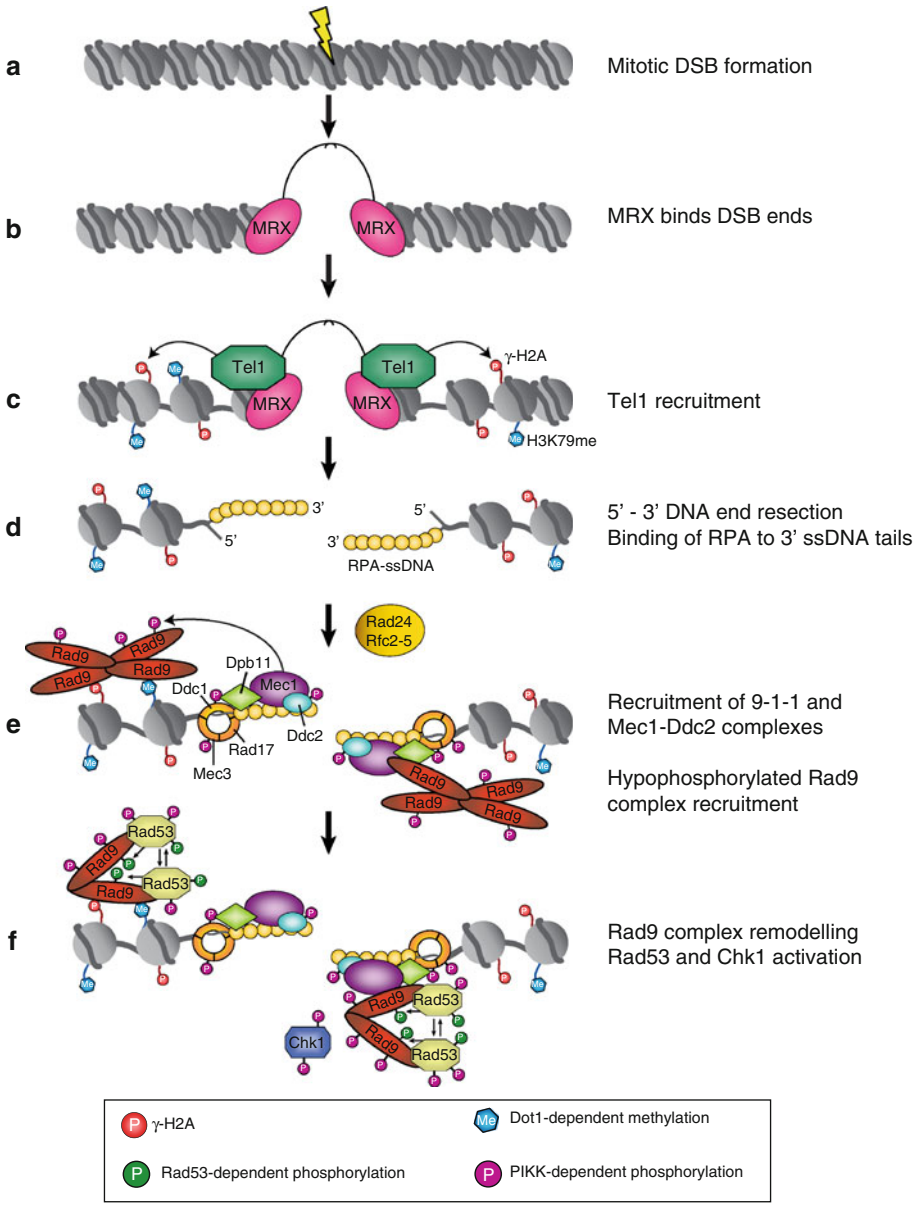

Fig. 3 DSB-induced checkpoint activation in *S. cerevisiae*. Following DSB formation (**a**), the DDR signalling cascade is initiated by binding of MRX to DSB ends (**b**). MRX recruits Tel1, which phosphorylates histone H2A (**c**). After extensive resection, the ssDNA is rapidly coated with RPA (**d**). This structure promotes the independent recruitment of the Ddc1-Rad17-Mec3 complex, which is loaded onto DNA by the Rad24-Rfc2-5 complex, and Mec1-Ddc2 (**e**). Dpb11 is recruited by binding to phosphorylated Ddc1. Subsequently, the hypophosphorylated Rad9 complex is recruited by either binding to γ-H2A and methylated H3K79 or through its association with Dpb11. PIKK-dependent phosphorylation promotes Rad9 complex remodelling. Hyperphosphorylated Rad9 facilitates the PIKK-dependent phosphorylation of Rad53 and Chk1 (**f**). Activated Rad53 and Chk1 phosphorylate downstream effectors, resulting in activation of the major biological responses to DNA damage, including cell cycle arrest, DNA damage-induced transcription, DNA repair and slowing of DNA replication

[75]. Following DSB formation (Fig. 3a), binding of MRX to DNA ends (Fig. 3b) promotes the recruitment of Tel1 to the DSB (Fig. 3c) via a direct interaction between Tel1 and the C-terminus of Xrs2 [76], leading to Tel1-dependent checkpoint activation prior to DNA end processing [77–79]. In contrast to Mec1, checkpoint-dependent activation of Tel1 has not been extensively studied. One reason for this being that it was long considered to be redundant with Mec1; a *tel1Δ* mutant is checkpoint proficient and does not exhibit increased sensitivity to genotoxic agents, while additional deletion of *TEL1* enhances the sensitivity of *mec1* mutants, suggesting some degree of functional redundancy between these genes [80–83]. The apparent minor role of Tel1 in the DDR may be somewhat explained by the capability of yeast to efficiently activate DSB signalling and rapidly convert DSB ends into substrates (i.e. ss-DNA) that preferentially stimulate Mec1 kinase activity. Further insight into Tel1 activation was recently gained from a study showing that Tel1 kinase activity is stimulated by binding of the MRX complex to DNA–protein complexes at DNA ends [84]. This study provides several interesting avenues for future investigation of the exact DNA-end structures that stimulate MRX-dependent Tel1 activation and how MRX specifically modulates Tel1 catalytic activity. Tel1 activity is required for the DNA damage-induced phosphorylation of Xrs2, Mre11 and Sae2, promoting their functions in DNA repair and checkpoint activation [77, 78, 85, 86]. Like its *S. cerevisiae* homologue, vertebrate ATM (*sc*Tel1) also recognises unprocessed DSBs. Unlike Tel1, ATM exists as an inactive

dimer that undergoes DNA damage-induced *in trans* autophosphorylation to form partially active monomers [87]. ATM monomers are recruited to DSBs through an interaction with the NBS1 subunit of the MRN complex and, once recruited, are also activated by MRN bound to DNA [88–90] as well as early processing events induced by MRN [91, 92]. It is proposed that full activation of ATM also requires prior acetylation of ATM by the acetyltransferase Tip60 [93, 94]. Although these activities all contribute to efficient ATM activation, the exact molecular mechanisms of Tel1/ATM activation remain to be elucidated.

Chromatin modulation and the DNA damage response

The phosphorylation of histone H2A on S129 (γ-H2A) by Tel1 is an early event in the activation of the DDR [95–99]. The discovery of this Tel1/Mec1-dependent modification was an important landmark in the DNA damage checkpoint field as it revealed the importance of chromatin context for DNA damage detection and the related downstream DDR processes. Since this discovery, numerous studies have highlighted the role of chromatin manipulation via covalent histone modifications, ATP-dependent chromatin remodelling and the incorporation of histone variants into nucleosomes to induce an efficient DDR [100, 101]. These mechanisms, more complex in vertebrate cells than in yeast due to a higher order of chromatin organisation, are still poorly understood. This field is under extensive investigation and has been recently reviewed elsewhere [102, 103].

