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Chapter

Checkpoint Control of DNA Repair in Yeast

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Abstract

Budding yeast has been a model organism for understanding how DNA damage is repaired and how cells minimize genetic instability caused by arresting or delaying the cell cycle at well-defined checkpoints. However, many DNA damage insults are tolerated by mechanisms that can both be error-prone and error-free. The mechanisms that tolerate DNA damage and promote cell division are less well-understood. This review summarizes current information known about the checkpoint response to agents that elicit both the G2/M checkpoint and the intra-S phase checkpoint and how cells adapt to unrepaired DNA damage. Tolerance to particular bulky DNA adducts and radiomimetic agents are discussed, as well as possible mechanisms that may control phosphatases that deactivate phosphorylated proteins.

Keywords: DNA damage tolerance, checkpoint, budding yeast, phosphatase

1. Introduction

DNA repair involves the recognition and excision of DNA damage followed by template-directed DNA synthesis using an undamaged strand (for reviews see [1]). Major repair mechanisms include base excision repair (BER, [2]), nucleotide excision repair (NER, [3]), and double-strand break (DSB) repair [4]; In budding yeast, homologous recombination (HR) is the preferred pathway for repair of DSBs. When the DNA replication apparatus bypasses DNA lesions on either the leading or lagging strand, single-strand gaps are created, and the resulting repair of gapped sister chromatids is referred to as postreplication repair (PRR); postreplication repair pathway involves DNA synthesis by low fidelity polymerases or template switch mechanisms. These studies have determined the identity of multiple components of these DNA repair pathways and demonstrated a remarkable conservation between both "simple" eukaryotes, such as budding yeast, and higher eukaryotes, including humans. Yeast studies, therefore, have a direct impact on understanding the molecular basis of inheritable DNA repair deficiencies in humans, many of which are associated with cancer (for review, see [5]).

One unifying theme of DNA repair is the redundancy of DNA repair mechanisms for specific DNA lesions. In budding yeast, the pathway choice may depend on the context of the DNA lesion, the stage of the cell cycle, and the ploidy of the strain. For example, DSBs can be repaired by non-homologous end joining (NHEJ), single-strand annealing (SSA), and HR [4]). HR is enhanced [6, 7] while NHEJ is suppressed in MATa/MATα diploid strains [8] where the HR repair can use an undamaged homolog as a repair template. Secondly, specific DNA base lesions, such

as abasic sites can be repaired by both bases excision repair (BER) or nucleotide excision repair (NER, [2, 9, 10]). Thirdly, postreplication repair (PPR) pathways can be both error-prone and error-free [11]. While some DNA lesions are repaired by redundant mechanisms, others, such as inter-strand DNA cross-links that impede DNA polymerases, require components of multiple pathways, including NER, postreplication repair, and HR [12].

Cells adapt to unrepaired DNA lesions and rely on DNA damage tolerance mechanisms to maintain viability. For example, exposure to 150 J/m^2 UV, generates 3×10^4 cyclobutane pyrimidine dimers (CPD) per yeast cell, or approximately one UV-induced dimer per 400 bp of yeast DNA [13]. The pyrimidine-pyrimidone (6–4) photoproduct is also abundant but eightfold less present after exposure to UVB (280–320) [14]. The efficiency of CPD repair depends on the surrounding chromatin and whether the damaged strand is transcribed (for review, [15]). Since not all UV-induced damage is repaired within the period of a single cell cycle, cell viability depends on DNA damage tolerance and adaptation.

The purpose of this review is to summarize mechanisms by which checkpoint activation and DNA damage tolerance confer resistance for particular DNA lesions, and to summarize more recent data concerning complex carcinogen-associated lesions. The importance of this topic is underscored by observations that tolerance of DNA damage may reduce the efficacy of chemotherapeutic drugs, such as cisplatin, while increasing genetic instability. We present studies that suggest that DNA damage tolerance can be influenced by multiple factors, including the nutritional status of the cell and signaling from both the Target of Rapamycin (TOR) and the protein kinase A (PKA) pathways.

The orchestration of DNA repair pathways is especially critical when the replication fork progression is blocked by bulky DNA adducts [16]. Stalled replication forks can generate DNA secondary structures that trigger genome instability. While particular mechanisms to bypass such adducts, such as template switching and translesion DNA synthesis, may be error free, there is a risk that toxic recombination intermediates can either impede DNA replication progression or lead to replication fork collapse [17]. Replication-associated DNA breaks, in turn, may re-initiate replication on an undamaged chromosome or chromatid, referred to as break-induced replication (BIR, [18]). Chromosome breaks, if unrepaired, can be aberrantly rejoined forming dicentric chromosomes, leading to further breakage and instability, often referred to as the breakage-fusion-bridge cycle, a phenomena suggested to account for gross genome rearrangements in yeast [19] and in cancer cells [20, 21].

To suppress genetic instability and facilitate DNA repair, cell cycle checkpoints trigger arrest at defined stages in the cell cycle to ensure that DNA damage is repaired before the damage is replicated or inherited in the next cell cycle (for review, see [22]). These checkpoints are referred to as the G1-S checkpoint, the intra S checkpoint, and the G2/M checkpoint. In brief, phosphoinositide three-kinase-related kinase (PI3K)-like kinases, referred to as apical or sensor kinases, initiate signaling after recruitment to DNA damage or stalled replication forks, checkpoint kinases, referred to as effector or downstream kinases, then amplify and transmit the checkpoint signal, and effectors that catalyze covalent protein modifications [23], resulting in activation or degradation of cellular targets. Besides ensuring that the cell cycle is delayed so that adequate time is available for DNA repair [24], effectors also modify DNA repair proteins [25, 26]), upregulate the synthesis of deoxynucleotides (dNTPs, [27]), regulate transport of tRNA from the nucleus to and from the cytoplasm, trigger autophagy [28], regulate histone levels [29], and cross-talk with other stress-induced pathways to ensure survival [30]. The totality of the response is generally referred to as the DNA

