Mechanism of Dun1 Activation by Rad53 Phosphorylation in Saccharomyces cerevisiae

Despite extensive studies, the molecular mechanism of DNA damage checkpoint activation remains incompletely understood. To better dissect this mechanism, we developed an activity-based assay for Dun1, a downstream DNA damage checkpoint kinase in yeast, using its physiological substrate Sml1. Using this assay, we confirmed the genetic basis of Dun1 activation. Rad53 was found to be directly responsible for Dun1 activation. We reconstituted the activation of Dun1 by Rad53 and found that phosphorylation of Thr-380 in the activation loop of Dun1 by Rad53 is responsible for Dun1 activation. Interestingly, phosphorylation of the evolutionarily conserved Thr-354 in the activation loop of Rad53 is also important for the regulation of Rad53 activity. Thus, this conserved mode of activation loop phosphorylation appears to be a general mechanism for the activation of Chk2 family kinases.

In Saccharomyces cerevisiae, an evolutionarily conserved kinase cascade, consisting of Mec1, Tel1, Chk1, Rad53, and Dun1, is responsible for cellular responses to DNA damage (1). Although Mec1 and Tel1 are involved in the initial detection of DNA damage (2), Rad53 and Dun1 function as effector kinases to regulate many downstream cellular processes. Both Rad53 and Dun1 belong to the Chk2 family kinases, which contain a Forkhead-associated (FHA) domain and a serine/threonine kinase domain. DNA damage-induced hyperphosphorylation of Rad53 and Dun1 in response to DNA damage depends on Mec1 (3). Furthermore, adaptor proteins Rad9 and Mrc1 are important for DNA damage-induced hyperphosphorylation of Rad53 (4–7). Rad9 and Mrc1 become hyperphosphorylated in response to DNA damage treatment in a Mec1- and Tel1-dependent manner (5, 8–10).

After DNA damage, Rad53 binds to hyperphosphorylated Rad9 and Mrc1 via its FHA domains. It was thus proposed that this interaction might be important for the activation of Rad53 in vivo. Rad53 activation appears to be accompanied by its autophosphorylation (11, 12). On the other hand, DNA damage-induced phosphorylation of Dun1 was shown to depend on Rad53, and Rad53 could phosphorylate Dun1 in vitro (13, 14). However, the activity of Dun1 was not measured directly. Because Dun1 acts genetically downstream of Mec1, Tel1, Rad9, Mrc1, and Rad53, understanding the regulation of Dun1 should further help to understand the activation of Rad53 and its upstream kinases Mec1 and Tel1. To this end, it is important to develop an activity-based assay for Dun1 and its upstream kinases using its physiological substrate.

Dun1 was originally identified as a mutant showing defect in the up-regulation of a transcription of genes encoding ribonucleotide reductase (RNR) in response to DNA damage (15). It has been suggested that Dun1 is involved in various DNA damage responses, including G2 arrest after DNA damage (13, 16), in addition to the regulation of dNTP level after DNA damage. The RNR complex catalyzes the rate-limiting step in the synthesis of dNTP from NTP, which is crucial for DNA replication and repair. Multiple mechanisms were identified to regulate the dNTP level via the regulation of the RNR complex in the yeast S. cerevisiae. First, transcription of the RNR genes is up-regulated in response to DNA damage, which is regulated by Dun1 (17). Second, Sml1, an inhibitor of RNR, is degraded in response to DNA damage (18, 19). Importantly, Dun1 appears to be directly responsible for Sml1 phosphorylation and its subsequent degradation (20). Finally, Dun1-dependent regulation of the localization of different RNR subunits in response to DNA damage serves as additional mechanisms for the regulation of RNR activity in the cells (21, 22). Taken together, it appears that an important function of Dun1 is the regulation of dNTP level in the cells. Several lines of evidence further suggested that Sml1 is a physiological substrate of Dun1. First, Sml1 was known to undergo Dun1-dependent degradation in response to DNA damage (20). Second, Dun1 was shown to phosphorylate Sml1 efficiently in vitro (20). Third, Dun1 interacts with Sml1 (23). Thus, phosphorylation of Sml1 by Dun1 may provide an assay to measure the kinase activity of Dun1.

