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Checkpoint Responses to DNA Double-Strand Breaks

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Abstract

Cells confront DNA damage in every cell cycle. Among the most deleterious types of DNA damage are DNA double-strand breaks (DSBs), which can cause cell lethality if unrepaired or cancers if improperly repaired. In response to DNA DSBs, cells activate a complex DNA damage checkpoint (DDC) response that arrests the cell cycle, reprograms gene expression, and mobilizes DNA repair factors to prevent the inheritance of unrepaired and broken chromosomes. Here we examine the DDC, induced by DNA DSBs, in the budding yeast model system and in mammals.

Contents

1. INTRODUCTION

DNA damage is a ubiquitous fact of life. Human cells encounter up to 100,000 instances of DNA damage per day (1), caused by a range of endogenous and exogenous insults. Even unperturbed DNA replication is intrinsically genotoxic and a major source of mutations and chromosome breakage (2). Failure to respond to DNA damage has dire consequences and can result in mutations, gross chromosomal rearrangements, and/or aneuploidy, which lead to disease, loss of fitness, and death. In eukaryotes, cellular responses to most types of DNA damage involve a signaling transduction pathway, referred to as the DNA damage checkpoint (DDC), which is responsible for sensing DNA damage and coordinating DNA repair transactions with the cell cycle and other key cellular processes.

1.1. Types of DNA Damage

Chromosomes are subjected to different forms of DNA damage, and the type of damage incurred dictates the subsequent repair mechanism. When damage is limited to one strand, the lesion is often repaired by excision of the damaged base followed by DNA synthesis using the opposite strand as a template. These single-stranded forms of DNA damage—chemical base modifications or misincorporation of ribonucleotides—are frequent and are efficiently corrected by the cell using either base excision repair or nucleotide excision repair mechanisms (reviewed in 3, 4). However, photodimers and intra- and interstrand cross-links, among other obstacles, can block replication and may result in double-strand breaks (DSBs) (5) (**Figure 1**). Moreover, single-strand nicks can be converted into one-ended DSBs upon replication fork passage. DSBs can arise directly from many additional sources, including ionizing radiation, the excision of transposable elements, failures of type II topoisomerases, and the action of site-specific endonucleases. Vertebrate cells suffer a dozen or more chromatid breaks every replication cycle. This is evident from the high frequency

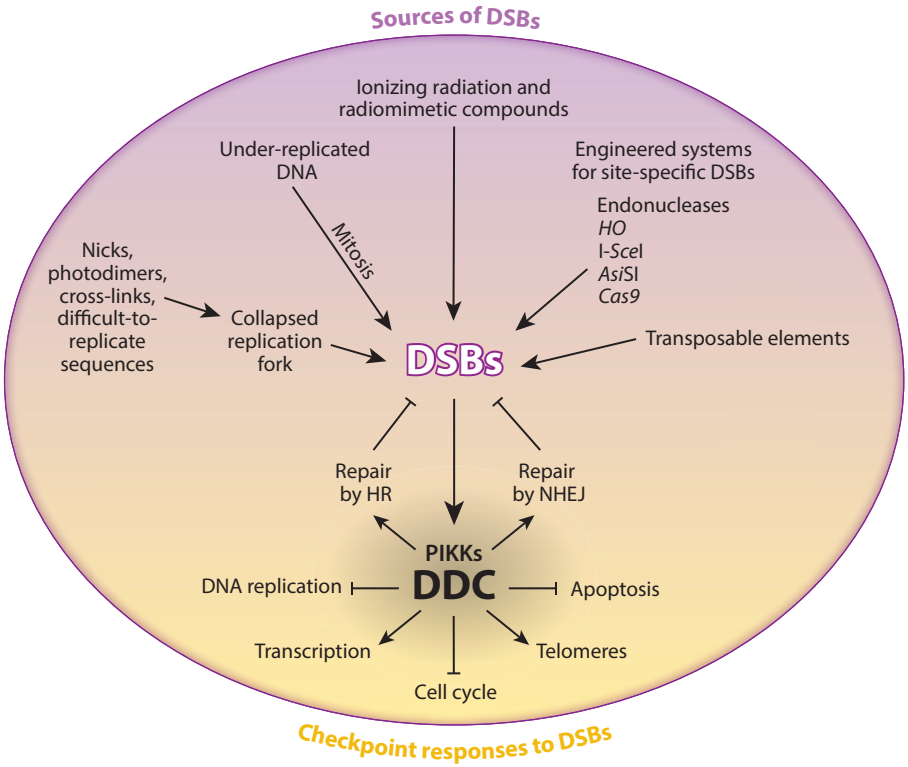

Figure 1

Sources of DSBs and the responses mediated by DDC kinases. DSBs originating from endogenous or exogenous sources trigger the activation of DDC kinases that coordinate an intricate cellular response that includes cell-cycle arrest and the mobilization of DSB-repair pathways. Abbreviations: DDC, DNA damage checkpoint; DSB, double-strand break; HR, homologous recombination; NHEJ, nonhomologous end joining; PIKK, phosphoinositide 3-kinase-related kinase.

of chromatid breaks when the key Rad51 repair protein is depleted from chicken DT40 cells (6) and from the frequency of sister-chromatid exchanges in humans when the BLM helicase is absent (7). Unlike single-stranded DNA damage, a DSB cannot be repaired by using the complementary strand as a template and, instead, one of two repair mechanisms are employed; nonhomologous end joining (NHEJ) or homologous recombination (HR) (8). Site-specific endonucleases, which make DSBs at specific locations within the genome, have been the workhorses used to detail the mechanisms behind HR and NHEJ.

1.2. Site-Specific Endonucleases as Tools to Study Responses to DSBs

Our understanding of the DNA damage response has also greatly been aided by site-specific endonucleases. The budding yeast *Saccharomyces cerevisiae* possesses two endogenous endonucleases, HO and I-*Sce*I, that have been the mainstay of experiments creating site-specific DSBs. I-*Sce*I recognizes an 18-bp sequence and triggers a gene-drive event in mitochondria to spread an intron encoding the endonuclease (9). The creation of a synthetic I-*Sce*I gene compatible with nuclear gene expression and mRNA translation has led to its use in many organisms, including mammals. The nuclear-encoded *HO* gene controls mating type switching in which a DSB in the *MAT* locus

is repaired by HR using a donor sequence that introduces sequences of the opposite mating type (reviewed in 10). HO requires a 24-bp sequence that can be moved to other locations to study various types of DSB repair, including NHEJ, microhomology-mediated end joining (MMEJ), and various forms of HR, including single-strand annealing (SSA), gene conversions with and without an accompanying crossover and break-induced replication (reviewed in 11, 12). Creation of an inducible *HO* gene fused to a galactose-inducible promoter makes it possible to induce synchronous cleavage of one or several chromosomal sites in less than 60 min. A special advantage of HO is that it is rapidly degraded, allowing the damage to be inflicted transiently (13). Induction of I-*Sce*I is less efficient and the protein is not rapidly degraded. Recently, CRISPR-Cas9-mediated DSBs have also been employed in yeast (14).

Site-specific endonucleases including I-*Sce*I have also been used to study both repair and DNA damage responses in mammals. *Asi*SI, which cleaves approximately 100 sites in the mammalian genome, has proven to be particularly useful in distinguishing roles of different DNA damage-associated kinases, including ataxia-telangiectasia-mutated (ATM) and DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) (15). The attachment of an auxin-induced degron to *Asi*SI has also allowed the rapid depletion of the nuclease after cleavage (16). The advent of Cas9-directed cleavage has stimulated an explosion of papers concerned with gene editing by both NHEJ and HR mechanisms (reviewed by 17). Cas9 has also been used to demonstrate that even a small number of such DSBs are sufficient to retard cell-cycle progression, by activation of DNA damage signaling (18).

1.3. Repair of DSBs

We refer the reader to several recent reviews that explore DSB repair in detail (8, 11, 12, 19, 20). Here, we confine our introduction to identifying key processes that are implicated in the generation and regulation of the DDC.

The core machinery in both NHEJ and HR is evolutionarily conserved, although mammalian repair processes are overlaid with several proteins absent in budding yeast (e.g., DNA-PKcs in NHEJ and BRCA1 and BRCA2—among others—in HR). In G1 cells, DSBs are predominantly repaired by NHEJ, because the 5′ to 3′ resection of DSB ends that is required for HR and MMEJ is inhibited. DSB ends are recognized by both the MRE11-RAD50-NBS1 complex [Mre11-Rad50-Xrs2 (MRX) in budding yeast], which recruits the ATM (yeast Tel1) kinase, and KU70-KU80 (hereafter Ku) proteins. Ku binding recruits DNA ligase 4 and its associated XRCC4 (yeast Lif1) and XLF (yeast Nej1) proteins to the break site to effect NHEJ. NHEJ continues to function throughout the cell cycle, but once 5′ to 3′ resection is initiated in cells that have entered S phase, HR is enabled, principally to repair replication-induced breaks by recombination with a sister chromatid, but also between homologous chromosomes or with a homologous sequence located at an ectopic site. 5′ to 3′ resection leads to 3′-ended single-strand DNA (ssDNA) that is initially coated by the three-member ssDNA-binding protein complex replication protein A (RPA). The activation of ATM and Rad3-related (ATR; budding yeast Mec1) depends on the binding of its obligate heterodimer partner, ATR-interacting protein (ATRIP; budding yeast Ddc2), to RPA. Rad51 displaces RPA on ssDNA then initiates strand exchange between the broken end(s) and a homologous donor sequence. In budding yeast, Rad51 is loaded on to the DNA by the mediator Rad52 and a set of Rad51 paralogs. In mammals the principal loader of RAD51 is BRCA2, along with a set of RAD51 paralogs.