damage response (DDR) [22]. Checkpoint recovery occurs once the DNA damage is repaired or the replication block has been circumvented [31]; however, adaptation to persistent DNA lesions, such as DSBs, can also occur. In either case, the phosphorylated and activated checkpoint proteins are either dephosphorylated or degraded and subsequently rendered inactive. Depending on the DNA damage and time period of exposure, peak activation occurs within 2–4 hours after acute DNA damage exposure [32], with simultaneous upregulation of dNTP levels and DNA damage-inducible genes. Repression of late origins of replication, inhibition of replication, and prevention of anaphase can last for additional hours [33], while adaptation can be observed after 12 hrs. The presence of DNA repair foci, such as Rad51, can thus last hours after the initiation of the DNA damage insult. Thus, aspects of the DNA damage response can persist hours after the initial genomic insult and after DNA repair is completed.

2. Checkpoint activation initiated by DSBs

Checkpoint activation triggered by DSBs, and subsequent recovery or adaptation has been extensively studied in strains containing either uncapped telomeres or chromosomal DSBs that cannot be repaired by HR. An unrepaired DSB occurs when HO endonuclease cleaves the recognition sequence at the MAT locus but silent mating type locus has been deleted [34]. Uncapped telomeres occur when either the Cdc13-Stn1-Ten1 (CST) complex or the Ku complex, composed of yKu70 and yKu80, is defective. At restrictive (elevated) temperatures in either *cdc13* or *yku70* mutants, extensive tracts of single-stranded DNA complex are generated [35]. While two DNA ends are revealed by a single unrepaired DSB at the MAT locus, at the non-permissive temperature in *cdc13* mutants single-stranded DNA is revealed at the telomeres of sixteen chromosomes, thus amplifying the DNA damage signal.

A single DSB occurring in G1 does not trigger cell cycle arrest at the G1/S or intra S phase checkpoint [36], but instead the cell cycle progresses through S phase and into G2 phase, where cells arrest. Repair of DSBs can occur at any time in the cell cycle by NHEJ; however, in budding yeast, NHEJ is favorable when the single-strand overhangs are short [37]. However, DSBs, will trigger a partial DNA damage response in G1 cells [38], and recombination proteins, such as Rad51 and Rad54, are still induced [39] and Rad55 is phosphorylated [38] .

The orchestration of checkpoint signaling has been well described in current reviews [40] and is briefly summarized (**Figure 1**). Mre11/Rad50/Xrs2 (MRX) and Tel1 (ataxia telangiectasia mutated (ATM) ortholog) bind to the ends of the DSB, which facilitates the juxtaposition of the ends of the breaks. NHEJ requires yKu70 and yKu80. However, if NHEJ is not successful, cyclin dependent kinase I (Cdk1 or Cdc28), which has high activity in G2, phosphorylates Sae2 and the 5′ to 3′ exonuclease Dna2 [41]. Sae2 phosphorylation activates the Mre11 endonuclease activity that ejects yKu70 from the ends of the DSB. Together with Sgs1/Dna2 and Exo1, the ends are further degraded in a 5′ to 3′ direction. NHEJ and resection require chromatin remodeling factors, including the Ino80 complex [42, 43], Rsc complex [44], and Fun30 [45, 46]. Resection is generally slow and proceeds at 1–2 nucleotide per minute [40]. Resection of the ends reveals single stranded DNA (ssDNA), which is then coated by single strand binding protein (RPA), which serves as a general sensor for DNA damage. The RPA-coated ssDNA is a binding site for Ddc2-Mec1 (ataxia telangiectasia mutated and rad3-related (ATR ortholog). Rad24/Rfc facilitates the binding of trimeric Rad17/Mec3/Ddc1 (9–1-1) protein which recognizes the junction between the single stranded DNA and the double-stranded DNA [47]. Thus, ssDNA serves as a general signal for checkpoint signaling [48].

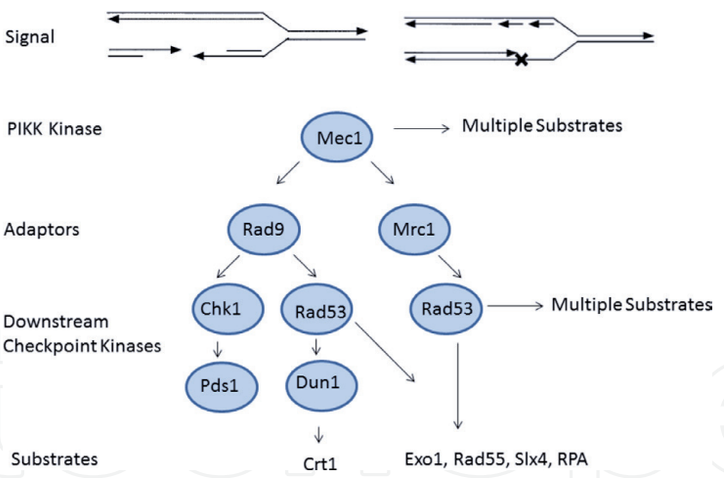


Figure 1.
A pathway for checkpoint pathway commences with a DNA damage signal that triggers the PIKK kinase, Mec1. Downstream checkpoint kinases are activated, as facilitated by the adaptors Rad9 and Mrc1. Kinase substrates are identified for Rad53 and Dun1, but both Mec1 and Rad53 phosphorylate multiple substrates that are not shown.

Mec1, a sensor or apical serine/threonine kinase phosphorylates downstream kinases, DNA repair proteins, and histones, preferably at SQ/TQ sites [49]. Both Mec1 and Tel1 phosphorylate histone γ-H2A for ~50 kb on either side of the DSB, which serves to recruit other checkpoint protein, such as the adaptor, Rad9 (53BP1 ortholog). Mec1 regulates checkpoint signaling by autophosphorylation on the S1964 residue [50] and phosphorylation of Ddc2, which destabilizes unbound Ddc2 and limits the amount of bound Ddc2-Mec1. Mec1 also phosphorylates Exo1 [51], which limits the amount of single-stranded DNA that could serve as a signal for checkpoint activation. Thus, Mec1's activity serves to not only activate downstream kinases but also dampen the checkpoint response.

