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Saccharomyces cerevisiae Rad9 Acts as a Mec1 Adaptor to Allow Rad53 Activation

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Summary

Background: The DNA damage checkpoint is a protein kinase-based signaling system that detects and signals physical alterations in DNA. Despite having identified many components of this signaling cascade, the exact mechanisms by which checkpoint kinases are activated after DNA damage, as well as the role of the checkpoint mediators, remain poorly understood.
Results: To elucidate the mechanisms that underlie the *MEC1* and *RAD9*-dependent activation of Rad53, the *Saccharomyces cerevisiae* ortholog of Chk2, we mapped and characterized in vivo phosphorylation sites present on Rad53 after DNA damage by mass spectrometry. We find that Rad53 requires for its activation multisite phosphorylation on a number of typical and atypical Mec1 phosphorylation sites, thus confirming that Rad53 is a direct target of Mec1, the mammalian ATR homolog. Moreover, by using biochemical reconstitution experiments, we demonstrate that efficient and direct phosphorylation of Rad53 by Mec1 is only observed in the presence of purified Rad9, the archetypal checkpoint mediator. We find that the stimulatory activity of Rad9 requires a phospho- and FHA-dependent interaction with Rad53, which allows Rad53 to be recognized as a substrate for Mec1.
Conclusions: Our results indicate that Rad9 acts as a bona fide signaling adaptor that enables Rad53 phosphorylation by Mec1. Given the high degree of conservation of checkpoint signaling in eukaryotes, we propose that one of the critical functions of checkpoint mediators such as MDC1, 53BP1, or Brca1 is to act as PIKK adaptors during the DNA damage response.

Introduction

The DNA damage checkpoint is a signal transduction system that detects and signals physical alterations in DNA as well as monitoring the integrity of replication forks [1]. When this highly conserved genome surveillance mechanism is disrupted, cells become sensitive to DNA damaging agents and DNA replication inhibitors and accumulate genetic lesions at high rates [2]. In human cells, disruption of components of the DNA damage checkpoint, such as ATM, Chk2 and p53, is associated with an aberrant response to DNA damage, resulting in genome instability and heightened predisposition to cancer [3].

In the budding yeast *Saccharomyces cerevisiae*, the DNA damage checkpoint is in large part under the control of the PI(3) kinase-like kinase (PIKK) Mec1, the ortholog of the ATR Ser/Thr protein kinase [4, 5]. In complex with its interacting protein Ddc2, Mec1 has been proposed to detect processed DNA lesions via an interaction with RPA-coated single-stranded DNA [6]. Upon Mec1 activation, the tandem BRCT domain-containing protein Rad9 becomes highly phosphorylated on putative Mec1 consensus sites characterized by Ser/Thr-Gln (S/T-Q) motifs [7]. Rad9 phosphorylation triggers the binding of Rad53, the Chk2 ortholog, to Rad9 in a manner that depends on both the N-terminal (FHA1) and C-terminal (FHA2) FHA domains of Rad53 [7–11]. Rad9 is essential for DNA damage signaling in the G1 and G2/M phases of the cell cycle, whereas in S phase, its function in the replication checkpoint is dispensable and appears to be substituted by Mrc1, a replication fork-associated protein [12, 13].

In vertebrates, there is no single Rad9 ortholog readily identifiable, but rather a family of tandem BRCT domain-containing proteins that act in the checkpoint response. These Rad9 homologs are Brca1, 53BP1, and MDC1 and are commonly referred to as checkpoint mediators [14]. Like Rad9, human checkpoint mediators localize directly to sites of DNA damage, are often substrates of PIKKs, and are required for the downstream propagation of the DNA damage signal [15–17]. The shared properties between the budding yeast Rad9 and human checkpoint mediators suggest that a common underlying role in the checkpoint response may govern checkpoint mediator action.

Two main models attempt to explain the requirement for checkpoint mediators in Chk2-like kinase activation. The first model, based on a study by Gilbert et al. [18], proposes that Rad9 activates Rad53 in a manner akin to a solid-state catalyst by increasing the local Rad53 concentration. The resulting increase in Rad53 concentration is thought to be sufficient to trigger *trans* autophosphorylation and catalytic activation. This model (called herein the "solid-state catalyst" model) was inferred from hydrodynamic data of the Rad9 complex and on the observation that recombinant Rad53 produced from *Escherichia coli* is extensively phosphorylated and active. However, the solid-state catalyst model of Rad53 activation does not satisfyingly account for the observation that Rad53 is phosphorylated in response to DNA damage independently of its kinase activity ([19–21] and below) or that covalent linking of Rad53 to Ddc2 partially alleviates the requirement of Rad9 for Rad53 activation [22]. A second model has

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been proposed since the realization that Rad9 (or other checkpoint mediators) are critical for the PIKK-dependent phosphorylation of Chk2-like kinases in response to DNA damage. This second model suggests that the main role of Rad9 is to act as a protein that recruits Rad53 to Mec1. In this model, Rad9 acts as a bona fide signaling adaptor, which can be defined as a protein lacking known catalytic domains that physically link at least two other proteins to facilitate a signaling event [23]. However, direct biochemical evidence to support the adaptor model has remained elusive.

In this study, we investigated how Rad53 phosphorylation influenced its catalytic activation. By using mass spectrometry coupled to metal-affinity enrichment of phosphopeptides, we mapped unambiguously phosphorylation sites on Rad53 purified from cells subjected to DNA damage. Results from the mutational analysis of these sites are consistent with a model whereby PIKKs play a direct and essential role in converting a catalytically latent Rad53 enzyme to an active state by phosphorylating multiple sites on Rad53. Furthermore, we have tested the adaptor model in biochemical reconstitution assays by using purified Mec1, Rad53, and Rad9, and our results indicate that Rad9 acts as a signaling adaptor necessary for efficient Rad53 phosphorylation by Mec1. Because MDC1, Brca1, and 53BP1 are analogous to Rad9 in many respects and are required for multiple PIKK phosphorylation events, our work supports a model whereby BRCT domain-containing checkpoint mediators act as PIKK phosphorylation adaptors to ensure the proper spatiotemporal regulation of the DNA damage response.

Results

In this study, we examined the contribution of the Rad53 FHA domains to the regulation of Rad53 kinase activation in response to DNA damage. We mutated a critical Arg residue in each of the Rad53 FHA domains to Ala (R70A in FHA1 and R605A in FHA2; Figure 1A) in order to impair the phosphopeptide binding activity of the mutated FHA domain [11, 24]. These mutations were generated alone or in combination to yield the *rad53*^{FHA1}, *rad53*^{FHA2}, or *rad53*^{FHA1,2} alleles. We also generated a kinase-dead allele of *RAD53*, *rad53*^{D339A} [25]. Each plasmid-borne allele (including the wild-type [wt] allele) was introduced in a *rad53*Δ *sml1* strain (*SML1* mutation is required to maintain the viability of the *RAD53* deletion; [26]). DNA damage-induced Rad53 activity was elicited by addition of the UV-mimetic drug 4-nitroquinoline oxide (4-NQO), and Rad53 activity in the resulting strains was monitored by in situ kinase assays (ISA; [19], or by examining protein gel mobility shifts due to phosphorylation). As shown in Figure 1B, we observe that Rad53 catalytic activation absolutely requires at least one functional FHA domain, an observation also corroborated by others [8, 27]. This result indicates that FHA-dependent binding of Rad53 to upstream activators is required for its conversion from a latent state to an active one.