2. THE DNA DAMAGE CHECKPOINT (DDC)

The first mutants found to be defective in mitotic delay following UV damage were uncovered in the fission yeast *Schizosaccharomyces pombe* by Hannan et al. (21). Painter & Young (22) later

reported that cell lines derived from ataxia-telangiectasia patients underwent DNA synthesis after X-radiation, whereas healthy cells did not. Weinert & Hartwell (23) began dissecting the DDC in *S. cerevisiae* using a UV-sensitive mutation of *RAD9* that failed to arrest in the G2/M phase of the cell cycle before completing DNA repair. Subsequent work by many investigators over the following three decades has revealed the general mechanism of checkpoint activation, maintenance, and deactivation. In this review, we focus on studies in budding yeast and in mammals. Important contributions have also been made using fission yeast, especially in the study of checkpoint responses to DNA replication stress (24), which is beyond the scope of this review.

2.1. The Cell Cycle and the DDC

In budding yeast, the DDC can be activated in three phases of the cell cycle. DNA damage incurred in G1 activates a transient DNA damage response that temporarily delays S-phase onset, providing extra time for DNA repair before replication (25, 26). Damage that arises during S phase slows replication and triggers a coordinated effort between replication fork and DNA repair machinery to resolve the damage (27). DNA lesions still present after completion of DNA replication activate the G2/M checkpoint, which stalls cell division until the damage is repaired (23). If repair is successful, the DDC is extinguished, and cells proceed through mitosis in a process known as recovery (28). However, sustained DNA damage does not prevent cell division indefinitely, as both budding yeast and metazoans will deactivate the checkpoint and proceed through cell division without repairing DNA, a process called adaptation (29, 30).

DDC in yeast is quite sensitive, with a single DSB being capable of activating the DDC and evoking a robust G2/M arrest (31). In mammalian cells, a few DSBs (1–4 breaks) can mildly activate the DDC and result in only minor delays in cell-cycle progression (18). As Harrigan et al. (32) and Lukas et al. (33) have documented, mammalian cells can often carry DNA lesions induced by replication stress into the following G1 cycle, likely reflecting the higher DNA damage thresholds required for DDC activation and imposition of cell-cycle arrest in mammals. As a consequence, vertebrates are likely more prone to encounter unrepaired DNA in the following G1 phase and could be more reliant on G1 checkpoints for inducing cell-cycle arrest upon low levels of DNA damage (34).

2.2. Primary Control of the DDC: PIKK Kinases

In mammals, DNA damage signaling is initiated by two members of the phosphoinositide 3-kinase-related kinase (PIKK) family of proteins, ATM and ATR. A third PIKK, DNA-PKcs, is also involved (35), although its role in the control of DDC signaling is less well understood. Both ATM and ATR phosphorylate SQ/TQ residues in effector proteins to launch a cascade of signals that establishes a transcriptional program and prevents cell division (**Figure 2**) (36, 37). Budding yeast homologs of ATM (Tel1) and ATR (Mec1) carry out similar roles, although, as discussed below, they have exchanged some roles compared to their mammalian homologs. Budding yeast lack DNA-PKcs. **Table 1** lists key proteins that control the DNA damage response.

2.3. ATM and ATR Structures

ATM and ATR are part of the PIKK family, all of which are very large proteins whose structures have only recently been solved at atomic or near-atomic resolution. All of these proteins have a similar amino acid sequence architecture, with a large series of HEAT (Huntingtin, elongation factor EF3, PR65/A subunit of protein phosphatase 2A and the yeast kinase TOR1) repeats at the

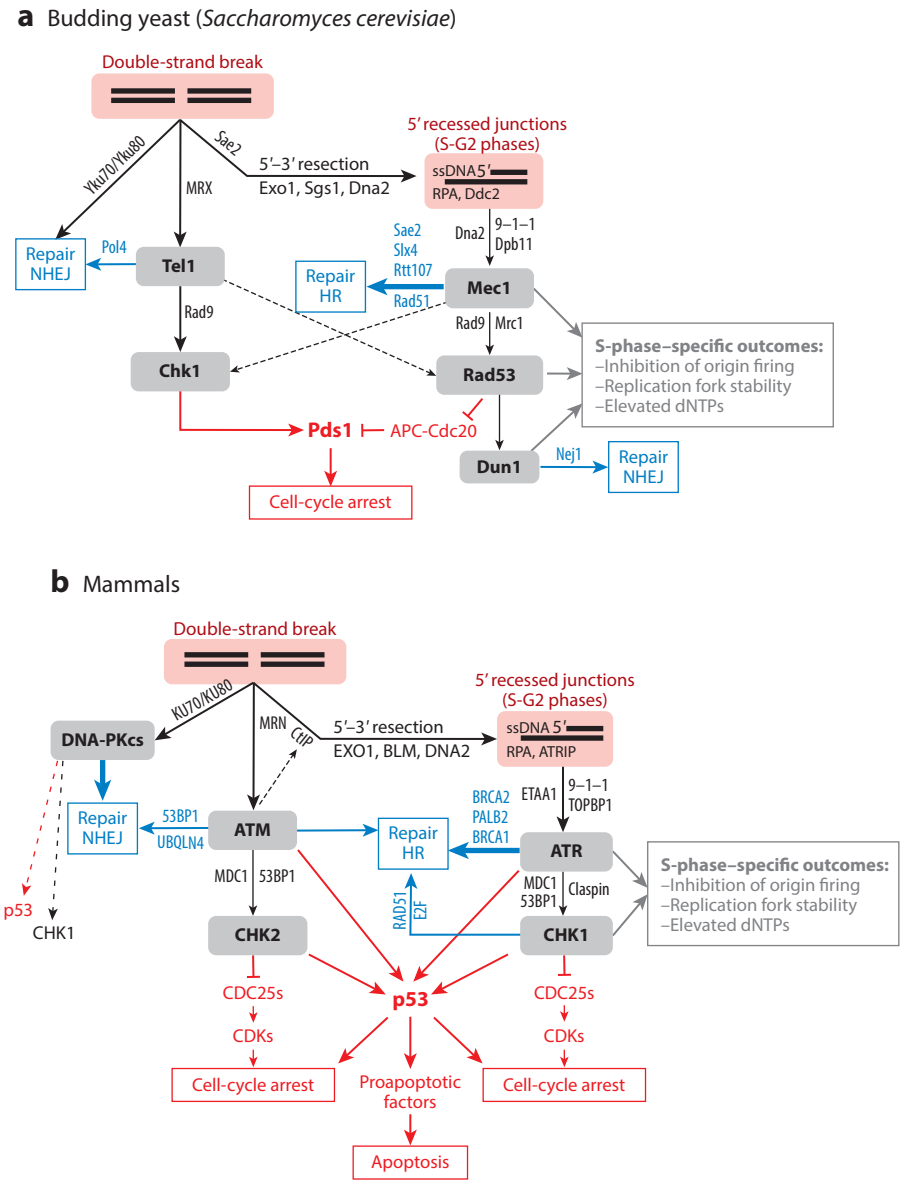

Figure 2

The DDC signaling network in (*a*) budding yeast and (*b*) mammals. Simplified network view of the role of DDC kinases with their main cofactors, regulators, adaptors, and substrates. Blue indicates key effectors through which DDC kinases regulate DNA repair of DSBs. Red indicates key effectors through which DDC kinases mediate cell-cycle arrest and apoptosis. Reference 198 provides more information about substrates involved in DNA repair. Abbreviations: 9–1–1, RAD9A-HUS1-RAD1; APC, anaphase promoting complex; CDK, cyclin-dependent kinase; CHK, checkpoint kinase; DDC, DNA damage checkpoint; DNA-PKcs, DNA-dependent protein kinase, catalytic subunit; dNTPs, deoxynucleoside triphosphates; DSB, double-strand break; HR, homologous recombination; MRN; MRE11-RAD50-NBS1; MRX, Mre11-Rad50-Xrs2; NHEJ, nonhomologous end joining. Figure adapted with permission from Wang et al. (47).

