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Perspectives on the DNA damage and replication checkpoint responses in *Saccharomyces cerevisiae*

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Abstract

The DNA damage and replication checkpoints are believed to primarily slow the progression of the cell cycle to allow DNA repair to occur. Here we summarize known aspects of the *Saccharomyces cerevisiae* checkpoints including how these responses are integrated into downstream effects on the cell cycle, chromatin, DNA repair, and cytoplasmic targets. Analysis of the transcriptional response demonstrates that it is far more complex and less relevant to the repair of DNA damage than the bacterial SOS response. We also address more speculative questions regarding potential roles of the checkpoint during the normal S-phase and how current evidence hints at a checkpoint activation mechanism mediated by positive feedback that amplifies initial damage signals above a minimum threshold.

Introduction

The concept of DNA damage checkpoints was first developed through the identification of G₂/M arrest after X-ray irradiation in the budding yeast *Saccharomyces cerevisiae*. This arrest required the *RAD9* gene, and the sensitivity of *rad9* mutants was reduced by delaying the onset of mitosis after irradiation [1]. This led to the view that *RAD9* and similar genes define DNA damage checkpoints, which delay specific cell cycle transitions in response to DNA damage to provide time for DNA repair to occur. In *S. cerevisiae*, DNA damage checkpoints delay the G₁/S transition and block the G₂/M transition of the cell cycle [1,2]. In addition, two types of S-phase checkpoints have been defined: the DNA replication checkpoint, which arrests cell cycle progression and inhibits firing of late replication origins in response to replication stress [3], and the intra-S checkpoint, which slows DNA replication and cell cycle progression in response to DNA damage [4]. The different DNA damage checkpoints share many components and are now known to target many aspects of cellular metabolism besides cell cycle transitions. These checkpoints also likely respond to endogenous sources of DNA damage as well as exogenous sources, as checkpoint defects result in increased spontaneous genome instability [5].

A “central dogma” for the DNA damage cell cycle checkpoints has been commonly presented:

damage signals → damage sensors → signal transducers → effectors

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In this scheme, damage is sensed by sensors, and this information is communicated through signal transducers to effectors that mediate the physiological response of the cell to the damage, including arresting or slowing the cell cycle and activating or repressing other pathways required for the eventual recovery of the cell. It has been convenient to think of checkpoints as unidirectional pathways, but this is an oversimplification. DNA repair proteins, for example, can act as both sensors and effectors. Similarly, checkpoint proteins that are components of replication complexes are both sensors and transducers and might even be effectors. This complexity suggests checkpoint responses likely involve complex regulatory networks that incorporate both feedback loops and threshold responses.

Here, we will summarize the core checkpoint machinery in *S. cerevisiae* to serve as a framework for examining some key features of checkpoint responses in greater detail. We will consider how checkpoints are activated and what is known about the effectors that are targeted by checkpoint activation. We will also consider the transcriptional response to DNA damage by reviewing which aspects of cell metabolism are transcriptionally regulated and how much of this actually represents a checkpoint response. Finally, we will also address more speculative questions regarding the potential roles of the DNA damage checkpoint during normal regulation and the use of positive feedback and threshold responses by checkpoint functions. We are particularly interested in how these checkpoint responses prevent genome instability. However, our goal is not to provide a comprehensive review of checkpoints but rather to highlight areas that are presently less well understood.

1. The central checkpoint pathway in *S. cerevisiae*

The key components of the common signaling pathway are several phosphoinositol-3-kinase-related kinase (PIKK) family members. *S. cerevisiae* has two PIKK proteins, Mec1, the homolog of human ataxia-telangiectasia and Rad3-related (ATR), and Tel1, the homolog of human ataxia telangiectasia mutated (ATM), that function as both damage sensors and signal transducers, but lacks a homolog of the DNA-dependent protein kinase, DNA-PKcs (Table 1). Loading of Mec1 and Tel1 onto damaged DNA does not appear to be mediated by direct recognition of DNA damage but rather by the recognition of complexes recognizing DNA damage and intermediates generated by the activities of DNA repair processes. Mec1 binds to Ddc2, the homolog of the human ATR interacting protein (ATRIP), which recognizes single-stranded DNA (ssDNA) bound by replication protein A (RPA) [6], and Tel1 binds to the DNA end-binding Mre11-Rad50-Xrs2 complex [7]. Genetically *MEC1* and *TEL1* are partially redundant; the *mec1Δ tel1Δ* double mutant cannot maintain telomeres via telomerase unlike the single mutants [8,9], has a synergistically increased sensitivity to DNA damaging agents [10], and has a synergistically increased rate of spontaneous genome rearrangement relative to the respective single mutants [5]. Both kinases preferentially phosphorylate serines and threonines preceding a glutamine residue on numerous target proteins in response to damage. For example, phosphorylation of histone H2A at Ser129 (formation of γ -H2AX) by Mec1 and Tel1 has important roles for the checkpoint response detailed below [11]. The identity of all Mec1 and Tel1 targets and phosphorylation sites is certainly not known; however, improved proteomics approaches have led to considerable progress in identifying these targets. Despite their similarities, Mec1 and Tel1 are not completely redundant. Tel1 is more important for maintaining normal telomere lengths than Mec1 [8,12]. Tel1 is more important in γ -H2AX formation at sites of DSBs in G₁, and Mec1 is more important during S and G₂ [13]. These cell cycle-dependent differences may be due to the yeast cyclin-dependent kinase (CDK) Cdk1 (also known as Cdc28) activating resection from DNA breaks via the nuclease Sae2 and thereby generating damage recognized by Mec1-Ddc2 [13,14].