Rad9, as an adaptor protein and 53BP1 ortholog, is required to bring the effector (transducer) kinases in contact with Mec1. Rad9 binding to chromatin is mediated by its BRCT and tudor domains that interact with phosphorylated and trimethylated histone H3, respectively [52]. While histone phosphorylation is induced by DNA damage, Dot1-mediated histone H3 methylation is constitutive [53]. Localization to damaged DNA is facilitated by binding to Rtt107/Dbp11. Both Mec1 and Cdk1 phosphorylate Rad9 on separate domains [40]. In turn, oligomers of phosphorylated Rad9 bind to Rad53 and facilitate Mec1-mediated phosphorylation. A Rad53 phosphorylated heterodimer then autophosphorylates; the hyper-phosphorylated Rad53 can, in turn, rapidly diffuse throughout the nucleus and phosphorylate multiple substrates, including Dun1 and Asf1. Similarly, Rad9 facilitates Mec1-mediated Chk1 phosphorylation; the activated Chk1 phosphorylates Pds1, which prevents its degradation by the anaphase promoting complex (APC). In turn, sister chromatid cohesion is maintained and anaphase is prevented [54]. Activated Rad53 also inhibits the APC from degrading securin [54]. A Rad53-mediated pathway inhibits Cdc5, a polo-like kinase that functions in mitotic exit through the regulation of spindle pole body separation [30]. While Pds1 phosphorylation can also be triggered by the Mad2-mediated spindle checkpoints, Rad53 phosphorylation is only triggered by DNA damage or stalled replication forks. While the single mutants are partially defective in DNA damage-induced G2 arrest, *rad53 pds1* and *rad53 dun1* double mutants are fully deficient [55].

Partial to full checkpoint activation will also occur when DNA damage processing is rendered less efficient. For example, mating-type switching in a *rad1* mutant, defective in removal of 3′ non-homologous ends, will trigger a

checkpoint-dependent cell cycle delay [56]. Interestingly, Rad53 phosphorylation was not abundant in the *rad1* mutant; however, the cell cycle delay was not observed in the *rad9* strain, and was shortened in the *mad3* mutants, defective in spindle checkpoint. The authors speculated that checkpoint activation occurred when H2A phosphorylation extended through centromeric chromatin, triggering a spindle pole checkpoint response [56]. These studies indicate that spindle pole checkpoints also participate in the DNA damage response, depending on the context of the DSB.

While the G2 checkpoint is critical for HR repair of DSBs, Mec1 and Mec1-signaling pathway also phosphorylate additional DNA repair functions that facilitate DSB repair and damage incurred by radiomimetic agents [40]. For example, Mec1 phosphorylates Rad51 [57] and Rad55 [58]. Phosphorylation of Rad51 enhances its activity and is required for resistance to recombinagens, such as methyl methane sulfonate (MMS) [57]. In addition, Mec1 phosphorylates Slx4, which binds to Rad1/Rad10 and facilitates single-strand annealing by cleaving non-homologous tails [59]. The studies indicate that the checkpoint pathway directly phosphorylates repair proteins to enhance their function. While there are many proteins that are phosphorylated in response to DNA damage [60, 61], the functional significance of the phosphorylation of many of these proteins has yet to be determined (**Table 1**).

Protein Phosphorylated	**Kinase**	**Effect**	**Reference**
Checkpoint Signaling			
Rad9	Mec1 /Tel1	Rad53 docking and Rad9 multimerization	[40, 60]
Rad53	Mec1	Activation of Rad53 autophosphorylation	[40, 60]
Rad53	Tel1	Activation of Rad53	[40, 60]
Chk1	Mec1	Phosphorylation of Pds1	[30, 54]
Pds1	Chk1	APC-associated degradation of Pds1 is inhibited	[54, 60]
Mec1-Ddc2	Mec1	Attenuation of Mec1 kinase activity	[50]
Dun1	Rad53	Activation of Dun1 kinase activity	[40]
Nucleases			
Sae2	Cdk1	Cell cycle regulation limiting resection to G2/M	[41]
Dna2	Cdk1	Cell cycle regulation limiting resection to G2/M	[62]
Exo1	Mec1	Inhibition of Exo1 5′-3′ exonuclease activity	[51]
Transcription inhibitors			
Crt1	Dun1	Crt1 phosphorylation leads to degradation, and subsequent Rnr transcriptional activation	[60, 63]
Protein Inhibitors			
Sml1	Dun1	Sml1 phosphorylation leads to degradation and release from Rnr1 subunit and subsequent increase in dNTPs	[27]
Dif1	Dun1	Allows for transport of RNR into the cytoplasm	[64]
Recombination Proteins			
Rad55	Rad53	Enhances recombination in rad5 mutants	[58]
Rad51	Mec1/Rad53	Enhances activity	[57]
Rev1	Mec1	Facilitates binding to ssDNA	[26]

Protein Phosphorylated	Kinase	Effect	Reference
Resolvases			
Yen1	Cdk1	Inhibits function in S phase by transportation to the cytoplasm	[62]
Mus81/Mms4	Cdk1, Dbf4 Cdc7	Regulation of cleavage of Holliday and branch junctions	[62, 65]
Helicases			
Pif1	Rad53	Inhibits fork unwinding, promotes DNA damage tolerance by HR	[66]
Rrm3	Rad53	Inhibits fork unwinding	[66]
Srs2	Cdk1	Promotes adaptation by removal of Rad51 filaments	[62, 67]

Table 1.
Proteins phosphorylated by DDR.