DSB-induced γ-H2A spreads over an approximate 50-kb domain of chromatin surrounding the break [96, 99]. This mark is conserved in mammalian cells where ATM-dependent phosphorylation occurs on S139 of the H2A variant, H2AX (γ-H2AX) [104], spreading over an approximate 2-Mb domain of chromatin surrounding the break [105]. In *S. cerevisiae*, *h2a-S129* mutants exhibit significant sensitivity to DSB-inducing agents as well as impaired DSB repair [96, 106, 107]. Furthermore, *h2a-S129* mutants are defective in G1 checkpoint activation in response to DSBs due to impaired recruitment of the Rad9 checkpoint protein to sites of damage, which binds γ-H2A via its tandem BRCT domain [108, 109]. Mammalian cells that are deficient in H2AX are radiosensitive and display elevated genomic instability and defects in sister chromatid recombination [110–113]. In addition, $H2AX^{-/-}$ mouse embryonic fibroblasts are defective in G2-M checkpoint activation following low doses of IR [114].

Several studies have demonstrated that γ-H2A(X) promotes efficient DSB repair by regulating chromatin modulation in response to DNA damage through the recruitment of cohesin, histone modifiers and chromatin remodelling complexes in both yeast and vertebrates [102, 115]. It also facilitates the accumulation and stable retention of checkpoint and repair proteins at the site of damage [116]. This latter function of γ-H2A(X) is key for the activation of a robust checkpoint response as suggested by the fact that co-localisation of checkpoint and repair proteins on either yeast or vertebrate chromatin is sufficient to activate a strong checkpoint response in the absence of DNA damage [117, 118].

Although γ-H2AX is dispensable for the initial recruitment of NBS1 and the putative *sc*Rad9 orthologues BRCA1 and 53BP1 to DSBs in mammalian cells [119], their accumulation and stable retention is dependent upon the interaction between MDC1 (another *sc*Rad9 orthologue) and γ-H2AX, which promotes further recruitment of ATM to the vicinity of the break leading to the spread of γ-H2AX along chromatin [120, 121]. The localisation of DNA damage mediator proteins of the Rad9 family at sites of damage has been extensively studied in vertebrate cells and has revealed an unexpected importance of ubiquitination in mediating the formation of a complex scaffold of DDR proteins, which is sufficient to trigger the checkpoint cascade [122]. Several ubiquitin ligases have been identified at foci in mammalian cells including RNF8, RNF168, BRCA1, RAD18 and HERC2 [123–130]. Identifying substrates for these ligases, in particular the RNF8/RNF168/BRCA1 pathway, and elucidating how these processes are regulated is an important area for future investigation. In *S. cerevisiae*, extensive ubiquitination events at sites of DSBs have not been observed and Rad9 accumulation at sites of DSBs seems to be the functional equivalent to the complex focal accumulation of vertebrate DDR proteins.

DNA double-strand break resection

DSB resection is a ssDNA generating process necessary to switch from Tel1/ATM to Mec1/ATR signalling as well as for homologous recombination mechanisms. A significant advancement in our understanding of the molecular mechanisms leading to DSB resection has recently taken place. It is now known that DNA end resection occurs via a two-step mechanism [131, 132]. The first step is initiated by the MRX complex and Sae2 (vertebrate CtIP), which mediate the removal of hairpins, bulky adducts and other aberrant DNA end structures [133–136]. Initial processing by Sae2/CtIP and MRX/MRN results in the removal of approximately 50–100 nucleotides from the 5′ ends of the break, giving rise to short 3′ ssDNA tails [131, 132]. The Ku complex has a low affinity for ssDNA [137], thus resection reduces its binding capability which prevents repair by NHEJ and instead facilitates extensive end processing, an event necessary for HR-mediated DSB repair and robust Mec1/ATR-dependent checkpoint signalling [53].

In the second step, partly reconstituted in vitro [138], the partially resected DNA ends are the substrate for further extensive nucleolytic degradation which is carried out via two redundant pathways. One pathway is dependent on the exonuclease Exo1 while the other is mediated by the helicase-topoisomerase complex Sgs1-Top3-Rmi1 in collaboration with the 5′ flap endonuclease Dna2 [131, 132, 139]. Recent studies in *S. cerevisiae* have greatly contributed to our understanding of how the transition from initial to long-range resection occurs [138]. MRX and Sae2 act cooperatively to stimulate Exo1-dependent resection [55, 140]. Interestingly, this does not require the nuclease activity of Mre11 nor is it dependent on interactions between MRX or Sae2 and Exo1 [55, 140]. Instead, it is proposed that MRX and Sae2 facilitate Exo1 recruitment partially by suppressing Ku accumulation at DSBs and also through creation of a specific DNA structure (most likely a branched structure) that results in a high-affinity binding site for Exo1 which promotes its recruitment to DNA ends and stimulates its nuclease activity [55, 140, 141]. In addition to this, it has recently been proposed that Exo1 nuclease activity is negatively regulated by 14-3-3 proteins (*sc*Bmh1, Bmh2) [142]. Exo1 physically associates with 14-3-3 proteins and it is thought that this interaction modulates Exo1 phosphorylation which could function to prevent Exo1 nuclease activity under certain conditions, for example at stalled replication forks [142]. Both MRX and Top3-Rmi1 also promote DSB resection by Sgs1-Dna2-RPA by facilitating the recruitment of Dna2 to DNA ends and increasing the DNA-binding affinity of Sgs1, which stimulates its helicase activity and promotes DNA unwinding [55, 141, 143, 144]. Resection of DNA ends by Sgs1-Dna2-RPA is confined to 5′-terminated ssDNA as RPA inhibits Dna2-dependent degradation of 3′ ends while stimulating degradation of 5′ ends [143, 144]. This specificity ensures the correct directionality of DNA end processing which is essential for the formation of long 3′ ssDNA overhangs.

Recent in vitro studies have shown that processive resection in vertebrates also occurs via two pathways similar to that described for *S. cerevisiae* above [145]. In one, BLM (*sc*Sgs1) and DNA2 (*sc*Dna2) physically interact and function in the 5′–3′ resection of DNA ends, a process dependent on the helicase and nuclease activity of BLM and DNA2, respectively [145]. This process also requires RPA which stimulates the helicase activity of BLM and ensures a strict 5′-3′ polarity of resection by DNA2 [145]. MRN also stimulates BLM–DNA2-mediated resection by recruiting BLM to DNA ends [145]. In a second EXO1 (*sc*Exo1)-dependent pathway, MRN, RPA and BLM stimulate resection by promoting the recruitment of EXO1 to DNA ends [145, 146] and, in the case of MRN, by enhancing the processivity of EXO1 [145]. Analogous DSB resection processes have also been reported in Archaea and Bacteria, demonstrating once again the conservation of essential mechanisms required for cell viability and genome stability [138, 147].