Activation of PIKK family members is also influenced by the action of other damage sensors. The PCNA-like Ddc1-Mec3-Rad17 complex (the *S. cerevisiae* homologs of the Rad9-Hus1-

Rad1 or 9-1-1 complex) is loaded onto partial duplex DNA via the Rad24-Rfc2-5 alternative replication factor C (RFC) complex independently of Mec1-Ddc2 [15,16]. Colocalization of Mec1-Ddc2-RPA and the 9-1-1 complex in the context of partial duplex DNA or to chromosomal arrays of Lac operator sequences in the absence of DNA damage results in Mec1 activation [17,18], indicating that DNA plays a passive scaffolding role in checkpoint activation. These results make the lack of checkpoint activation from normal telomeres that bind numerous DNA repair and DNA damage checkpoint proteins even more surprising [19]. Activation of Mec1 is also mediated by Dpb11, the *S. cerevisiae* homolog of TopBP1, and Dpb11 is synergistic with the 9-1-1 complex [20,21]. The combination of the 9-1-1 complex and Dpb11 in activation of Mec1 is highly conserved, and differs from *fission yeast*, *Xenopus*, and human systems only in that the 9-1-1 complex in those organisms cannot activate the Mec1 homolog in the absence of the Dpb11 homolog [22-24].

In addition to phosphorylating effectors of the checkpoint response (described below), the PIKK proteins also activate downstream kinases, including Chk1 and Rad53 (the *S. cerevisiae* homolog of mammalian Chk2), which presumably diffuse away from the site of their activation. In other organisms, Chk1 homologs are required to inhibit CDKs to prevent cell cycle progression in the presence of DNA damage; however, budding yeast lacks this requirement [25,26]. Budding yeast Chk1 does, however, have direct roles in suppressing the cell cycle in the context of DNA damage as described below. Phosphorylation of the Rad53 protein kinase by Mec1 and Tel1 leads to its activation and subsequent autophosphorylation; the resulting hyperphosphorylated Rad53 is frequently used as an experimental surrogate for monitoring activation of the DNA damage response. At least some inactive, hypophosphorylated Rad53, but not active hyperphosphorylated Rad53, is bound to the chromatin assembly factor Asf1 [27-29]. Rad53 also inhibits Asf1-mediated chromatin deposition *in vitro*. Despite the the binding of inactive Rad53 by Asf1, deletion of *ASF1* does not give rise to a checkpoint response [27], but rather causes defects in checkpoint shut off due to its roles in chromatin assembly and modification [30].

Activation of Rad53 also depends upon Mec1 or Tel1 phosphorylating a scaffolding protein, Rad9 or Mrc1, which then binds the forkhead-associated (FHA) domains of Rad53; simultaneous deletion of both *RAD9* and *MRC1* extensively phenocopies a deletion of *RAD53* [31], and mutagenesis of Mec1 and Tel1 consensus phosphorylation sites in Rad9 and Mrc1 prevent Rad53 activation [32,33]. A Ddc2-Rad53 fusion construct alleviates many of the defects of a *rad9Δ mrc1Δ* double mutation [34], and a similar construct suppresses *mrc1Δ* defects in fission yeast [35]. Mrc1 is a component of the replication fork, has no identifiable protein domains or motifs, and seems to specifically signal replication stress [31,33,36]. Rad9, on the other hand, is more important for other types of DNA damage, although it is required for Rad53 hyperphosphorylation late in S-phase in *mrc1Δ* strains [33], which could indicate that DNA repair processes convert replication stress into damage recognized by Rad9. Rad9 contains tandem BRCT domains that bind phosphorylated residues, mediate dimerization after DNA damage-induced phosphorylation [37], and direct binding to phosphorylated histone H2A [38]. Rad9 also contains tandem Tudor domains, the latter of which recognize methylated histones [39]. In the G₁/S and intra-S checkpoints, phosphorylation of Rad9 is dependent upon methylation of histone H3 at Lys79 by Dot1 or Lys4 by Set1, which in turn is dependent upon ubiquitination of histone H2B at Lys123 by Rad6 and the Bre1/Lge1 complex [40,41]. Strains with deletion of *RAD6*, *BRE1*, both *DOT1* and *SET1*, or strains encoding the histone H2B Lys123Arg variant have defects in the G₁/S and the intra-S checkpoint after UV treatment and fail to hyperphosphorylate Rad53 [40,41]. Surprisingly, deletion of *BRE1* or *DOT1* causes only minor defects in the G₂/M checkpoint in response to a DSB, despite the role of *BRE1* in G₁/S and the intra-S checkpoints [40], and its role in conjunction with *DPB11* in M phase [42]. Intriguingly, the mitotic roles for a phosphorylated adaptor protein appears to be conserved for

the activation of the meiotic Rad53 homolog, Mek1, by phosphorylation of Hop1 by Mec1/Tel1 [43].