We also noticed that in stark contrast to the lack of a DNA damage-induced mobility shift displayed by Rad53 in the *rad53*^{FHA1,2} strain (Figure 1B, lane 10, bottom), Rad53^{D339A} displays a noticeable mobility shift after DNA damage, albeit not at the level seen with the wild-type allele (Figure 1B, lane 4, bottom). This mobility shift, also observed in [21], is abrogated by treatment with λ protein phosphatase (data not shown), indicating that the mobility shift is due to a phosphorylation event independent of Rad53 catalytic activity but which requires the phosphothreonine binding activity of the FHA domains. Because both Rad53 FHA domains are required for optimal binding to Rad9 ([8] and data not shown), we next examined whether the Rad53 kinase-independent phosphorylation event requires Rad9 residues necessary for its interaction with Rad53. Thus, the kinase-dead *rad53*^{D339A} allele was introduced in a *RAD9*$^{+}$ strain, a strain lacking *RAD9* (*rad9*Δ), or in a

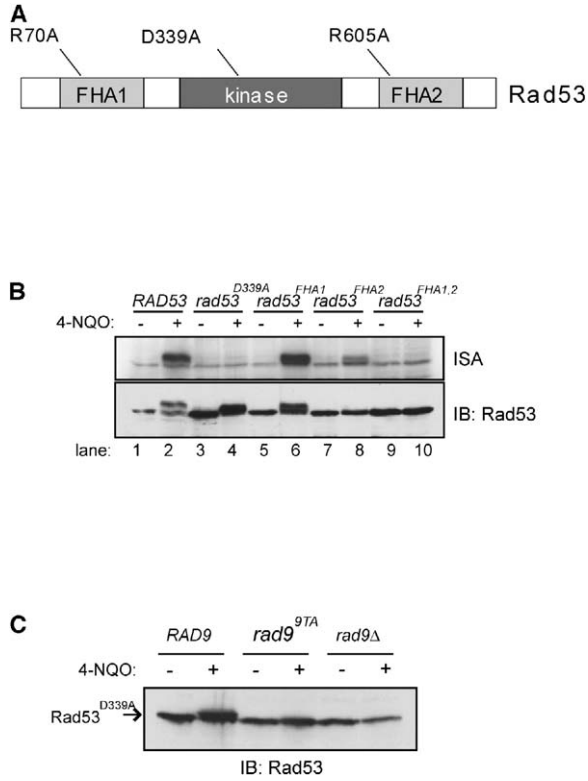


Figure 1. Rad53 Is Modified in Two Distinct Phosphorylation Events

(A) Domain architecture of Rad53 showing the two FHA domains (FHA1 and FHA2) flanking the central catalytic protein kinase domain. Also indicated are the sites of mutations abolishing phosphopeptide binding activity in the FHA1 (R70A) or FHA2 (R605A) domains. The site of the D339A mutation in the Rad53 kinase domain, which abolishes kinase activity, is also indicated.

(B) Analysis of Rad53 activity and phosphorylation after DNA damage. Strains harboring the indicated *RAD53* alleles were (+) or were not (–) treated with the UV-mimetic drug 4-NQO. Whole-cell extracts (WCE) were prepared by glass bead lysis in TCA, and proteins were separated by SDS-PAGE. Rad53 activity was measured by in situ kinase assays ([ISA], top), and Rad53 phosphorylation was estimated by examining gel mobility shifts after immunoblot analysis (bottom).

(C) Rad53 kinase-independent phosphorylation depends on Rad9. *rad53*^{D339A} cells containing either the *RAD9*, *rad9*Δ, or *rad9*^{9TA} alleles (the *rad9*^{9TA} allele contains T→A mutations on nine TQ motifs) were arrested in G1 by exposure to α factor and then treated with 4-NQO. Phosphorylation of Rad53 was examined by immunoblot as described above. Rad53^{D339A} phosphorylation is dependent on the PIKK phosphorylation sites on Rad9.

rad9^{9TA} strain that harbors mutations in nine TQ motifs required for the phosphorylation-dependent Rad53-Rad9 interaction [7]. As shown in Figure 1C, the DNA damage-induced phosphorylation of the Rad53^{D339A} protein in G1 (a cell cycle phase where Rad53 activation is totally dependent on *RAD9*) is totally abolished in the *rad9*^{9TA} strain, indicating that this Rad53 kinase-independent phosphorylation event is entirely dependent on a functional Rad9-Rad53 interaction. Furthermore, as described in the Supplemental Data available with this article online, this phosphorylation step is dependent on Mec1 and generates phospho-S/T-Q epitopes, suggesting that Mec1 may be the protein kinase responsible for this phosphorylation event (Figure S1).

Mapping of Rad53 Phosphorylation Sites In Vivo Using Mass Spectrometry

Because phosphorylation of Rad53^{D339A} after DNA damage is dependent on the same genetic determinants required for Rad53 activation, we hypothesize that the *trans*-phosphorylation event observed participates in Rad53 catalytic activation. We therefore sought to identify the phosphorylation sites involved in this event in order to characterize their function. As outlined in Figure 2A, our strategy was to map phosphorylation sites on Rad53^{D339A} in order to identify sites that are independent of Rad53 kinase activity. In parallel, we also mapped sites on wt Rad53 after DNA damage in order to deduce which sites are likely to represent autophosphorylation events. Therefore, to map Rad53 phosphorylation sites in vivo, we introduced FLAG epitope-tagged *RAD53* or *rad53*^{D339A} alleles under the control of the inducible *GAL1/10* promoter into a *rad53Δ sml1* strain to facilitate purification. The C-terminal FLAG epitope does not alter Rad53 function as the *RAD53-FLAG* plasmid restores tolerance to DNA damage when introduced in a *rad53Δ* strain (Figure S2). Rad53-FLAG and Rad53^{D339A}-FLAG were both immunopurified from 4-NQO-treated, asynchronously dividing cells to identify phosphorylation sites that are dependent or independent of Rad53 catalytic activity according to the scheme described in Figure 2B.

After immunopurification (Figure 2C), Rad53-FLAG or Rad53^{D339A}-FLAG was digested with trypsin or a combination of trypsin-Glu-C or trypsin-chymotrypsin proteases. The resulting peptides were split in two aliquots. One aliquot was analyzed by tandem mass spectrometry to estimate protein coverage. Our analysis indicates that we obtained peptide coverage of 74.7% of the total protein sequence (data not shown). The other aliquot was enriched for phosphopeptides by passing the peptide mixture over a Fe (III)-IMAC column, and phosphorylation sites were identified by tandem mass spectrometry [28]. By using this method, we unambiguously detected 42 phosphorylated peptides (Tables S1–S3) representing 14 sites on the Rad53^{D339A} sample (Figure 2E). In addition to identifying phosphopeptides corresponding to most of the sites found on the Rad53^{D339A} sample (boxed residues, Figure 2E), we identified 13 additional sites on the wild-type protein (Figure 2E).

Analysis of the phosphorylation sites mapped on Rad53^{D339A} reveals that five of the 14 sites mapped conform to the S/T-Q consensus for PIKK phosphorylation (Table S1). However, we also noted four additional sites that contain a hydrophobic residue immediately after the phosphoacceptor residues (S/T-ψ; where ψ is either Ala, Val, Ile, Leu, Phe, or Trp residues), a motif that is found phosphorylated by PIKKs in vitro and in vivo (see [29]). Of the remaining five sites, one appears to be a proline-directed site (S375), perhaps indicative of Cdk-dependent phosphorylation. As for the 13 sites found solely in the Rad53 wt sample, we predict that most correspond to autophosphorylation sites. Satisfyingly, two of the 13 sites (S350 and T354) map to the predicted activation segment of Rad53 (Table S1 and Figures 2D and 2E), a functionally relevant motif in kinases that is often phosphorylated. In addition, many of the phosphorylation sites are found as clusters (e.g., S745, S746, S748, and S750). Whether Rad53 acts as a processive kinase and prefers previously phosphorylated substrates or whether the observed clusters of phosphorylation sites represent particularly accessible regions of the protein is unknown due to the paucity of bona fide Rad53 substrates. Lastly, from the analysis of these 13 sites, we could not detect any obvious Rad53 consensus phosphorylation site or any site that conforms to the Rad53 phosphorylation consensus derived from the analysis of Swi6 phosphorylation [30].