In this study, we established an in vitro assay to quantify the activity of Dun1 for Sml1 hyperphosphorylation. Using this assay, we found that the activity of Dun1 is strongly induced after DNA damage treatment. Importantly, phosphorylation of Dun1 is required for this activity. Furthermore, we confirmed and expanded the roles of Mec1, Tel1, Rad9, and Mrc1 in the activation of Dun1. Interestingly, deletion of RAD53 completely abolished the activity of Dun1 for Sml1 hyperphospho-
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Yeast Strains, Plasmids, and Genetic Methods—Standard yeast growth and genetic methods are used in this work. Strains used in this work are listed in supplemental Table S1. Plasmids used in this work are listed in supplemental Table S2. GFP-Sml1 was first cloned into the pFA6a plasmid using PacI and AscI and then introduced into yeast cells via homologous recombination (24). C-terminal TAF-tagged Dun1 was cloned into a pFA6a plasmid, yielding pFA6a-Dun1-TAF-KanMX6 plasmid. Site-directed mutagenesis was used to generate various Dun1 mutants in this plasmid, which were then used to generate endogenous Dun1 mutants using homologous recombination. The TAF tag contains a His6-FLAG-protein A, with a TEV protease cleavage sequence between the FLAG sequence and protein A. This allows the elution of Dun1 using TEV protease. After TEV cleavage, a remaining FLAG sequence at the C terminus of Dun1 allows the detection of Dun1 using anti-FLAG Western blot. Deletion of Sml1 suppresses the lethality of MECl1 and RAD53 deletion, as well as synthetic lethality of RAD9 and MRC1 deletion. For Dun1 activity study, sml1Δ background was therefore used unless noted otherwise (other studies indicated that deletion of SML1 does not affect Dun1 activity in WT cells).3 All mutations or integrations introduced in yeast cells were confirmed by DNA sequencing.

Sml1 was cloned into pGEX-4T1 using BamHI and EcoRI sites. Recombinant Rad53 was cloned into a pET21a plasmid with an N-terminal protein A tag using EcoRI and NotI sites. Site-directed mutagenesis was used to generate Rad53-T5A, T8A, T12A, and T15A mutant. To generate Rad53-4TA, which contains a quadruple alanine mutation, purified from either yeast or bacteria has a drastically reduced activity. For Dun1 activity study, sml1Δ background was therefore used unless noted otherwise (other studies indicated that deletion of SML1 does not affect Dun1 activity in WT cells).3 All mutations or integrations introduced in yeast cells were confirmed by DNA sequencing.

Protein Purification Techniques and CIP Treatment—For purification of Dun1, 50 ml of yeast culture was used. Cells were either untreated or treated with 0.05% MMS for 2 h and then harvested. Dun1-TAF in various yeast mutants was affinity-purified using IgG resin and eluted after TEV protease cleavage. For CIP treatment, Dun1-TAF bound to the IgG resin was incubated with 10 units of CIP (New England Biolabs) in 50 ml of buffer 3 at 37 °C for 1 h (New England Biolabs), washed by Tris-buffered saline, and then eluted after cleavage using 10 units of TEV protease at 30 °C for 1 h. The amount of Dun1 was quantified and normalized using quantitative anti-FLAG Western blot and silver staining. To purify recombinant Rad53, N-terminal protein A-tagged Rad53 was affinity-purified using IgG resin and eluted after TEV protease cleavage. To purify recombinant GST-Sml1, glutathione-affinity chromatography was used according to the manufacturer’s recommendation.

In Vitro Kinase Assay—In a typical kinase assay, 50 mCi Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 0.2 mM ATP, 10 mM MgCl2, 10 μCi of [32P]ATP were used. 2 ng of Dun1 and 2 μg of GST-Sml1 were always used unless indicated otherwise. The amount of Rad53 or Dun1 used is indicated in the figure legends. A 1-h kinase reaction at 30 °C was always used.

