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Surviving the Breakup: The DNA Damage Checkpoint

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Abstract

In response to even a single chromosomal double-strand DNA break, cells enact the DNA damage checkpoint. This checkpoint triggers cell cycle arrest, providing time for the cell to repair damaged chromosomes before entering mitosis. This mechanism helps prevent the segregation of damaged or mutated chromosomes and thus promotes genomic stability. Recent work has elucidated the molecular mechanisms underlying several critical steps in checkpoint activation, notably the recruitment of the upstream checkpoint kinases of the ATM and ATR families to different damaged DNA structures and the molecular events through which these kinases activate their effectors. Chromatin modification has emerged as one important component of checkpoint activation and maintenance. Following DNA repair, the checkpoint pathway is inactivated in a process termed recovery. A related but genetically distinct process, adaptation, controls cell cycle re-entry in the face of unrepairable damage.

Contents

INTRODUCTION

DNA damage resulting from radiation, reactive oxygen species, and replication across nicked DNA is a fact of cellular life. To survive and generate viable progeny, cells must assess the damage and then either repair it or trigger the apoptotic program. A major component of the response is the DNA damage checkpoint, which arrests the cell cycle to provide time in which to carry out DNA repair. As some repair events are slow, the checkpoint-triggered arrest can last for the length of several cell cycles. Proper control of the activation and inactivation phases of the checkpoint is essential to prevent the segregation of broken chromosomes that can generate aneuploid progeny by chromosome loss and the formation of nonreciprocal translocations. When repair has been completed, the checkpoint arrest signal must be extinguished so that cells can re-enter the cell cycle.

DNA damage checkpoint: a stress response pathway triggered by chromosomal DNA damage. Its best-understood output is cell cycle arrest, which in yeast is typically just before anaphase onset

The first notion of a checkpoint to control cell cycle progression was suggested by Rao & Johnson (115), who fused mammalian cells at different stages of the cell cycle and showed that a nucleus still engaged in DNA replication transmitted a signal through the cytoplasm to prevent initiation of mitosis in another nucleus that had completed replication. Working in budding yeast, Weinert & Hartwell (156) provided the first experiments to show that DNA damage caused by UV irradiation provoked cell cycle arrest prior to the execution of mitosis. They further identified *RAD9* as the first of many genes necessary for DNA damage-induced arrest.

Genetic and biochemical studies have established a general framework for DNA damage checkpoint signaling (**Figure 1**). The central player is the phosphatidylinositol 3′ kinase-like kinase (PIKK), Mec1 (see **Table 1**). Mec1 is part of a sensor mechanism that detects DNA damage in the form of single-stranded DNA (ssDNA) and relays the checkpoint signal to a pair of transducing kinases, Rad53 and Chk1. These kinases amplify the signal and regulate the cell cycle machinery to effect checkpoint arrest prior to mitosis. Yeasts lack p53 and do not have either a long checkpoint arrest in G_1 or a robust apoptotic pathway that can eliminate damaged cells. In budding yeast the checkpoint arrest occurs prior to anaphase (in a phase often termed G_2/M), with the sister centromeres of replicated chromosomes attached to the mitotic spindle and under tension. Anaphase is prevented primarily by the persistence of securin, which prevents the separase enzyme from cleaving cohesin and releasing the sister chromatids into anaphase, but there are likely to be other restraints as well. In mammalian cells, DNA damage during interphase prevents the accumulation of mitotic CDK activity and triggers a checkpoint-mediated arrest in G_2, before nuclear envelope breakdown and

chromosome condensation. In cells with repairable DNA damage, the checkpoint arrest is maintained until repair is completed but is then released, allowing cells to complete mitosis and re-enter the proliferative cycle. Although checkpoint activation does promote the repair of some lesions (135, 145), the establishment and maintenance of cell cycle arrest are the essential roles of the checkpoint in the presence of DNA damage. The DNA damage response also includes the induction and repression of many genes as well as posttranslational regulation of protein abundance.

Studies of cancer-predisposition syndromes and sporadic tumors in humans have identified mutations in many DNA damage checkpoint genes, underscoring the importance of the checkpoint response (127). Recent work has also shown that the checkpoint is activated in early cancerous lesions and may function more generally to prevent human tumorigenesis (9, 56).

We divide the molecular events of the checkpoint into activation, maintenance, and inactivation phases. In the cell these phases translate into cell cycle arrest, maintenance of the arrest, and recovery, or re-entry into the cell cycle. This review focuses on results from budding yeast but with reference to studies in fission yeast and in animal cells. For greater detail on these systems, we direct the reader to four excellent recent reviews (81, 105, 120, 155).

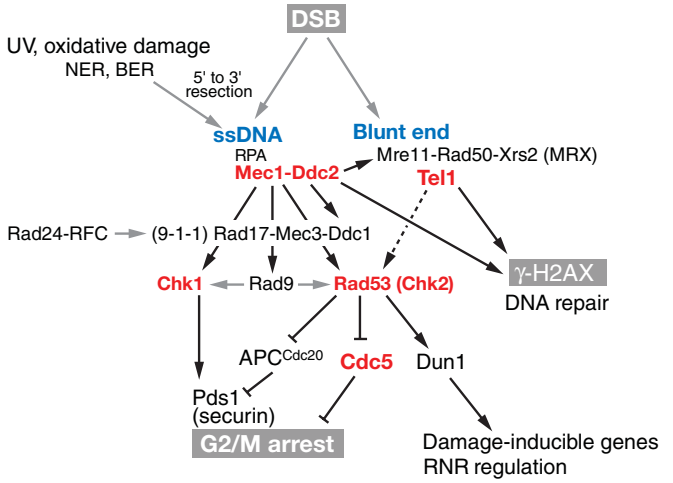

Figure 1

The DNA damage checkpoint in *Saccharomyces cerevisiae*. This review focuses primarily on DNA damage in the form of DSBs. Resection of the DSB end yields long 3′-ended ssDNA tails that trigger the Mec1-Ddc2-dependent DNA damage checkpoint kinase cascade. Mec1 is also activated by ssDNA gaps arising by nucleotide excision repair (NER) or base excision repair (BER). Unresected, blunt-ended DNA also activates a DNA damage response, primarily through the Tel1 protein kinase and its associated MRX complex. Kinases in the cascade are indicated in red. Under some circumstances where Mec1 is absent, Tel1 can activate the S-phase checkpoint involving Rad53 and other kinases, as indicated by a dotted line. There are three important outputs of DNA damage signaling: phosphorylation of histone H2AX (γ-H2AX) and associated increases in some DSB repair events; arrest of the cell cycle prior to anaphase (G_2/M arrest); and induction of damage-inducible genes as well as posttranslational regulation of ribonucleotide reductase (RNR). Black arrows indicate protein kinase phosophorylations of several target proteins that activate downstream events, whereas a black line terminated in a bar indicates an inhibitory modification. Gray arrows protein interactions that facilitate checkpoint activation.

ARREST

Signals and Sensors

All aspects of the DNA damage checkpoint in eukaryotes depend on members of the PIKK family, most famously human ATM and ATR (1). ATM (Ataxia-Telangiectasia Mutated) was identified as the gene mutated in the inherited cancer predisposition syndrome ataxia telangiectasia (A-T), and is a key player in the response to double-strand chromosomal breaks (DSBs) (1). ATR (Ataxia-Telangiectasia mutated and Rad3-related) is also a critical component of checkpoint pathways, especially in response to lesions generating ssDNA, for example, stalled replication forks (1). Budding yeast Mec1 is more similar to ATR and Tel1 is more similar to ATM (see **Table 1**), but these comparisons are not entirely helpful. In the checkpoint response to DSBs, for example, Mec1 alone is responsible for the checkpoint-induced cell cycle arrest.