Cell cycle regulation of DSB signalling

A characteristic of eukaryotic systems is the CDK-dependent regulation of DSB resection. The discovery of the key role of CDK in regulating DSB resection, checkpoint activation and DNA repair was another major milestone in the understanding of DDR activation [24, 69]. It appeared that CDK control of DSB resection also modulates the balance between NHEJ and HR, ensuring that the cell engages in the most appropriate DSB repair pathway based on its position in the cell cycle [148]. It was first shown that DSB processing is less efficient in G1 phase where cells display a reduced rate and/or processivity of resection due to low CDK activity, in contrast to the robust DNA resection that is initiated in S/G2 phases when CDK activity is high [68, 69, 149]. Interestingly, although resection is less efficient in G1 phase, evidence (including IR-induced Rfa1, Ddc1 and Ddc2 foci formation) suggests that limited processing does occur following IR-induced DSB formation and is confined to regions close to the DNA ends [68, 69, 149]. This limited processing is likely to explain the Mec1-dependency of the G1 checkpoint. Over recent years, CDK targets involved in controlling DSB resection in both G1 and G2 phase have been identified in both yeast and vertebrates. In *S. cerevisiae,* Sae2 is a major controller of DSB resection in G2 cells and its CDK phosphorylation at a conserved CDK consensus site (S267) promotes DNA end resection [71]. The *sae2-S267A* mutant exhibits impaired generation of 3′ ssDNA and reduced HR-mediated DSB repair [71]. This CDK phosphorylation site is also conserved in CtIP (T847; analogous to S267 in *sc*Sae2) [71]. A second vertebrate-specific CDK site (S327) in CtIP is also phosphorylated in S and G2 phases [150, 151] and promotes the association of CtIP with the putative *sc*Rad9 orthologue BRCA1 [152]. The BRCA1-CtIP interaction facilitates BRCA1-mediated ubiquitination of CtIP, which promotes its association with chromatin following DNA damage [153]. Furthermore, interaction of BRCA1 with the MRN complex, with CtIP acting as a bridging factor, is also dependent on CDK activity [150]. Both interactions are important for maintaining the stability of the BRCA1-CtIP-MRN complex at sites of damage, promoting efficient DSB resection [150]. CDK-dependent regulation of DSB resection is highly conserved as mutation of T847 and S327 in CtIP produces a similar phenotype to that observed in *S. cerevisiae sae2* mutants [154, 155]. In addition to *sc*Sae2, Dna2 has also been recently identified as a direct CDK target [156]. CDK-dependent phosphorylation of Dna2 on several residues

(T4, S17 and S237) stimulates its role in DSB resection by promoting its nuclear localisation and recruitment to DSBs. The modest defect in resection observed in a *dna2-3A* mutant (T4, S17 and S237 mutated to alanine) is greatly augmented by additional deletion of Exo1 indicating that resection in *dna2-3A* cells is Exo1-dependent and that the Exo1 and Dna2/Sgs1 pathways cooperate to mediate efficient DSB resection [156].

The Ku proteins and the Rad9 mediator are two factors that negatively regulate DSB resection [53, 68, 157, 158]. In the absence of the Ku complex or the Rad9 mediator, resection and checkpoint activation observed in *yku* and *rad9* mutants are no longer CDK-dependent suggesting that the Ku and Rad9 proteins might be directly regulated by CDK to control DSB processing [53, 159]. In vertebrates, similar regulation of resection exists since the Ku complex has been shown to prevent HR by blocking the access to DSB [160]. Additionally, both ubiquitination and PARylation have been shown to be involved in the regulation of Ku retention at DSBs [160, 161]. The exact mode of regulation of resection by CDK remains to be elucidated and several interesting questions remain to be answered, notably how CDK phosphorylation affects Sae2/CtIP activity, how it potentially mitigates Ku inhibition of DSB resection and how it regulates the Rad9 mediator protein in preventing DSB resection.

DNA damage signal transduction

Processive resection coincides with the dissociation of Sae2, MRX and Tel1 from the DSB [15] and concomitant binding of RPA to the resultant 3′ ssDNA tails (Fig. 3d) [52]. The appearance of RPA-coated ssDNA provides increased loading sites for Mec1 [38, 158], with extensive resection of the DSB ends likely promoting the transition from Tel1/ATM-dependent to robust Mec1/ATR-dependent checkpoint signalling [80, 92]. Mec1 associates with Ddc2 (hATRIP) [162–164], which recognises and binds RPA-coated ssDNA thereby localising the Mec1-Ddc2/ATR-ATRIP complex to sites of damage [38, 165–168]. In addition, the 9-1-1 checkpoint clamp (*sc*Ddc1-Rad17-Mec3; human RAD9-RAD1-HUS1) and clamp loader (*sc*Rad24-Rfc2-5; human RAD17-RFC2-5) are recruited to RPA-coated ssDNA independently of the Mec1-Ddc2/ATR-ATRIP complex (Fig. 3e) and are required to initiate Mec1/ATR-dependent checkpoint signalling in both G1 and G2 phases [163, 166, 167, 169]. The 9-1-1 checkpoint clamp is structurally related to the replication clamp PCNA [170, 171], while the Rad24-Rfc2-5 complex is similar to the PCNA loader except that Rad24 replaces the Rfc1 subunit [172]. Studies have shown that the structure that facilitates Mec1 recruitment on ssDNA is a 5′ ssDNA/dsDNA junction since RPA restricts the loading of the 9-1-1 clamp by the Rad24-Rfc2-5 complex specifically on this type of junction [173–175]. Evidence suggests that both the presence of 5′ ss/dsDNA junctions and the amount of ssDNA control ATR checkpoint activation [176]. In G1 phase, slow accumulation of these types of structures could explain the requirement of the Mec1 signalling pathway in this phase of the cell cycle.

In *S. cerevisiae*, co-localisation of Mec1-Ddc2 and 9-1-1 complexes at sites of damage facilitates a stable association between Mec1-Ddc2 and Ddc1 [118, 163, 177]. The 9-1-1 complex promotes Mec1-dependent phosphorylation of its targets [162, 163, 178–180] and stimulates Mec1 kinase activity via a direct interaction with Ddc1 both in vitro and in vivo leading to activation of the G1 checkpoint [181]. Unlike *S. cerevisiae*, evidence demonstrating that the 9-1-1 clamp in vertebrates can similarly directly stimulate ATR kinase activity is lacking despite the fact that this would be structurally possible [182]. Rather, the 9-1-1 clamp functions to recruit TopBP1 (*sc*Dpb11) to sites of damage via an interaction between phosphorylated RAD9 (*sc*Ddc1) and TopBP1 [183, 184]. Casein kinase 2 (CK2) has been identified as a RAD9 kinase that could stimulate this interaction [185, 186]. This interaction facilitates the association of TopBP1 with ATRIP which stimulates ATR kinase activity [187, 188]. This mechanism of Mec1/ATR activation is also conserved in *S. cerevisiae* (Figs. 3e and 4) in G2/M phase, and is dependent on the interaction of the replication protein Dpb11 with Ddc1, which promotes Mec1 kinase activity [177, 189–191]. A recent study has identified two specific residues in the C-terminal tail of Dpb11 (W700 and Y735) as being required for activation of Mec1 in vitro, and these residues significantly contribute to activation of the DNA damage checkpoint in G2/M [181]. Subsequently, Mec1/ATR phosphorylates and activates Rad9 (Fig. 3e), a key adaptor protein in the DNA damage checkpoint [81, 192, 193].