3. Checkpoint recovery and adaptation from double-strand break

Once cells have repaired the DSB, recovery involves reversal of protein modifications and chromatin restoration. While the DNA damage may no longer be present, protein modifications are still present that signal checkpoint activation. To inactivate the G2/M checkpoint and resume division, Rad53 must be dephosphorylated. Two phosphatases involved in the inactivation of Rad53 include phosphorylated versions of the type 2C protein phosphatases (PP2C), Ptc2 and Ptc3 [68–70]; these phosphatases are also involved in inactivating other stress induced pathways, such as the Hog1-mediated osmotic stress induced pathway [71], while Ptc2 dephosphorylates Cdk1. Casein kinase II (Ck2) phosphorylates Ptc2, which specifically binds to the Rad53 FHA1 domains [72]. Interestingly, CK2 mutants are more defective in adaptation than *ptc2* mutants, suggesting that CK2 may control additional genes involved in adaptation [68].

Pph3, a member of the PP4 family, is important in maintaining full recovery; the triple mutant (*ptc2, ptc3, pph3*) is severely defective in DSB repair when the repair pathway is slow [70]. This may be partially explained by observations that Pph3 functions to dephosphorylate γ-H2A, which serves as a signal for activation of checkpoint proteins, cohesins, and chromatin remodelers [73]. However, the mechanism by with chromatin associated gamma γ-H2A is fully dephosphorylated is still being explored.

Chromatin restoration requires Asf1 and Caf1 which reassemble chromatin on DNA (Kim and Haber [74]). Asf1 binds histone H3 triggering acetylation by the histone acetyltransferase, Rtt109, and further ubiquitylation by Rtt101 [75]. This, in turn promotes the binding of the histone H3 and H4 heterodimer by Caf1. Interestingly, Asf1 also functions to bind Rad53, thus serving a role to sequester dephosphorylated Rad53. Thus Asf1 functions both in reassembling chromatin and stabilizing dephosphorylated Rad53 [75].

If a DSB is not repaired, cells will either resume the cell cycle or die. The resumption of the cell cycle is referred to as adaptation. Similar to recovery, adaptation involves both chromatin remodeling and phosphatases that deactivate the Rad53 kinase and Cdk1 kinase. This adaptation is blocked in *yku80* mutants [69], deficient in NHEJ, and *cdc5-ad*, which is defective in mitotic exit. *Yku80* mutants exhibit twice the rate of resection of the DSBs, resulting in more single-stranded DNA

and thus the potential for more Rad53 checkpoint signaling. This interpretation is supported by observations that overexpression of Ptc2 is sufficient to suppress the adaptation defect of both *yku70* and *yku80* [69]. However, the role of resection in checkpoint adaptation is complicated by the identification of chromatin remodelers, such as Fun30 [76–78], which are required for adaptation but enhance resection. One possibility is that Fun30-associated resection in γ -H2A-modified chromatin antagonizes the checkpoint protein Rad9 from binding and signaling downstream checkpoint effectors [78].

Additional genes function to remove recombination proteins from chromatin. Removal of Rad51 filaments is facilitated by the chromatin modifier Tid1 (Rdh54) [79] and the Srs2 helicase, the former is phosphorylated by Mec1 and the latter is phosphorylated by Cdk1 [67]. Both *rdh54* and *srs2* mutants are defective in adaptation (reference [79, 80]). These studies present additional evidence *MEC1* functions both in the triggering of checkpoint arrest as well as recovery from checkpoint arrest.

4. Uncapped telomeres, checkpoint activation, and adaptation

While single-stranded DNA is present at telomeres, it is normally "capped" by a RPA-like structure, referred to as Cdc13-Stn1-Ten1 (CST), and by Ku (yKu70/yKu80) complex [35]. During replication, Cdc13 is phosphorylated by Cdk1 and recruits telomerase [81]. Telomere ends are susceptible to nucleases in yeast mutants defective in proteins that bind to chromosome ends, such as *yku70*, and that are defective in recruiting telomerase, such as the *cdc13–1* mutant at the restrictive temperature. Pif1 helicase inhibits telomerase and leads to slow resection at the telomere end [82, 83]. Resection is also slowed by binding of Rif1 and Rap1, which bind specifically to single stranded telomere sequences and inhibit the binding of the checkpoint activators, RPA and Rad24 [84, 85]. In the *cdc13–1* mutant, resection is extensive and largely performed by Exo1, leading to ssDNA bound to RPA, the 9–1-1 complex, and Rad9. Similarly, in *yku70* mutants, ssDNA is generated, but it takes several generations for ssDNA to accumulate [35]. The 9–1-1 complex is apparently not involved in eliciting a checkpoint response but Chk1 activation is required for Exo1-mediated resection [86]. In *yku70* mutants, resected telomeres elicit both a spindle and DNA damage checkpoint activation. However, unlike HO-induced DSBs, Mec1 binding does not lead to rapid resection but rather an inhibition of resection through subsequent binding of Rad9 and Rad53 [87]). Resection of the telomere, in turn, may facilitate recombination or break-induced replication (BIR, [88]) using an undamaged chromosomal end as a template for replication to the end of the chromosome. BIR is facilitated by activated Pif1 [89]. Thus, checkpoint activation at uncapped telomeres enables alternative mechanisms of telomere lengthening.

Adaptation to shortened telomeres was first noted by Sandell and Zakian [90] and require CKII and Cdc5 [91]. CKII directly phosphorylates Ptc2, which is required for tolerating shortened telomeres [92]. In addition, phosphorylated Cdc13 can be dephosphorylated by Pph3/Psy3, resulting in the segregation of uncapped chromosomal ends [35]. Over-expression of Cdc5 also decreases Rad53 phosphorylation [93]. Thus, as in HO-induced DSBs, there are multiple phosphatases and kinases that modulate adaptation.