Rad53 recruits Dun1 through binding by the Dun1 FHA domain [44], and Rad53 activates the Dun1 protein kinase through phosphorylation of the activation loop [45]. Activation by Rad53 is the principle way in which Dun1 is activated; however, the phenotypes of mutations affecting the FHA domain of Dun1 have been used to argue for some Rad53-independent roles of Dun1 [44]. The best characterized Dun1 substrate is the ribonucleotide reductase (RNR) inhibitor Sml1, which upon phosphorylation is targeted for degradation; consequently Sml1 is a key experimental target for following DNA damage signaling [46]. Deletion of *RAD53* or *MEC1* is lethal without co-deletion of *SML1* or overexpression of RNR [47]; however, deletion of *DUN1* does not require co-deletion of *SML1* for viability, suggesting either that deficiency in Mec1 or Rad53 but not Dun1 causes defects requiring increased RNR function or that other pathways redundant with Dun1 inactivate Sml1, albeit without mediating Sml1 degradation. The net effect of activating the central kinase cascade in the DNA damage response is to amplify an initial chromatin-associated signal via the action of chromatin-bound and diffusible protein kinases that affect other processes to facilitate repair of the damage.

2. Targets of the DNA damage and replication checkpoint pathways

Identifying targets of checkpoint pathways is complicated by the fact that protein phosphorylation is a ubiquitous modification in the cell. In addition to genetic screens and candidate gene studies, a number of mass spectrometry experiments have identified phosphorylation sites dependent upon checkpoint activation [48,49]. The overlap between these global screens and traditional studies is imperfect, possibly due to the complexity of the peptide mixtures being analyzed by mass spectrometry; however, these studies have identified new candidate targets for checkpoint pathways. Checkpoint-dependent phosphorylation sites are not necessarily functionally significant, however, as some proteins may be adventitious targets. Verification of the roles of individual targets is complicated by several factors, including multiple redundant phosphorylation sites, cell-cycle phase dependence, potential dependence on the types of DNA lesions, and the need to analyze both non-phosphorylatable and phosphomimicking amino acid substitutions at many different positions within an individual protein. The identification and validation of checkpoint targets remains an important area of study.

2.1 Cell-cycle targets of the checkpoint response

The arrest of the cell cycle is believed to be a primary role of checkpoint responses, and the checkpoint protein kinases play multiple roles in suppressing progression of the cell cycle through mitosis. Phosphorylation of Pds1, an anaphase suppressing protein, by Chk1 blocks its APC-dependent degradation [50]. Pds1 is further stabilized through Rad53-dependent inhibition of the Chk1-Cdc20 interaction, which recruits the APC ubiquitin ligase [51]. In addition, both Rad53 and Chk1 also appear to suppress the later stage of mitotic exit by preventing the release of the Cdc14 phosphatase from a complex with Net1 in the nucleolus thus inhibiting the mitotic exit network (MEN) and Cdc-fourteen early anaphase release (FEAR) pathways, respectively [52]. An additional role for Rad53 in suppressing MEN through regulation of the Cdc5 POLO-like kinase has been proposed [50]; however, this is controversial [52]. Release of Cdc14 marks the end of mitosis through dephosphorylation of Cdk1 targets, leading to the degradation of mitotic cyclins, induction of transcription of the *SIC1* gene encoding a Cdk1 inhibitor, and stabilization of the expressed Sic1 protein [53]. The DNA damage checkpoint also appears to interact with the spindle assembly checkpoint, as Rad9 and Rad53 are phosphorylated after nocodazole arrest, which both activates the spindle assembly checkpoint and prevents the degradation of Pds1. This phosphorylation is independent of Mec1 and Tel1, but is abolished by deletion of the spindle assembly checkpoint genes *MAD2* or

BUB1 [54]. These types of checkpoint crosstalk may provide a mechanism for generating a more robust cell cycle arrest phenotype.

Checkpoint activation also impinges upon the Dbf4-dependent kinase (DDK), which activates origins of replication [55]. In budding yeast, Dbf4 is phosphorylated in a *MEC1*-dependent fashion in strains with persistent DNA damage [56]. Similarly, Dbf4 phosphorylation after HU treatment is *RAD53*-dependent [57]. The *in vivo* role of these modifications has not been established, but it is attractive to speculate that this modification might suppress late origin firing in the replication checkpoint response. Remarkably in both budding and fission yeasts, it has been suggested that complete activation of Rad53 requires DDK [58,59], consistent with a notion that Rad53 activation is sensitive to the efficiency of origin firing [60]. This result, however, is controversial and several studies have shown efficient cell cycle arrest in the DDK deletion background [57,61]. Remarkably, bypassing the lethality of DDK deletions via *mcm5^{bob1}* requires the kinase activity of Rad53, but not Mec1 or Tel1, which has been used to argue that Rad53 has a DNA damage-independent role in cellular replication [60]; however, it is not clear if this activity is important in wild-type cells.