Both Mec1 and its signaling target Rad53 are essential for cell viability even in the absence of DNA damage. The inviability of *mec1*Δ or *rad53*Δ cells is suppressed by increasing the activity of cellular ribonucleotide reductase (RNR) rather than by restoring

PIKK: phosphatidylinositol 3′ kinase-like kinase. A family of protein kinases structurally related to phosphatidylinositol 3′ kinase and playing a prominent role in the DNA damage checkpoint

ssDNA: single-stranded DNA

Table 1 DNA damage checkpoint proteins

Budding yeast	Fission yeast	Human
PIKK	Mec1	Rad3	ATR
PIKK	Tel1	Tel1	ATM
Adaptor	Rad9	Crb2	53BP1, MDC1, BRCA1?
Rfc1 homolog	Rad24	Rad17	Rad17
9-1-1 clamp	Rad17	Rad9	Rad9
	Mec3	Hus1	Hus1
	Ddc1	Rad1	Rad1
MRX complex	Mre11	Mre11	Mre11
	Rad50	Rad50	Rad50
	Xrs2	Nbs1	Nbs1
BRCT domain adaptor?	Dpb11	Rad4/Cut5	TopBP1
Signaling kinase	Rad53	Cds1	Chk2
Signaling kinase	Chk1	Chk1	Chk1
Polo kinase	Cdc5	Plo1	Plk1
Securin	Pds1	Cut2	Securin
Separase	Esp1	Cut1	Separase
APC-targeting subunit	Cdc20	Slp1	$p55^{CDC}$

Recovery: checkpoint inactivation and cell cycle re-entry following successful DNA repair

DSB: double-strand DNA break

checkpoint function (33, 164), and the essential role of Mec1 and Rad53 during normal cell growth appears to be in stabilizing stalled replication forks (84, 138). Mammalian ATR is also essential for cell viability (14, 30), though whether this reflects a conserved role in fork stabilization or RNR regulation is unknown. More likely, perhaps, is that the rigors of replicating larger genomes necessitate an ATR-dependent response to ssDNA or double-strand breaks (DSBs) in almost every cell cycle (133).

A very important development was the realization that creation of a single unrepaired HO endonuclease-induced DSB in budding yeast is sufficient to cause prolonged, Mec1-dependent cell cycle arrest (75, 122, 139). The intensity of the checkpoint proved to be stronger with two such DSBs, where cells remained permanently arrested, whereas a single DSB caused a 12–14 h G_2/M arrest (75). Site-specific cleavage of DNA has also made it possible to observe the assembly of checkpoint proteins, recombination proteins, and domains of modified chromatin at a defined DSB in vivo.

Ddc2 is a Required Partner for Mec1 Function

Recruitment of the checkpoint PIKKs to DNA is considered the most upstream event that triggers pathway activation and cell cycle arrest. These PIKKS bind DNA with the obligatory assistance of one or more partner proteins. For the Mec1/Rad3/ATR proteins this partner protein is Ddc2/Rad26/ATRIP (**Table 1**). Ddc2 and Mec1 form a complex in cells independently of DNA damage or other checkpoint genes, and *ddc2*Δ cells show the same defects seen in *mec1*Δ cells, namely a complete loss of checkpoint arrest; failure to phosphorylate Rad9, Rad53, Ddc1, Chk1, or Pds1; and sensitivity to DNA-damaging agents, although Mec1 kinase activity after immunoprecipitation is intact (107, 118, 153). Mec1 kinase activity is not obviously regulated by DNA damage, and Ddc2's role in checkpoint activation is thought to be recruitment of Mec1 to damaged DNA. This suggests that Mec1 can only interact with its substrates while bound to damaged DNA, which may help prevent spurious checkpoint activation.

The Interaction Between Mec1-Ddc2 and RPA-Coated ssDNA Triggers the Checkpoint

Much evidence suggests that the molecular species recognized by the Mec1-Ddc2 complex is ssDNA. ssDNA is a useful common checkpoint signal as it is formed during nucleotide and base excision repair, and at stalled replication forks (16, 132). That ssDNA could trigger checkpoint activation was directly demonstrated over a decade ago in the context of the S-phase checkpoint in *Xenopus* egg extracts (70), and recent work has solidified the view that ssDNA recruits Mec1-Ddc2 to trigger DNA damage checkpoint activation in vivo (**Figure 2*b***). Studies

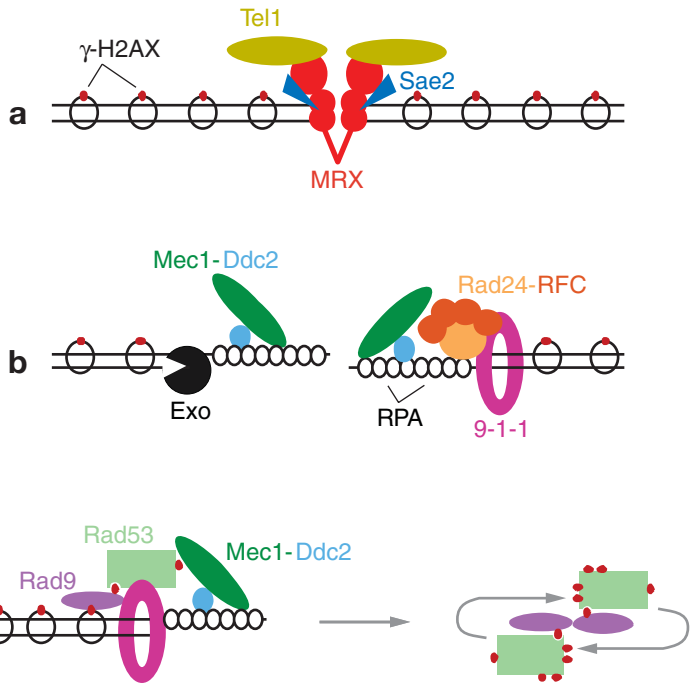

Figure 2

Checkpoint protein association with DSB. (*a*) The first important checkpoint proteins interacting with DSB ends are Mre11, Rad50, and Xrs2 (the MRX complex; *red*). MRX recruits the PIKK Tel1 (*dark yellow*) which can phosphorylate histone H2A in chromatin to create a region of γ-H2AX (*red spot* on nucleosome). Sae2 (*blue wedge*) stimulates the nuclease activity of the MRX complex and also promotes the removal of MRX and Tel1 from DNA thus limiting their signaling potential. (*b*) DNA resection at a DSB is carried out by MRX, Exo1, and an unknown nuclease (collectively indicated in *black*). Resection leaves a region of 3′ ended ssDNA which is rapidly coated by the RPA heterotrimer (represented as *white circles*). Rad24 (*orange*), in complex with Rfc2-5 (*dark orange*), binds at the ssDNA/dsDNA junction and loads the 9-1-1 clamp (*magenta*). This clamp can slide over dsDNA but not over RPA-coated ssDNA. RPA-coated ssDNA also recruits the Mec1-Ddc2 heterodimer (*green* and *light blue*), which activates the checkpoint cascade. Like Tel1, Mec1 can generate a region of γ-H2AX around the DSB. (*c*) Activation of the checkpoint cascade. Rad9 (*purple*) is recruited to DNA via its interactions with the modified histones including γ-H2AX. Rad9 is then phosphorylated (*red dot*) by Mec1 (*green*). Phosphorylated Rad9 recruits Rad53 (*avocado*) for phosphorylation (red dot) by Mec1. Both of these events are facilitated by the 9-1-1 complex, though the precise interactions have not been elucidated. A complex of Rad9 and phosphorylated Rad53 then dissociates from Mec1 and multimerizes in order to allow further trans-autophosphorylation and full activation of Rad53 (see 110a). Chk1 is activated by Mec1 and Rad9 via a similar but molecularly distinct mechanism.

Resection: the 5′ to 3′ nucleolytic degradation of one DNA strand at a DSB end that results in a long region of ssDNA with a 3′ end

RPA: replication protein A

in yeast have also shown a strong connection between the exposure of ssDNA at DSBs and at unprotected telomeres and activation of the DNA damage checkpoint (50, 75). At DSBs ssDNA is generated by 5′ to 3′ resection, leaving long 3′ ended tails (50, 157). Generation of ssDNA at a DSB occurs at twice the normal rate in a *yku70*Δ mutant; this increased resection causes a permanent arrest, whereas a slowing of 5′ to 3′ degradation caused a shortening of arrest, further linking ssDNA generation and checkpoint activation (75).