Multisite Phosphorylation Is Required for Rad53 Function

We next turned our attention to the sites mapped on kinase-dead Rad53^{D339A} after DNA damage, as they may represent sites that directly participate in Rad53 transactivation. To determine whether these phosphorylation sites play a critical role in catalytic activation of Rad53, we mutated each of the 14 phosphorylation sites individually or in pairs. Rad53 catalytic activity was then evaluated by ISA, mobility shift on SDS-PAGE, and by examining the ability of the phosphorylation site mutants to complement the DNA damage sensitivity of the *rad53Δ sml1* strain. None of the single or double site mutants displayed sensitivity to DNA damage or loss of catalytic activity, as measured by ISA (Figures 3A and 3B and data not shown). However, mutation of residues S485/489 (Rad53^{A1}) and S560/563 (Rad53^{A5}) leads to a reproducible reduction in Rad53 mobility shift after DNA damage, suggesting that these sites are indeed phosphorylated in vivo (Figure 3B, bottom).

Next, we considered the possibility that multiple sites contribute to Rad53 kinase activation. Therefore, we combined phosphorylation site mutations and analyzed their phenotypes, as described above. To our surprise, even though we could nearly abolish the mobility shift of Rad53 after DNA damage when we mutated a total of six sites (Rad53^{A6} mutant), its activity as measured by ISA (Figure 3B, top) and DNA damage sensitivity (Figure 3C) remained similar to wt levels. However, when we mutated additional residues resulting in mutations of eight or nine of the 14 sites (e.g., the Rad53^{A8} or Rad53^{A9} mutants), we reached a critical point where Rad53 cannot be converted into an active protein kinase after exposure to DNA damaging agents, as demonstrated by the lack of activity in the ISA (Figure 3B, top) and DNA damage sensitivity (Figure 3C). These results suggest that Rad53 activation is dependent on the contribution of multiple phosphorylated residues.

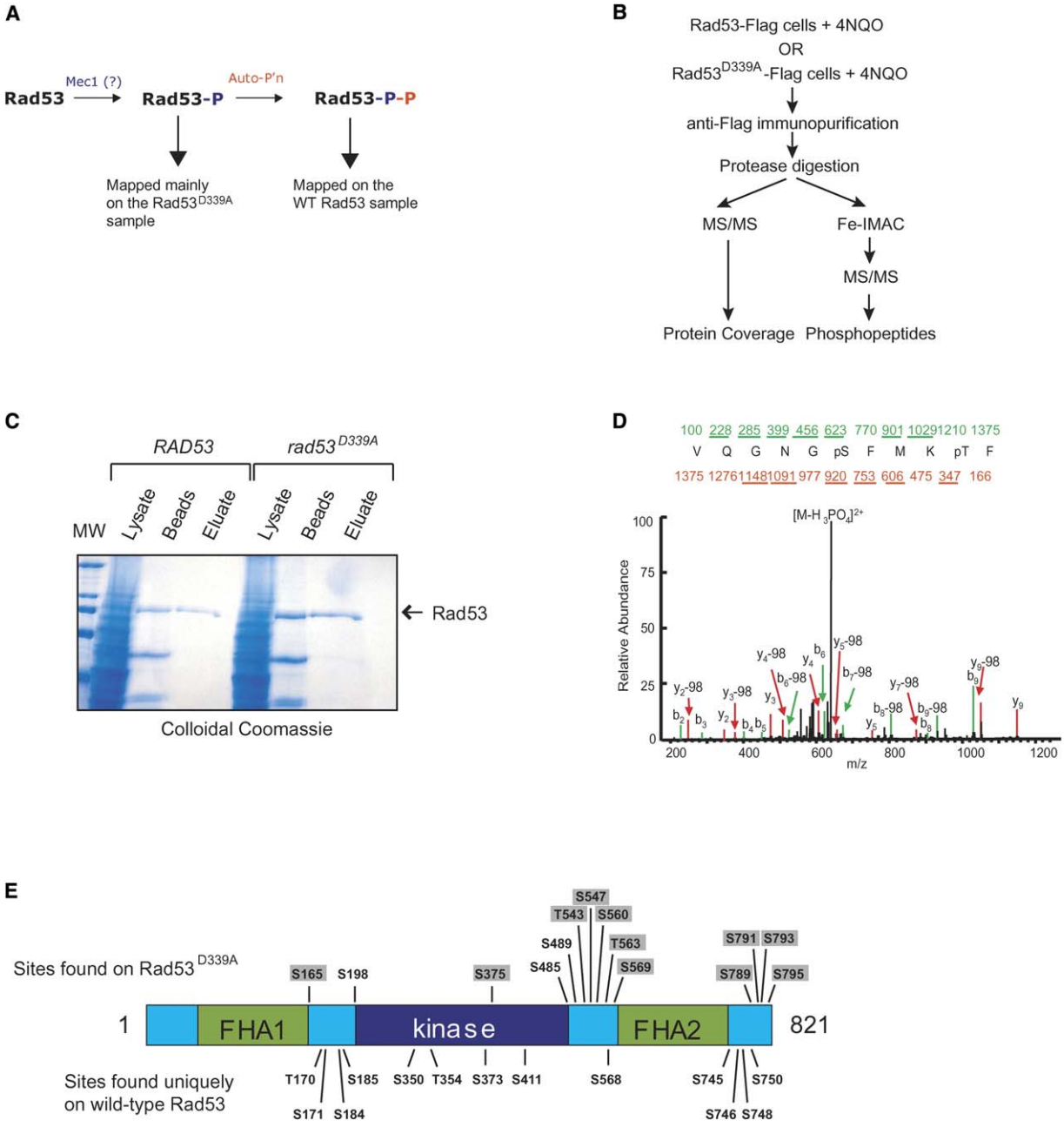


Figure 2. Phosphorylation Site Mapping of Rad53

(A and B) Strategy for the identification Rad53 phosphorylation sites by mass spectrometry. Rad53 phosphoregulation occurs in two distinct steps. (A) The Rad53^{D339A} kinase-dead variant was used to identify sites that are phosphorylated independently of Rad53 catalytic activity. Conversely, sites that are present only on wild-type (wt) Rad53 are likely to represent autophosphorylation sites. (B) After immunopurification of Rad53, the purified protein was digested by a combination of proteases and the sample was subjected either to tandem mass spectrometry to estimate coverage or to Fe(III)-IMAC chromatography to enrich for phosphorylated peptides that were subsequently identified by mass spectrometry.

(C) Immunopurification of Rad53 proteins. Equal fractions of crude cell lysates, proteins bound to anti-FLAG beads, and proteins eluted from the beads were stained with colloidal Coomassie. The eluate fraction corresponds to 5% of the total quantity of protein used for phosphorylation site mapping by mass spectrometry.

(D) Typical MS/MS spectrum obtained from phospho-Rad53 showing a fragmentation pattern identifying doubly phosphorylated Ser350 and phosphorylated Thr354 residues in the activation segment of Rad53. Please refer to Tables S1–S3 for a complete list of the peptide sequences and the position of the phosphorylation sites.

(E) In vivo phosphorylation sites identified by mass spectrometry from the Rad53^{D339A} sample (sites above the drawn Rad53 molecule) and wt Rad53 (sites below). Boxed residues represent sites that we also found in the wt Rad53 sample. Sequences of the sites are found in Table S1; full sequences of the corresponding peptides in which these sites were identified can be found in Table S3.