2.2 DNA repair targets of checkpoint activation

The arrest of the cell cycle is likely required to allow DNA repair to occur. Numerous proteins directly involved in this repair have been identified as targets of the checkpoint kinases (Table 2). In some cases, such as Rad55, Rtt107, and Nej1, the phosphorylation appears to play important functional roles [62-65]. In others, such as Rfa1, Rfa2, and Xrs2, the role of the phosphorylation during mitotic DNA repair is less clear [66-69]. Taken as a whole, the data accumulated to date (Table 2) are insufficient to define the specific pathways targeted by checkpoint activation. Similarly, the data are not sufficient to discern potential differences in the downstream responses to specific types of DNA damage. The data do, however, indicate that the activities of at least some DNA repair proteins in *S. cerevisiae* are modified after DNA damage, suggesting a post-translational regulatory mechanism for DNA repair proteins rather distinct from the classic transcriptional SOS response in bacteria driven by DNA damage [70].

2.3 Chromatin targets of the damage checkpoint

One of the more important direct substrates for the activated Mec1 and Tel1 is histone H2A Ser129 [11], which is equivalent to phosphorylation of the alternative metazoan H2A subunit H2AX on Ser 139, called γ -H2AX, and has been extensively used as an experimental surrogate for checkpoint activation. In response to an induced DSB, Mec1 and Tel1 generate a unique γ -H2AX-containing chromatin domain that can extend at least 10 kb from the lesion [71]. In metazoans, this modification suppresses the formation of chromosomal rearrangements due to DSBs [72] but as yet there is little evidence for this in budding yeast [73].

In yeast, γ -H2AX domains recruit the Ino80, Swr1, and NuA4 chromatin modifying complexes via the Nhp10 and/or Arp4 subunits [71,74,75]. It has been suggested that Ino80 and the Swr1 chromatin remodeling complexes act antagonistically, with Swr1 replacing γ -H2AX with the alternative H2A subunit Htz1, although accumulation of Htz1 near the break only occurs in an *ino80 Δ* strain [76]. In contrast, other experiments have suggested that Ino80 facilitates ssDNA formation by Mre11-Rad50-Xrs2 at DSBs to promote HR and that Swr1 complex recruits Ku80 to promote NHEJ [77]. Disruption of the NuA4, Swr1, and Ino80 complexes had no effect on cell cycle arrest at G₁/S, although a NuA4 mutation caused a persistent checkpoint response [78]. The Ino80 complex subunit Ies4 is also a Mec1 and Tel1 substrate [79]; however, the role of this phosphorylation remains unclear. Overall, the exact roles of these chromatin remodeling complexes as part of the damage response is also currently unclear; mutations affecting these complexes have had little or no effect on assayed DNA repair reactions.

Post-replicative assembly of cohesin domains holding together sister chromatids occurs after treatment with ionizing radiation or induction of a DSB and is also dependent upon γ -H2AX domain formation and is important for efficient homologous recombination [80,81]. Cohesin recruitment at the break site is also dependent upon the cohesin loading gene *Scs2*, *Mre11* and partially on *Rad53* [81]. Cohesin recruitment induced by breaks is propagated throughout the entire genome in a *MRE11*-, *MEC1*-dependent and a *RAD52*- and *RAD9*-independent fashion and also requires the acetyltransferase function of *Eco1/Ctf7* [82,83], which is dispensable for normal cohesin loading during S-phase [84]. Inhibiting genome-wide cohesion between sister chromatids causes unbroken chromosomes to be lost at three-fold higher rates [83]. Phosphorylation of *Mdc1* (*Scs1*) by *Chk1* also appears to be required for DSB-induced sister chromatid cohesion [85] and directly links the checkpoint kinases to this process.

2.4 Cytoplasmic targets of the checkpoint response

The known checkpoint targets described above are nuclear, and the introduction of a single unreparable DSB within one nucleus of a budding yeast heterokaryon has established that the resulting G₂/M arrest does not influence DNA replication in the other nucleus in the cell [86]. Despite this, the effects of the checkpoint activation are integrated with a cytoplasmic response; *rad53Δ* strains have significant morphological defects [87], and *Rad53* phosphorylates a number of cytoplasmic targets including septins [48]. Deletion of *RAD53* or *CHK1* also cause inappropriate movement of the nucleus into the bud neck in a dynein-dependent fashion prior to anaphase [88]. Thus it is clear that *Rad53* is a component that helps coordinate the checkpoint response with the larger cell cycle response of the entire cell.