Rad9 recruitment to sites of damage by histone-dependent and -independent pathways

In undamaged cells, hypophosphorylated Rad9 (so named as to distinguish the cell cycle modified forms from DNA damage-induced hyperphosphorylated Rad9 [81, 193]) exists as a large complex (≥850 kDa) containing the Ssa1 and/or Ssa2 chaperone proteins that promote the stability of the complex [194, 195]. It is proposed that Ssa1/Ssa2 facilitate Mec1-dependent remodelling of the Rad9 complex although this remains to be formally tested [194]. A small proportion of hypophosphorylated Rad9 is associated with chromatin in G1 and G2/M phases and it is suggested that this dynamic association could enhance the speed and efficiency of the Rad9-dependent DNA damage checkpoint

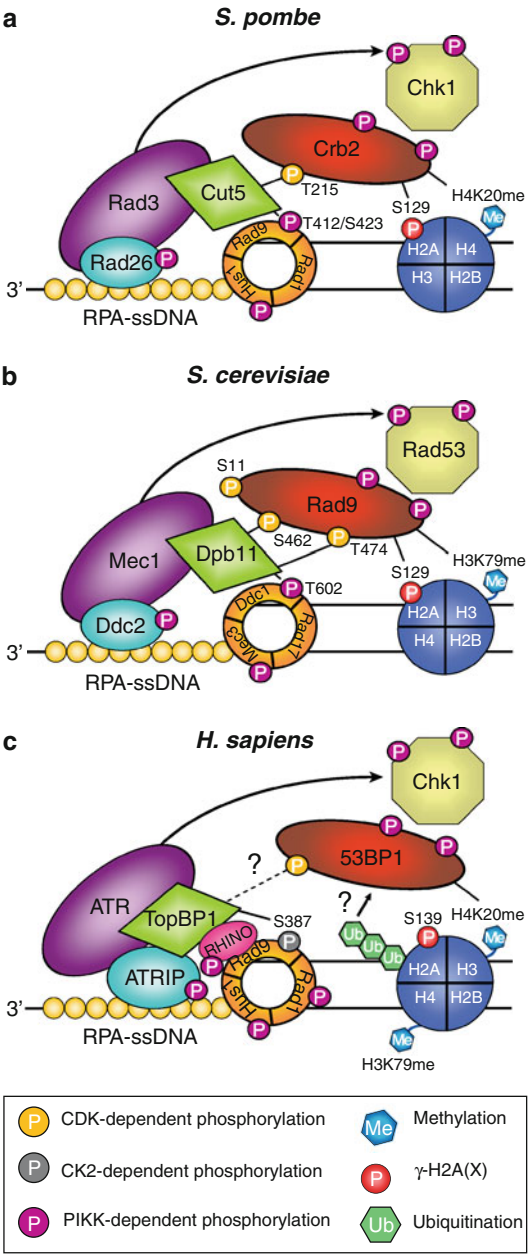

Fig. 4 Two parallel pathways operate to recruit *sp*Crb2 and *sc*Rad9 ▶ to sites of damage. Histone modification-dependent and -independent pathways promote the recruitment of *sp*Crb2 and *sc*Rad9 to sites of damage. **a** In *S. pombe*, Crb2 is recruited to chromatin via binding of its Tudor and BRCT domains to methylated H4K20 and γ-H2A, respectively. Crb2 is also recruited via an interaction between the phosphorylated T215 residue of Crb2 and the two N-terminal BRCT domains of Cut5. **b** Similar mechanisms exist in *S. cerevisiae*, where recruitment of Rad9 to chromatin requires binding of its Tudor and BRCT domains to methylated H3K79 and γ-H2A, respectively. Rad9 can also be recruited by an alternative pathway, which requires CDK-dependent phosphorylation of Rad9. Recruitment of Rad9 to sites of damage occurs through a direct interaction between the phosphorylated S462 and T474 residues of Rad9 and the two N-terminal BRCT domains of Dpb11. CDK-dependent phosphorylation of Rad9 S11 may indirectly promote the Dpb11-Rad9 interaction, however the exact mechanism by which this residue contributes remains to be determined. **c** A similar pathway may exist in vertebrates. 53BP1 is recruited to chromatin via binding of its Tudor domain to methylated H3K79 and/or H4K20. The first two BRCT domains of TopBP1 interact with the phosphorylated S387 residue of RAD9. Whether CDK-dependent phosphorylation of 53BP1 promotes its association with TopBP1 at sites of damage remains to be determined

response [108, 196]. Following DNA damage, two parallel pathways operate to recruit Rad9 to chromatin (Figs. 3e and 4); one is dependent on histone modifications (γ-H2A and H3K79 methylation) [108, 197, 198], while the other (which is important in G2 phase) is independent of histone modifications and instead requires an interaction between Ddc1 and Dpb11 [199].

In contrast to histone H2A, which is phosphorylated in a DNA damage-dependent manner, methylation of histone H3K79 (H3K79me) is constitutive [200] and is mediated by the conserved histone methyltransferase (HMT) Dot1 [200, 201]. H3K79me is buried in the nucleosome core [202] and is inaccessible until DNA damage-induced chromatin remodelling exposes the mark, providing a docking site for checkpoint proteins at the site of damage [116]. In *S. cerevisiae*, H3K79me is essential for checkpoint activation in both G1 and S phase; *dot1Δ* and *h3K79A* mutants are defective in G1 and intra-S phase checkpoint activation [197, 203, 204]. Furthermore, cells harbouring a mutated Rad9 Tudor domain, which mediates the interaction between Rad9 and H3K79me in vitro [197, 198], are G1 checkpoint-defective and exhibit minor sensitivity to various genotoxic treatments [197, 205]. Moreover, epistasis analyses revealed that the Rad9 Tudor domain and Dot1 operate in the same pathway to mediate resistance to DNA damage [197] and belong to the same epistasis group as H2A-S129 and the Rad9 BRCT domains with respect to checkpoint activation [107, 108]. Collectively, these studies support a model whereby, following DNA damage in G1 phase, Rad9 is recruited to chromatin via binding of its Tudor and BRCT domains to H3K79me and γ-H2A, respectively. This recruitment also occurs in the absence of DSB resection and can be induced by the Tel1 kinase [77, 80]. This is demonstrated by the fact that, in G1-arrested cells lacking Mec1, Rad53 is activated [80]. Therefore, in this condition, Tel1 is also able to activate Rad9 by mechanisms that may be similar to those described for Mec1 [206] but are currently unknown. In wild-type G1 cells, gradual DSB end processing/accumulation of Mec1-activating structures will eventually induce a switch from Tel1 to Mec1 signalling. The molecular events involved in Mec1 signalling in G1 phase are not well understood but they only seem to rely on the histone modification-dependent recruitment of Rad9. In contrast, loss of either histone modification does not significantly perturb G2/M checkpoint activation, indicating that an

alternative mechanism for Rad9 recruitment to sites of damage must operate in G2/M phase independently of H3K79me and γ-H2A [197, 203, 204].