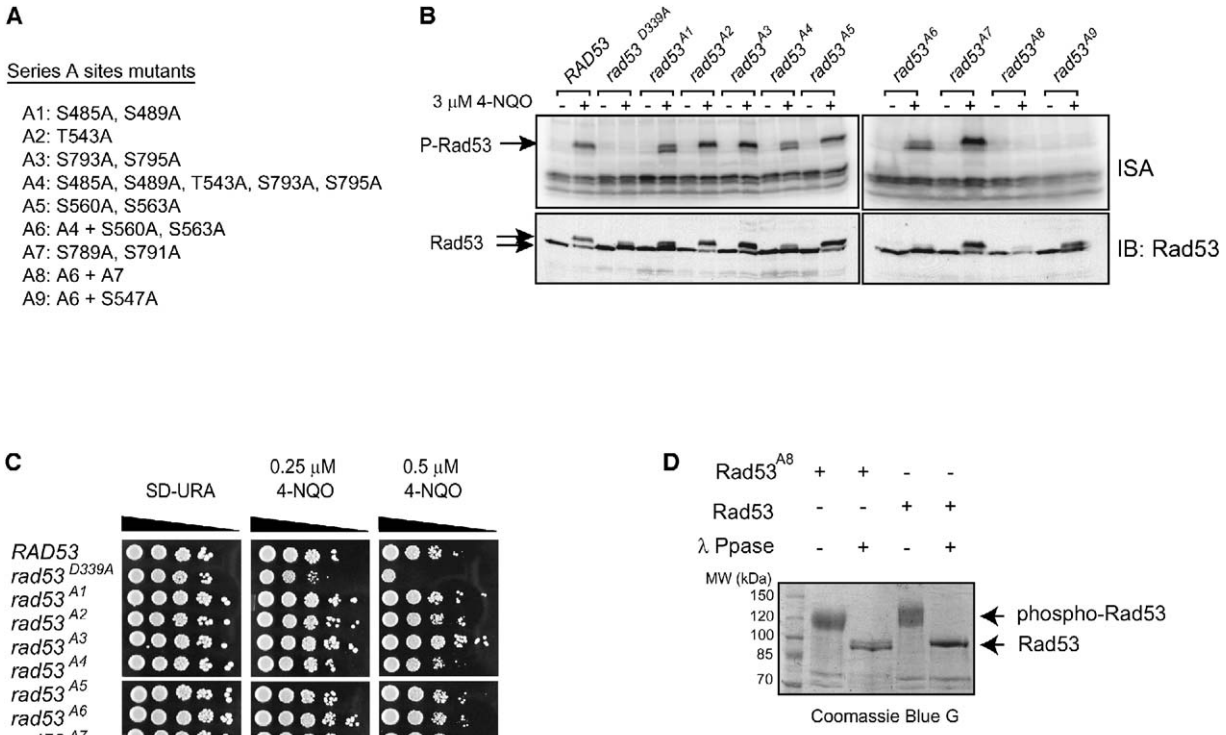


Figure 3. Putative Mec1 Phosphorylation Sites Are Required for Rad53 Activity

(A) Series A mutations.

(B) Strains harboring the indicated *RAD53* alleles were (+) or were not (–) treated with 3 μM 4-NQO. WCE were prepared and proteins separated by SDS-PAGE. Rad53 activity was monitored by ISA (top), and Rad53 phosphorylation was estimated by gel mobility shift after immunobloting (bottom). Combinations of eight or nine mutations lead to a defect in Rad53 activation (mutants $Rad53^{A8}$ and $Rad53^{A9}$). However, note that some combinations of mutants affect Rad53 mobility shift after DNA damage without affecting its kinase activity.

(C) Strains containing the alleles described above were diluted serially 10-fold and spotted on agar plates containing 4-NQO at the indicated concentrations.

(D) Bacterially produced $Rad53^{A8}$ has protein kinase activity. His-tagged Rad53 and $Rad53^{A8}$ were expressed and purified in *E. coli* as described in the Supplemental Experimental Procedures. Note that $Rad53^{A8}$ displays an extensive mobility shift comparable to wt Rad53, indicating that it has protein kinase activity.

The $Rad53^{A8}$ and $Rad53^{A9}$ mutants are expressed at wt or near wt levels in yeast cells and interact with Rad9 (data not shown). These observations rule out the possibility that the defect in Rad53 activity is simply due to loss of protein expression or loss of interaction with Rad9. Importantly, expression of $Rad53^{A8}$ in bacteria results in an active protein kinase as demonstrated by the substantial autophosphorylation observed on $Rad53^{A8}$ (Figure 3D). This latter result indicates that the *rad53*^{A8} allele is likely to be inactive in yeast due to the disruption of regulatory residues rather than protein misfolding. Based on the above results, we conclude that *MEC1*-dependent Rad53 *trans* phosphorylation is required to convert the latent Rad53 enzyme into an active protein kinase.

Rad9 Stimulates Mec1 Phosphorylation of Rad53 In Vitro

The observation that putative Mec1 phosphorylation sites are required for Rad53 activation in vivo suggests a role for Mec1 in Rad53 activation that is upstream of the solid-state catalyst model. In this model, Rad9 is proposed to catalyze in *trans* autophosphorylation of Rad53 in soluble Rad9-Rad53 complexes that have been released from the lesion at which the Mec1 kinase is tethered [18, 31]. However, considering our new data on the phosphoregulation of Rad53, we reasoned that instead of acting solely as a solid-state catalyst, Rad9 may also be acting to recruit Rad53 to sites of DNA damage to allow it to be efficiently phosphorylated by Mec1.

To directly test the adaptor model, we first examined whether Mec1 can phosphorylate Rad53 directly in vitro. To do so, we optimized a purification procedure to produce full-length, recombinant Rad9 from yeast and performed large-scale immunoprecipitations of FLAG-tagged Mec1 and kinase-dead Mec1 proteins (Mec1-kd, Figures 4A and 4B). With these purified proteins, we examined the capacity of Mec1 to phosphorylate $Rad53^{D339A}$ directly in vitro. We incubated purified Mec1 with increasing amounts of bacterially produced $Rad53^{D339A}$ or the model PIKK substrate PHAS-I [32].