2.5 Transcriptional targets of the damage checkpoint

The transcriptional response of a number of individual *S. cerevisiae* genes relevant to repair of DNA damage has been well characterized as part of the checkpoint response. The *RNR1*, *RNR2*, *RNR3*, and *RNR4* genes, which encode subunits of ribonucleotide reductase, are normally repressed by *Crt1*, and this repression is alleviated by hyperphosphorylation by *Dun1* [89,90]. Similarly, repression of *PHR1*, encoding photolyase, is alleviated during DNA damage by phosphorylation of *Rph1* in a manner dependent on *RAD17*, *MEC1*, *RAD9*, and *RAD53* and independent of *CHK1* [91]. *MAG1*, encoding 3-methyladenine DNA glycosylase, and *DDI1* are expressed from a single divergent promoter, and *MAG1* expression has some dependence on the central checkpoint kinases and *Rad6*-*Rad18*-dependent ubiquitination of *Rad17* [92-94], although transcription factors mediating this response have not been identified. Additionally, expression of *CLN1* and *CLN2*, encoding cyclins, and transition through start are inhibited by methylmethanesulfonate (MMS) treatment in a mechanism partially dependent upon *RAD53* and putative *Rad53* phosphorylation sites on the *Swi6* transcription factor [95, 96].

The relevance of the individually analyzed transcriptional targets to the DNA repair response has led to a number of unfortunate parallels with the highly specific and coordinated bacterial SOS response to DNA damage [70], which uses a single mechanism to activate transcription of a number of critical operons encoding DNA repair factors. An early observation arguing against yeast employing a SOS-like response was a lack of concordance between genes upregulated in response to DNA damaging agents and the genes that conferred resistance to those agents [97]. Numerous large-scale transcriptional profiles of *S. cerevisiae* under conditions of DNA damaging agents have now been undertaken (for example [89,98,99]), and analysis of one study [99] that identified 293 up-regulated genes (Table 3) and 220 down-regulated genes (Table 4) in the cellular response to MMS exposure is illustrative.

In the 513 genes with altered transcription, only 10 up-regulated genes are involved in DNA replication and repair processes (*CDC13*, *DIN7*, *MAG1*, *MDC1*, *MSH2*, *POL30*, *RAD51*,

RAD54, *RFA1*, and *RFA2*; Table 3). The vast majority of genes appear to result from multiple independent responses. Around a fourth of the genes are cell-cycle regulated [100] and therefore might be affected indirectly by DNA damage, including 6 of the 10 DNA replication and repair genes (*MSH2*, *POL30*, *RAD51*, *RAD54*, *RFA1*, and *RFA2*). The MMS response also partially overlaps with the ~900 genes in the “environmental stress response” (ESR) that display differential expression in response to a variety of external stresses [101]. Roughly half of the genes affected by MMS are independent of transcription factors encoded by genes that when deleted cause MMS sensitivity [99]. In fact, a major aspect of the MMS response is halting protein synthesis and activating proteolysis, consistent with results from other studies involving multiple DNA damaging agents [98,102].

Some, but not all, of the above transcriptional responses are likely due the influence of the checkpoint response. Mutations of *MEC1* and *DUN1* affected the transcriptional response of roughly half of the ~2000 genes altered by ionizing radiation and MMS treatment in another study [89]. Of the ~1000 checkpoint-dependent genes, only 9 (*DIN7*, *DUN1*, *PCM2*, *RAD51*, *RAD54*, *RNR2*, *RNR4*, *YBR070C*, and *YER004W*) could not be attributed to a general stress response or to indirect regulation through loss of checkpoint function. Importantly, induction of the ESR genes was *MEC1*-dependent, suggesting that the DNA damage and replication checkpoints are responsible for more than simply halting the cell cycle, whereas other responses such as induction of protein degradation genes were unaffected by deletion of *MEC1* or *DUN1*.

Taken together, these data provide evidence that distinguish the eukaryotic transcriptional response to DNA damage from the bacterial SOS response, despite claims to the contrary [94]. Overall, the induced genes have very little to do with DNA repair *per se*, which suggests that the checkpoint control of DNA repair is primarily mediated by post-translational modifications of pre-existing proteins (described above). Additionally, these transcriptional responses lack a single master regulator of the response similar to LexA in the bacterial SOS response. Neither *MEC1* [89] nor *RAD6* [94] are completely responsible for the diverse range of observed transcriptional effects, and there is evidence for multiple downstream transcription factors [89]. Finally, the transcriptional response appears coordinated with more general stress responses and may reflect the tendency for DNA damage to coincide with other stresses in environments outside of the laboratory, similar to the fact that the resistance of the bacterium *Deinococcus radiodurans* to ionizing radiation is likely incidental to its ability to survive prolonged desiccation [103].

3. Do DNA damage and replication checkpoints have non-damage related roles during DNA replication?

To date, most studies on induction of checkpoint responses have relied upon a number of methods for artificially perturbing cells, such as induction of DSBs via endonucleases, induction of DNA damage, or induction of replication stress. These methods have been very useful for identifying key genes and mechanistic features of checkpoint responses; however, the artificial nature of these treatments may be obscuring the normal role of checkpoint signaling in a typical cell cycle.