Recruitment of the Mec1/Ddc2 complex to ssDNA generated at a DSB requires the single-strand binding protein complex RPA (167). A specific point mutation in the large subunit of RPA, Rfa1-L45E (*rfa1-t11*), shows a partial reduction both in Ddc2 ChIP and in localization of a Ddc2-GFP fusion protein (82, 99, 167). However, *rfa1-t11* cells suffering one unrepaired DSB still exhibit a robust checkpoint arrest (75, 78). Similarly, in human cells depletion of the large RPA subunit RPA70 reduces focus formation by ATR and ATRIP after irradiation and reduces phosphorylation of the ATR target Chk1 (167). RPA is also required for the interaction between the ATR/ATRIP complex and ssDNA in vitro (167).

Studies have differed on whether Ddc2 alone can interact with RPA-coated ssDNA or whether the interaction between Mec1 and Ddc2 is required for their recruitment to a damage site (94, 119). A recent study has shown that the conserved FAT domain of Mec1 interacts with RPA in a two-hybrid assay, and mutations in the FAT domain eliminate not only the Mec1-RPA coimmunoprecipitation (co-IP) but also the Ddc2-RPA co-IP, though the Mec1-Ddc2 co-IP is still intact (100). Additionally, the FAT mutant prevents recruitment of Mec1 and Ddc2 to a DSB, suggesting that the complex of Mec1 and Ddc2, rather than Ddc2 alone, is most effective as the sensor for binding RPA-coated ssDNA (7, 100). Ddc2 appears to contain its own domain for interacting with DNA, a conserved cluster of basic residues. This region is dispensable for the Ddc2-Mec1 interaction but is essential for the binding of Ddc2 to DNA and for a functional DNA damage checkpoint (119, 153). Detailed structural studies will be required for a more complete understanding of the interdependencies among RPA, Mec1, and Ddc2 in their interaction with ssDNA. No other checkpoint proteins are required for Mec1 and Ddc2 to interact with the site of DNA damage (69, 94, 166), demonstrating that the Mec1-Ddc2 complex is an authentic damage sensor and that RPA-coated ssDNA is the damage signal that triggers checkpoint activation.

Very recent evidence demonstrates that *Xenopus* ATR is catalytically activated by DNA damage with the help of TopBP1 (72, 160). It will be important to determine if this is a conserved feature of ATR-family kinases, though the domain of TopBP1 required for this activation is apparently limited to vertebrates.

ssDNA is Generated by Multiple Exonucleases

Generation of ssDNA at a DSB requires the activity of a 5′ to 3′ exonuclease or a helicase/endonuclease similar to bacterial RecBCD. The budding yeast Mre11-Rad50-Xrs2 (MRX) complex is responsible for some of this activity, as the deletion of any of these proteins results in a twofold reduction in resection in cycling cells (63, 99, 142). In nocodazole-arrested cells, however, the absence of MRX eliminates nearly all resection (34). Paradoxically, MRX's in vitro exonuclease has 3′ to 5′ activity rather than the 5′ to 3′ activity seen during DSB resection (44, 109, 141). Mre11 also exhibits endonuclease activity, which could still be compatible with a helicase/endonuclease mode of resection (44, 109, 141); but more problematic is that the Mre11-H125N mutation, which eliminates in vitro nuclease activity, has no effect on the production of ssDNA at HO-induced DSBs (74, 83, 96). Mre11 is also dispensable for ssDNA generation at deprotected telomeres (90).

The MRX complex appears to be regulated by the Sae2 protein, and deletion of Sae2 phenocopies specific mutations in Rad50 and Mre11 called *rad50-S* and *mre11-S* (15, 93, 98, 104, 114). Sae2 has recently been shown to play a minor role in the resection of HO-induced DSBs in mitotic cells (23), presumably by regulating an MRX-dependent nuclease activity. Deletion of Sae2 or elimination of Mre11's nuclease activity results in the persistence of Mre11-GFP foci at DSBs in vivo as well as a prolongation of the DNA damage checkpoint (8, 24, 82). Thus, Sae2 may help limit checkpoint activation by the MRX complex (and Tel1, see below) by stimulating nuclease activity and the subsequent dissociation of MRX from DNA. It is possible that MRX control of nuclease activity is indirect, involving a separate bona fide nuclease.

Various studies of the connection between MRX activity and checkpoint activation have not yielded a coherent picture. Following induction of a single HO-induced DSB in a normal chromosomal location, cells lacking any of the MRX proteins exhibit a significant G_2/M arrest, though perhaps shorter than wild type cells (75; F. J. Dotiwala & J. E. H., unpublished). However, when prearrested in G_2/M by nocodazole treatment, these same cells fail to generate the characteristic hyperphosphorylation of Rad53 that normally reflects checkpoint activation (57, 62). This failure may reflect the absence of 5′ to 3′ resection during nocodazole arrest in the absence of the MRX proteins (34).

Resection of an HO-induced DSB is also partially reduced by deletion of the exonuclease Exo1, and this effect is most pronounced at distances beyond 2.3 kb from the HO-induced DSB (83). Similarly, for telomeres deprotected by the *cdc13–1* mutation, Exo1 is not essential for resection of the telomeric TG_{1-3} repeat or the terminal Y′ sequences, but it is strongly required for resection that progresses into subtelomeric X elements and unique chromosomal sequences (169). These data suggest that there may be a hand-off from MRX-dependent resection close to the DSB to Exo1 as resection progresses. Accordingly, while deletion of MRX proteins reduces the rate of resection near the DSB, the resection of more distant regions is unaffected (G. Ira & J. E. H., unpublished). The combined deletion of Exo1 and either Mre11, Xrs2, or Sae2 results in the very strong (though not complete) reduction of DSB resection (24, 99). Predictably, this resection defect results in reduced Mec1 association with the damage site, reduced phosphorylation of Rad53 and Chk1, and a much shorter cell cycle arrest (99).

In *Schizosaccharomyces pombe*, activity of Exo1 is likely to be regulated by the DNA end-binding Ku protein complex. Deletion of the Ku proteins suppresses the DNA damage sensitivity in cells lacking a functional MRX complex. This suppression requires Exo1 and suggests that Exo1 can only function efficiently at damage sites when Ku is absent (140). As mentioned above, deletion of Yku70 in budding yeast increases the rate of resection at a DSB (75). Whether this effect is due to increased activity of Exo1 toward a DSB end that is no longer bound by the Ku complex is unknown.

Another factor governing the generation of ssDNA at DSBs is the phase of the cell cycle. There is little or no resection in budding yeast cells arrested in G_1 by mating pheromone (which prevents activation of the B-type cyclins, or "Clbs"). A similar block in resection can be achieved by overexpression of the Clb inhibitor Sic1, or by inhibition of an analog-sensitive allele of the Cdk1 kinase (4, 62, 169). These conditions also prevent Rad53 phosphorylation (62, 112). Why Cdk1/Clb activity is required to activate 5′ to 3′ resection (which appears to involve several different nucleases) remains a mystery. There are over 200 in vitro targets of Cdk1 phosphorylation (144), including Mre11 and Xrs2, but mutation of the Cdk1 phosphorylation sites in Mre11 and Xrs2 did not affect resection and none of the other targets obviously contribute to DSB resection (62). Inhibition of Cdk1 in checkpoint-arrested cells is sufficient to stop ongoing resection of a DSB and to turn off Rad53 phosphorylation, suggesting that

RFC: replication factor C

9-1-1 clamp: PCNA-like checkpoint "clamp" made up of the Rad9-Hus1-Rad1 proteins in *S. pombe* and animal cells and the Rad17-Mec3-Ddc1 proteins in *S. cerevisiae*

γ-H2AX: a species of the histone variant H2A(X) that is phosphorylated at a C-terminal SQEX motif

continuous Cdk1-dependent resection is required for checkpoint maintenance (62). One barrier to resection may be the displacement of nucleosomes; indeed, deletion of the Arp8 subunit of the Ino80 chromatin remodeling complex reduces resection at a DSB by approximately twofold (147), though this result was not found by Tsukuda et al. (143).