Evidence for a parallel pathway operating in G2/M first came from studies in *S. pombe* where recruitment of Crb2 to sites of damage is mediated by two partially redundant mechanisms [207]. It is worth noting that H3K79me has not been reported in *S. pombe* [207], but effects on checkpoint activation and repair that resemble those of H3K79me in *S. cerevisiae* have been shown to be mediated by H4K20me [208]. Conversely, there is no evidence that H4K20me occurs in *S. cerevisiae* [209, 210] and strains in which H4K20 cannot be methylated are checkpoint proficient [197]. Analogous to that described for *sc*Rad9 above, one pathway for Crb2 recruitment is regulated by H4K20me and γ-H2A (Fig. 4a) and is dependent upon the Tudor and BRCT domains of Crb2 which bind H4K20me and γ-H2A, respectively [207, 208, 211–214]. The second pathway is independent of histone modifications and instead requires phosphorylation of a single CDK consensus site (T215) in the N-terminus of Crb2 [207]. Cells harbouring a Crb2 Tudor domain mutation or mutations abolishing either H4K20me or γ-H2A display synergistic genetic interactions with a *crb2-T215A* mutant, demonstrating that H4K20me, γ-H2A and the Tudor domain of Crb2 act cooperatively with phosphorylated Crb2-T215 to recruit Crb2 to sites of damage [207, 208, 215]. The model for Crb2 recruitment to sites of damage in the absence of histone modifications is as follows: Rad3-dependent phosphorylation of T412/S423 in the C-terminus of the Rad9 (*sc*Ddc1) subunit of the 9-1-1 complex promotes its interaction with the replication factor Cut5 (*sc*Dpb11; human TopBP1), which binds these phosphorylated residues via its two C-terminal BRCT domains [216]. The Rad9–Cut5 interaction promotes the recruitment of Crb2 to sites of damage through an interaction between the two N-terminal BRCT domains of Cut5 and phosphorylated T215 in the N-terminus of Crb2 [207, 217, 218]. This interaction facilitates the Rad3-dependent phosphorylation of Crb2 and subsequent activation of Chk1 [216, 218].

Recent studies in *S. cerevisiae* have described a similar histone modification-independent mechanism for the recruitment of Rad9 to sites of damage [191, 196, 199]. It relies on Mec1-dependent signalling that induces phosphorylation of T602 in the C-terminus of Ddc1 providing a docking site for Dpb11 (Fig. 4b), which binds phosphorylated T602 via its two C-terminal BRCT domains [199, 219]. However, a recent study has shown that Dpb11 is proficient for focus formation in zeocin-treated *mec1Δ tel1Δ sml1Δ* cells, suggesting that PIKK-dependent phosphorylation of Ddc1 is not absolutely required for the association of Dpb11 with the 9-1-1 complex [220]. Similarly, in *Xenopus*, TopBP1 (*sc*Dpb11) can be recruited to chromatin by a mechanism that is dependent on the 9-1-1 complex but independent of RAD9 (*sc*Ddc1) S387 phosphorylation [221]. It is likely that the Ddc1-Dpb11 interaction promotes the recruitment of Rad9 to sites of damage via a direct interaction between the N-terminal BRCT domains of Dpb11 and two recently identified CDK phosphorylation sites (S462 and T474) on Rad9 [191, 199]. CDK-dependent phosphorylation of Rad9 S462 and T474 creates a binding site for Dpb11 and the subsequent Dpb11-Rad9 interaction functions to recruit the Rad9 mediator to sites of damage [191]. Phosphorylation of a CDK consensus site in the N-terminus of Rad9 (S11) has also been implicated in promoting the Dpb11-Rad9 interaction albeit indirectly [196]. However, the exact mechanism by which Rad9 S11 phosphorylation contributes to this interaction remains to be determined. Once recruited to sites of damage, Mec1-dependent phosphorylation of Rad9 facilitates the recruitment of Rad53 and promotes its Mec1-dependent activation [192, 195, 206]. The Rad9 protein contains 20 putative sites for phosphorylation by CDK, 9 of which conform to the strict consensus sequence [(S/T)-P-X-(R/K)]. Mass spectrometric analyses identified 15 of these CDK sites to be phosphorylated in vivo [23, 222, 223]. While particular functions have been attributed to specific sites as discussed above, at present the functional roles of the remaining residues remain elusive. The complex CDK-dependent phosphorylation of Rad9 is likely to be very important for all its DDR functions, including its role in the inhibition of DSB resection.

Analogous to *sc*Rad9 and *sp*Crb2, mammalian 53BP1 functions as a molecular adaptor at sites of damage, facilitating IR-induced phosphorylation of a subset of ATM targets including CHK2, BRCA1 and SMC1 [114, 224–228]. 53BP1 is recruited to chromatin via binding of the 53BP1 Tudor domain to H4K20me and/or H3K79me [198, 211, 229], which could possibly only be recognised following prior RNF8-RNF168-UBC13-mediated polyubiquitination of histone H2A and H2AX [123–127]. The accumulation and stable retention of 53BP1 in ionising radiation-induced foci (IRIF) is dependent on γ-H2AX and its role in recruiting MDC1 [115, 119, 121, 230, 231]. Similar to the Rad9-Cut5 (*sc*Dpb11) interaction described above for *S. pombe*, the first two BRCT domains of TopBP1 (*sc*Dpb11) in vertebrates interact with a C-terminal phosphorylation site (S387) on RAD9 (*sc*Ddc1) which is constitutively phosphorylated during the cell cycle [183, 184], possibly by casein kinase 2 [185, 186]. In addition, a novel player in ATR-mediated checkpoint signalling, RHINO (Rad9, Rad1, Hus1 interacting nuclear orphan), has recently been identified [232]. Recruitment of RHINO to sites of damage is dependent on the 9-1-1 complex, and mutants that are unable to bind the 9-1-1 complex are checkpoint defective [232]. RHINO also independently associates with TopBP1 [232]. It is suggested

that the RHINO-TopBP1 interaction may function to promote TopBP1 recruitment to the 9-1-1 complex at sites of damage possibly through a mechanism that is independent of RAD9 S387 phosphorylation; however, this remains to be determined [232]. The TopBP1-RAD9 interaction (Fig. 4c) promotes the TopBP1-dependent activation of ATR, which is mediated by the ATR activation domain (AAD) of TopBP1, facilitating the ATR-dependent activation of CHK1 [187, 188]. TopBP1 colocalises with 53BP1 following IR-induced DNA damage and interacts with 53BP1 in vitro [233, 234]. Similar to *sc*Rad9, the 53BP1 protein, like BRCA1 and MDC1, is regulated by CDK activity during a normal cell cycle and several of its CDK consensus sites have been shown to be phosphorylated in vivo [235–247]. 53BP1 contains 42 putative CDK phosphorylation sites and mass spectrometric analysis identified 27 of these sites to be phosphorylated in vivo [235–242]. Furthermore, 5 of these sites (S294, S380, S552, S1028, S1114) are phosphorylated during an unperturbed cell cycle [236]. Although there is clear functional similarity between 53BP1 and its yeast orthologue, it remains to be determined whether CDK-dependent phosphorylation of 53BP1 promotes its association with TopBP1 leading to efficient DNA damage checkpoint activation.