Table 1 Selection of DNA damage checkpoint and repair proteins

Category	*S. cerevisiae*	Humans	Note
PI3K-like kinases	Mec1	ATR	PIKK; initiates checkpoint signaling
	Tel1	ATM	PIKK; initiates checkpoint signaling
Clamp loader	Rad24	RAD17	9–1–1 clamp loader in complex with Rfc2–5
9–1–1 clamp	Ddc1	RAD9	Clamps ds/ssDNA junctions
	Mec3	HUS1	
	Rad17	RAD1	
Adaptor proteins	Rad9	53BP1, MDC1	Facilitates activation of downstream checkpoint kinases
	Dbp11	TOPBP1	Activator of Mec1/ATR; also functions as adaptor to couple 9–1–1 to checkpoint adaptors and DNA repair proteins
Sensor	Ddc2	ATRIP	Obligate binding partner of Mec1; recruits Mec1 to RPA-coated ssDNA
MRX/MRN complex	Mre11	MRE11	Nuclease
	Rad50	RAD50	
	Xrs2	NBS1	
Downstream checkpoint kinases	Rad53	CHK2	Primary kinase responsible for checkpoint signal propagation
	Chk1	CHK1	Stabilizes Pds1 by phosphorylation
Securin	Pds1	Securin	Inhibitor of Esp1/separase; degraded by APC-Cdc20
Separase	Esp1	Separase	Cleaves cohesin rings for anaphase onset
Spindle assembly checkpoint	Cdc20	p55	Specificity factor for APC E3 ubiquitin ligase
	Mad2	MAD2L1	Mitotic spindle assembly checkpoint protein
Repair proteins	Yku70/Yku80	KU70/KU80	DSB sensor; required for NHEJ
	Rad51	RAD51	Recombinase; binds RPA-coated ssDNA; performs homology search during HR
	Rad52	BRCA2	Deposits Rad51; stimulates strand exchange
Resection	Dna2	DNA2	5′–3′ endonuclease (budding yeast Dna2 is also an activator of Mec1)
	Exo1	EXO1	5′–3′ endonuclease; cooperates with Sgs1 helicase
	Sae2	CtIP	Endonuclease; cooperates with MRX to initiate end processing
Phosphatases involved in DDC deactivation	Ptc2, Ptc3	PP2C	Type 2C phosphatase (PP2C)
	Pph3	PP4	Pph3 is the catalytic subunit of budding yeast type 4 phosphatase (PP4)
	Glc7	PP1, PP2A	Type 1 and 2A phosphatase complexes
Adaptation kinase	Cdc5	PLK1	Polo-like kinase required for adaptation to DNA damage

Abbreviations: ATM, ataxia-telangiectasia-mutated; ATR, ATM and Rad3 related; CHK, checkpoint kinase; DDC, DNA damage checkpoint; DSB, double-strand break; HR, homologous recombination; MRN, MRE11-RAD50-NBS1; MRX, Mre11-Rad50-Xrs2; NHEJ, nonhomologous end joining; PIKK, phosphoinositide 3-kinase-related kinase; PI3K, phosphatidylinositol 3-kinase; PLK1, Polo-like kinase 1; RPA, replication protein A.

N terminus followed by a FAT (focal adhesion targeting) domain, the kinase domain and a FATC (FAT-associated C terminal) domain (**Figure 3*a*,*b***). The HEAT repeats consist of units containing two alpha helices joined by a short linker. ATR has 45 such repeats and ATM even more (38). These repeats can form a highly ordered structure that facilitates monomer-monomer interactions and likely the binding of other proteins; however, in the case of ATR, a single amino acid change in HEAT repeat 27 results in hyperactivation of the kinase (39). The FAT domain

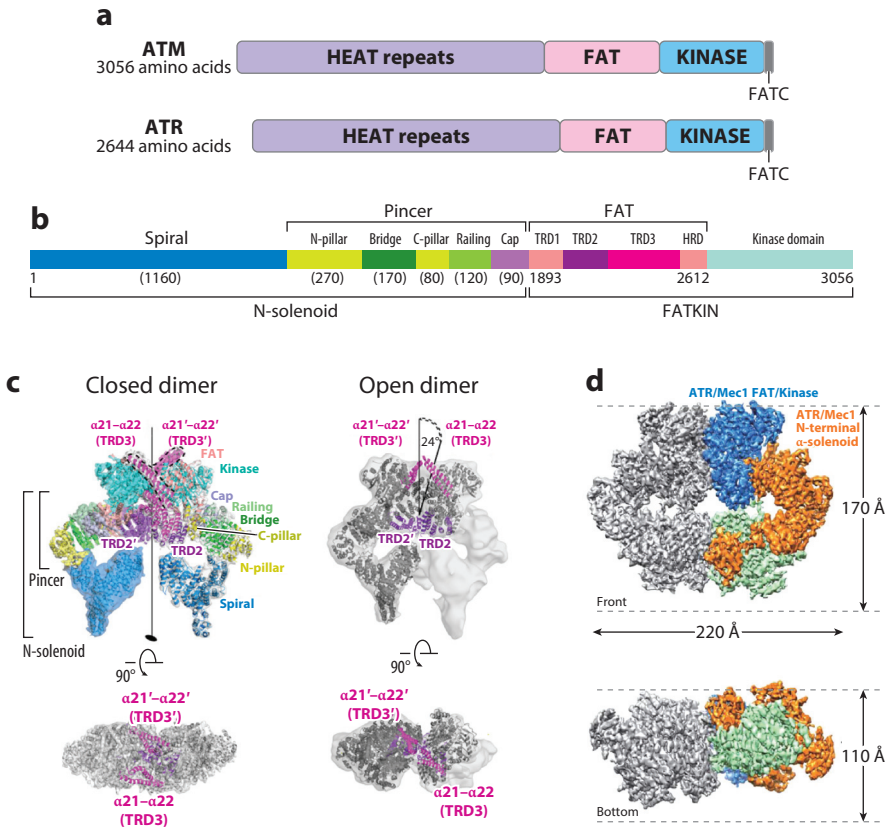

Figure 3

ATM and ATR structures. (*a*) Overall domain structure of ATM and ATR. (*b*) A more detailed structure of ATM, including structural features identified by cryo-electron microscopy (45). (*c*) Cryo-electron microscope–derived open (presumably active) and closed structures of ATM, as determined by Baretic et al. (45). The FAT and kinase region (FATKIN) has been solved at a higher resolution than the N-terminal solenoid domain. In the closed form, the conformation of the active site is maintained by interaction with a long helical hairpin in TRD3. (*d*) Electron microscope–derived structure of yeast Mec1-Ddc2 (ATR-ATRIP), determined by Wang et al. (47). Abbreviations: ATM, ataxia-telangiectasia-mutated; ATR, ATM and Rad3 related; ATRIP, ATR-interacting protein; FAT, focal adhesion targeting; FATC, FAT-associated C terminal; FATKIN, FAT and kinase region; HEAT, Huntingtin, elongation factor EF3, PR65/A subunit of protein phosphatase 2A and the yeast kinase TOR1; HRD, HEAT-related domain; TRD, tetratricopeptide repeat domain. Panel *d* adapted with permission from Wang et al. (47).

is a conserved ~500-aa domain shared by several kinases [DNA-PKcs, ATM, ATR, and transformation/transcription domain-associated protein (TRRAP)] (40); its function is poorly defined. In ATM, a conserved serine 1961 is phosphorylated after ATM activation, but how this modification leads to dissociation of the dimer is not known (41). The actual kinase domain of these PIKKs constitutes only a small fraction of the total protein; single amino acid mutations replacing a conserved aspartate are kinase inactive. Within the kinase domain is a PIKK regulatory domain that is required for activity and for interaction with ATR's coactivator, TOPBP1 (DNA topoisomerase 2–binding protein 1) (42).

The C terminus of these proteins includes a FATC domain for which several functions have been noted. FATC domains of DNA-PKcs, ATM, and TRRAP all interact with the chromatin

remodeler histone acetyltransferase Tip60 (43). In addition, the C terminus of budding yeast's ATM homolog, Tel1, appears to be required for the protein's association with broken chromosome ends and for full kinase activity (44).

Both mTOR and DNA-PKcs have been solved by X-ray crystallography and those data, combined with recent advances in electron microscopy, have yielded our first images of ATM (45, 46) and ATR (47) structures. ATM proves to be a front-to-front homodimer, as illustrated in **Figure 3*c***. The arrangement of the two monomers does not reveal how the kinase domain would contact serine 1961, whose autophosphorylation has been correlated with kinase activation. ATR is found as a dimer of heterodimers with the ATRIP cofactor that is required for ATR to bind to broken DNA ends. The most detailed structural information has come from studying the budding yeast proteins Mec1/ATR and Ddc2/ATRIP (47). This 3.9 Å structure reveals the amino acids needed for both ATRIP-ATR association and dimer stability (**Figure 3*d***). The structure of the active site suggests that several HEAT repeats come into contact (including the repeat analogous to the human repeat 27 whose S1333A mutation was implicated in hyperactivating the kinase).

2.4. Activation of the DDC: Detection of DSBs

Detection of broken DNA ends is the initiating event for both DSB repair and checkpoint activation (**Figure 4**). Experimentally, DSBs are created by ionizing radiation, by clastogens such as bleomycin, and by site-specific endonuclease cleavages (**Figure 1**). Breaks created by ionizing radiation may be more complex, terminating in glycosidic fragments of the deoxyribose-phosphate backbone, whereas chemically and enzymatically created ends have 5′ phosphates and 3′ hydroxyls at the sites of cleavage. Hence, the recognition of the broken ends may differ between "clean" and "dirty" DSBs (48). In yeast, chromosome breaks created by rupture of a dicentric chromosome proved to have the same pattern of repair as those induced by HO endonuclease (49).

A key modulator of repair and damage signaling is the MRN (MRE11-RAD50-NBS1; MRX in budding yeast) complex, which plays multiple roles in DNA end recognition and end processing. Dirty ends and ends that are blocked by protein adducts can be "cleaned up" by the action of the Mre11-Rad50 nuclease complex (50). Mre11-Rad50, along with an adaptor/chaperone protein, Nbs1/Xrs2, associate with an enhancer of the nuclease activity of the complex, CtIP/Sae2 (51).

The MR complexes also can bridge DSB ends and play an important but not exclusive role in tethering the two ends of a DSB together (52). Consequently, when a DSB in budding yeast is flanked with LacO arrays to which LacI-YFP can bind (53) or when Rad51-GFP binds to resected DSB ends (54), one sees only a single fluorescent focus, indicating that the ends are tethered. Disruption of the MRX complex results in a modest fraction of the ends forming two distinct foci (55), but there are other, undefined mechanisms that hold broken ends together as well.