RESULTS

Activity of Dun1 Is Induced by DNA Damage Treatment and Depends on Its Phosphorylation—To dissect the regulation of Dun1, we sought to develop an activity assay for Dun1, using its physiologic substrate Sml1. To ask whether DNA damage treatment could activate Dun1, we compared the phosphorylation of Sml1 by Dun1-TAF that is epitope-tagged and integrated into the chromosomal locus, purified from either untreated or MMS-treated cells (we chose to analyze Dun1-TAF, because previous data indicated that Dun1 overexpressed from either yeast or bacteria has a drastically reduced activity for Sml1 hyperphosphorylation).3 As shown in Fig. 1A, MMS treatment strongly enhanced the activity of Dun1 for Sml1 hyperphosphorylation. Importantly, prior treatment of the purified Dun1, using CIP, abolished the activity of Dun1 entirely. Therefore, phosphorylation of Dun1 is essential for its ability to hyperphosphorylate Sml1 (kinase-dead mutant of Dun1, purified using the same method, cannot hyperphosphorylate Sml1; see Fig. 4C). An important feature of Sml1 hyperphosphorylation by Dun1 is a characteristic slower gel shift of the hyperphosphorylated Sml1 compared with unphosphorylated Sml1 (see Fig. 1A, top panel). The 32P incorporation of Sml1 is attributed to this hyperphosphorylated Sml1 (see Fig. 1A, middle panel).

3 S-H. Chen, M. B. Smolka, and H. Zhou, unpublished observations.
Genetic Analysis of Dun1 Activation Reveals the Roles of Various DNA Damage Checkpoint Genes—Mec1 and Tel1 are known to function at the top of the kinase cascade in the DNA damage checkpoint. We therefore asked how Dun1 activity might depend on Mec1 and Tel1. As shown in Fig. 2A, deletion of MEC1 greatly diminished the activity of Dun1 in either untreated or MMS-treated cells, compared with WT cells. On the other hand, deletion of TEL1 diminished the activity of Dun1 in the absence of MMS treatment. After MMS treatment, Dun1 was activated in tel1Δ cells, similar to that in WT cells. Interestingly, deletion of both MEC1 and TEL1 completely abolished the activity of Dun1 (also see Fig. 2D). The 32P incorporation in the phosphorylated Sml1, excised from the gel, was quantified using scintillation counting, which confirmed the relative contributions of Mec1 and Tel1 in Dun1 activation.

Rad9 and Mrc1 are adaptor proteins in the DNA damage and replication checkpoints. We next asked whether they could play a role in Dun1 activation using the same Sml1 phosphorylation assay. As shown in Fig. 2B, deletion of RAD9 reduced the activity of Dun1 in both untreated and MMS-treated cells, compared with that in WT cells. Interestingly, deletion of MRC1 alone led to a much higher activity of Dun1 than that of Dun1 in WT cells in the absence of MMS treatment. MMS treatment of the mrc1Δ cells resulted in a higher activity of Dun1, which is similar to that of WT cells after MMS treatment. Furthermore, deletion of both RAD9 and MRC1 greatly diminished the activity of Dun1, although a residual activity of Dun1 in rad9Δ mrc1Δ cells does exist (Fig. 2D). Therefore, Rad9 and Mrc1 act redundantly for Dun1 activation in vivo. Although Rad9 appears to only promote Dun1 activation in response to DNA damage, Mrc1 has a dual role. First, Mrc1 prevents Dun1 activation in the absence of exogenous DNA damage treatment. Second, Mrc1 acts redundantly with Rad9 to facilitate DNA damage-induced Dun1 activation. Together, Rad9 and Mrc1 are critical for the activation of Dun1.