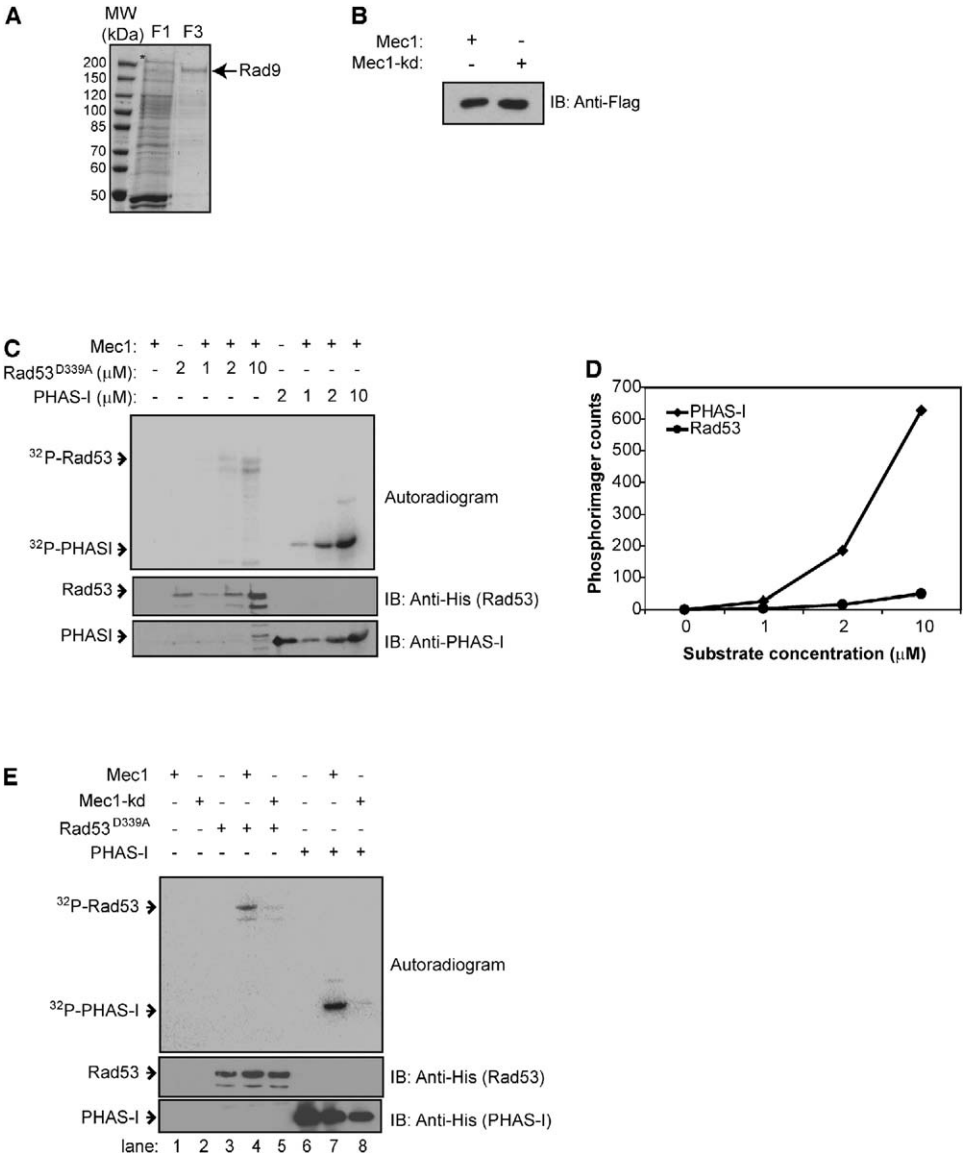


Figure 4. Direct Phosphorylation of Rad53 by Mec1

(A and B) Purified recombinant proteins used in the in vitro phosphorylation assays of this study. Full-length GST-TEV-Rad9 (A) was purified from yeast cells by a three-step chromatography protocol that included heparin-sulfate chromatography as a first purification step (F1). The Rad9-containing eluate was then bound to glutathione-Sepharose beads and, finally, Rad9 was released after TEV protease cleavage of the fusion protein (F3). Full-length FLAG-His_{10}-Mec1 or FLAG-His_{10}-Mec1-kd (B) was purified from yeast cells by using Ni^{2+}-NTA-agarose affinity columns followed by FLAG immunopurification. The residue mutated in the kinase-dead *mec1-kd* allele is D2224A [50].

(C) Rad53 is a poor Mec1 substrate relative to PHAS-I. In vitro Mec1 kinase assays using equimolar concentrations of $Rad53^{D339A}$ or PHAS-I as substrates were performed in the presence of [γ-^{32}P]ATP. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane, and radioactivity incorporation was measured by phosphorimaging (top). The presence of Rad53 and PHAS-I was monitored by immunoblotting using anti-His tag and anti-PHAS-I antibodies, respectively (bottom panels).

(D) Phosphorimager quantification of the in vitro kinase assays performed in (B). Note that at equimolar concentrations, Rad53 is markedly less efficiently phosphorylated than PHAS-I.

(E) Kinase reactions as described in (B) using 2 μM of the indicated substrates were carried out by using either wt Mec1 or Mec1-kd. The presence of substrate was monitored by immunoblotting using an anti-His tag antibody which recognizes either substrates (bottom panels).

The protein kinase reactions were carried out in the presence of radiolabeled ATP, and phosphorylation was detected and quantitated by phosphorimaging. At equimolar concentrations, Rad53 is consistently phosphorylated less than PHAS-I (Figures 4C and 4D) even though there is only one PIKK site, S111, on PHAS-I [5] compared to 14 potential phosphoacceptor sites on Rad53 (i.e., all sites mapped on $Rad53^{D339A}$). The observation that Rad53 behaves as a poor Mec1 substrate relative to the nonphysiological PIKK substrate PHAS-I is clearly at odds with the quantitative phosphorylation of Rad53 observed in vivo (Figure 1). As a control, we ensured that the phosphorylation of both Rad53 and PHAS-I is dependent on Mec1 kinase activ-

ity by carrying out kinase assays with the Mec1-kd protein instead of wt Mec1. As expected, Mec1-kd is unable to support efficient phosphorylation of either PHAS-I or Rad53 (Figure 4E, lanes 5 and 8).

In vivo, Rad53 is efficiently phosphorylated in a *RAD9*-dependent manner and in a Rad53 FHA domain-dependent manner (Figure 1). Therefore, the observation that Rad53 is a relatively poor Mec1 substrate prompted us to test whether Rad9 can stimulate phosphorylation of Rad53^{D339A} by Mec1. Kinase assays were performed with fixed amounts of Rad53^{D339A} and Mec1 and increasing amounts of Rad9. Under these conditions, Rad9 is clearly able to stimulate Rad53 phosphorylation in a dose-dependent manner (Figure 5A). Intriguingly, we observe that Rad9 does not appear to incorporate as much ^{32}P as Rad53 in these assays. This observation can be reconciled with our finding that Rad9 purified from yeast cells is substantially phosphorylated on multiple sites, including at some S/T-Q residues (Figure S3), a condition that may preclude additional Mec1 phosphorylation. Nevertheless, the stimulation of Rad53 phosphorylation is not due to contaminating kinase activity in the Rad9 preparation because: (1) omission of Mec1 does not lead to appreciable Rad53 phosphorylation (Figure 5A); (2) Rad53 phosphorylation is not observed when Mec1-kd is used instead of wt Mec1 (Figure 5B); and (3) Rad53 phosphorylation by Mec1 is inhibited by the PIKK inhibitor wortmannin (Figure 5D). We therefore conclude that Rad9 facilitates Rad53 phosphorylation by Mec1 in vitro.

Two possible models can explain the observed effect of Rad9 on Rad53 phosphorylation by Mec1. Firstly, Rad9 may directly stimulate Mec1 catalytic activity. If this is the case, one would expect that phosphorylation of other substrates such as PHAS-I may also be stimulated by Rad9. Secondly, Rad9 may act as an adaptor (or scaffold) to bring Rad53 in close proximity to Mec1. To test the first model, we examined the effect of Rad9 on the phosphorylation of PHAS-I by Mec1. As shown in Figures 5B and 5C, addition of Rad9 does not lead to appreciable stimulation of PHAS-I phosphorylation, indicating that Rad9 does not act by increasing Mec1 catalytic activity.