Intermediates formed during the normal replication process, such as ssDNA, are substrates known to activate the DNA damage checkpoints. This might be a coincidence due to the structure of DNA itself; however, Mec1 and Rad53 appear to play roles affecting DNA replication in the absence of exogenous damage. These kinases are antagonistic to origin firing: late firing replication origins fire earlier in strains with *rad53* and *mec1* mutations [3], the *mec1-21* mutation suppresses the origin firing defects of an *orc2-1* mutation [104], and strains with deletions of *MEC1* or *RAD53* complete S-phase more quickly than wild-type cells during

MMS treatment only due to the firing of late and cryptic origins [105]. These kinases also have roles in replication fork progression: *mec1* and *rad53* mutations have slower fork progression, elongation defects, and difficulty in completely duplicating DNA [105,106], and *MEC1* prevents fork stalling and chromosome fragmentation at “replication slow zones”, which may be due to dNTP depletion at the end of S-phase as these defects are suppressed by *SML1* deletion [107]. In addition to *Mec1* and *Rad53*, *Dun1* increases dNTP pools by degrading *Sml1* and by promoting the transcription of RNR subunits [46,89,90]. Moreover, numerous proteins implicated as damage sensors or signal transducers are components of the replication fork or are involved in polymerase loading, including *Mrc1*, *Tof1*, *Csm3*, *Pol2*, *Pri1*, *Rfc2*, *Rfc5*, *Dpb11*, and *Sld2* [108-110]. Components of the DNA replication checkpoint may be part of a normal mechanism that allows active replication forks to regulate the replication of adjacent DNA, but likely do not signal the completion of DNA replication. Strains with *smc6* mutations initiate anaphase prior to completion of the replication of the entire genome, despite the presence of a functional DNA damage checkpoint response [111]. One intriguing possibility is that the difference between “replication control” and “damage signaling” may be the persistence of the signal at any site along the DNA, providing an elegant mechanism for recognizing inappropriately halted replication forks. Consistent with this, checkpoint defects result in spontaneous genome rearrangements in the absence of exogenously induced DNA damage [5].

Insights into the role of checkpoint signaling during normal replication may be provided through studies that stall replication forks via nucleotide depletion due to HU treatment. Mutants lacking *RAD53* and *MEC1* are highly sensitive to transient exposure to HU [112], and treatment of *rad53* mutants with HU is associated with the formation of “regressed forks” observed by electron microscopy [113]; however, these structures are formed in cells that never recover from HU and therefore do not necessarily represent repair intermediates. In the presence of functional checkpoints, HU treatment does not induce increased negative supercoiling during plasmid replication, indicating that the *Mcm2-7* replicative helicase is associated with the rest of the fork [114], and genetic results are consistent with a role of *MRC1* in arresting *Mcm2-7* and *Cdc45* in response to HU treatment in fission yeast [115]. Remarkably, deletion of *MEC1* or *RAD53* does not affect pausing of replisomes stalled at protein barriers or recovery from this pausing [116], which contrasts greatly from the failure of *mec1* and *rad53* mutants to recover after transient HU exposure. Thus, the ultimate target for replisome stabilization by checkpoints may be maintenance of the association of the *Mcm2-7* and/or *Cdc45* with the rest of the replication fork: the helicase is present at stably stalled forks [116]; these complexes are the major regulatory target for preventing re-replication of the genome [117]; and temperature shifting the degron-tagged version of the *Mcm2-7* complex in S-phase, but not G₁-phase is lethal, indicating that loss of the complex during DNA replication is irreversible [118]. That *S. cerevisiae* appears to lack a reloading mechanism for the replisome helicase reminiscent of the multiple PriABC pathways in bacteria [119] may not be so surprising given that bacteria have only a single bidirectional origin whereas *S. cerevisiae* has replication origins roughly every 40 kbp [120], making avoidance of re-replication, and not fork reloading, the primary concern during DNA replication in *S. cerevisiae*.

4. Do DNA damage and replication checkpoints function by positive feedback?

The checkpoint signal leading to cell cycle arrest must either be on or off. Mechanistically, both electronic and biological circuits mediate these responses through positive feedback [121], which feeds the downstream signal back into upstream amplification components. Positive feedback has recently been established in *S. cerevisiae* in the control of the G₁/S transition in coordinating the expression of S-phase specific genes [122] and the initiation of

anaphase in coordinating the degradation of Pds1, leading to sister chromatid separation [123]. Although this has not been directly tested, there is some evidence that would be consistent with a positive feedback mechanism in DNA damage and replication checkpoint responses.