5. Intra-S phase checkpoint and stabilization of the replication fork

The purpose of the intra-S phase checkpoint is to maintain replication fork integrity so that replication can be completed; collapsed replication forks are a

major source of genetic instability [94]. Replication forks stall because of limiting amounts of dNTPs or when DNA damage, resulting from a bulky adduct or cross-links, block progression of a high fidelity polymerase (for review, see [95]). The precise number of stalled forks to trigger the intra S-phase checkpoint is unknown [96]. Uncoupling of the helicase and DNA polymerase activity generates single strand gaps on both the leading and lagging strands. Checkpoint responses serve to maintain the stability of the replication fork in part by blocking the formation of toxic secondary DNA structures and replication fork reversal, degrading excessive histones, and inhibiting the firing of late replication forks so that replication can be resumed if stalled replication forks become permanently arrested [97]. In S phase checkpoint mutants, such as *rad53*, regressed replication forks, referred to as "chicken feet" structures, can be visualized [98]. Severe deficiencies can lead to mitotic catastrophe and subsequent lethality.

The extensive tracts of single-stranded DNA generated at stalled forks signal a checkpoint response. Similar, to checkpoint signaling at DSBs, the 9–1-1 complex is loaded and facilitates binding of Ddc2-Mec1. Rad18, which monoubiquitinates trimeric PCNA at K164 position at stalled replication forks, also monoubiquitinates the 9–1-1 complex leading to enhanced recruitment of Ddc2-Mec1 [99]. Checkpoint activation at stalled forks can also be facilitated by Elg1, which removes PCNA from stalled forks [100]. The Mrc1 (claspin) functions as an adaptor, analogous to Rad9, in the phosphorylation of Rad53; however, unlike Rad9, Mrc1 is associated with the replication forks [101]. Full checkpoint activation requires BLM homolog Sgs1 [102]. Rad53 phosphorylation in turn serves to promote histone degradation, inhibit late origin firing, and increase the levels of dNTPs. The inhibition of late origin firing maintains RPA and allows replication restart from other replication origins.

Deoxynucleotide levels (dNTPs) increase 7–8 fold after DNA damage by upregulating the activity of ribonucleotide reductase (Rnr) activity [103]. Upregulation of Rnr activity is achieved at the transcriptional, translational, and the posttranslational levels. At the transcriptional level, phosphorylated Rad53 activates Dun1 kinase, which deactivates Crt1 transcriptional repressor [63]. At the translational level, TRM9, which functions to methylate the uridine wobble base of tRNA-Arg (UCU) and tRNA-Glu (UUC), facilitates the translation of the Rnr1 transcript [104]; however, how checkpoint signaling enhances *TRM9* function is unclear. At the post-translational level, Dun1 inactivates the Sml1 protein inhibitor by phosphorylation [27]. Besides increasing the transcription of Rnr subunits, the Rnr inhibitor Sml1 and its paralog Dif1 are degraded, subsequently the Rnr subunits are shuttled to the cytoplasm where they form an active enzyme complex [64]. In addition to increasing the overall level of dNTPs, the Rnr3 large subunit forms an alternative ribonucleotide reductase complex that has relaxed dATP negative feedback regulation [105]. This ensures that adequate levels of dNTPs are available during times of unscheduled DNA synthesis.

While high levels of dNTPs facilitate replication fork progression [106]; abnormally high or low levels of dNTPs can promote genetic instability. High levels of dNTPs reduce the fidelity of polymerase epsilon [107]. Low levels of dNTPs correlate with hyper-recombination, as has been observed in *dun1* null mutants and in mec1 hypomorphs; these phenotypes can be suppressed by higher basal levels of dNTPs conferred by a *SML1* deletion [108]. These studies suggest that there is a range of dNTP concentrations that correlate with replication fork stability; however, the mechanisms by which higher dNTP levels decrease replication fork collapse are unclear.

In contrast to DSBs, where HR processes are facilitated, there are redundant mechanisms to prevent recombination at stalled forks; these mechanisms include disassembly of Rad51 filaments, helicases that abort recombination intermediates,

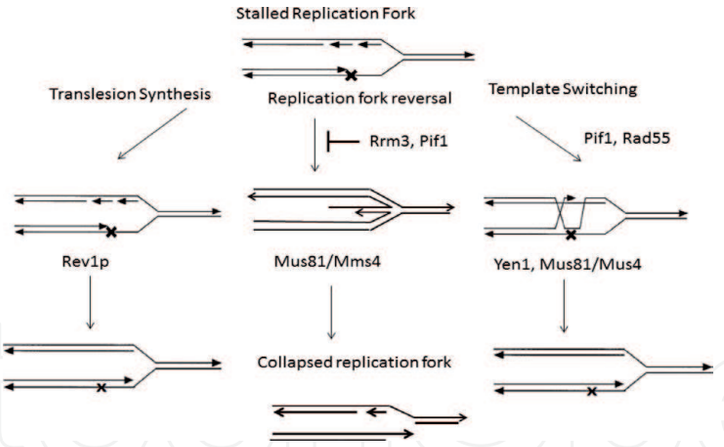


Figure 2.
The pathways of tolerating DNA damage at a stalled fork are shown by the arrows. The 5′ to 3′ polarity of the DNA is designated by an arrow. The Rrm3 and Pif1 helicases inhibit replication fork reversal, while the Pif1 helicase promotes template switching. Phosphorylated Rev1 binds to single-stranded DNA and promotes replication bypass.

and nucleases that degrade aberrant structures. PCNA is SUMOylated (SUMO-PCNA), facilitating the binding of the helicase Srs2, which suppresses recombination by disassembling Rad51 filaments (for review, see [109]. Recombination intermediates are aborted by the BLM ortholog Sgs1 helicase; indeed, the *sgs1 srs2* double mutants is not viable but viability is rescued when HR is defective [110]. Additional helicases, including Pif1 and Rrm3 can unwind and reverse "chicken feet" structures and reversed forks [66]. Nucleases, such as Exo1, function to degrade reversed forks, although excessive Exo1 activity can lead to replication fork collapse [62]. Finally, enzymes which cleave aberrant secondary structures, such as Yen1 and Mus4/Mus81, are inhibited or rendered less active by phosphorylation and Sumoylation (SUMO) [65]). Thus, there are multiple mechanisms that prevent aberrant structures from accumulating at replication blocks (**Figure 2**).