Next, we examined whether Rad9 stimulation of Rad53 phosphorylation in vitro depends on the same determinants for Rad53 activation in vivo, namely a requirement for functional Rad53 FHA domains and Rad9 phosphorylation. First, we employed the kinase assay described above but in some reactions substituted Rad53^{D339A} with a mutant version of this protein incorporating the R70A and R650A mutations, which disable the Rad53-Rad9 interaction in vivo (Rad53$^{D339A,\ FHA1,2}$). As shown in Figure 5D (lane 8), the Rad9 stimulation of Rad53 phosphorylation clearly requires the Rad53-Rad9 phospho-dependent interaction, as mutation of the Rad53 FHA domains completely abrogates the effect of addition of Rad9. Secondly, we examined whether dephosphorylation of Rad9 (Figures S3B and S3C) prior to kinase assays affects its ability to stimulate phosphorylation of Rad53 by Mec1. As shown in Figure 5E, phosphorylation of Rad9 is essential for its ability to stimulate Rad53 phosphorylation by Mec1. We therefore conclude that Rad9 stimulates Rad53 phosphorylation by Mec1 in vitro via an FHA domain-dependent interaction between Rad53 and phospho-Rad9, thus recapitulating the requirement for the Rad9-Rad53 interaction essential for Rad53 activation in vivo.

In Vitro Rad53 Phosphorylation Sites Overlap with Those Observed In Vivo

To verify that the reconstitution of Rad9-dependent Rad53 phosphorylation by Mec1 recapitulates Rad53 phosphorylation after DNA damage, we examined whether the sites phosphorylated by Mec1 in vitro overlap with the sites mapped on Rad53 purified from yeast cells. First, we tested whether the Rad53$^{D339A,\ A8}$ mutant, which is not appreciably phosphorylated in vivo (Figure 3), can act as an efficient substrate in Mec1 kinase reactions. As shown in Figure 6A, substitution of Rad53^{D339A} by the Rad53$^{D339A,\ A8}$ mutant in Mec1 kinase assays (in the presence of Rad9) results in a marked decrease in incorporation of radioactivity onto Rad53, suggesting that some of the sites mapped in vivo are essential for Rad53 phosphorylation by Mec1 in vitro.

In parallel to these experiments, Rad53^{D339A} was phosphorylated by Mec1 in the presence of Rad9 on a preparative scale (Figure 6B), and Rad53 phosphorylation sites were then mapped by mass spectrometry, as described above. Satisfyingly, we unambiguously identified 13 sites, seven of which were also identified in vivo (Figure 6C and Table S2). Of these seven sites, two correspond to S/T-Q sites, three to S/T-ψ sites, and two to S-S motifs (Table S2). Interestingly, some of the sites mapped in our in vitro kinase reactions were previously mapped on wt Rad53 and not on Rad53^{D339A} (see Table S1 and Figure 2E), perhaps indicating that some sites were missed in our analysis of the Rad53^{D339A} sample. Therefore, we conclude that we are, at least partially, able to reconstitute the phosphorylation of Rad53 observed in vivo after DNA damage. Overall, the reconstitution assays described herein support the adaptor model for Rad9 function, indicate that Mec1 directly phosphorylates Rad53 in vitro and in vivo, and suggest that Mec1 phosphorylation of Rad53 is essential for its subsequent catalytic activation.

Discussion

Rad9 was the first checkpoint protein identified in yeast by the pioneering work of Weinert and Hartwell [33]. As the founding member of the group of checkpoint mediator proteins, the elucidation of the function of Rad9 may bring new light to the role of checkpoint mediators in checkpoint signaling. In this study, we found that direct phosphorylation of Rad53 by Mec1 is required for its catalytic activation. Furthermore, based on biochemical reconstitution experiments, our results suggest that an important role of Rad9 in Rad53 activation is to promote phosphorylation of Rad53 by its upstream activator, Mec1. In that sense, the function of Rad9 in Rad53 activation is more akin to classical signaling adaptors. To our knowledge, our data present the first direct biochemical evidence supporting the adaptor model of checkpoint mediator function. However, our data do not exclude a mixed model whereby

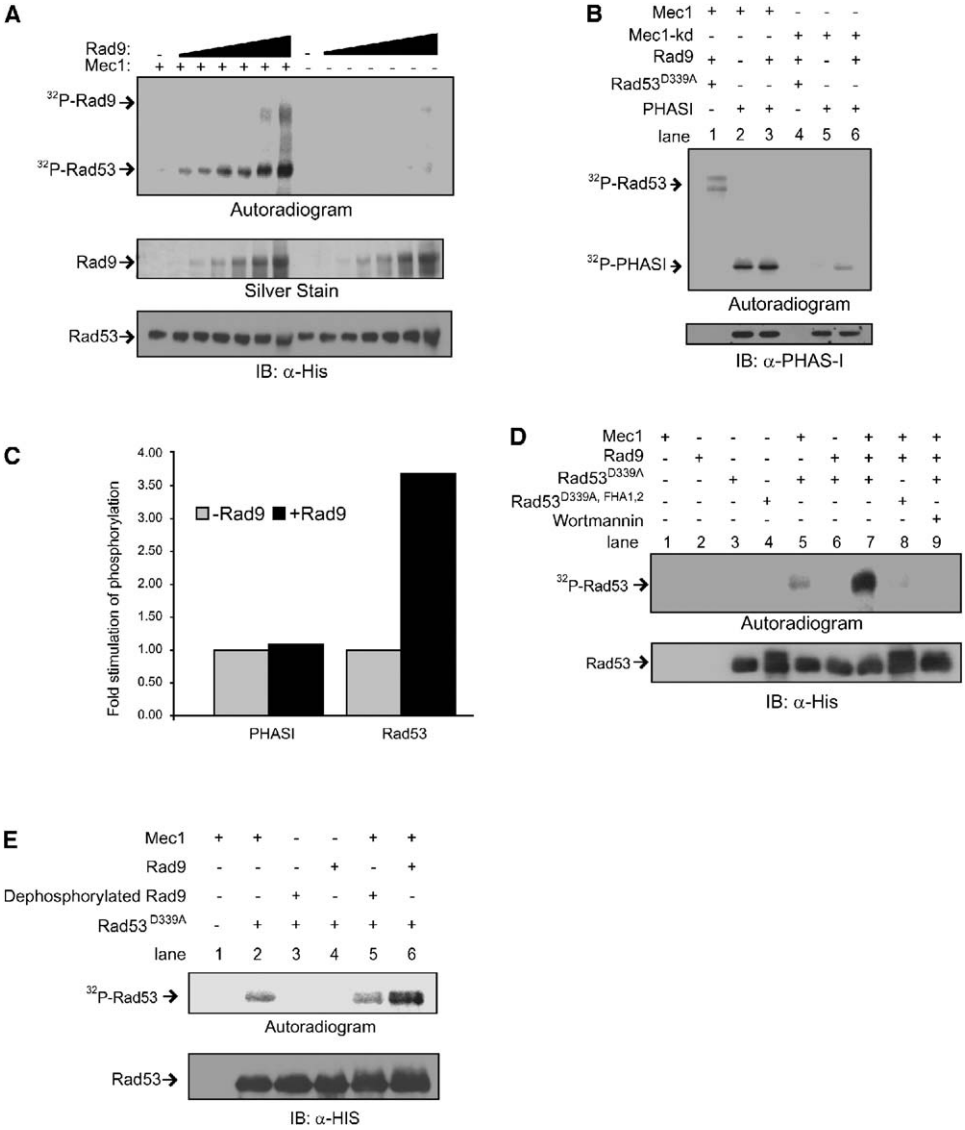


Figure 5. Rad9 Acts as an Adaptor to Facilitate Rad53 Phosphorylation by Mec1 In Vitro

(A) Rad9 stimulates phosphorylation of Rad53 by Mec1 in vitro. In vitro kinase assays were carried out by using a constant amount of Mec1 and Rad53^{D339A} in the absence or increasing amounts of purified Rad9. The presence of Rad9 was monitored by silver staining, and the levels of Rad53 were monitored by anti-His tag immunoblotting (bottom panels).

(B) Rad9 does not increase Mec1 intrinsic kinase activity. Protein kinase reactions were carried out as above with the exception that phosphorylation of PHAS-I was also measured in the presence and absence of Rad9. Addition of Rad9 does not significantly increase Mec1 activity toward PHAS-I.