Resection at telomeres differs in some ways from resection at endonuclease-induced DSBs. One intriguing feature of telomere resection is the more notable role played by checkpoint proteins, especially the Rad24/RFC and the 9-1-1 checkpoint clamp loader and sliding clamp (see below), which promote resection (66, 87), presumably by recruiting an unknown exonuclease to deprotected telomeres. Genetic studies have excluded Exo1 and the MRX complex from this role as well as the Rad2, Din7, Yen1, and Nuc1 exonucleases (169); the identity of "ExoX" is unknown. The Rad9 checkpoint adaptor protein has the opposite effect on resection, especially at telomeres, as the rate of 5′ to 3′ resection is accelerated in its absence (87). Neither MRX nor Exo1 is responsible for this acceleration. Resection is nearly wild type in a *rad9*Δ *rad17*Δ double mutant, suggesting that Rad9 and the 9-1-1 clamp may regulate the same target (87). Increased resection has also been seen in *rad9*Δ cells at an HO-induced DSB within the *LEU2* gene, but curiously, not at DSBs induced at two other chromosomal locations (M. Vaze & J. E. H., unpublished). Again, resection is slower in *rad9*Δ *rad17*Δ than in *rad9*Δ (though not in wild type), and is neither Exo1- nor MRX-dependent; moreover, in this case the increased resection is Mec1-dependent.

In summary, most evidence suggests that Exo1 and the MRX complex (assisted by Sae2) play significant roles in resection of chromosomal DSBs. At "clean" endonuclease-induced DSBs, these factors appear to be largely redundant, but the sensitivity of MRX mutants to ionizing radiation suggests that this complex may be more important for processing "multiply damaged" DSB ends that do not end in 3′ OH and 5′ phosphates. Other nucleases clearly contribute both at telomeres and at DSBs, but whether these are typical exonucleases, collaborations between helicases and endonucleases, or perhaps the proofreading exonuclease domains of DNA polymerases remains to be determined.

Tel1 is Recruited to DSB Ends by the MRX Complex

Like Mec1 and ATR, the checkpoint PIKKs Tel1 and ATM are recruited to free DSB ends. Rather than using Ddc2/ATRIP, these kinases bind DNA through their interaction with the DNA-binding MRX/MRN complex (**Figure 2*a***) (43, 101). Studies in both human and yeast cells showed that Tel1/ATM binds a common motif in the C terminus of Xrs2/Nbs1, and that this interaction is specifically required for Tel1/ATM recruitment to a DSB (43, 101). Although Tel1 does not usually play a role in the checkpoint response to DSBs, introduction of the *rad50-S* allele or deletion of Sae2 (both of which prolong MRX occupancy at DSBs) can partially suppress the MMS sensitivity of *mec1*Δ, and this suppression requires Tel1 (146). Sae2 deletion also causes a prolongation of Rad53 and Mre11 phosphorylation following global DNA damage, likely due to excess Tel1 activation (8).

In contrast to the Mec1-Ddc2 complex, which is activated by its interaction with RPA-coated ssDNA, MRX and Tel1 appear to be recruited to blunt or minimally processed DSB ends. This is demonstrated both by the very rapid formation (and gradual disappearance) of Mre11-GFP and Tel1-GFP foci in response to DSBs and by the specific role of Tel1 in γ-H2AX formation in G_1-arrested cells where resection is minimal and Mec1 does not contribute (62, 82, 128). Additionally, neither Tel1 nor MRX requires RPA or any other checkpoint protein for focus formation, though Tel1 requires Mre11. Finally, cells treated with ionizing radiation, which generates both single-strand and double-strand breaks, show incomplete overlap of Mre11

and RPA foci (82). Presumably this reflects RPA at ssDNA and Mre11 (and Tel1) at unprocessed DSB ends. ChIP analysis shows a steady increase of Tel1 at a DSB over time instead of the rapid peak and decline seen for Tel1-GFP foci (101).

Dissociation of Mre11 from DSB ends is inhibited in *sae2*Δ, *rad50-S*, and *mre11-H125N* cells and by inhibition of Cdk1/Clb activity (62, 82). These results suggest that Sae2 and Clb/Cdk1 stimulate MRX-associated nuclease activity to promote MRX dissociation from DNA. It also suggests that in cells competent to resect DSB ends (i.e., cells outside G_1 phase) signaling by Tel1 is typically restricted to the short period before resection (23, 62, 82). However, in cycling cells lacking Mec1, where resection is normal, Tel1 is sufficient to phosphorylate histone H2AX as it does in G_1 where resection is blocked (J.-A. Kim & J. E. H., unpublished). Additionally, cohesin binding around a DSB is only partially reduced in *mec1*Δ cells arrested in G_2/M (145). As this cohesin binding requires Mec1- or Tel1-dependent phosphorylation of histone H2AX (see below), this result suggests that Tel1 can function outside of G_1 and at resected ends in some situations.

Mammalian ATM appears to play a more prominent checkpoint role than does Tel1. This could result from a longer persistence of unresected DSB ends in mammalian cells than in yeast (48). Recent data in human cells suggest that ATM triggers DSB resection and the consequent RPA loading and leads directly to ATR activation (28, 64). This important finding strongly challenges the current view of the division of labor between the checkpoint PIKKs, and it likely explains the importance of ATM in mammalian cells.

In vertebrates, DNA damage also triggers autophosphorylation of ATM on serine 1981 (S1981). Phosphorylation of this FAT domain residue governs ATM dimerization via the interaction between the FAT domain of one ATM molecule and the kinase domain of another (6). Accordingly, ATM dimers are dissociated following cellular irradiation and S1981 phosphorylation. At least half of cellular ATM is phosphorylated within minutes of irradiation at 0.5 Gy, which is predicted to cause only 18 DSBs per cell (6). The authors posit that ATM can be activated by chromatin alterations occurring in a large chromosomal domain rather than only at a DSB.

In contrast, in vitro studies using either purified human proteins or using the *Xenopus* egg extract have found an essential role for the MRN complex in ATM activation (73, 162). As the MRN complex is believed to bind primarily to DSBs it is unclear how it could activate ATM in response to chromatin disruption that did not cause DSBs. These studies have further shown that activation of dimeric human ATM requires the full MRN complex, including ATPase-proficient Rad50, and DNA (73). In the *Xenopus* egg extract, ATM S1981 phosphorylation also requires an intact MRN complex (162). These findings have led to a model in which DSB binding by MRN activates Rad50 ATPase activity, resulting in a conformational change in the MRN complex that then triggers activation of ATM (73, 162). Clarifying the relationship between the MRN-ATM interaction, ATM S1981 autophosphorylation, and ATM activation toward other substrates in vivo is an immediate goal for the field.

CHECKPOINT CLAMP AND CLAMP LOADER

Checkpoint activation and cell cycle arrest strongly require the 9-1-1 complex. This heterotrimeric complex is made up of Rad17, Mec3, and Ddc1, all of which show limited sequence homology to the PCNA clamp (152), and is therefore referred to as a checkpoint clamp. The 9-1-1 complex is loaded onto DNA by a "checkpoint clamp loader"—a form of RFC in which Rad24, instead of Rfc1, forms a complex with the Rfc2–5 subunits (**Figure 2*b***) (11, 41, 88, 152). Biochemical analysis of Rad24/RFC and the 9-1-1 complex has shown that Rad24/RFC interacts with the

dsDNA: double-stranded DNA

SQ/TQ: serine or threonine residues immediately followed by a glutamine residue; the amino acid motif most commonly phosphorylated by PIKKs

9-1-1 complex and recruits it to DNA (11, 41, 88).