Generally, replication blocks that impede DNA polymerases can be bypassed by two mechanisms: 1) lesion bypass inserts a base opposite the replication block using error-prone or error-free translesion polymerases, and 2) template switch mechanisms [111] utilize recombination so that DNA polymerase bypasses DNA lesions on an undamaged template. Factors recruited to stalled forks would initially suggest that checkpoint signaling might favor lesion bypass by translesion polymerases. For example, Rad5 binding to stalled forks facilitates the recruitment of Rev1, even in the absence of DNA damage [112]. In addition, Rev1 is also phosphorylated by Mec1, which increases its affinity to ssDNA [26]. In vitro replication experiments have demonstrated that other error-prone polymerases can "jump start" replication, even without association of the replicative helicase [113]. However, there is no evidence that high levels of dNTPs would facilitate translesion synthesis mediated by error-prone polymerases, such as polζ [114], and the mechanism for jump start is unclear.

6. Bypass of single-strand gaps and replication blocks by template switch mechanisms

Template switch mechanisms also allow polymerases to bypass replication forks and resume DNA synthesis; these mechanisms are generally thought to occur on both leading and lagging strands. Template switching is orchestrated by proteins that modify the DNA polymerase processivity factor, PCNA. When the high fidelity

polymerase stalls at the replication block, Rad18/Rad6 monoubiquitinates PCNA at the K164 position; monoubiquitinated PCNA can facilitate polymerase switching from to a translesion polymerase of lower fidelity and processivity. PCNA may further become polyubiquitinated at position K164 by combined action of Ubc13/Mms2/Rad5 (for review, see [115]). Rad5 also contains a helicase function that catalyzes replication fork reversal and is required for template switch mechanisms on the lagging strand [116]. While Rad5 does not require DNA damage at the stalled replication fork for recruitment [112], Rad5 over-expression can trigger genome instability [117]. The checkpoint signaling cascade, mediated by the Dun1 kinase, regulates Rad5 at the post-transcriptional level by destabilizing Rad5 mRNA [118]. These studies indicate that *RAD5* function is regulated.

However, checkpoint signaling may also facilitate template switch mechanisms. Rad53 is required for DNA damage-associated unequal SCE after exposure to MMS [119] and Rad53-mediated Rad55 phosphorylation confers enhanced MMS resistance when RAD5 is also defective [25]. The Rad9 checkpoint protein binds to persistent single strand gaps on the lagging strand, inhibiting the RecQ-like Sgs1 anti-recombination function. In addition Pif1, which is phosphorylated by Rad53, functions in template switching [120]. With longer term checkpoint-mediated G2 arrest, however, Rev1 protein levels accumulate [121, 122], suggesting that error-prone polymerases may serve as the ultimate backup in postreplication repair after error-free mechanisms have failed.

7. Choice of DNA damage tolerance pathway is influenced by the DNA lesion

Multiple tolerance pathways can confer resistance to particular types of DNA damage and the pathway preference depends on the DNA damaging agent. For example, MMS exposure generates by ^{7}Me-Guanine and ^{3}Me-Adenine lesions; while the ^{7}Me-Guanine is mutagenic, the ^{3}Me-Adenine blocks replication [123]. Replication bypass can occur by error-prone or error-free polymerases, or by template switching. While all three pathways are involved in bypass of ^{3}Me-Adenine lesions [124], template switch mechanisms are preferred [125]. Checkpoint signaling facilitates template switch mechanisms after exposure to MMS [125, 126]. These studies suggest that template switch mechanisms may be the preferred pathway for bypassing particular lesions that block DNA replication.

The preference of template switch mechanisms or translesion pathways may depend on the efficiency of bypass and repair for large bulky adduct or cross-links. Particular UV-associated DNA cross-links are efficiently bypassed using either polε [127] or a two-step mechanism involving polε and polζ [128]. However, error-free bypass of 4–6 pyrmidine-pyrimidone lesions, present on a plasmid, occurs by template switch mechanisms after their introduction in a NER deficient yeast strain [129]. Likewise, 4-NQO induces bulky damage and stimulates template switch mechanisms [126]. These studies indicate that template switch mechanisms are likely used in error-free postreplication repair pathways [130].

8. Attenuation of the S phase checkpoint activation

In order for the cell cycle to resume and chromatids to separate the checkpoint activation needs to be downregulated and joint molecules need to be resolved. Once replication is completed, Mrc1 functions as an adaptor for Mec1-mediated checkpoint signaling is diminished since there are no more replication forks [33].

Resumption of the cell cycle is accomplished by dephosphorylating Rad53 [131]. However, single-strand gaps on sister chromatids can still function to trigger Rad9-mediated checkpoint signaling. To dampen Rad9's adaptor function in mediating Mec1 catalyzed Rad53 phosphorylation, competitive scaffolds compete with Rad9 binding to chromatin [132]. For example, the Mec1-mediated phosphorylation of Slx4 enables an association with Rtt107/Dpb11, which provides a competitive scaffold for the interaction of Rad9 with Dpb11 [133]. These mechanisms thus prevent Rad53 hyperphosphorylation.

Cleavage of joint DNA strands, or Holliday structures, is timed just before anaphase so that cleavage does not occur during S phase. Both kinases and phosphatases fine tune the timing of joint molecule cleavage. Cdk phosphorylates structure-specific nucleases Slx1/Slx4 and Mus81/Mus4 in late G2 and M phases respectively [134]. Whereas Mec1 phosphorylates and subsequently inactivates Yen1, Cdc14 dephosphorylates the inactivated form in mitosis, ensuring that joint molecules do not hinder sister chromatid division [134].