The first feature of positive feedback is that the signal needs to be amplified by upstream components. Maintenance of a *MEC1*- and *RAD53*-dependent G₂/M arrest due to an induced double-strand break is dependent upon resection of the break in a Cdk1 and Mre11-Rad50-Xrs2 dependent fashion, which presumably continuously generates more RPA-ssDNA filaments [13,14]. Elimination of Cdk1 activity abolishes the checkpoint and continued resection from the DSB; however, it remains unclear whether newly liberated ssDNA and additional Mec1-Ddc2-RPA binding or some aspect of continued resection is the amplified signal. Similarly in metazoans, ATM (the Tel1-homolog) generates phosphorylated γ -H2AX, and ATM is also recruited to DNA marked by phosphorylated γ -H2AX via the adaptor protein MDC1, which provides a mechanism for amplifying the ATM signal [124]. Moreover, signal amplification would be anticipated to combine multiple input signals. In an *orc2-1* background, which has 30% of the normal replication forks, activation of the intra-S checkpoint shows a dosage dependence for the number of stalled forks and can be combined with bleomycin-induced damage to enhance the overall signaling [60].

The second feature of biological positive feedback is a mechanism to squelch the signal until it crosses an activating threshold. An argument can be made for this aspect of the mechanism based on the fact that the DNA damage driving the signal appears to function only as a passive scaffold to mediate colocalization of DNA damage sensors [18,125]. Thus, transient colocalization of damage sensors in cells could lead to inappropriate activation of the checkpoint if a silencing mechanism did not exist to dampen the positive feedback response until the initiating signal reached an activating threshold. A number of candidate proteins for suppressing the DNA damage signal exist, including the PP2A protein phosphatase complex Psy2-Psy4-Pph3, which dephosphorylates Rad53 and γ -H2AX when γ -H2AX is displaced from chromatin [126,127], and the PPC2-like protein phosphatases Ptc2 and Ptc3, which are required for “adaptation” to a constitutively activated DNA damage checkpoint and may also dephosphorylate Rad53 [128]. Remarkably, overexpression of Rad53 suppresses the ability of cells to adapt [129]; however, it remains to be seen if the genetic requirements for adaptation match those for suppression of a DNA damage positive feedback loop.

The third and final feature required for positive feedback during checkpoint activation is distinct from the positive feedback loops identified in the G₁/S transition and the initiation of anaphase: the DNA damage signal must be reversible. Thus, the “sensors” for the DNA damage must also be an integral part of the feedback amplification so that the signal can be silenced when repair is complete. Intriguingly, shifting a temperature-sensitive degron-Mec1 fusion to the non-permissive temperature decreases Rad53 activity even in the presence of persistent DNA damage [129], and inhibition of oligomerization of Rad9 via interactions of Rad9 BRCT domains with Rad9 molecules phosphorylated at Mec1/Tel1 consensus sites is required for the maintenance but not initial activation of a DNA damage checkpoint response [130].

The presence of a positive feedback loop in the DNA damage checkpoint changes the nature of the experiments that need to be pursued. First, the proteins that control the normally suppressive environment need to be closely investigated. Although we know some details of the phenotypes of some of the candidate genes that could be involved in suppressing the positive feedback signal, until their roles are clearly established, a diagnostic phenotype caused by loss of function of the genes mediating basal checkpoint suppression has not been identified; it is currently unclear if these genes should be essential, show synthetic lethality with adaptation mutations, show basal activation of the DNA damage response, and/or show hypersensitivity

to DNA damaging agents due to an inability to inactivate a checkpoint after damage is repaired. Second, the biological implications of modification of “upstream” sensors in checkpoint maintenance, such as RPA and replication fork subunits, need to be explored. Finally, in both studies that identified a positive feedback response in the G₁/S transition and anaphase initiation, the heterogeneity in population studies averaged out the evidence for positive feedback, suggesting that future studies may also require monitoring individual cells [122, 123].

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Table 1
Homologs of the components of the central kinase cascade

Class	<i>S. cerevisiae</i>	<i>S. pombe</i>	<i>H. sapiens</i>
Sensors	MEC1	RAD3	ATR
	DDC2	RAD26	ATRIP
	RAD24	RAD17	RAD17
	DDC1	RAD9	RAD9
	MEC3	HUS1	HUS1
	RAD17	RAD1	RAD1
	DPB11	CUT5/RAD4	TOPBP1
	TEL1	TEL1	ATM
	MRE11	MRE11	MRE11
	RAD50	RAD50	RAD50
	XRS2	NBS1	NBS1
	-	-	DNA-PKcs
Adaptors	RAD9	CRB2	53BP1
	MRC1	MRC1	CLSPN
Effector Kinases	CHK1	CHK1	CHK1
	RAD53	CDS1	CHK2