In vivo, Rad24, Rad17, Mec3, and Ddc1 are recruited to a DSB, as shown by ChIP and GFP fusion protein analysis. As predicted, Ddc1 and Mec3 recruitment requires Rad24, but the converse is not true (69, 82, 94). Ddc1 focus formation also does not require Mec1, Ddc2, Rad53, Rad9, or Tel1, suggesting that the 9-1-1 clamp functions as a third independent damage sensor (69, 82, 94). 9-1-1 complex loading in vivo does require RPA as Ddc1 ChIP to either an HO-induced DSB or a stalled replication fork is reduced by *rfa1-t11* (86, 99, 168), and Ddc1-GFP foci are not seen in Rfa1-depleted cells (82).

Despite the role for RPA in 9-1-1 DNA loading, extensive DSB resection is not required. Cells lacking both Xrs2 and Exo1 have strongly reduced resection and little Mec1 association with a DSB, but Ddc1 association is not obviously reduced (99). Even minimal resection will create the ssDNA/dsDNA junction at which the 9-1-1 clamp is likely to be loaded, which may explain this result (**Figure 2*b***).

How the 9-1-1 clamp promotes checkpoint activation in vivo is not yet fully understood, but it likely recruits Mec1 substrates for phosphorylation. Phosphorylation of Rad9 and Rad53 is reduced in *9-1-1*Δ and *rad24*Δ mutants (42), leading to a very strong checkpoint defect. Using a crippled 9-1-1 clamp (containing a partially active Mec3 fusion to the LexA DNA-binding domain), Giannattasio et al. (54) have found weak phosphorylation of Rad9 by Mec1 and formation of the Rad9-Rad53 complex, but in these cells Mec1 cannot phosphorylate Rad53. In contrast, Tel1 can phosphorylate Rad9 and Rad53 in these cells. This result suggests a role for the 9-1-1 clamp in both Rad9 phosphorylation and the subsequent Rad53 phosphorylation, and it further suggests that Tel1 might be significantly less reliant on the 9-1-1 clamp than Mec1 during Rad53 phosphorylation (54). Consistent with that observation, Tel1-dependent suppression of the MMS sensitivity of *mec1*Δ cells also does not require Rad24 (146). The 9-1-1 clamp may also promote the phosphorylation of other proteins by Mec1 and Tel1. For example, *S. pombe* Cut5 interacts with the 9-1-1 complex, and this interaction is required for its phosphorylation by Rad3 (45).

ADAPTORS AND SIGNAL TRANSDUCING KINASES

Mec1 Activates Rad53 via Rad9

After damage detection by upstream sensors, a kinase cascade amplifies and relays the signal to checkpoint targets, notably the cell cycle machinery. In budding yeast the primary transducer is the Chk2-family kinase Rad53 whose activation requires Mec1 and the Rad9 "adaptor" protein (**Figure 2*c***). Unlike *mec1*Δ, *rad53*Δ and *rad9*Δ mutant cells do have some cell cycle arrest in response to a single DSB though it is strongly compromised (49, 121; F. J. Dotiwala & J. E. H., unpublished). During checkpoint activation, Rad53's phosphothreonine-binding FHA domains interact with PIKK-phosphorylated Rad9 leading to catalytic activation of Rad53 and extensive Rad53 autophosphorylation (**Figure 2*c***) (39, 136). The two FHA domains of Rad53 are only partially redundant for its activation. In DNA damage checkpoint assays loss of either FHA domain shortens the normal arrest time, and the double FHA1,2 mutant is as strongly checkpoint-defective as the Rad53-kd (kinase-dead) allele (113, 125). Mutation of just the the FHA2 domain, which strongly interacts with Rad9, reduces Rad53 phosphorylation and the Rad53-Rad9 interaction in MMS-treated cells but not in HU-treated cells (125, 136). Mutation of FHA1, which binds more strongly to Rad53 itself and to the S-phase regulators Asf1 and Dbf4, slightly sensitizes cells to HU and impairs the S-phase checkpoint. (38, 125, 136).

Rad53's FHA domains are likely to interact with a cluster of 7 SQ/TQ motifs in Rad9's central region, and mutation of the first 6 of

these is sufficient to prevent Rad9 phosphorylation, Rad9-Rad53 binding, Rad53 activation, and checkpoint arrest in damaged cells (124). Why the checkpoint is not governed by a simple interaction between Mec1 and Rad53 is unclear, but presumably the elaborate activation mechanism requiring Rad9 and the 9-1-1 complex (see above) allows greater regulatory flexibility and may reduce spurious signaling.

Recent in vitro studies have clearly demonstrated the adaptor function of Rad9 in the phosphorylation of Rad53 by Mec1 (137). Two studies have also mapped a large number of Rad53 phosphorylation sites before and after damage using mass spectrometry techniques (131, 137). The two studies did not find many of the same phosphorylated sites after damage. Whether this is due to different detection techniques or different damage stimuli (MMS vs 4NQO) is not clear (131, 137). Several predicted CDK target sites on both Rad9 and Rad53 are also phosphorylated in vivo, even in the absence of DNA damage (131, 137). We have previously argued that the rapid inactivation of Rad53 that follows CDK inhibition is the result of blocked resection (62), but it is also possible that direct phosphorylation of Rad9 or Rad53 by Cdk1 contributes to normal checkpoint activation.

A different approach to identify phosphorylation sites required for Rad53 activation has been to mutate the clusters of SQ and TQ residues that are found in Rad53 (79). Rad53 contains an N-terminal cluster of TQ sites and a C-terminal cluster of SQ sites. Both the TQ and SQ motifs contribute to Rad53 phosphorylation in vivo, and mutation of both clusters eliminates most phosphorylation of Rad53 by Mec1 in vitro (79). The TQ phosphorylation cluster interacts with the FHA domain of its signaling target Dun1 and is essential for Dun1 phosphorylation. The TQ cluster is also bound by the Rad53 FHA1 domain, presumably to promote Rad53 oligomerization and activation (10, 79). Both the SQ and TQ phosphorylation clusters also contribute to Rad53 autophosphorylation in response to DNA damage stimuli (79).Thus, PIKK-mediated (and perhaps CDK-mediated) phosphorylation serves several functions in Rad53 activation: activation of Rad53 kinase activity, promotion of oligomerization and trans-autophosphorylation, and creation of an interface for the Rad53-Dun1 interaction (79, 124).

Activated Rad53 also interacts with the nuclear import factors Srp1 and Kap95, and a major damage-induced phosphorylation site is found within Rad53's bipartite NLS (131). Mutation of this site prevents the threefold increase in Rad53 levels that is observed after MMS treatment. This suggests that Rad53 activation in part requires nuclear import and promotes Rad53 accumulation (131).

Mec1 Activates Chk1 via Rad9

Like Rad53, Chk1 requires Rad9 for its activation and the *chk1*Δ mutant only partially reduces the checkpoint arrest in response to a single DSB (49, 121). Unlike Rad53, however, Chk1 has no FHA domain, and it can be activated by an allele of Rad9 that lacks the SQ/TQ cluster and cannot activate Rad53 (124). Conversely, an N-terminal truncation of Rad9 prevents phosphorylation of Chk1 but not of Rad53 (13).

Both *S. cerevisiae* Rad9 and *S. pombe* Crb2 dimerize via C-terminal BRCT motifs, and this dimerization is required for checkpoint function in vivo (26, 37, 95, 134). Replacement of the BRCT domains with heterologous dimerization domains largely rescues Crb2 checkpoint function but does not rescue Crb2's IR-induced focus formation or full Crb2 phosphorylation (37). Addition of a dimerization domain to *S. pombe* Chk1 suppresses the UV sensitivity of a Crb2 allele lacking its N terminus (37). This suggests that Chk1, like Rad53, requires oligomerization and probably autophosphorylation for full activity, and accordingly, bulk phosphorylation of a Chk1 kinase-dead allele is strongly reduced after DNA damage (95).