Rad53 and Chk1 are thought to be effector kinases in the DNA damage checkpoint. Rad53, in particular, was shown to interact with Dun1 and to be able to phosphorylate Dun1 in vitro (13, 14). We thus examined the roles of Rad53 and Chk1 in the activation of Dun1. The activities of Dun1 in rad53Δ, chk1Δ, and rad53Δ chk1Δ mutants were quantified. Deletion of RAD53 completely abolished the activity of Dun1 in both untreated and MMS-treated cells, whereas deletion of CHK1 alone had no detectable effect (see Fig. 2C). Therefore, Rad53 is required for Dun1 activation in vivo. To better examine the residual activity of Dun1 in rad53Δ, mec1Δ, mec1Δ tel1Δ, and rad9Δ mrc1Δ cells, 5-fold more Dun1 (10 ng), compared with the amount used in Fig. 2, A–C, was used to phosphorylate Sml1. As shown in Fig. 2D, Dun1 purified from rad53Δ cells is completely defective in Sml1 phosphorylation, although a hypophosphorylated Sml1 was detected. Similarly, Dun1 purified from mec1Δ tel1Δ cells is completely defective in Sml1 hyperphosphorylation. Interestingly, Dun1 has a weak but readily detectable activity for Sml1 hyperphosphorylation in mec1Δ cells, which is induced by MMS treatment. This activity is presumably dependent on Tel1 because deletion of both MEC1 and TEL1 abolished the activity of Dun1 completely (see Fig. 2D). Finally, Dun1 in rad9Δ mrc1Δ cells could still hyperphosphorylate Sml1. This

To determine the efficiency of Sml1 hyperphosphorylation by Dun1, we examined the dependence of Sml1 hyperphosphorylation on varying concentrations of active Dun1 purified from MMS-treated cells. As shown in Fig. 1B, increasing amounts of Dun1 led to more Sml1 hyperphosphorylation and its slower gel shift. Quantification of hyperphosphorylated Sml1 revealed that there is an approximately linear relationship between the amount of hyperphosphorylated Sml1 and the concentration of Dun1 when the Dun1 concentration is less than 1.6 nm (see Fig. 1C). This is expected because unphosphorylated Sml1 is in great excess compared with Dun1. At higher Dun1 concentrations, phosphorylation of Sml1 appeared to reach saturation, and most of Sml1 was converted into a hyperphosphorylated form (see Fig. 1B). Therefore, for accurate comparison of Dun1 activity, the amount of Dun1 used here is always in the linear range where approximately half of Sml1 remains unphosphorylated.
FIGURE 2. Analysis of Dun1 activity purified from various mutant backgrounds. A, upper panel, Coomassie staining of Sml1. Middle panel, autoradiography of Sml1. Bottom panel, anti-FLAG Western blot shows the loading control of Dun1 used. A, effect of MEC1 and TEL1 deletion on Dun1 activity. B, effect of RAD9 and MRC1 deletion on Dun1 activity. C, effect of RAD53 and CHK1 deletion on Dun1 activity. D, closer examination of Dun1 activity in various checkpoint mutants, using 5-fold more (10 ng) of Dun1 purified from each mutant.
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FIGURE 3. A, Coomassie staining of purified recombinant Rad53, including wild type, 4TA mutant, and kinase-dead mutant. B, in vitro activation of Dun1 using Rad53. 1 ng of WT or 4TA mutant Rad53 was used. C, phosphorylation maps of Dun1 revealed that Rad53 is entirely responsible for Dun1 phosphorylation. D, summary of autophosphorylation sites of Dun1 and the Rad53-induced transphosphorylation sites of Dun1 identified using quantitative MS. N/D indicates not determined. # indicates that the corresponding phosphopeptide without Rad53 phosphorylation was not detected; thus the value refers to signal to noise ratio. + indicates the phosphorylated Ser/Thr. * indicates the residue at the +1 position.
observation suggested that Mec1 and Tel1 could bypass the requirement of Rad9 and Mrc1 to activate Rad53 and Dun1; however, this activation is far less than that in WT cells.

In Vitro Activation of Dun1 by Rad53 for Sml1 Hyperphosphorylation—Because Rad53 is required for Dun1 activation in vivo, we next asked whether recombinant Rad53 could activate Dun1 for Sml1 hyperphosphorylation in vitro (recombinant Rad53 alone does not hyperphosphorylate Sml1). As shown in Fig. 3A, WT Rad53 shows a reduced electrophoretic mobility compared with Rad53-KD, which is due to its autophosphorylation. Rad53-4TA has a similar electrophoretic mobility as WT Rad53. As shown in Fig. 3B, recombinant WT Rad53 activated WT Dun1, purified from rad53Δ cells, for Sml1 hyperphosphorylation, whereas Dun1-D328A or Dun1-KD, failed to be activated by WT Rad53 (Fig. 3B). Thus, the kinase activity of Dun1 is required for Sml1 hyperphosphorylation.