MRX also plays a role in displacing tightly bound proteins from DSB ends by nicking the DNA and then resecting in a 3′ to 5′ direction, thus removing a short (20–50 nt) region of one strand and creating a 3′ overhang on the adjacent strand (56). The Ku70/Ku80 heterodimer is displaced in this way, allowing for access to the DSB end by the Exo1 5′ to 3′ exonuclease (57, 58). Both Ku and MRX are rapidly recruited to DSB ends (59). A similar role for Mre11-Rad50-Nbs1 in displacing Ku (and MRN itself) has been shown in *S. pombe* (60).

2.4.1. Resection of the broken ends. Activation of PIKKs at DSBs is profoundly impacted by resection of DSB ends. Loss of CtIP in vertebrate cells strongly prevents resection and the formation of RPA foci (61); however, in budding yeast, loss of MRX/Sae2 only retards resection near the DSB by a factor of two and does not impair long-range resection (62–64). In G2/M-arrested budding yeast cells, loss of the MRX complex blocks resection profoundly (65), suggesting that

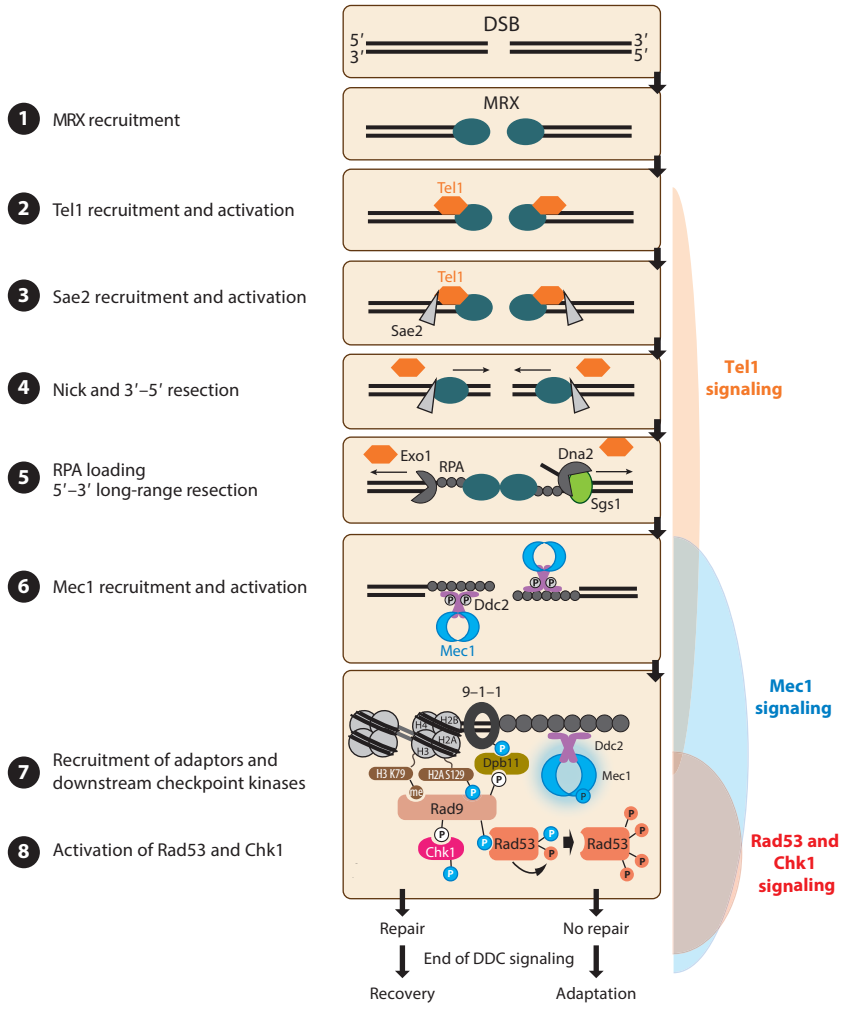

Figure 4

Detection of DSBs by PIKKs and downstream DDC signaling in budding yeast. DSBs are first recognized by the MRX complex, which binds to the broken ends. MRX recruits the PIK kinase Tel1 and Sae2 to begin DSB end processing by Exo1 and Dna2. RPA coats ssDNA, leading to the recruitment of Mec1-Ddc2 dimers. The 9–1–1 clamp loader assembles the 9–1–1 clamp at the 5′ recessed end of the dsDNA/ssDNA junction. Dpb11 is recruited to 9–1–1 via a Mec1-dependent phosphorylation in the Ddc1 subunit. Mec1 and Tel1 phosphorylate histone H2A on S129 (γ-H2AX). Rad9 bound to Chk1 is recruited to γ-H2AX through Rad9's BRCT domain and histone H3K79me through Rad9's Tudor domain. Rad9 is shown here as monomeric for simplicity. Mec1 then phosphorylates and activates Chk1 and phosphorylates Rad9, priming Rad9 for Rad53 recruitment. Rad53 binds phosphorylated Rad9, allowing for Mec1-dependent phosphorylation and activation. Activated Rad53 phosphorylates and activates other Rad53 molecules, leading to full checkpoint activation. Tel1 in yeast plays a very modest role in activating Rad53 and Chk1. The bottom-most panel shows key interactions and phosphorylation events involved in activation of the DDC. Color of phosphorylation sites (circles with "P") refers to the kinase responsible: blue, Mec1; red, Rad53; white, CDK. See text for additional details. Abbreviations: CDK, cyclin-dependent kinase; DDC, DNA damage checkpoint; DSB, double-strand break; MRX, Mre11-Rad50-Xrs2; PIKK, phosphoinositide 3-kinase-related kinase; RPA, replication protein A.

there is an MRX-independent resection process in S phase. Long-range 5′–3′ resection in both budding yeast and mammals is carried out by two partially redundant exonucleases, Exo1 and a complex containing the endonuclease Dna2/DNA2 with the Sgs1/BLM helicase, which is associated with Top3 and Rmi1 (56, 64, 66). In budding yeast, these two activities act independently to resect DNA at the slow rate of approximately 1 nt/s (~4 kb/h) (67). In mammals, it has been difficult to measure a resection rate; one estimate by measuring RPA binding to ssDNA suggests a rate of only 0.2 kb/h (68). However, as in yeast, resection in mammalian cells can continue for tens of kilobase pairs as measured by SSA assays (69). Exo1 removes single nucleotides from the DSB end, whereas the Sgs1/BLM- and Dna2-dependent process involves a helicase/endonuclease cleavage that liberates short oligonucleotides (70–72). In budding yeast, deleting either *EXO1* or *DNA2* results in a slight defect in resection but inactivation of both severely cripples resection, preventing DDC activation (73). In mammalian cells, there is a different relationship of the BLM helicase to resection: BLM promotes both EXO1 and DNA2's cleavage of DNA (74). RPA stimulates resection activity initially, but later checkpoint kinase-dependent phosphorylation of RPA appears to retard subsequent resection (75).

Resection is also tightly controlled during the cell cycle. Both Sae2 and Dna2 are targets of Cdc28 (budding yeast cyclin-dependent kinase, CDK1) for activation by phosphorylation (66, 76). Alanine substitution of CDK phosphorylation sites within Sae2 markedly reduces resection (66), whereas similar substitutions of three CDK phospho-sites in Dna2 has a mild effect, as Exo1 is still active (76). The G1 block to resection in budding yeast can be overcome by inactivating Ku proteins, enabling Exo1 to access the DSB end and thereby allowing resection even though CDK remains inactive (77).

In mammals, resection is under similar cell-cycle regulation, with CDK also being crucial to promote resection by phosphorylating CtIP, the functional ortholog of Sae2 (61). Overall, in both yeast and mammals, cell-cycle-dependent control of resection becomes a major determinant of how, and which, PIKKs are activated and the type of DDC response that is mounted.

2.4.2. Activation of Tel1/ATM and Mec1/ATR. MRX/MRN plays a key role in the recruitment and activation of Tel1/ATM to a blunt, unresected, DSB end. In both budding yeast and mammals, the C terminus of Xrs2/NBS1 appears to harbor a Tel1/ATM-interacting domain (78), but recent work suggests that in mammals, ATM activation is a function of MRE11-RAD50 themselves (79). In contrast, Mec1/ATR is not recruited to blunt or nearly blunt ends. Instead, Mec1/ATR recruitment requires that the DSB ends be resected (**Figure 4**); consequently, in G1-arrested cells and in other conditions where resection of DSB ends is blocked, Mec1/ATR is not activated (80, 81). Mec1 can be activated in the absence of a DSB, in G1-arrested cells, by treating cells with the UV-mimetic drug 4NQO when there are multiple RPA-bound single-stranded regions resulting from nucleotide excision repair that could attract Ddc2-Mec1 to these regions and activate the checkpoint (81). In fact, Mec1 can be activated even in the absence of DNA damage by tethering multiple copies of LacI-Ddc2 and LacI-Ddc1 to an array of LacO sequences (82). This activation can occur in G2/M-arrested cells and requires the scaffold protein Rad9 (discussed in more detail below) to mediate phosphorylation of Rad53. Rad9's recruitment presumably requires that Mec1 phosphorylates nearby histone H2A (γ-H2AX), because the level of phosphorylation of Rad53 is markedly less when cells carry a nonphosphorylatable histone H2A-S129 mutation.