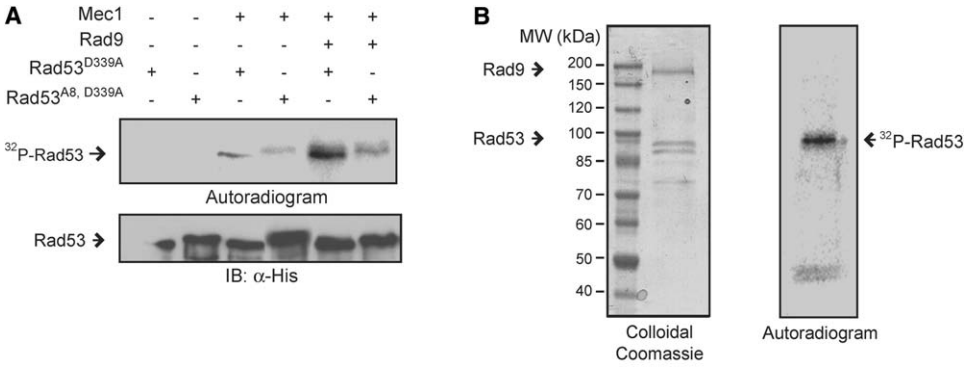
(C) Quantification of the PHAS-I/Rad53 kinase assays described in (B). Please note that the data for Rad53 phosphorylation +/– Rad9 were quantitated from the kinase reaction shown in (D) (lane 7 versus 5).

(D) In vitro Mec1 kinase assays using equimolar concentration of Rad53^{D339A} or Rad53$^{D339A, FHA1,2}$ as substrates were performed in the presence of [γ-^{32}P]ATP in the presence or absence of Rad9. In one reaction (lane 9), the PIKK inhibitor wortmannin was also included. Proteins were separated on SDS-PAGE and transferred onto a PVDF membrane, and radioactivity incorporation was measured by phosphorimaging. The presence of Rad53 was monitored by immunoblotting using anti-His tag antibody (bottom). Please note that the apparent "shift" displayed by the Rad53$^{D339A, FHA1,2}$ protein is due to a longer hexahistidine tag.

(E) In vitro Mec1 kinase assay was performed in the presence of Rad53^{D339A} and equimolar concentration of purified Rad9, dephosphorylated Rad9, or Rad9 subjected to a mock dephosphorylation reaction (see Figures S3B and S3C). The proteins were incubated in the presence of [γ-^{32}P]ATP, separated by SDS-PAGE, and transferred to PVDF membrane. The presence of radioactivity incorporation was monitored by phosphorimaging (top), and the presence of Rad53^{D339A} was monitored by immunoblotting using an anti-His antibody (bottom).

Rad9 would play two distinct roles during Rad53 activation: first as a Mec1 adaptor and then as a facilitator of Rad53 autophosphorylation, perhaps of the activation segment, by increasing the local concentration of Rad53 on the Rad9 surface.

Mec1 therefore plays two distinct roles in the Rad53 activation cycle (Figure 7A). Firstly, after detection of DNA damage, Mec1 phosphorylates multiple Rad9 residues at S/T-Q sites [7]. Rad9 phosphorylation presumably occurs at regions of DNA damage [34, 35],



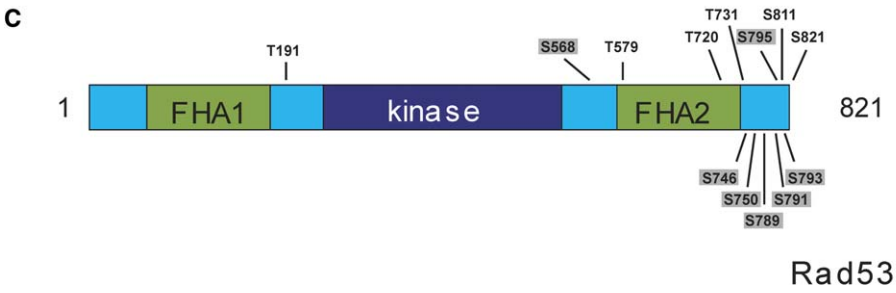


Figure 6. In Vitro Rad53 Phosphorylation Sites Overlap with Those Observed In Vivo

(A) The Rad53$^{D339A,\ A8}$ mutant is not efficiently phosphorylated by Mec1. In vitro Mec1 kinase assays using equimolar concentration Rad53^{D339A} or Rad53$^{D339A,\ A8}$ as substrates were performed in the presence of [γ-^{32}P]ATP. Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane, and analyzed by phosphorimaging (top) or immunoblotting by using an anti-His tag antibody (bottom). The difference in size between Rad53^{D339A} or Rad53$^{D339A,\ A8}$ is due to an additional 31 amino acids residue between the C-terminal hexahistidine sequence and the stop codon (see Supplemental Experimental Procedures).

(B) Recombinant Rad53^{D339A} was phosphorylated by Mec1 in the presence of Rad9 on a preparative scale. After termination of the kinase reaction, proteins were separated by SDS-PAGE and visualized by colloidal Coomassie-staining (left). In a separate reaction, radiolabeled [γ-^{32}P]ATP was added to monitor phosphorylation by phosphorimaging (right).

(C) Phosphorylated residues identified on Rad53^{D339A} phosphorylated by Mec1. Sites that were also identified in vivo are boxed. The sequence of these sites can be found in Table S2.

and multisite-phosphorylated Rad9 recruits Rad53 to DNA lesions in an FHA-dependent manner [8–11, 35]. Mec1 then acts a second time to phosphorylate Rad9 bound Rad53 on multiple sites. This multisite phosphorylation of Rad53 presumably contributes to the relief of catalytic autoinhibition, allowing Rad53 to become active. The relief of catalytic autoinhibition by protein phosphorylation is often used as a catalytic switching mechanism by protein kinases [36]. After Mec1 phosphorylation, Rad53 autophosphorylates itself on many residues, including S350 and T354 in the activation segment. We propose that activation segment autophosphorylation completes the Rad53 activation process, as in most kinases [37]. Active Rad53 is finally released from Rad9 [18], liberating Rad9 for another round of Rad53 activation or for inactivation by the action of protein phosphatases (Figure 7A). The role of autophosphorylation outside the activation segment remains to be ascertained, but it is tempting to speculate that at least some of these sites promote the release of Rad53 from Rad9 or promote the phosphorylation-dependent Rad53-Dun1 interaction.

PIKK-Dependent Activation of Rad53 Orthologs

In both fission yeast and in human cells, the Rad53 orthologs (Cds1 and Chk2, respectively) are also subject to direct phosphorylation by PIKKs. However, Cds1 and Chk2 are phosphorylated by PIKKs on important N-terminal TQ motifs (T11 in *S. pombe* and T68 in human cells) in response to DNA damage [38, 39]. In human cells, T68 phosphorylation of Chk2 by ATM is thought to promote the formation of Chk2 multimers, which are then competent to autoactivate via *trans* autophosphorylation [40]. The formation of these multimers is dependent on the Chk2 FHA domain. However, in the case of Rad53, the analogous residues appear rather to be involved in the interaction with Asf1 and Dun1 [20] and not directly in Rad53 activation. In fact, Rad53 does not appear to form multimers in response to DNA damage (F.D.S. and D.D., unpublished data). Furthermore, despite detecting the phosphorylation of the analogous T12 and T15 residues in bacterially produced hyperphosphorylated Rad53, we have failed—despite multiple attempts—to detect any phosphorylation on these residues from Rad53 purified from yeast