Several protein-protein interactions are required during the activation of Chk1 in *S. pombe*, including Crb2/Rad3, Crb2/Chk1, Rad3/Chk1, Crb2/Cut5, and Cut5/9-1-1 (45, 95). The interactions between Rad3 and both Crb2 and Chk1 are dissociated by DNA damage while the Crb2/Chk1 and the Crb2/Cut5 interactions are increased (95). Similarly, in human cells Chk1 dissociates from chromatin after UV damage, and all activated Chk1 is soluble (130). This dispersal is required for normal checkpoint activation as a Chk1 fusion protein tethered to DNA can be activated by DNA damage but does not have full checkpoint function (130). Further study will be required to understand the dynamics of the various interactions and their significance to the checkpoint response.

Effectors of Cell Cycle Arrest

Chk1 regulates the stability of Pds1. Following activation of the checkpoint signaling kinases, cell cycle arrest is effected by direct regulation of the cell cycle machinery (**Figure 1**). Yeast securin, Pds1, is required for normal cell cycle arrest in response to DNA damage (25, 159). After DNA damage, Pds1 is hyperphosphorylated in a Mec1-, Rad9-, and Chk1-dependent, but Rad53-independent manner (25). Pds1 is also essential for prevention of anaphase during the spindle assembly checkpoint, but Pds1 hyperphosphorylation is specific to DNA damage (25, 121). In the unperturbed cell cycle, Pds1 protein is degraded at the entry into mitosis after being ubiquitinated by the Anaphase Promoting Complex (APC) in complex with its specificity factor Cdc20, but after DNA damage Pds1 is stabilized by phosphorylation that blocks its ubiquitination (2, 121). In DNA damage checkpoint assays, *pds1*Δ and *chk1*Δ cells show a partial defect, and these mutants are largely, but not entirely, epistatic, that is, the double mutant behaves very similarly to the more affected single mutant (49, 121; F. J. Dotiwala & J. E. H., unpublished). Furthermore, elimination of the predicted Chk1 phosphorylation sites on Pds1 strongly impairs the checkpoint and leaves Pds1 susceptible to proteolysis in vivo (154). Accordingly, Chk1-phosphorylated Pds1 is resistant to Cdc20-/APC-dependent ubiquitination in vitro (2).

Rad53 regulates Pds1 stability but also regulates mitotic exit. Like Chk1, Rad53 regulates Pds1 stability but does so by specifically blocking the interaction between Pds1 and Cdc20 in vivo (2). The molecular mechanism is unknown, but one site on Cdc20 has been identified as a likely substrate of Rad53 phosphorylation (106). Protein kinase A (PKA) has also been suggested to phosphorylate Cdc20 at this and one other site, and mutation of these two sites prevents the normal inhibition of Cdc20 during the checkpoint (126). The relative contributions of Rad53 and PKA to Cdc20 regulation during the checkpoint are not known, but Cdc20 appears to be an underappreciated target of DNA damage checkpoint signaling. Regulation of Cdc20 protein abundance is also seen in the yeast S-phase checkpoint and spindle-assembly checkpoint. suggesting that Cdc20 is a common regulatory target to prevent anaphase (22, 108).

Genetic studies suggest that Rad53 has targets other than Pds1 stability because *rad53*Δ cells have a more severe checkpoint defect than *pds1*Δ cells (49, 121; F. J. Dotiwala & J. E. H., unpublished). While Pds1 regulates mitotic entry, Rad53 also inhibits mitotic exit. Rad53 (but not Chk1) is required to maintain CDK activity during the checkpoint arrest and likely does so through inhibition of Cdc5 (19, 121). Cdc5 inhibits the Bub2/Bfa1 complex (52, 59, 60), which in turn inhibits the mitotic exit network [MEN; reviewed in (29)]. Rad53-dependent inhibition of Cdc5 could therefore inhibit progression through mitosis and help maintain checkpoint arrest.

Despite its prominent role, we understand surprisingly little about Rad53's targets in cell cycle control. Cdc5, Cdc20, Dun1, and perhaps Pds1 are likely targets of Rad53, but the molecular details are unknown. Additionally,

it is unclear whether Rad53 might have cell cycle targets other than Pds1 stability and MEN inhibition. We have recently found, however, that whereas *bub2*Δ and *pds1*Δ single mutant cells have a moderate checkpoint defect in response to a single DSB, *bub2*Δ *pds1*Δ double mutants are as defective as *rad53*Δ cells (F. J. Dotiwala & J. E. H., unpublished). Rad53's best-understood target is the kinase Dun1. As *dun1*Δ cells are nearly as checkpoint defective as *rad53*Δ cells, it is possible that Dun1, rather than Rad53, regulates many cell cycle targets (49; F. J. Dotiwala & J. E. H., unpublished).

In some situations, the spindle assembly checkpoint appears to contribute to cell cycle arrest following DNA damage. After microtubule damage, the Mad2 protein triggers preanaphase arrest by inhibiting Cdc20 and stabilizing Pds1. *mad2*Δ can also reduce cell viability and attenuate the DNA damage checkpoint in cells experiencing nucleotide depletion, DNA-damaging agents, an unrepaired DSB, or deprotected telomeres (3, 47, 90). Whether these Mad2-dependent arrests reflect authentic activation of the spindle assembly checkpoint is not known. Alternatively, deletion of Mad2 may free up more Cdc20/APC to promote Pds1 ubiquitination and mitosis. A role for Mad2 does not require damage to centromeric DNA or kinetochore disruption as *mad2*Δ shortens cell cycle arrest in cells experiencing a single DSB far from the centromere (F. J. Dotiwala, A. Arbel-Eden, J. C. H., M. Vaze, & J. E. H., unpublished data).

Using single cell checkpoint assays we have found that even *rad53*Δ *chk1*Δ cells enact a statistically significant delay in response to a single DSB (F. J. Dotiwala, A. Arbel-Eden, J. C. H., M. Vaze, & J. E. H., unpublished). The triple mutant combinations of *rad53*Δ *chk1*Δ with either *rad9*Δ, *rad17*Δ, or *pds1*Δ fully eliminate the residual arrest (F. J. Dotiwala, A. Arbel-Eden, J. C. H., M. Vaze, & J. E. H., unpublished). How these factors contribute to a Rad53 and Chk1-independent arrest remains to be determined.

Dun1 and damage-inducible genes. DNA damage provokes a significant transcriptional response. The best-characterized aspect of this response is the induction of elevated transcription of ribonucleotide reductase (RNR) genes (40). Some of the regulation of RNR also occurs posttranscriptionally as the checkpoint induces proteolysis of the RNR inhibitor Sml1. These responses are largely under the control of the Dun1 kinase (40, 163, 165). Maintenance of adequate RNR activity is essential for cell viability, and the inviability of *mec1*Δ and *rad53*Δ can be suppressed by overexpressing an RNR gene, by ablating Sml1, or by deletion of Yku70 or Yku80 (27, 33, 164).

A single unrepaired DSB provokes a global transcriptional response. In G_2-arrested cells, where there are no changes in gene expression due to the progressive arrest of logarithmic cells prior to mitosis, about 150 genes are either induced or repressed. No genes important for DSB repair were found to be induced under these conditions (76). Moreover, although a 50–100-kb region of chromatin surrounding the DSB becomes modified by histone phosphorylation (see below), there are no significant local changes in gene expression until the region is degraded by 5′ to 3′ exonucleases (76). Thus chromatin modifications in yeast do not serve the purpose of shutting off local transcription that might interfere with DSB repair. Two studies of the transcriptional response of cycling yeast cells to ionizing radiation or MMS reported a much larger set of transcriptionally responsive genes (51, 65).

γ-H2AX and other histone modifications. In addition to regulation of signaling proteins, DNA damage also leads to Mec1- and Tel1-dependent phosphorylation at serine 129 of the histone variant H2AX. Phosphorylated H2AX, termed γ-H2AX, is detected very soon after DNA damage and is found over a large region of chromatin flanking the DSB—approximately 1 Mb in mammalian cells and 50–100 kb in yeast (117, 128). γ-H2AX has been shown to contribute to DNA repair in

both fungal and animal cells and is required for full viability of yeast and animal cells in the presence of DNA damaging agents (17, 18, 36, 102, 116). γ-H2AX also plays a conserved role in the DNA damage checkpoint; yeast or animal cells that cannot generate γ-H2AX display mild checkpoint defects (17, 102). In both systems it has been proposed that γ-H2AX functions primarily in checkpoint maintenance because in its absence the checkpoint is activated normally but extinguished prematurely (17, 102).