Activation of Rad53 and Chk1 effector kinases

Once recruited to sites of damage, *sc*Rad9 is hyperphosphorylated in a PIKK-dependent manner [81, 193] which promotes remodelling of the large hypophosphorylated complex into a smaller 560-kDa complex comprising hyperphosphorylated Rad9, Ssa1/2 and Rad53 [81, 193–195] (Fig. 3e). Hyperphosphorylated Rad9 acts as a molecular adaptor/scaffold (Fig. 3f) to facilitate amplification of the checkpoint signal [195, 206], and its molecular role in Mec1-dependent Rad53 activation, partially reconstituted in vitro [206], is the best understood. Phosphorylation of several SQ/TQ sites on Rad9 provides a docking site for Rad53, which binds these phosphorylated residues via its two FHA domains [192, 206, 248–250]. Rad9 functions as a molecular adaptor (Fig. 3f) to bring Rad53 into close proximity to Mec1 at sites of damage to facilitate the Mec1-dependent phosphorylation of Rad53 [206]. Rad9 also functions as a molecular scaffold to bring Rad53 molecules into close proximity, thus catalysing Rad53 *in trans* autophosphorylation [195]. Vertebrate CHK2 is also known to dimerize and trans-autophosphorylate in an ATM-dependent manner, but a molecular role for DNA damage mediators in this activation remains to be investigated [251]. Fully activated Rad53 is then released from the hyperphosphorylated Rad9 complex in an ATP-dependent manner [195]. As well as mediating the interaction between Rad9 and Rad53, phosphorylation of the Rad9 SQ/TQ cluster domain (SCD) promotes Rad9 oligomerisation at sites of damage via an interaction between the BRCT domains of Rad9 and the phosphorylated SCD [252]. Although Rad9 oligomerisation appears to be dispensable for initial Rad53 activation, it is required for the maintenance of Rad53 activation and checkpoint-induced cell cycle arrest [252]. Rad9 oligomerisation promotes its accumulation at sites of damage, allowing amplification of the DNA damage signal and sustained activation of Rad53 [252]. It is also proposed that a negative feedback loop exists in which fully activated Rad53 phosphorylates the Rad9 tandem BRCT domains to attenuate the BRCT-SCD interaction, mediating the turnover of hyperphosphorylated Rad9 by promoting its dissociation from sites of damage and subsequently dampening Rad53 activity [252].

Rad9 also facilitates the DNA damage-induced phosphorylation of the parallel downstream kinase Chk1 (Fig. 3f), where Mec1-dependent Chk1 activation requires the Chk1 activation domain (CAD) of Rad9 [44, 253]. Mechanistic activation of Chk1 by Rad9 remains a mystery to date since the physical interaction between Rad9 and Chk1 demonstrated by yeast two-hybrid analyses [44, 254] has never been corroborated by other biochemical assays. Rad9-dependent activation of Chk1 may occur in a manner analogous to Rad9′s role as a molecular adaptor in the activation of Rad53: Chk1 could bind to the Rad9 CAD domain, promoting its recruitment to sites of damage and subsequent Mec1-dependent phosphorylation. Additionally, the existence of budding yeast *chk1* mutants that can be activated in a Rad9-independent manner [255] suggests that Rad9 might facilitate the conformational change shown to be required for Chk1 activation [256]. Once activated, Rad53 and Chk1 phosphorylate several downstream targets that are involved in cell cycle control and transcriptional regulation.

In contrast to *S. cerevisiae* where Rad53 (CHK2 in humans) is the principal effector kinase, CHK1 is the primary effector of both the DNA damage and replication checkpoints in vertebrates, with CHK2 playing a subsidiary role [43]. CHK1 activation is mediated by ATR and the adaptor protein Claspin (*sc*Mrc1) and occurs primarily in response to replication stress and UV-induced DNA damage [251]. In contrast to vertebrates, *sc*Mrc1 regulates activation of Rad53 (the main effector kinase in *S. cerevisiae*) in response to replication stress [257]. Chk1 only becomes activated under these conditions when Mrc1 is absent and the replication stress signal is converted into a DNA damage signal [257, 258], leading to Rad9-mediated activation of both Rad53 and Chk1 [257]. It is proposed that Mrc1 mediates the Mec1-dependent phosphorylation of Rad53 by acting as a molecular adaptor in a manner analogous to the role of Rad9 in responding to DNA damage [259], as Mrc1 physically interacts with Rad53

following MMS-induced DNA damage [260, 261]. In vertebrates, ATR-dependent phosphorylation of S317 and S345 on CHK1 reportedly promotes CHK1 activation by inducing a conformational change that relieves the inhibition of the N-terminal kinase domain by the C-terminal regulatory domain [262–265] and stimulates the release of CHK1 from chromatin [266, 267]. CHK1 activation is also known to be dependent on 53BP1, BRCA1 and MDC1 [43]. Whether this dependency relies on direct molecular mechanisms, rather than the role of the Rad9-family DNA damage mediators in the focal organisation of DDR proteins essential for checkpoint maintenance, is currently unknown. CHK1 phosphorylates several downstream targets, including Cdc25A, Cdc25C and Wee1, which are key regulators of the cell cycle [268–270]. The recent identification of novel putative CHK1 substrates involved not only in the DDR (e.g. KU70, FEN1 and RIF1) but also in other cellular processes including RNA metabolism [271] will provide key insights into the physiological functions of CHK1.

Targets of the DNA damage checkpoints

The G1 checkpoint

The G1 checkpoint induces cell cycle arrest at or prior to START, before cells become irreversibly committed to the next cell cycle (Fig. 1). Rad53 directly phosphorylates the Swi6 regulatory subunit of the SBF [Swi4/6-dependent cell cycle box (SCB) binding factor] transcription factor, which inhibits transcription of the G1/S cyclins (Cln1 and Cln2) [272, 273]. Furthermore, Rad53-dependent checkpoint signalling promotes the cytoplasmic accumulation of the Los1 tRNA export factor, leading to activation of the Gcn4 transcription factor, which contributes to the G1 checkpoint by delaying the accumulation of Cln2 [274]. This reduced level of G1/S cyclin activity prevents the destruction of the B-type cyclin inhibitor Sic1, thus inhibiting progression through the G1/S transition [275, 276]. DNA damage-dependent phosphorylation of Chk1 in G1-arrested cells [79] suggests that it also participates in the G1 checkpoint. However, the mechanistic role of Chk1 in the G1 checkpoint has not been explored.

In vertebrates, the G1 checkpoint is very robust and a two-wave model of checkpoint activation has been proposed [277]. The first response requires ATM-dependent phosphorylation of CHK2 on T68 which facilitates CHK2 homodimerization and *in trans* autophosphorylation [278–280]. Activated CHK2 phosphorylates the Cdc25A phosphatase targeting it for proteosomal degradation [270, 281, 282]. Cdc25A functions to remove inhibitory phosphorylation on T14/Y15 residues of CDK2 [16], and thus loss of Cdc25A activity prevents dephosphorylation and activation of the CDK2-cyclinE kinase complex which is required for S phase entry [282, 283]. A second response is dependent upon ATM- and CHK2-mediated phosphorylation of the tumour suppressor protein p53 [284–287], which reduces binding of the negative regulator MDM2 to p53 and stimulates p53 activation [285, 286, 288–290]. In addition, phosphorylation of the p53 regulator MDMX by ATM and CHK2 promotes its MDM2-mediated ubiquitination and degradation [291]. Together, these events lead to the stabilization and nuclear accumulation of p53 [292]. p53 promotes the transcriptional induction of the CDK inhibitor p21, which inhibits CDK2-cyclinE activity [293, 294]. Although the p53-dependent response is slower, it is proposed that it functions to maintain the G1 arrest initiated by the Cdc25A pathway [277].