Table 2
Known DNA repair substrates of checkpoint activation

Substrate	Kinase	Effect of mutagenizing sites	References
Rfa1	Mec1, Tel1	Meiotic crossover effects ($^{122}\text{SQ} \rightarrow \text{DQ}$); no effect ($^{122}\text{SQ} \rightarrow \text{AQ}$)	[66,67]
Rfa2	Mec1, Tel1	No effect ($\text{SQ/TQ} \rightarrow \text{AQ}$) [*]	[67,68]
Xrs2	Tel1	No effect ($\text{SQ/TQ} \rightarrow \text{AQ}$)	[68,69]
Mre11	Tel1	Untested	[68,131]
Rad55	Rad53; possibly Mec1, Tel1	Slow S-phase traversal during damage ($\text{S} \rightarrow \text{A}$)	[62]
Slx4	Mec1, Tel1	Defect in single-strand annealing, but no defect with <i>sgs1Δ</i> or sensitivity to DNA damaging agents ($\text{SQ} \rightarrow \text{AQ}$)	[132]
Rtt107 (Esc4)	Mec1; requires Slx4	Sensitivity to DNA damaging agents ($\text{S} \rightarrow \text{A}$)	[63,64]
Nej1	Dun1	Decrease in NHEJ ($\text{S} \rightarrow \text{A}$), No effect ($\text{S} \rightarrow \text{E}$)	[65]
Exo1	Kinase pathway; possibly Rad53	Growth defects with <i>cdc13-1</i> ($\text{S} \rightarrow \text{A}$); No effect with <i>cdc13-1</i> ($\text{S} \rightarrow \text{E}$)	[133]
Srs2	Kinase pathway + Cdk1	Untested	[134]
Sae2	Mec1, Tel1	MMS hypersensitivity, synthetic lethality <i>rad27Δ</i> , decreased mitotic recombination ($\text{SQ/TQ} \rightarrow \text{AQ}$)	[135]
Cdc13	Mec1, Tel1	Telomere shortening, senescence ($\text{SQ/TQ} \rightarrow \text{AQ}$); Stable short telomeres ($\text{SQ/TQ} \rightarrow \text{DQ/EQ}$)	[136]
Mdc1 (Scc1)	Chk1	Defect in forming DSB-induced cohesion ($^{83}\text{S} \rightarrow \text{A}$); Cohesion formed in absence of DSB ($^{83}\text{S} \rightarrow \text{D}$)	[85]

^{*} In mammalian cells, phosphomimicking mutations have impaired ability to associate with replication centers; checkpoint phosphorylation proposed to contribute to inhibition of DNA replication [137].

Table 3
Genes upregulated under MMS treatment

Category	Total	Cell cycle*		ESR**		TF***	
		Yes	No	Yes	No	Yes	No
Energy metabolism	37	6	31	25	12	22	15
Fatty acid and lipid metabolism	5	0	5	2	3	1	4
Amino acid biosynthesis and metabolism	11	3	8	1	10	9	2
Nucleotide biosynthesis and metabolism	6	3	3	1	5	6	0
DNA replication/repair and chromosome structure	10	7	3	1	9	4	4
Transcription, RNA metabolism, and modification	9	1	8	5	4	8	1
Protein translation and ribosome biogenesis	0	0	0	0	0	0	0
Protein folding and trafficking	14	0	14	8	6	6	8
Proteolysis	32	2	30	6	26	9	23
Signaling	10	1	9	5	5	9	1
Stress responses (oxidative, osmotic, drug, general)	39	7	32	22	17	26	13
Organelle and vesicle transport	12	0	12	3	9	4	8
Cell structure	7	2	5	1	6	4	3
Other	18	5	13	3	15	8	10
Unknown	83	10	73	42	41	56	27
All	293	47	246	125	168	174	119

* Genes shown to have cell-cycle regulated transcription [100].

** The ~900 genes associated with the Environmental Stress Response [101].

*** Transcriptional response affected by deletion of one of several genes encoding transcription factors that contribute to MMS resistance [99].

Table 4
Genes downregulated under MMS treatment

Category	Total 1	Cell cycle *		ESR **		TF ***	
		Yes	No	Yes	No	Yes	No
Energy metabolism	17	4	13	1	16	8	9
Fatty acid and lipid metabolism	12	5	7	2	10	5	7
Amino acid biosynthesis and metabolism	9	4	5	2	7	5	4
Nucleotide biosynthesis and metabolism	12	1	11	6	6	7	5
DNA replication/repair and chromosome structure	4	4	0	0	4	1	3
Transcription, metabolism, and modification	18	6	12	7	11	7	11
Protein translation and ribosome biogenesis	65	0	65	53	12	32	33
Protein folding and trafficking	2	0	2	0	2	1	1
Proteolysis	5	2	3	1	4	3	2
Signaling	11	8	3	1	10	4	7
Stress responses (oxidative, osmotic, drug, general)	3	1	2	0	3	2	1
Organelle and vesicle transport	3	2	1	1	2	2	1
Cell structure	16	13	3	3	13	4	12
Other	8	4	4	2	6	11	5
Unknown	35	10	25	12	23	15	20
All	220	64	156	91	129	106	114

* Genes shown to have cell-cycle regulated transcription [100].

** The ~900 genes associated with the Environmental Stress Response [101].

*** Transcriptional response affected by deletion of one of several genes encoding transcription factors that contribute to MMS resistance [99].