A major role for γ-H2AX is the recruitment of chromatin remodelers, including the Ino80, Rvb1, NuA4, and Swr1 complexes to the DSB (12, 17, 35, 97, 110, 147). None of these factors is known to be strongly required for proper checkpoint function, however (97, 147). γ-H2AX also recruits cohesin and the Smc5/6 complex to DSBs, and both of these SMC complexes cover a similarly large chromosomal region as γ-H2AX (135, 145) (L. Aragon and C. Sjøgren, personal communications).

In yeast the only aspect of DSB repair that appears to be affected by γ-H2AX formation is repair between sister chromatids (135, 145). Cells expressing the nonphosphorylatable histone H2A-S129A have normal repair of meiotic DSBs as well as normal HO-induced recombination but exhibit a fourfold reduction in γIR-induced repair (135, 145; R. Shroff & M. Lichten, personal communication).

A second damage-induced histone phosphorylation, at serine 1 of histone H4, has been seen in budding yeast (20). This phosphorylation appears more slowly than γ-H2AX and depends on casein kinase II (CKII). How CKII is activated by DNA damage is not yet known, nor is it clear if this modification contributes to DSB repair or to checkpoint-mediated arrest.

Other chromatin modifications do contribute to checkpoint function. Methylated lysine 79 of histone H3 (H3-K79^{Me}) is bound by the Tudor domain of the yeast and human adaptor proteins Rad9 and 53BP1 and contributes to their recruitment to DSBs (61, 158). In budding yeast, elimination of H3-K79^{Me} by deletion of the Dot1 methyltransferase results in defects in several checkpoints, presumably due to aberrant recruitment of Rad9 (53, 158; F. J. Dotiwala & J. E. H., unpublished).

In fission yeast, methylation of lysine 20 of histone H4 (H4-K20^{Me}) also contributes to the checkpoint response by recruitment of the Crb2 adaptor protein to damaged DNA (123). Neither H4-K20^{Me} in *S. pombe* nor H3-K79^{Me} in *S. cerevisiae* is stimulated by DNA damage but exists at a basal level in normal chromatin (123, 148, 158). This suggests that the recruitment of Rad9-related adaptors by these histone modifications may require local chromatin decondensation for exposure (123). Acetylation of histone H3 lysine 56 (H3-K56^{Ac}) may also promote DNA accessibility in chromatin, though in this case the relevant targets appear to be repair rather than checkpoint proteins (91). In summary, several different chromatin modifications contribute to checkpoint arrest. Two of them are implicated in the recruitment of checkpoint adaptor proteins to DNA. Whether these modifications play an authentic role in checkpoint maintenance or a partially redundant role in checkpoint activation is unknown. Recent work in mammalian cells has shown that gamma-H2AX directly interacts with the checkpoint adaptor protein MDC1, a BRCT domain-containing protein that may function analogously to Rad9 (84a, 135a).

CHECKPOINT MAINTENANCE AND REPRESSION

Maintenance

Although ssDNA is required to activate the checkpoint, it is insufficient to maintain it. Several hours after the checkpoint has been activated by a single DSB, Rad53 hyperphosphorylation rapidly disappears when ongoing 5′ to 3′ resection is arrested by Cdk1 inhibition, despite the continuing presence of extensive ssDNA (62). One interpretation of these

results is that the proteins bound to newly generated ssDNA are different from those associated with "old" ssDNA. There could be a difference in the state of modification of ssDNA binding proteins such as RPA; alternatively, RPA could be displaced by Rad51 on "old" ssDNA. Another possibility is that the 5′ to 3′ exonuclease clips off di- or trinucleotides, an unusual DNA metabolite whose continuing generation might be needed to maintain an active DNA damage checkpoint. Continuous Mec1 activity is also needed for checkpoint maintenance as Mec1 inactivation either by the PIKK inhibitor caffeine or through use of a Mec1-degron releases the checkpoint arrest (111, 150).

Adaptation

In the presence of an unrepairable DSB, yeast cells enact a long checkpoint arrest lasting 12–14 h but then re-enter the cell cycle, or "adapt," despite the persistence of unrepaired DNA (75, 122, 139). Several proteins are required for adaptation, and their mutation prevents Rad53 inactivation and cell-cycle re-entry. Many of these proteins function in chromatin regulation and recombination, such as Yku70 and Yku80, the Swi2/Snf2/Rad54 homolog Tid1, Rad51, the Srs2 helicase, and Sae2. Others have checkpoint or mitotic roles, such as the PP2C-family phosphatases Ptc2 and Ptc3, the CKII subunits Ckb1 and Ckb2, and the Polo kinase Cdc5 (24, 75, 77, 78, 80, 111, 139, 150). The adaptation defect in *yku70*Δ cells is apparently the result of significantly increased resection at an unrepairable DSB and is comparable to that seen in cells resecting two DSBs at a normal rate (75). Reducing this resection by deletion of Mre11 suppresses the *yku70*Δ adaptation defect, suggesting that the rate or extent of resection contributes to maintenance of the checkpoint signal and therefore to adaptation (75). No other adaptation mutant is known to have increased resection, however, suggesting that a variety of factors govern adaptation. Dephosphorylation of checkpoint proteins clearly contributes to adaptation, and the phosphatases Ptc2 and Ptc3 are responsible for at least one important dephosphorylation event (80). Ptc2 interacts with the FHA1 domain of Rad53 and presumably inactivates Rad53 by dephosphorylation (80). Ptc2 phosphorylation by CKII (which includes the Ckb1 and Ckb2 subunits) promotes its interaction with Rad53 in vitro, and these interactions likely explain the adaptation and recovery roles of Ptc2, Ptc3, and CKII (see below) (80).

Adaptation: checkpoint inactivation and cell cycle re-entry in the presence of DNA damage

In strains suffering an unrepairable DSB, Ddc2-GFP foci are maintained during the entire checkpoint arrest. At the time of adaptation, however, these foci show reduced intensity and in many cases disappear (94). In contrast, Ddc1-GFP foci do not dissociate but maintain intensity or brighten during and beyond adaptation (94). These results suggest that regulation of Mec1-Ddc2 rather than the 9-1-1 clamp is likely to govern the timing of adaptation. One possible Mec1 regulator in this process is Sae2. As mentioned above, Sae2 promotes the dissociation of the MRX complex from DNA. *sae2*Δ cells, which frequently fail to adapt, maintain Rad53 phosphorylation in the presence of a single DSB and can do so in the absence of either Mec1 or Tel1 (but not both). Additionally, overexpression of Sae2 can override the checkpoint arrest following UV irradiation and can do so in the presence or absence of Tel1 (24). These results suggest that Sae2 may function to inhibit Mec1-Ddc2, perhaps by removing the complex from DNA.

The essential role of the DNA damage checkpoint is to prevent the segregation of broken or damaged chromosomes. Adaptation promotes the mis-segregation of acentric chromosome fragments in as many as 95% of divisions, and the mis-segregation of even centric chromosome fragments is seen in 42% of divisions (67). This clearly leads to increased genomic instability as has been demonstrated for both chromosome loss and translocations (46). Despite these phenotypes, adaptation is required for full viability of yeast

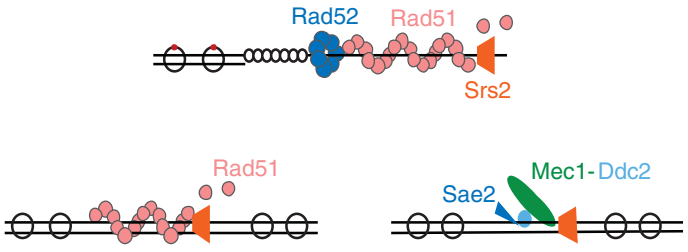

Figure 3

Models for the role of Srs2 in checkpoint recovery. (*a*) Rad51 (*pink*) is loaded onto ssDNA by Rad52 (*blue*) and displaces RPA. The helicase Srs2 antagonizes Rad51 by removing it from DNA. (*b*) Following successful repair, Srs2 (*orange*) promotes checkpoint recovery, apparently by removing Rad51 (*pink*) from DNA. Whether this Rad51 is bound to paired duplex dsDNA (as shown) or intact but unpaired ssDNA strands at the repair site is unknown. Neither is it known how Rad51 promotes checkpoint maintenance. Srs2 may also promote recovery by removing checkpoint signaling proteins from DNA following repair, for example, Mec1-Ddc2. Sae2 (*blue*) promotes checkpoint recovery and also may do so by negatively regulating Mec1 association with DNA.

cells in response to persistent DNA damage, suggesting that very slow or delayed repair of DNA damage, even after adaptation, aids cell viability (46).