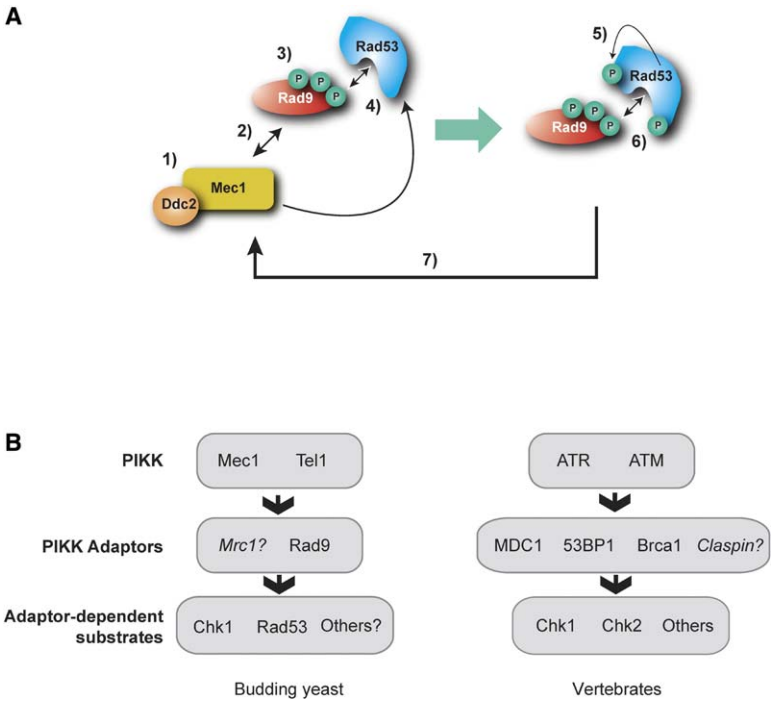


Figure 7. Models of DNA Damage Checkpoint Activation

(A) Model of Rad53 activation. After Mec1/Ddc2 activation by DNA lesions (1), Mec1 phosphorylates the adaptor molecule Rad9 on multiple S/T-Q motifs (2). Phospho-Rad9, in turn, recruits Rad53 in an FHA-dependent manner to DNA lesions (3). We propose that Rad53 recruitment leads to direct phosphorylation of Rad53 by Mec1 (4). Mec1 phosphorylation of Rad53 leads to Rad53 activation and subsequent autophosphorylation (5). Hyperphosphorylated Rad53 is then released from Rad9 (6), freeing Rad9 to perform another round of Rad53 activation (7).

(B) We propose that checkpoint mediators act as PIKK adaptors during the DNA damage response, linking PIKKs to their substrates. The diversity of mediators in metazoans suggests that the spatio-temporal control of the checkpoint response is regulated by a network of protein-protein interactions controlled by checkpoint mediators.

cells (data not shown). The bacterially produced Rad53 protein is hyperphosphorylated to a great extent, and many of these autophosphorylation sites seem to be artifactual, as illustrated by the identification by mass spectrometry of at least 70 distinct phosphorylation sites on recombinant Rad53 (F.D.S., F.Y., D.D., and D.F.H., unpublished data). Therefore, bacterially produced Rad53 appears to be a poor model to investigate the phosphoregulation of Rad53. In any case, our data suggest that phosphorylation of this cluster is not a universally conserved mechanism for the regulation of Chk2 orthologs.

Physiologically Relevant Noncanonical PIKK Phosphorylation

Many members of the PIKK family such as Mec1 (ATR), Tel1 (ATM), or DNA-PK prefer to phosphorylate S/T-Q sites [7, 41, 42]. Such substrate selectivity has predictive value, and a number of investigations have taken advantage of this preference to identify biologically relevant PIKK phosphorylation events (e.g., see [7, 13, 43]). In this study, we present evidence that Mec1 can phosphorylate Rad53 in vivo and in vitro on sites that do not conform necessarily to the S/T-Q consensus. Of these, most contain hydrophobic residue following the phosphoacceptor site (the S/T-ψ motif). Although uncommon, non-S/T-Q site phosphorylation is also observed for PIKKs other than Mec1. For example, ATM phosphorylates Brca1 in vitro and in vivo on residue Ser1542 at a S-G motif [44]. In addition, DNA-PK phosphorylates the Ku heterodimer on a number of sites that do not conform to the Ser/Thr-Gln consensus [29]. Why S/T-ψ phosphorylation is observed on some substrates and not on others is puzzling. S/T-ψ may represent suboptimal PIKKs phosphorylation sites, and their phosphorylation by PIKKs may indicate that additional conditions must be met before they can be phosphorylated. For example, one can speculate that physical colocalization of PIKKs and substrates may result in such a phosphorylation event. Colocalization of PIKK and substrate is clearly expected in the case of Ku and DNA-PKcs or Brca1 and ATM [45]. The fact that Rad53 requires binding to Rad9 in order to be efficiently phosphorylated by Mec1 and that Mec1 and Rad9 colocalize at sites of DNA damage may support this possibility. If physical colocalization is required for phosphorylation of non-S/T-Q sites, these noncanonical phosphorylation sites on Rad53 may provide a mechanism to ensure that Rad53 activation only occurs after the proper physical assembly of the checkpoint signaling apparatus.

Checkpoint Mediators as PIKK Adaptors

In "classical" signal transduction pathways such as those operating at the cell surface, signaling adaptors are usually polypeptides that lack known catalytic domains but possess at least two protein-protein interaction domains, usually of a modular nature [46]. In a general sense, signaling adaptors function to couple upstream signaling with downstream events by bridging at least two distinct proteins. The modular nature of signaling adaptors enables the use of a relatively small subset of protein domains and target motifs to construct elaborate signaling networks. In such networks, adaptors also act as regulatory nodes where signaling can be regulated via signal-dependent protein-protein interactions.

Based on the above considerations and on the following observations, we propose that Rad9 acts as a bona fide signaling adaptor during the DNA damage

response: (1) Rad9 physically colocalizes with Mec1 at sites of DNA damage, suggesting that both proteins may physically interact [34]; (2) the Rad9-Rad53 interaction is essential for Rad53 activation in vivo [8, 9]; and (3) Rad9 greatly stimulates Rad53 phosphorylation by Mec1 in vitro (this study). We propose that Rad9 functions to physically bridge Rad53 to Mec1 and that Rad9 may be able to physically interact with Mec1, at least transiently. It is tempting to speculate that this latter interaction may be mediated via the tandem BRCT domains of Rad9, as the Rad9 BRCT domains are required for checkpoint signaling in vivo [47]. Interestingly, tandem BRCT domains can act as phosphopeptide recognition modules [48, 49], but whether the Rad9 BRCT domains act as bona fide phosphopeptide recognition modules to bind to Mec1 or any other protein remains to be determined.

Finally, given the similarities between Rad9 and human checkpoint mediators containing tandem BRCT domains, we propose that Brca1, MDC1, and 53BP1 also act as PIKK adaptors during the DNA damage response and that this activity represents a common function of checkpoint mediators. Interestingly, each known mammalian checkpoint mediator harbors a unique protein-interaction module such as the FHA domain (Mdc1), RING finger (Brca1), or the Tudor domain (53BP1), indicating that they promote either a unique set of protein-protein interactions or engage an overlapping set of proteins in response to different upstream signals. The requirement for multiple checkpoint mediators in mammals may reflect the increased complexity of the mammalian genome or may indicate that novel layers of regulation were added during metazoan evolution. By virtue of these properties, we therefore propose that checkpoint mediators are PIKK adaptors that orchestrate the DNA damage response in space and time.

Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, Supplemental References, three figures, and three tables and are available with this article online at http://www.current-biology.com/cgi/content/full/15/15/1364/DC1/.

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