The intra-S phase checkpoint

Two checkpoints operate during S phase to signal the presence of DNA damage (intra-S) or replication stress (replication checkpoint) [295]. Although genetically separable, these checkpoints partially overlap as many checkpoint proteins function in both pathways due to the occurrence of similar or common DNA structures [296]. Faithful replication of the genome is paramount to the maintenance of genomic stability, thus it is not surprising that the multiple checkpoint and repair pathways that act in S phase display considerable complexity and redundancy [297]. Recently, it has been shown that two independent pathways function in the activation of Mec1 in response to replication stress [298]. One pathway is dependent on the 9-1-1 complex and the other requires the leading strand polymerase DNA polymerase epsilon (pol ε) and the replication factors Dpb11 and Sld2 [298]. It is proposed that the 9-1-1 complex, which loads specifically onto 5′ junctions [174, 175], signals replication stress on the lagging strand, while pol ε and the leading strand factors Dpb11 and Sld2 detect replication perturbations on the leading strand, with both pathways independently leading to Mec1 activation [298]. In *S. cerevisiae*, checkpoint activation in S phase fully depends on the Mec1 and Rad53 kinases [32]. Mec1-dependent activation of Rad53 in response to replication stress or DNA damage is mediated by the adaptor proteins Mrc1 [257] and Rad9 [299], respectively. The critical role of Rad53 and Mec1 in promoting cell viability following DNA damage is to stabilise DNA replication forks [300–302]. In addition, Mec1 and Rad53 activation inhibits firing of late replication origins when replication from already fired origins is stalled due to DNA damage [303, 304], and prevents mitotic entry until DNA replication is complete [305].

The rate of S phase progression is decreased in response to DNA damage partly by regulating the phosphorylation

status of RPA [180, 306, 307] and inhibiting DNA polymerase α-primase (pol α-primase) activity [308, 309]. DNA pol α-primase is required for both G1 and S-phase checkpoints, where activated Rad53 inhibits its phosphorylation, thus preventing initiation of DNA synthesis downstream of the lesion [308, 309]. DNA polymerase ε is also required for activation of the S-phase checkpoint. It acts as a sensor of DNA replication and links the DNA replication machinery to the checkpoint [298, 310, 311].

Rad53 also targets components of the CDK- and DDK (Dbf4-dependent kinase)-dependent pathways which are required for the firing of early and late origins of replication [312]. The recent discovery that the replication initiation protein Sld3 and the Cdc7 kinase regulatory subunit Dbf4 are Rad53 substrates and are phosphorylated in a Rad53-dependent manner in response to DNA damage was a significant advancement in our understanding of the molecular mechanisms governing the DDR in S phase [313, 314]. Rad53 directly phosphorylates Sld3 in vitro indicating that it is a direct target of Rad53 in vivo. Rad53-dependent phosphorylation of Sld3, which has been already phosphorylated by Clb5/6-Cdk1 [315, 316], prevents its interaction with the Dpb11 and Cdc45 replication proteins which are both required for the initiation of DNA replication, thus preventing late origin firing [313, 314]. Inhibiting downstream components of the CDK-dependent pathway, and not targeting CDK complexes directly, ensures that CDK activity remains high in S phase in the presence of DNA damage, which is crucial to prevent the re-licensing of early firing origins [313, 314]. Rad53-mediated phosphorylation of Dbf4 also blocks late origin firing, yet how this phosphorylation event inhibits DDK activity remains to be elucidated [313, 314].

Another recently identified target of the intra-S phase checkpoint is Exo1 [317, 318], which is recruited to stalled replication forks [319]. Deletion of *EXO1* almost completely suppresses the sensitivity of *rad53Δ* cells to a range of DNA-damaging agents, suggesting that Exo1 might be a primary target of Rad53 [317]. Indeed, Exo1 is phosphorylated in a Rad53-dependent manner in response to MMS treatment [318, 320]. These data suggest that Rad53-dependent phosphorylation of Exo1 inhibits Exo1-dependent resection events at the fork and subsequent replication fork breakdown by limiting ssDNA accumulation and DNA damage checkpoint activation [317, 318]. Furthermore, in the absence of Rad53, Chk1 also plays a role in the stabilisation of replication forks. This function is independent of its role in inhibiting anaphase entry and only occurs in the absence of Exo1 [317].

Rad53 also targets transcription via Dun1, a protein kinase required for the transcriptional induction of several DNA damage-inducible genes and genes encoding ribonucleotide reductase (RNR) subunits involved in the modulation of dNTP pools [321–323]. Dun1 induces *RNR* gene transcription by phosphorylating and inhibiting the repressor Crt1, promoting its dissociation from the promoter region of *RNR2*, *RNR3* and *RNR4* [324]. Dun1 also phosphorylates the RNR inhibitor Sml1 and targets it for degradation [325]. Furthermore, Dun1 directly phosphorylates Dif1, a protein involved in regulating the nuclear import of the Rnr2-Rnr4 subunits [326]. Dun1-dependent phosphorylation of Dif1 promotes its degradation, allowing Rnr2-Rnr4 to relocalise to the cytoplasm where it binds Rnr1 to form an active complex [326].

In vertebrates, ATR is the primary PIKK activated in response to replication perturbations, with ATM playing a minor role in response to DSBs [327]. The intra-S phase checkpoint functions to suppress origin firing and stabilise stalled replication forks to maintain replication fork integrity [328–331]. ATR-dependent phosphorylation of CHK1 is dependent on the mediator proteins TopBP1 and Claspin. As discussed above, TopBP1 is recruited to sites of damage by the 9-1-1 complex, where it functions to stimulate ATR kinase activity [183, 184, 187, 188]. The replication fork-associated protein Claspin functions to recruit CHK1 to stalled replication forks, facilitating ATR-dependent phosphorylation and activation of CHK1 [332–335]. ATR-CHK1 signalling in response to replication stress also requires the mediator proteins Timeless and Tipin (timeless-interacting protein), and loss of either protein impairs intra-S phase checkpoint activation [336–338]. Tipin binds RPA-coated ssDNA [336, 337] and it is thought that Timeless-Tipin may function to recruit Claspin and CHK1 to stalled replication forks [337, 339]. These interactions likely serve to increase the local concentration of CHK1 at sites of damage, facilitating efficient ATR-mediated phosphorylation of CHK1. Activated CHK1 dissociates from chromatin to phosphorylate its substrates [266], including the CDC25 phosphatases [340, 341]. In response to replication perturbations, the ATM/ATR-CHK2/CHK1-Cdc25A-CDK2 pathway prevents the initiation of DNA replication by inhibiting loading of the replication initiation factor Cdc45 onto replication origins [268, 282, 342]. Furthermore, ATR-CHK1-mediated signalling inhibits an interaction between Cdc45 and the Mcm7 subunit of the MCM helicase complex at origins of replication via a CDK2-independent mechanism [343]. Additionally, a distinct pathway dependent on ATM, NBS1, BRCA1 and SMC1 mediates the ATM-dependent phosphorylation of the SMC1 and SMC3 subunits of the cohesin complex [344–347], which promotes chromosomal repair and enhances cell survival [346, 347]. The exact mechanism by which these phosphorylation events contribute to intra-S phase checkpoint activation remains to be elucidated, although it is proposed that they may function to modulate the rate of DNA synthesis or regulate recombinational repair following DNA damage [345–347].