Because it promotes genomic instability, adaptation has been considered unlikely in metazoans, but recent work in the *Xenopus* egg extract has demonstrated adaptation to the S-phase checkpoint. In response to the replication inhibitor aphidicolin, cell cycle arrest is mediated by ATR-dependent activation of Chk1 with the assistance of the adaptor protein claspin. ATR also phosphorylates claspin, and this phosphorylation facilitates the interaction between claspin and the Polo kinase Plx. Plx then phosphorylates a neighboring site on claspin, which promotes claspin's dissociation from chromatin and the attenuation of Chk1 signaling that allows adaptation (161). Whether the yeast Polo kinase Cdc5 contributes to adaptation by a similar mechanism is unknown.

Recovery

When DSB repair is successful, cells turn off the checkpoint and re-enter the cell cycle in a process termed checkpoint recovery. Genetic analysis has shown that many adaptation-defective mutants, including *yku70*Δ, *tid1*Δ, and *cdc5-ad*, are not defective in recovery (150). Some adaptation mutants exhibit slow recovery (*ckb1*Δ, *ckb2*Δ, and *rad51*Δ) (150; M.-C. Marsolier-Kergoat, personal communication), but only *srs2*Δ, *ptc2*Δ *ptc3*Δ, and *sae2*Δ have a strong recovery defect (24, 80, 150; J. C. H. & J. E. H., unpublished). Biochemical analysis of the Srs2 helicase shows that it can remove Rad51 from ssDNA in vitro, and deletion of Rad51 substantially alleviates *srs2*Δ's recovery defect (**Figure 3*a*, *b***) (71, 150, 151). One possibility is that Rad51 remains on DNA in *srs2*Δ mutant cells, even after successful repair, and promotes maintenance of the DNA damage checkpoint signal through an unknown mechanism. Given that DSB repair products are intact and apparently lack ssDNA (150), it is possible that Rad51 is associated with dsDNA (**Figure 3*b***).

In contrast, the PP2C-family phosphatases Ptc2 and Ptc3 (and perhaps CKII) work at the level of Rad53 (see above) to extinguish the checkpoint signal. The human homolog of Ptc2 and Ptc3, Wip1/PPM1d, has also been implicated in checkpoint recovery. Wip1 expression is induced after DNA damage in a p53-dependent manner, and Wip1 subsequently reverses PIKK-mediated phosphorylation of both p53 and Chk1 (85). Depletion of Wip1 leads to prolonged phosphorylation of both p53 and Chk1 after DNA damage and prolongs the checkpoint arrest by maintaining inhibition of Cdc2 (85). Similarly, depletion of the human Polo kinase Plk1 also impairs checkpoint recovery (149). Plk1 promotes the degradation of the CDK-inhibitory kinase Wee1, and thereby allows Cdc2 activation and mitotic entry after successful DNA repair (149). The activity of Plk1 is known to be inhibited by DNA damage (129), and it will be of great interest to determine whether regulation of Plk1 (and perhaps Cdc5) activity as DNA repair is completed governs the timing of checkpoint recovery.

In *S. pombe*, the PP1-family phosphatase Dis2 controls the timing of checkpoint

recovery by dephosphorylating and inactivating Chk1 (32). Dis2 phosphatase activity is not obviously regulated by DNA damage (31). This suggests that basal Dis2 activity promotes checkpoint inactivation only when the acute stage of checkpoint activation (and presumably Rad3 activation) is over. In budding yeast, PP1 governs recovery from a checkpoint monitoring repair of meiotic DSBs (5, 58), and we have found that overexpression of the PP1 catalytic subunit Glc7 can hasten the onset of adaptation (J. C. H. & J. E. H., unpublished). How PP1 promotes adaptation or recovery is unclear, though dephosphorylation of Rad53, Rad9, or Chk1 is an obvious possibility. *Xenopus* PP1 promotes mitotic entry by dephosphorylation of the CDK activator Cdc25 (89), but the budding yeast homolog of Cdc25, Mih1, is unlikely to be relevant to recovery as it is not known to participate in any aspect of the DNA damage checkpoint.

Dephosphorylation of γ-H2AX also influences the duration of the checkpoint. Studies in yeast have identified a novel, evolutionarily conserved PPP4C phosphatase complex, consisting of Pph3, Psy2, and Ybl046w, that dephosphorylates γ-H2AX in vitro and in vivo (55, 68). Cells lacking any of these subunits have excess γ-H2AX even in the absence of DNA damage and show persistent γ-H2AX foci in irradiated cells. Additionally, the DNA damage checkpoint is significantly prolonged despite normal DSB repair (68). Detailed studies have shown that γ-H2AX is removed from chromatin during homologous repair of a DSB in both wild-type and *pph3*Δ cells. Whether this γ-H2AX maintains checkpoint activity while soluble, or whether it is reincorporated into chromatin at other loci has not yet been determined (68). Studies in human cells have identified the PP2A phosphatase complex as the relevant γ-H2AX phosphatase. Unlike in yeast, however, the prolonged checkpoint in mammalian cells with excess γ-H2AX is apparently due to defects in DNA repair (21).

An allele of *S. pombe* Cdc20, *slp1-362*, was identified as a recovery mutant that specifically prevents recovery after UV irradiation but not HU arrest (92), further underscoring the importance of Cdc20 regulation in cell cycle arrest and re-entry.

Finally, retrograde vesicular transport is essential for both adaptation and recovery in budding yeast. We have found that deletion of either the ARF-GAP Gcs1 or any member of the golgi-associated retrograde protein (GARP) complex prevents adaptation, and that the GARP mutants block checkpoint recovery (J. C. H., A. Arbel-Eden, V. Ranade & J. E. H., in preparation). These factors function in different retrograde vesicular transport events (golgi to ER and endosome to golgi, respectively), and their mutation may disrupt checkpoint inactivation by altering the subcellular localization of an adaptation or recovery protein. One possibility is that the secretory pathway defect activates the arrest of secretion response (ASR). This stress response pathway has been shown to respond to secretory blockage by reducing nuclear import (103), and it is possible that a protein whose nuclear localization is required for adaptation and recovery is sequestered in the cytoplasm. Indeed, overexpressing Gsp2, the Ran GTPase that promotes protein import into the nucleus, suppresses the adaptation defect of *gcs1*Δ cells.

SUMMARY POINTS

1. ssDNA generation is essential for activation of the DNA damage checkpoint.
2. Activation of Mec1/ATR depends on recruitment to ssDNA by RPA.
3. The 9-1-1 checkpoint clamp facilitates Mec1 phosphorylation of multiple substrates, including adaptors and checkpoint transducing kinases.

4. Rad53 and Chk1 are activated by distinct Mec1 and Rad9-dependent mechanisms.
5. Chromatin modifications, especially histone phosphorylation and methylation, contribute to checkpoint activation and maintenance.
6. Rad53 and Chk1 regulate cell cycle arrest by shared and distinct mechanisms.
7. Checkpoint recovery appears to require the active reversal of several checkpoint activation steps.
8. Checkpoint adaptation shares some, but not all, genetic requirements with recovery.

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