Once resection begins, the checkpoint signaling is switched from Tel1/ATM to Mec1/ATR dependence (**Figure 4**) (83). Mec1/ATR recruitment to the break is mediated by its obligate binding partner, Ddc2/ATRIP (also known as Lcd1), which binds to RPA bound to ssDNA (84). Following recruitment, at least two pathways exist for Mec1/ATR activation (reviewed in more detail in 85, 86). First, Mec1/ATR can be activated by the Dpb11/TOPBP1 scaffold, which is recruited

to DNA lesions at ss/dsDNA junctions created by end resection, mediated by the 9–1–1 checkpoint clamp complex (Ddc1-Rad17-Mec3/RAD9-RAD1-HUS1) (87, 88). In budding yeast, both Dpb11 and the 9–1–1 component Ddc1 have Mec1-activating domains, whereas in mammals an ATR-activating domain is present in TOPBP1, but absent in the 9–1–1 complex. A second mode of Mec1/ATR activation has been recently uncovered involving the replication/DNA damage-associated proteins Dna2 (yeast) and ETAA1 (mammals) (89–92). Interestingly, differently from TOPBP1, which depends on a ss/dsDNA junction for recruitment (via 9–1–1 loading), ETAA1, which interacts with RPA, was proposed to mediate ATR activation at long stretches of RPA-coated ssDNA, therefore providing a system capable of sensing ssDNA-containing structures that may activate ATR through TOPBP1.

2.5. A Cascade of Signaling

The activation of Tel1/ATM and Mec1/ATR launches a broad cellular response, resulting from the phosphorylation of several substrates, many of which are themselves kinases with different specificities. In mammals, the principal kinase activated by ATM is CHK2, whereas ATR activates CHK1 (**Figure 2*b***); both kinases are important in the damage response. In budding yeast, the division of labor is different; Mec1 (ATR) activates Rad53 (CHK2) and contributes to Chk1 activation, whereas Tel1 (ATM) normally contributes a very modest amount to activating the downstream DDC kinases (**Figure 2*a***). In *mec1*Δ mutants, however, deletion of Sae2 reveals an Mre11/Tel1-dependent checkpoint response (called the TM pathway) that leads to robust Rad53 phosphorylation; this Mec1 bypass has been little studied (93).

2.5.1. Phosphorylation of histone H2A. In mammals, one very rapidly appearing phosphorylation is that of the C-terminal SQ site of the histone variant H2AX, referred to as γ-H2AX. γ-H2AX appears within minutes of exposure to ionizing radiation and can spread over 1 Mb around the break site (94). All three human checkpoint PIKKs have been shown to carry out this modification. The magnitude of the γ-H2AX modification by ATM is facilitated by the γ-H2AX-binding protein MDC1, but it does not affect the extent of such spreading (95). In mammals, propagation of γ-H2AX appears to transiently inhibit transcription (96), but there are also new transcripts that appear associated with the DSB ends (97). There is no such transient inhibition of transcription in budding yeast, where gene expression around the DSB site continues until 5′ to 3′ resection renders genes single-stranded (98, 99).

In budding yeast, there is no distinct H2AX isoform, but core histone H2A is phosphorylated at the equivalent C-terminal SQ site. Spreading of γ-H2AX around a site-specific DSB in budding yeast occurs soon after DSB formation and, in approximately 30 min, reaches ~50 kb on either side of the DSB (80, 100). Spreading is not uniform: Transcriptionally active areas adjacent to the DSB are refractory to γ-H2AX modification, but when transcription is turned off, γ-H2AX is quickly established within the locus, specifically by Mec1 (101). How Mec1 and Tel1 coordinate their activity to rapidly spread γ-H2AX is unknown, but Mec1 is able to spread γ-H2AX to an adjacent unbroken chromosome in *trans* (101, 102). A detailed kinetic analysis of γ-H2AX suggests that Tel1 primarily moves down the chromatin (one-dimensional diffusion), whereas Mec1 either diffuses from its initial binding site or comes into contact by looping from the end (101, 103).

2.5.2. DDC adaptors. Transduction of signaling from PIKKs to downstream checkpoint kinases Rad53/CHK2 and Chk1/CHK1 requires adaptor proteins that recruit these kinases within close proximity of PIKKs engaged at the DSB ends or nearby ssDNA. The molecular events and determinants of such a transduction process are best understood in budding yeast. Although

attempts have been made to expand the findings from yeast to mammals, many gaps in our understanding of the mammalian system remain, likely due to the increased complexity and redundancy of the proteins and regulatory mechanisms involved.

2.5.2.1. Budding yeast Rad9: a checkpoint adaptor paradigm. Rad9 is a large (150 kDa) scaffold protein required for robust G2/M checkpoint activation (**Figure 4**). Cells lacking *RAD9* exhibit a very brief checkpoint arrest after a single DSB, whereas *mec1*Δ *sml1*Δ cells have no arrest at all (104, 105).

Rad9 contains two chromatin interacting domains, a Tudor domain that binds histone H3 trimethylated on lysine 79 (H3K79me3) and tandem BRCT domains that recognize γ-H2AX (106, 107). H3K79me3 is found constitutively throughout the genome, established by the histone methyltransferase Dot1 (108, 109). Full DDC activation requires both Rad9 domains and therefore both histone modifications H3K79me3 and γ-H2AX. It is unclear whether Rad9 binds two histones within the same nucleosome or adjacent nucleosomes.

Recruitment of Rad9 to DNA lesions is also dependent on the scaffold Dpb11 and the 9–1–1 clamp member Ddc1 (110, 111) (**Figure 4**). Dpb11 contains a pair of tandem BRCT domains that bind phosphoproteins—one binds Rad9 and the other binds Ddc1 (112, 113). Dpb11's interaction with Rad9 is also regulated by phosphorylation of two S/TP residues in Rad9, S462 and T474, both of which are CDK consensus sites (111). Originally, it was proposed that CDK phosphorylation of these sites restricts Rad9-Dpb11 binding to G2, when CDK activity is high. However, a recent report found that these residues are phosphorylated in Rad9 during G1 even upon direct CDK inhibition (114), suggesting additional kinases are involved and implying more complex cell-cycle regulation.

Upon recruitment to a DSB, Rad9 is phosphorylated by Mec1 and Tel1 on multiple SQ/TQ sites. These phosphorylations serve two purposes: to promote Rad9 multimerization mediated through its BRCT domains and to prime docking sites on Rad9 to which the Rad53 kinase binds (115, 116). Mutations that impair Rad9 oligomerization do not prevent Rad53 activation, but checkpoint maintenance is lost, indicating that oligomerization is needed to sustain checkpoint signaling (116).

2.5.2.2. Mammalian DDC adaptors. In mammals, the identity and precise roles of DDC adaptors are not as well defined as in yeast and remain somewhat controversial. 53BP1 and MDC1 were shown to participate in signal transduction from ATM and ATR to CHK2 and CHK1 (117–122). Both 53BP1 and MDC1 are large BRCT-domain-containing scaffolds that share functional similarities with Rad9, including the ability to directly bind to γ-H2AX (123–125) and to the Dpb11/TOPBP1 scaffold (126–128). 53BP1 is the mammalian ortholog of budding yeast Rad9, and both proteins also share key roles in limiting resection (129–131). A key difference between yeast Rad9 and 53BP1 is that the latter directly binds to N-terminally ubiquitylated H2A—a modification lacking in budding yeast—and uses this chromatin modification as a major recruitment mechanism (132). In both yeast and vertebrates, the Mrc1/Claspin adaptor has a well-established role in transducing Mec1/ATR signaling to Rad53/CHK1 at stalled replication forks (133, 134) and could in principle participate in the DDC response to a DSB occurring at the replication fork.

2.5.3. Yeast Rad53 and Chk1 checkpoint kinases. Rad53 serves as the primary DDC signal transducer in budding yeast. As with *MEC1*, *RAD53* is an essential gene, whose deletion can be propagated by raising the level of dNTPs (135), by overexpressing ribonucleotide reductase (RNR) (136), by deleting the RNR inhibitor gene *SML1* (137), or by deleting the RNR transcriptional repressor gene *CRT1* (138). Upon activation, Rad53 governs a widespread transcriptional

response (139), largely through the activation of MBF-dependent transcription (analogous to human E2F) (140, 141) and through activation of the downstream Dun1 kinase, which controls transcription factors such as Crt1 and Ndd1 (138, 142). Rad53 also triggers a Dun1-independent phosphorylation of the repressor Rph1, allowing upregulation of the Phr1 photolyase as well as several key genes in the autophagy pathway (143, 144). Rad53 helps to restrain mitosis (see Section 2.7 below), and *rad53*Δ mutants display a much-shortened length of checkpoint arrest following a DSB (105). Cells lacking Rad53 signaling are sensitive to numerous DNA-damaging agents (145, 146).

After Rad53 is recruited to SQ/TQ phosphorylation sites on Rad9 (115, 147, 148), its proximity to Mec1 allows for initial Mec1-dependent phosphorylation on Rad53's N-terminal SQ/TQ cluster domain (149). Once activated, Rad53 hyperphosphorylates other nearby Rad53 molecules in *trans*, resulting in a population of fully activated Rad53, which then dissociates from Rad9 (150). While Rad9 promotes Rad53 activation, Rad53 also limits Rad9 oligomerization by phosphorylating and disrupting Rad9's *trans* BRCT domain interactions, thereby limiting its own activation through a negative feedback loop (116). In vitro, Rad53 autophosphorylation and activation can be achieved simply by self-oligomerization, even in the absence of Rad9 and Mec1, suggesting that the role of Rad9 and Mec1 in Rad53 activation is to promote localized accumulation of Rad53 and not necessarily direct activation (151).