Similar to adaptions to DSBs, phosphatases deactivate Rad53 (**Figure 3**). These phosphatases include Pph3/Psy2 complex and Ptc1, 2. Interestingly, Pph3 directly interacts with Mec1/Ddc2 [135] at the replication fork, although the interaction does not rely on DNA damage [135]. Besides Rad53, other Mec1 substrates are likely dephosphorylated by Pph3, including phosphorylated Mec1. Thus Pph3 could potentially upregulate Mec1. However, the full range of Pph3 substrates is unknown [135].

Mutations in different phosphatases may confer sensitivities to different DNA damaging agents (**Table 2**). For example, *pph3* and *psy2* mutants are hypersensitive to phelomycin but not 4NQO, while *ptc2*, *ptc3* and *ptc2 ptc3* double mutants are not phleomycin sensitive and are not required for recovery from MMS-associated checkpoint delay [136, 142]. On the other hand *ptc2 ptc3* double mutants are hypersensitive to 4-NQO while *pph3* and *psy2* mutants are not sensitive. However, for particular agents, such as cisplatin, the triple *pph3 ptc2 ptc3* mutant, is synergistically more sensitive [140]. One idea is that phosphorylation of Rad53 is differentially patterned by particular DNA damaging agents, and that the phosphatases, Ptc2/Ptc3 and Pph3/Psy2 recognize different patterns [142]. This notion is supported by the identification of different MMS and 4-NQO associated Rad53 phosphorylation sites. The connection between Ptc2 and checkpoint activation is further strengthened by observations that over-expression of Ptc2 suppresses the lethality in a Rad53 dominant lethality mutant [69].

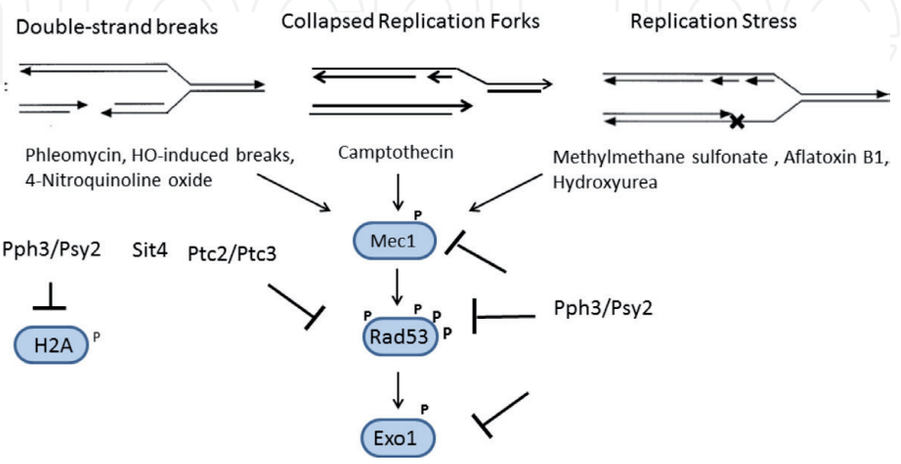


Figure 3.
Double-strand breaks, collapsed replication forks, and replication stress lead to checkpoint activation. Activated Rad53 is dephosphorylated by a series of phosphatases, depending on the signal induced by the DNA damaging agent, shown below the DNA damage. The 5′ to 3′ polarity of the DNA is designated by an arrow. The substrates of the phosphatases Pph3/Psy2 and Ptc2/Ptc3 include Mec1, Rad53, and Exo1.

DNA damaging agent or environmental condition	DNA damage	Phosphatase Required for Resistance or Adaptation	Phenotype of Δ *ptc2 ptc3 pph3*	Reference
Restrictive temperature for *cdc13* mutant	Long tracts of ssDNA	Ptc2	Unknown	[84]
HO Endonuclease	Double-strand break	Ptc2/Ptc3	Completely deficient in adaptation	[70]
4-Nitroquinoline oxide	Bulky adduct and oxidative damage	Ptc2/Ptc3, Sit4	Unknown	[136, 137]
Phleomycin	Single-strand and double-strand breaks	Pph3, Ptc2/Ptc3	Unknown	[136]
Methyl methanesulfonate	Major and minor groove alkylations	Pph3/Psy2	Synergistically sensitive	[70]
Aflatoxin B1	AFB1-N7-Gua, and AFB1 formamidopyrimidine	Pph3/Psy2	Unknown	[138]
Ultraviolet radiation	Pyrimidine dimers and Pyrimidine-pyrimidone cross links	Not required	Moderate sensitivity	[70]
Hydroxyurea	Stalled DNA replication forks, double-strand breaks	Pph3/Psy2, Pph2	Synergistically sensitive	[70, 139]
Camptothecin	Topo1 cross-link with DNA	Ptc2, Ptc3	Synergistically sensitive	[70]
Cisplatin	DNA cross links	Pph3/Psy2	Synergistically sensitive	[140]
Bleomycin	Single and double-strand break s	Pph3, Rts1 (regulator of Cdc55)	Unknown	[73, 141]

Table 2.
Phosphatases that function in checkpoint adaptation to specific DNA damaging agents.

Tolerance to MMS-induced DNA damage includes reactivation of stalled replication forks, which depends on the level of Rad53 phosphorylation [143]. Pph3/Psy2 phosphatase is the principle phosphatase that deactivates Rad53. In the absence Pph3/Psy2 replication restart can occur; however late origins are used to complete DNA replication. Interestingly, downregulation of Rad53 phosphorylation by a HA-Rad53 or a *dot1* deletion confers higher levels of MMS resistance, although at the sake of more Rev1 foci and mutagenesis [144]. These studies would suggest that MMS-induced checkpoint activation is a double-edged sword; limiting MMS-induced mutation may come at the cost of toxic recombination intermediates.