The G2/M checkpoint

DNA damage-induced G2 arrest is the most prominent checkpoint response in most eukaryotes, where entry into mitosis is prevented via inhibition of CDK activity. In *S. pombe* and vertebrates, CDK activity is regulated by inhibitory phosphorylation of a conserved tyrosine residue (Y15) of the *sp*Cdc2/human CDK1 kinase complexes [348–352]. Additional inhibitory phosphorylation can also occur on the adjacent threonine residue (T14) [348, 349, 353]. The phosphorylation status of these residues is reciprocally regulated by the Wee1 family of kinases (*sc*Swe1; *sp*Wee1 and Mik1; human Wee1 and Myt1) and the Cdc25 family of phosphatases (*sc*Mih1; *sp*Cdc25; human Cdc25A, Cdc25B, Cdc25C) [354]. In *S. pombe*, Chk1 inhibits Cdc2 activity by targeting both Wee1 and Cdc25 [355]. Chk1-dependent phosphorylation of Wee1 stabilises the protein, ensuring phosphorylation of Cdc2 on Y15 is maintained [355–357], while phosphorylation of Cdc25 by Chk1 promotes the association of Cdc25 with the 14-3-3 protein Rad24, which sequesters Cdc25 in the cytoplasm, thus preventing the dephosphorylation and activation of Cdc2 [358–360]. Either of these mechanisms are sufficient to elicit an efficient G2 DNA damage checkpoint response, preventing entry into mitosis until DNA repair is complete [355]. A similar mechanism exists in mammalian cells [7].

In *S. cerevisiae*, however, the DNA damage checkpoint does not control cell cycle progression by inhibitory phosphorylation of the equivalent tyrosine residue (Y19) of Cdk1 [361, 362]. Instead, this checkpoint induces a mitotic arrest by inhibiting the metaphase to anaphase transition [363]. Both Rad53 and Chk1 function in the G2/M checkpoint response to inhibit anaphase entry and mitotic exit in the presence of DNA damage [44, 364, 365]. This is accomplished, at least in part, by inhibiting the degradation of Pds1 [366]. Chk1-dependent phosphorylation of Pds1 prevents its degradation by the APC^{Cdc20} complex, thus inhibiting sister chromatid separation and preventing anaphase entry [44, 367, 368]. Rad53 also contributes to the stability of Pds1 by inhibiting the interaction between Pds1 and Cdc20 [368]. In addition to inhibiting mitotic entry, a parallel pathway dependent on the Rad53 kinase acts to prevent mitotic exit by maintaining high levels of mitotic CDK activity [44]. The Cdc5 polo-like kinase is a component of the mitotic exit network (MEN), which initiates mitotic exit by promoting the destruction of the mitotic cyclins [369, 370]. Recent major findings identified Rad53 as a Cdc5 substrate that is phosphorylated in a Cdc5-dependent manner during an unperturbed cell cycle [371–373]. Although the exact mechanism by which Cdc5 promotes checkpoint inactivation by targeting Rad53 is currently unknown, recent findings support a model in which Cdc5-dependent phosphorylation of Rad53 inhibits the ability of the Rad9 mediator to promote Rad53 autophosphorylation [372]. Another study suggests that Cdc5- and Cdk1-dependent Rad53 phosphorylation in G2/M may function to lower the threshold required for DNA damage checkpoint activation, contributing to the robustness and maintenance of the checkpoint [371]. Following checkpoint activation, Cdc5 is also phosphorylated in a Rad53-dependent manner [374], leading to Cdc5 inactivation and prevention of mitotic exit [44]. Rad53 also suppress the MEN by preventing the release of the Cdc14 phosphatase from the nucleolus in a Cdc5-independent manner [365]. Taken together, these studies demonstrate that *RAD53* and *CHK1* function in parallel, partially redundant, pathways that co-operate to ensure efficient G2/M checkpoint arrest.

Conclusions and future perspectives

Our knowledge of the complex regulatory mechanisms governing DNA damage checkpoint activation has advanced significantly over recent years. However, our understanding of these intricate pathways is still far from complete. Recent research has uncovered several elegant regulatory mechanisms that modulate divergent aspects of the DDR at different levels, in particular the early steps of checkpoint activation. CDK has fast emerged as a key post-translational regulator of the DDR [68–71, 118, 191, 375, 376]. Accumulating evidence suggests an expanding network of kinases and substrates being identified as novel players in the DDR. This further adds to the complexity of these signalling networks, especially in vertebrates where DDR signalling and cross-talk between proteins is more complex [46]. Recent phosphoproteomic screens have significantly impacted our understanding of how phosphorylation mediates DDR events, resulting in a major expansion of the kinase network through the identification of novel damage-induced phosphorylation events induced by non DDR protein kinases [377, 378]. Understanding the functional cross-talk between signalling proteins and pathways, also conserved in yeast, is a critical area of research that will yield important insights into how complex mechanisms governing the DDR are regulated.

Additionally to phosphorylation and other PTMs, microRNAs (miRNAs) and RNA-binding proteins (RBPs) represent an exciting post-transcriptional level of regulation for the DDR [379, 380]. miRNAs are often deregulated in cancer [381], and siRNA-mediated knockdown of Dicer (essential component in miRNA biogenesis) increases cellular sensitivity to cisplatin and UV, as well as perturbed cell cycle control following UV-induced DNA damage [382, 383]. It is likely that miRNAs and RBPs orchestrate the expression of several protein clusters

involved in cell cycle control and the DDR (recent evidence implicates miR-182 in the regulation of BRCA1 function [384]). Identification of novel players involved in miRNA-mediated gene regulation will be crucial to our understanding of the DDR and the potential role miRNAs play in cancer development and progression. Understanding the context-dependent regulation of DNA damage checkpoint activation at the molecular level will provide further insight into how defects in DNA damage signalling pathways can drive tumourigenesis. Ultimately, this knowledge will facilitate the development of more efficacious and targeted cancer therapies and will improve our knowledge of cancer initiation and progression.

Another emerging direction of research is the identification of novel functions for checkpoint proteins and their involvement in controlling nuclear dynamics in response to DNA damage. It was already shown that 53BP1 is a modulator of chromatin dynamics and plays an important role in uncapped telomere rejoining [385], long-range V(D)J recombination [386] and the repair of lesions induced within regions of heterochromatin [387]. More recently, it has been shown that the E3 ubiquitin ligase activity of the BRCA1 tumour suppressor protein has a novel role, key for genomic stability, in maintaining centromeric sites on chromosomes in a structurally condensed state known as pericentric heterochromatin [388]. The exact mechanism underlying these novel functions represents an important area for future investigation. The molecular mechanisms by which the putative *sc*Rad9 vertebrate orthologues mediate DSB signalling in this context are only beginning to emerge, and further studies should lead to a greater understanding of the role of nuclear dynamics in the DDR and the maintenance of genomic integrity.

Interestingly, and more specifically relevant to vertebrate cells, it has recently been discovered that several proteins required for the maintenance of genome stability also play key roles in other cellular contexts. For example, p53 and histone H2AX have been implicated in the regulation of developmental potency [389–393] and embryonic stem (ES) cell proliferation [394]. The observation that $GABA_A$ receptor activation can induce γ-H2AX foci in undamaged cells provides a potential paradigm shift across many areas of research: in addition to demonstrating a novel mechanism for regulating ES cell proliferation, the authors have shown that proteins involved in the DDR can be activated in contexts outside the remit of DNA damage [394]. It will be important to elucidate the molecular mechanisms that link GABA signalling to PIKK activation. It is also tempting to speculate that other key players in the DDR may be activated by additional receptor–ligand signalling loops and that checkpoint proteins likely have cellular roles outside the context of DNA damage. However, it will be important to explore these novel activation mechanisms in the context of genome instability. Ultimately, a comprehensive understanding of the cellular and molecular response to DNA damage will greatly impact our knowledge of cell biology in a wide variety of contexts, ranging from development to disease.

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Conflict of interest All co-authors have seen and agree with the contents of the manuscript. The authors certify that the submission is original work and is not under review at any other publication. The authors declare no conflict of interest.

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