Chromatin remodelers, which remove and restructure nucleosomes, have been implicated in regulating Rad53 activation (152, 153). The yeast INO80 chromatin remodeling complex interacts with Rad53 in vitro and in vivo following MMS (methyl methanesulfonate) treatment, depending on an SQ phosphorylation site within the INO80 subunit Ies4 (154), indicating that PIKK-dependent phosphorylation regulates INO80 activity. *ies4*Δ cells and Ies4 mutants lacking SQ phosphorylation sites display reduced Rad53 phosphorylation after MMS treatment. This defect is further exacerbated in *ies4*Δ *rad9*Δ double mutants, suggesting that INO80-dependent Rad53 activation functions in parallel to Rad9's activation (154).

Budding yeast Chk1 plays a relatively minor role in DNA damage signaling transduction. *chk1*Δ mutants display only a slight reduction in cell-cycle delay after a single DSB and *chk1*Δ *rad53*Δ double mutants exhibit similar short G2/M arrest as *rad53*Δ alone (104, 105). Chk1 activation requires Rad9 but not Rad9's SQ/TQ cluster, which is required for Rad53 activation (155). Instead, Chk1 activation requires Rad9's N-terminal Chk1 activation domain (155–157), which is phosphorylated independently of DNA damage during the S, G2, and M phases of the cell.

Some of the initial studies on the activation and subsequent adaptation of the DDC were performed by using a temperature-sensitive mutation of Cdc13, a component of the telomere end-protection complex in budding yeast (158). Cdc13 inactivation leads to the deprotection of telomeres, resulting in their 5′ to 3′ resection and the activation of the Mec1-dependent DDC. By and large, the behavior of cells triggered by unprotecting chromosome ends is similar to that achieved by creating a single site-specific DSB with HO endonuclease; for example, adaptation-defective mutations in casein kinase II (*ckb1*Δ) or a point mutation in the Cdc5 Polo-like kinase (*cdc5-ad*) that were identified in the *cdc13* system are also adaptation defective in the HO system. However, there are some differences worth noting. First, whereas Chk1 plays only a minor role in maintaining G2/M arrest in the HO system, it is much more important upon *cdc13* inactivation. Second, whereas two HO-induced DSBs are sufficient to block adaptation, the deprotection of 32 telomeres in *cdc13–1* at its restrictive temperature does not elicit such a strong response; cells still adapt. A likely explanation is that telomeres are constantly being restored by telomerase and that ends are not nearly as extensively degraded as are HO-induced DSBs (159). Indeed, when resection is impaired by together deleting Sgs1, Exo1, and Rad9, *cdc13–1* cells will continue to grow at their restrictive temperature, whereas under these conditions a single HO-induced DSB remains lethal.

2.5.4. Mammalian CHK1 and CHK2 checkpoint kinases. ATM and ATR activate the downstream checkpoint kinases CHK2 and CHK1, which together mediate key checkpoint outcomes including cell-cycle arrest and apoptosis (**Figure 2**) (160). The early checkpoint signaling response to DSBs is carried out predominantly through the ATM-CHK2 signaling axis. Once ends are resected, ATR and ATRIP are recruited to ssDNA via RPA, leading to activation of CHK1 (161, 162). In the canonical mode of activation, recruitment of CHK2 and CHK1 to DNA lesions via checkpoint adaptors allows their direct phosphorylation by ATM and ATR, causing the relief of the inhibitory domains of CHK2 and CHK1 and their activation (reviewed in 163). Similar to Rad53, overexpression of CHK2 in bacteria results in its trans-phosphorylation and autoactivation, supporting oligomerization and a concentration-dependent effect in promoting its activation (164, 165). Whereas insolubility issues have prevented expression of CHK1 in bacteria, overexpression of CHK1 in human cells resulted in only minor activation, much lower compared to activation of CHK2 in a similar system, suggesting differences in the regulatory mechanism of activation (166). DNA-damage-induced phosphorylation sites in CHK1 and CHK2 are commonly used as readouts of DDC activation in mammalian cells. Key markers of DDC activation include phosphorylation of CHK2 at threonine 68 (an ATM site) (165, 167) and CHK1 phosphorylation at serine 317 and serine 345 (ATR sites) (168, 169).

Intriguingly, although DNA-PKcs has little role in CHK2 activation, it was recently reported to promote CHK1 activation in cells lacking ATR signaling (170). Activation of DNA-PKcs in the absence of ATR signaling is dependent on ssDNA, structure-specific nucleases, and KU70, suggesting that DSBs formed through the processing of stalled replication forks activate DNA-PKcs. This finding reveals unexpected levels of complexity in the cross talk between PIKKs and downstream checkpoint kinases in mammals. Because inhibitors of DDC kinases are currently in clinical trials, understanding noncanonical mechanisms of signaling cross talk is relevant for therapeutic purposes. For example, the finding that DNA-PKcs can activate CHK1 helps explain why CHK1 inhibitors are more toxic than ATR inhibitors and provides a rationale for the combined use of inhibitors (170).

2.6. Enforcing Cell-Cycle Arrest and Apoptosis

In higher eukaryotes, cells suffering DNA damage are principally under the control of the transcription factor p53, which orchestrates the decision for cells to arrest the cell cycle, enter apoptosis, or senesce (reviewed in 171, 172). p53 is phosphorylated and activated by all DDC kinases, highlighting the centrality of p53 as a target through which the DDC can enforce an arrest in the cell cycle or, upon multiple and/or persistent unrepaired DSBs, apoptosis (**Figure 2**). DDC kinases CHK2 and CHK1 also promote cell-cycle arrest through multiple p53-independent mechanisms, such as inhibition of the CDC25 phosphatase, which drives CDK inhibition (**Figure 2**). Details of cell-cycle regulation by DDC kinases in mammals are the focus of numerous reviews (173–176).

Budding yeast lack a p53-like homolog. Instead, cell-cycle arrest triggered by the DDC is achieved largely through regulation of the molecular chaperone protein Pds1 (securin in mammals) and its binding partner, the cohesin protease Esp1 (separase) (177, 178). In undamaged cells, Pds1 binds Esp1, inhibiting its protease activity (179). During the metaphase to anaphase transition, Pds1 is ubiquitinated by the E3 ubiquitin ligase APC-Cdc20 and subsequently degraded by the proteasome (180). Degradation of Pds1 frees Esp1 to cleave cohesin rings, allowing sister chromatids to segregate. Upon DNA damage, Pds1 is phosphorylated by Chk1, blocking its interaction with APC-Cdc20 (177). Cdc20 is also phosphorylated, but instead by Rad53, to prevent APC-Cdc20 from ubiquitinating Pds1 (181). Together, both Pds1 and Cdc20 phosphorylation inhibit mitosis progression by keeping Esp1 inactive. Deletion of *PDS1* reduces the duration of

G2/M arrest by 60% while arrest is completely abrogated in *pds1*Δ *rad53*Δ (105, 182). In addition, DNA damage results in the sequestration of a fraction of Pds1 and Esp1 to the vacuole (183), adding an additional layer of control over cell-cycle progression.

2.7. Regulation of DNA Repair via DDC Signaling

Apical checkpoint PIKKs directly phosphorylate and regulate key proteins involved in the repair of DSBs (**Figure 2**). Such control of DSB repair largely occurs independently of downstream kinases, consistent with the view that Rad53/CHK2 and Chk1/CHK1 are mobile kinases that tend to coordinate global or "distant" responses (such as cell-cycle arrest) (184) and are therefore not best suited to control localized DNA repair transactions. Although unbiased proteomic analysis revealed local and distant roles for all DDC kinases (185), primary targets of these PIKKs in budding yeast and mammals are indeed highly enriched in proteins that localize at or close to DNA lesions (185). Among these local targets are numerous proteins involved in DNA repair.

DNA-PKcs and ATM play central roles in NHEJ-mediated repair in mammals, with DNA-PKcs signaling playing the principal role (186–188). Although several NHEJ factors are known to be phosphorylated by DNA-PKcs, the crucial substrates and phosphorylation events through which DNA-PKcs mediates NHEJ remain unknown. The only exception is DNA-PKcs itself, whose autophosphorylation is important to release DNA-PKcs from DNA ends to allow for ligation (189). ATM limits resection to favor NHEJ by two recently discovered mechanisms. First, ATM phosphorylates 53BP1 to assemble the 53BP1-RIF1-REV7-SHIELDIN antiresection complex (190). Second, ATM promotes the degradation of MRE11, by phosphorylating the proteasome-associated ubiquitin receptor UBQLN4 (191). Paradoxically, ATM also plays a proresection function through the phosphorylation of CtIP (192). Nonetheless, inhibition or loss of ATM only mildly impairs HR-mediated repair (193, 194), indicating that ATM is not crucial for promoting CtIP function, resection, or other key steps in HR.