While tolerance to MMS-induced DNA damage relies on dampening the checkpoint response, UV resistance heavily relies on checkpoint activation, as illustrated by observations that the *rad14 mec1* double mutant, defective in both NER and checkpoint signaling, is synergistically more UV sensitive [145]. In yeast, UV triggers the G1-S checkpoint when NER is functional, but unrepaired UV lesions trigger checkpoint responses in S and G2 cells [146]. Interestingly, chronic exposure to low dose UV does not elicit cell cycle arrest at the G2 checkpoint, suggesting that DNA replication machinery is not significantly impeded during chronic exposure [147].

P450-acitvated carcinogens may also elicit a strong DNA damage inducible effect. For example, aflatoxin B1 (AFB1), induces strong Rad53 activation in budding yeast, which generally occurs within two hours of exposure and then is gradually attenuated [148]). AFB1 exposure also upregulates the expression of DNA repair genes, including Rad51, Csm2, and Rad16 [149, 150]. Interestingly, AFB1 exposure elicits an S phase delay coinciding with the appearance of Rad51 foci [148]. This is consistent with AFB1 being a strong recombinagen but weak mutagen in yeast [151]. Interestingly, checkpoint signaling is required for stimulation of both AFB1-associated unequal sister chromatid recombination and mutation [152]. By profiling the yeast genome for AFB1 resistance using next generation sequencing, St. John *et al.* [138] identified both HR genes, including Rad54, Rad55, and Csm2, and those encoding error-prone polymerases. Similar to alkylated induced damage, the Csm2(Shu) complex favors an error-free template switch mechanism [153]; thus, csm2 mutants are deficient in sister chromatid recombination but exhibit higher frequencies of AFB1-associated mutations.

Genes the confer AFB1 resistance included *PSY3*, *CKB1* and *CKB2*, which function in DNA damage tolerance [138]. While the genes encoding the CKII substrates, Ptc2 and Ptc3, did not appear in the screen, the identification of both CKII and Pph3 suggest that tolerance to AFB1-associated DNA damage requires both phosphorylation and dephosphorylation of multiple proteins. The identity of these proteins may further elucidate how AFB1-associated DNA damage is tolerated.

Additional phosphatases that function in DNA damage tolerance include PP2A and PP2A-like phosphatases. These phosphatases are composed of catalytic subunits, such as Pph21 and Pph22, scaffolding subunits, and regulatory subunits, such as Cdc55 and Rts1. While a direct interaction with phosphorylated Rad53 has not been demonstrated, the PP2A phosphatase suppresses the checkpoint response after HU exposure [139]. While the identity of all of the PP2A substrates is unknown, PP2A is involved in both cytokinesis and mitosis [154]. Particular regulatory subunits are required for tolerance to different DNA damaging agents. For example, Rts1 is required for DNA damage tolerance after *rad51* cells are exposed to bleomycin [141] and Sit4, a PP2A-like phosphatase, is required for tolerance to 4NQO [137].

9. Nutrient sensing and the regulation of adaptation and the checkpoint response

One unifying theme in DNA damage tolerance to multiple types of DNA lesions is that nutrient sensing plays an important role in promoting downregulation of the checkpoint response. Deregulation of *IRA1* and *IRA2*, which control glucose-growth signaling, prevent adaptation to uncapped telomeres in *cdc13* strains [155]. Inhibition of TOR1 by rapamycin prevents adaptation and aneuploidy in rad52 diploid strains exposed to DNA damaging agents [156].

Nutrient sensing is also important in controlling the checkpoint response through type 2A protein phosphatases. In the presence of plentiful carbon and nitrogen, target of rapamycin (TORC1) activates Mec1-signaling pathway by inhibiting PP2A and PP2A-like phosphatases. PP2A activators include ceramide and S-adenosyl methionine (SAM) [139]. The effect of this signaling on the PP2C and PP4 phosphatases is unclear. Nonetheless, these studies illustrate that the DNA damage response requires an active growth signaling response [139]. Recent data also suggests that TORC1 inhibition results in lower levels of checkpoint proteins [157]. Thus, it may appear that TORC1 may be required for both checkpoint activation and for adaptation.

10. Concluding remarks

Adaptation to DNA damage is critical for cell survival. The simple, straightforward model is that DNA damage activates checkpoint signaling kinases and that phosphatases, which are constitutively expressed, serve to dephosphorylate and deactivate phosphorylated proteins. Once the DNA damage is repaired, the checkpoint signaling ceases and activated proteins are dephosphorylated. However, yeast can adapt to DNA damage caused by diverse damaging agents and individual phosphatases are controlled by different kinases. In addition, cells exposed to different DNA damaging agents exhibit distinct Rad53 phosphorylation patterns and rely more on particular phosphatases for checkpoint adaptation. The checkpoint pathway also autoregulates itself and dampens its signaling in coordination with Cdk1. Finally, adaptation to particular DNA damage requires TORC1 function, which senses nutrient abundance. Thus, simple models are likely complicated by the complexity of the checkpoint responses elicited by distinct DNA damaging agents.

11. Future directions and implications

Understanding DNA damage tolerance and repair will have a significant impact on elucidating the mechanisms by which DNA adducts cause mutations and genome instability. While DNA damage tolerance has been well-studied for particular types of DNA damage, such as that caused by UV and MMS, the mechanisms for DNA damage tolerance of more complex lesions is still in its infancy. The importance of studying DNA damage tolerance mechanisms for complex agents is underscored by the importance of cross-linking agents, such as cisplatin, in cancer chemotherapy. In addition, understanding how DNA damage is tolerated may have important impacts in assessing the efficacy of antifungal agents. Elucidating DNA damage tolerance mechanisms will also be important in understanding how mutations and genetic instabilities arise when cells are exposed to low doses of the DNA damaging agent. These studies should elucidate mechanisms on how cellular aging, ploidy, and cell type may affect DNA damage tolerance pathways.

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