ATR plays important roles in HR-mediated repair. This was recently demonstrated in human osteosarcoma cells in which Cas9 was used to induce HR-mediated DSB repair (193, 195). Buisson et al. (196) recently reported that phosphorylation of PALB2 by ATR promotes recruitment of the PALB2-BRCA2 complex to damaged sites via BRCA1, to promote RAD51 loading. However, the scenario is likely more complex, with ATR controlling multiple steps in HR. For example, chronic inhibition of ATR-CHK1 signaling strongly impairs HR efficiency by inhibiting E2F transcription, which depletes the abundance of key HR factors (197). Lanz et al. (198) provide a more detailed review on the role and mechanisms by which DDC kinases regulate DNA repair machineries in yeast and in mammals (198).

2.8. Checkpoint Maintenance

It would appear that the entire sensory apparatus to turn on the DDC has been identified; however, it is less certain, once the checkpoint has been activated, how it is maintained. Given that Rad53/CHK2 is heavily autophosphorylated, one might imagine that some of the proteins involved in initially detecting the damage would cease to be required. By using conditionally inactive proteins, such as those fused to auxin-inducible or temperature-sensitive degrons, it is possible to establish the DDC and then inactivate specific elements. For example, degradation of Ddc2 (199) or heat inactivation of Mec1 (200) rapidly extinguishes the checkpoint, suggesting that at least some of the proteins needed to establish the signal are needed to also maintain it. The situation is less clear in human cells as the effect of ATM and ATR chemical inhibitors after establishment of a DDC response results in variable results depending on the cell line used,

possibly due to the variable levels of expression of phosphatases involved in DDC downregulation in different cell lines (D. Dibitetto, C. Ascençao, J. Badar & M. Smolka, unpublished observation).

Unexpectedly, the maintenance of the DNA damage signal in budding yeast appears to require another checkpoint: the spindle-assembly checkpoint (SAC) (105, 201). Deletion of *MAD2* or other SAC genes markedly shortens the arrest in response to a single DSB and suppresses the permanent arrest induced in a variety of adaptation-defective mutants (see below) (105). The SAC appears to be activated by monitoring some change in the centromere region associated with the spread of γ-H2AX slowly down the chromosome until it reaches the kinetochore. Deletion of the SAC can be mimicked by deleting the centromere on the damaged chromosome but not when the damage is on a different chromosome. SAC is not strongly activated when the DSB is located several hundred kilobase pairs from its centromere.

2.9. Checkpoint Deactivation

With or without successful repair, the DDC is eventually terminated by dedicated deactivation mechanisms (**Figure 5**). Checkpoint deactivation is crucial to allow cell-cycle progression and cell proliferation, and mutants that fail to properly deactivate the DDC are sensitive to DNA damage (202–204). Deactivation of the DDC after successful repair is associated with the process of recovery, whereas DDC deactivation upon persistent damage is referred to as adaptation (see sections below). At the level of kinases and kinase targets, deactivation is achieved primarily by the action of phosphatases, including, but not limited to, PP2C and PP4 phosphatases in budding yeast (**Figure 5**) and PP2A phosphatases in mammals (205). These phosphatases remove activating phosphorylations from key checkpoint components, such as downstream checkpoint kinases and γ-H2AX. Because cell-cycle arrest is mostly established via the downstream checkpoint kinases Chk1/CHK1 and Rad53/CHK2, deactivation of these kinases allows rapid termination of the proarrest signaling. The checkpoint adaptors, such as budding yeast Rad9, offer another key point of regulation, as their degradation or disengagement from DNA lesions prevents new checkpoint kinases from becoming active to maintain the cell-cycle arrest. For example, in budding yeast the Slx4-Rtt107 repair factors compete with Rad9 at sites of DNA lesions to disengage Rad9 and dampen the checkpoint (204, 206, 207) (**Figure 5**). A similar competition-based mechanism of DDC downregulation has yet to be demonstrated in mammals.

2.9.1. Adaptation to DNA damage. In budding yeast, a single unrepaired DSB elicits robust DDC activation that persists for at least 9 h, after which, even without repair, cells adapt to the damage by switching off the DDC and proceed through several rounds of mitosis (31, 158, 208). A key feature of adaptation is that the cell becomes "deaf" to the presence of continued DNA damage; consequently, there is no arrest when cells traverse the next G2/M boundary. Although adaptation has traditionally been studied using site-specific endonucleases such as HO, adaptation has also been observed in aneuploid yeast cells, which frequently display Rad52-GFP foci in S phase, an indicator of DNA damage (209). Moreover, adaptation appears to be a conserved phenomenon. *Xenopus* egg extracts treated with the replication inhibitor aphidicolin eventually switched off checkpoint signaling, and human osteosarcoma cells were seen to enter mitosis despite the presence of γ-H2AX foci following gamma irritation (210).

How the checkpoint signal is extinguished during adaptation is slowly coming into focus. Recently, we found that Mec1 autophosphorylation at serine 1964 is required to turn off the signal (**Figure 5**); mec1-S1964A fails to adapt and Rad53 remains hyperphosphorylated (211). This modification appears only several hours after Mec1 has phosphorylated Ddc2, Rad9, and histone H2A; how S1964 is initially prevented from being phosphorylated remains unknown. Moreover,

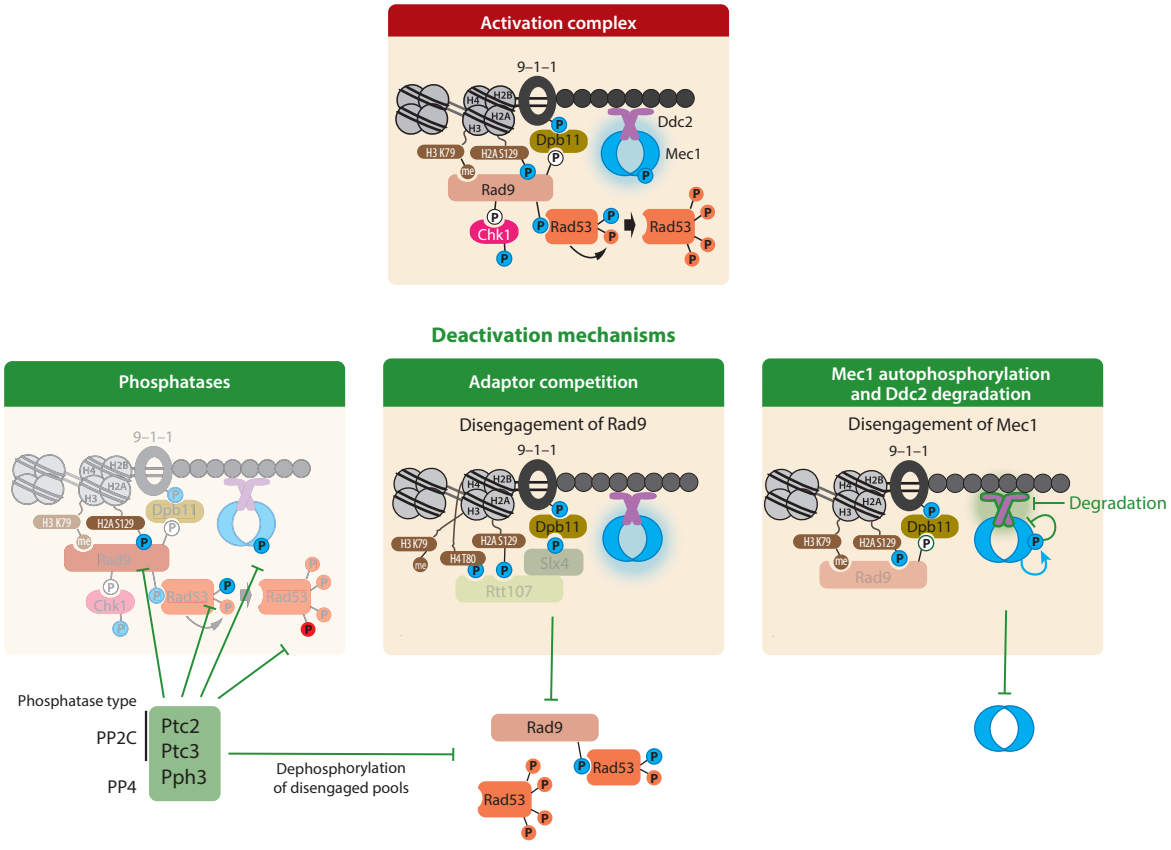

Figure 5

Mechanisms of DNA damage checkpoint (DDC) deactivation in budding yeast. (*Top*) Key interactions and phosphorylation events involved in activation of the DDC. Color of phosphorylation sites (circles with "P") refers to the kinase responsible: blue, Mec1; red, Rad53; white, CDK. (*Bottom*) Three modes of downregulating the DDC. Note that current evidence supports a model in which the PP4 phosphatase Pph3 removes Mec1 phosphorylation sites involved in Mec1 activation (238), and a later Mec1 autophosphorylation site triggers further Mec1 deactivation (211). For adaptor competition, recent evidence suggests that Sae2 may also compete with Rad9 (239, 240), although the mechanism is not understood. See text for additional details.

two serines in Ddc2 that are not SQ/TQ sites are required to adapt; these serines appear to be involved in regulating the stability of Ddc2. Ddc2 is normally a stable protein, but it is degraded at the time adaptation occurs (211).

Most adaptation-defective mutants exhibit hyperphosphorylated Rad53 that persists for >24 h after DNA damage, whereas adaptation results in the loss of Rad53 hyperphosphorylation approximately 9–12 h after creating the DSB. These mutants include, but are not limited to, deletion of the PP2C phosphatases, Ptc2 and Ptc3, which prevents dephosphorylation of Rad53 and presumably other Mec1 kinase targets (212, 213), and abrogation of casein kinase II, which is required to promote the association of Ptc2 with Rad53 via phosphorylation of a threonine in Ptc2 that is recognized by Rad53's FHA domain (212). However, the way in which mutations implicated in DSB repair (e.g., *rad51*Δ or *rdh54*Δ, but not *rad52*Δ or *rad54*Δ) (200, 214) prevent adaptation is not yet understood. Adaptation is also prevented by a mutation in the Cdc5 (Plk1) kinase that has altered activity, but, again, how this occurs is not understood (158, 215).

The relationship between the extent of 5′ to 3′ resection of DSB ends and adaptation is complex. The adaptation defect in y*ku70*Δ or y*ku80*Δ cells appeared to be related to the fact that resection is twice as fast as in wild type and that the adaptation defect of Ku mutants is suppressible by deleting *MRE11*, which slows resection (208). These observations led to the idea that the cell might monitor the rate of resection, for example by detecting short deoxyoligonucleotides that would be liberated by Mre11-Rad50 or by the helicase-endonucleases involved in long-range resection; indeed, in *Xenopus*, such short oligonucleotides stimulate the activation of ATM (216). But in yeast, neither elimination of the long-range helicase-endonuclease complex (e.g., deleting *SGS1*) nor slowing down resection by eliminating the Fun30/SMARCAD1 chromatin remodeler shortened arrest, but unexpectedly made cells adaptation defective (206, 217, 218). Moreover, H2BK123R, which abolishes histone H2BK123 ubiquitination, triggers early adaptation despite an elevated resection rate (D.P. Waterman & J.E. Haber, unpublished data). Therefore, resection cannot be the sole signal responsible for adaptation.

Noncanonical adaptation-defective mutants, which fail to resume cell-cycle progression despite dephosphorylation of Rad53, have been found in genes involved in processes outside the nucleus. Deletion of components of the Golgi-associated retrograde particle (GARP), such as *VPS51* or *YPT6* (183), displays other disruptions of normal adaptation, suggesting a complex cross talk between nuclear and cytoplasmic processes required for cell-cycle progression.

2.9.2. Recovery from DNA damage. After successfully repairing their DNA, cells rapidly switch off the DDC and reenter the cell cycle through a process known as recovery (28). Recovery has been best studied when there is a long delay between the induction of a DSB and the completion of repair, such as when a deletion occurs between repeated sequences flanking a DSB that are separated by 25 kb, such that resection—moving 4 kb/h—will only promote repair by SSA after 6 h (212). Only a fraction of adaptation-defective mutants are also recovery defective; these include *vps51*Δ (and other members of GARP), *sae2*Δ and *ptc2*Δ *ptc3*Δ (29, 183), suggesting that cell-cycle progression and completion of repair are two separate events.

2.10. DDC and Cancer

The DDC is often activated in preneoplastic cells in response to oncogene activation, establishing a barrier cells must overcome to enter a malignancy. Hyperactivation of the DDC, typically triggered by oncogene-induced replication stress (reviewed in 219) often leads to apoptosis or senescence, but cells may escape this terminal fate by gaining mutations or copy number variations in genes that either abolish checkpoint activation or cause its premature deactivation. Mutations in DDC genes such as ATM and TP53 are a central factor in contributing to genomic instability seen in tumors because they allow for the accumulation of more mutations over many cell generations (220, 221). Heterozygous mutations in ATM occur in ∼1% of the population and, like inherited TP53 mutations, predispose individuals to cancer (222, 223). Somatic TP53 mutations are by far the most frequently occurring across all tumor samples, present in nearly 40% (224), and ATM mutations are frequent in several cancers (222).

The role of other components of checkpoint signaling in cancer development is more complex. ATR is an essential kinase and its activity is important for the growth of many cancers (225). CHK1 exhibits both tumor-promoting and -inhibiting behaviors. In agreement with its role as a tumor suppressor, heterozygous deletion of CHK1 in mice together with heterozygous loss of Trp53 (mouse p53) potentiates mammary tumor formation (226). However, the same study found that homozygous loss of CHK1, expressed by a tissue-specific driver, inhibited mammary tumor development (226). Moreover, skin-specific homozygous deletions of CHK1 prevented tumor

formation in mouse skin cells exposed to chemical carcinogens (227). Therefore, it appears that although loss of some checkpoint factors increases the likelihood of cancer, amplification or gain-of-function mutations in others may help cells handle burdens imposed by oncogene activation and therefore prevent tumorigenesis.

2.11. Manipulation of Checkpoint Responses to DSBs: Applications and Perspectives

The past 15 years have witnessed a revolution in the development of specific inhibitors of DDC kinases. In 2004, Hickson et al. (228) developed a potent and highly selective inhibitor for ATM (KU-55933), ending the dark years of reliance on unspecific inhibitors such as caffeine and wortmannin. In 2011, Charrier et al. (229) developed the first potent and selective inhibitor for ATR, VE-821, with minimal cross-reactivity against ATM and DNA-PKcs. Since then, inhibitors with better bioavailability, potency, and selectivity have been developed for nearly all DDC kinases (230). These agents have allowed more careful studies on the architecture of the DSB-induced signaling network and the mechanisms by which DDC kinases control key functional outputs. Importantly, these inhibitors exhibit strong synergism with radiation therapy and DSB-inducing drugs, including topoisomerase inhibitors and PARP inhibitors, in sensitizing cancers, which led to numerous clinical trials currently underway (231). In addition to cancer therapy, the ability to manipulate the DDC may have a range of other applications, including improved strategies for precision genome editing. The efficiency of CRISPR-Cas9-mediated genome engineering was demonstrated to be compromised by DSB induction of a p53 response leading to cell-cycle arrest or apoptosis, especially in pluripotent stem cells and noncancerous cells (232, 233) as well as in pigs (234); however, the effect is not seen in other cell lines (235). This suggests that by manipulating the DDC response to Cas9-induced DSBs it should be possible to bypass the deleterious p53-dependent outcomes and improve genome editing efficiency. In fact, a transient inhibition of p53 was recently shown to reverse the constrained proliferation of edited pluripotent stem cells (236).

3. SUMMARY AND PERSPECTIVES

Since the first observations of cell-cycle delays in response to DNA damage, our understanding of the DDC has increased tremendously, from its activation, maintenance, and deactivation to its interplay with other cellular pathways. Broken DNA ends are substrates for recruitment and activation of the apical kinase Tel1/ATM. Once cells have committed to HR, ssDNA generated by 5′–3′ resection allows for robust checkpoint activation via Mec1/ATR-mediated signaling. Activation of apical kinases results in the phosphorylation of dozens of substrates within the vicinity of the DSB to create a region of chromatin "inflammation," orchestrate DNA repair, and trigger downstream signaling events. DDC kinases Chk1/CHK1 and Rad53/CHK2 amplify DDC signaling to mediate a response that stalls cell-cycle progression, rewires transcriptional programs, and impacts several other cellular processes. Upon successful DNA repair, the DDC is inactivated through the concerted action of phosphatases and the disengagement of kinases and DDC adaptors from the site lesion, allowing cells to progress through the cell cycle. But if repair fails, cells adapt to the damage by switching off checkpoint signaling and resume cell-cycle progression despite the continual presence of DNA damage.

Despite many advances in characterizing the DDC in the past four decades, several outstanding questions need to be addressed to complete a detailed picture. In particular, it remains largely unclear how the apical kinases coordinate DNA repair transactions necessary for suppressing

genomic instabilities upon DSBs. For example, how Mec1/ATR coordinates homology-directed DNA repair remains a fundamental knowledge gap in the field and an important barrier to better understanding how DNA-damage-induced signaling prevents chromosomal rearrangements. Multiple targets of Tel1/ATM and Mec1/ATR have been identified in the HR machinery, but we still do not have a comprehensive and mechanistic understanding of how, and which, defined sets of signaling events determine repair pathway commitment and coordinate steps in HR. Solving this question requires defining spatiotemporal signaling dynamics and dissecting the functionality of an extremely complex repertoire of phosphorylation events.

Another question that remains unanswered concerns what fraction of ATM and ATR are activated by one or a few DSBs. In yeast, downstream targets such as Rad53 have well-documented mechanisms of recruitment to the Rad9 scaffold and extensive autophosphorylation that can account for how most of the Rad53 in the cell is hyperphosphorylated when there is only a single DSB, assuming that there is robust turnover. Recent estimates of protein abundance in yeast (237) indicate that there are approximately 300 molecules of Mec1, 1,000 Rad9, and 2,100 Rad53; yet the great majority of both Rad9 and Rad53 migrate on western blots as hyperphosphorylated forms. Bakkenist & Kastan (41) suggested that mammalian ATM propagated its auto-activation by exchanging monomers (one activated monomer trans-phosphorylating a partner that would then diffuse away and allow another monomer to become associated with a presumably tethered active monomer). Yeast Mec1 has been shown to phosphorylate at least one site (not essential for its activity) in *trans* (211), and this may be a property of all such apical kinases.

Finally, a major outstanding question about DDC termination concerns exactly how checkpoint signaling is deactivated and phosphatase activity is regulated. In addition to addressing whether phosphatase activity is inducible or constitutive, it will be important to thoroughly explore if phosphatase action is out-competed by checkpoint activation/maintenance. It will also be crucial to explore whether mechanisms of DDC termination found in yeast (**Figure 5**) are also present in mammals.

DISCLOSURE STATEMENT

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