Next we asked whether a known interaction between the N-terminal 4TQ phosphorylation cluster of Rad53 and FHA domain of Dun1 is important for Rad53 to activate Dun1 (14). As shown above, several phosphorylation sites of Dun1 were found, including N-terminal region, the region between the FHA and kinase domains, the activation loop of the kinase domain, and the C-terminal region (see Fig. 3C). Next, we identified the phosphorylation sites of Dun1 (purified from rad53Δ cells) before and after Rad53 phosphorylation in vitro. Little phosphorylation of Dun1 was detected in rad53Δ cells. After in vitro phosphorylation by Rad53, the phosphorylation sites of Dun1 were again identified. Interestingly, the DNA damage-induced phosphorylation sites of endogenous Dun1 and the in vitro phosphorylation sites of Dun1 by Rad53 are essentially the same. Therefore, Rad53 is directly responsible for Dun1 phosphorylation.

To identify possible autophosphorylation sites of Dun1, we used the N-isotag technology to quantitatively compare the relative abundance between WT Dun1 and Dun1-KD, i.e. Dun1-D328A, after MMS treatment using MS (12). More than 20 phosphorylation sites were detected in four regions of Dun1, including the N-terminal region, the region between the FHA and kinase domains, the activation loop of the kinase domain, and the C-terminal region (see Fig. 3C). Next, we identified the phosphorylation sites of Dun1 (purified from rad53Δ cells) before and after Rad53 phosphorylation in vitro. Little phosphorylation of Dun1 was detected in rad53Δ cells. After in vitro phosphorylation by Rad53, the phosphorylation sites of Dun1 were again identified. Interestingly, the DNA damage-induced phosphorylation sites of endogenous Dun1 and the in vitro phosphorylation sites of Dun1 by Rad53 are essentially the same. Therefore, Rad53 is directly responsible for Dun1 phosphorylation.

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In the absence of MMS treatment, the abundance of Sml1 is reduced in mrc1Δ cells, compared with that in WT or rad9Δ cells (see Fig. 5C). Furthermore, Sml1 abundance in rad9Δ cells is higher than that in WT cells in the absence of MMS treatment. With the assumption that the abundance of Sml1 should be inversely correlated with Dun1 activity, these observations are consistent with the activities of Dun1 found in rad9Δ and mrc1Δ cells (see Fig. 2B). Importantly, Sml1 abundance in dun1-T380A cells is the same as that in dun1-KD and dun1Δ mutants, and it failed to be degraded after MMS treatment. Therefore, the ability of Dun1 to hyperphosphorylate Sml1 is inversely correlated with the abundance of Sml1, and it is required for MMS-induced Sml1 degradation in cells.

Phosphorylation of Thr-354 of Rad53 Is Important for Rad53 Activity and Functions in Vivo—Because Thr-380 of Dun1 locates in the activation loop of the Dun1 kinase domain and activation loop phosphorylation was often found to be important for activities of many kinases (25), we performed a sequence alignment of the activation loops of various Dun1 homologs and found that Thr-354 of Dun1 is strictly conserved in the Chk2 family kinases, including Thr-354 of Rad53 (see Fig. 6A). Interestingly, Thr-354 of Rad53 is followed by a hydrophobic phenylalanine at its +1 position. Phosphorylation of Thr-354 of Rad53 was found previously (26). We re-examined our previous data on the phosphorylation of Rad53 and found that phosphorylation of Thr-354 of Rad53 was induced by MMS treatment, and it is an autophosphorylation site of Rad53 (12). We next examined the activity of Rad53 to activate Dun1 for Sml1 hyperphosphorylation. As shown in Fig. 6B, hyperphosphorylation of Sml1 was greatly reduced when Rad53-T354A was used, compared with WT Rad53. Quantification of the 32P-labeled Sml1 using scintillation counting indicated a 20-fold reduction of the amount of hyperphosphorylated Sml1 when Rad53-T354A was used (see Fig. 6B, right panel). Although much reduced, Rad53-T354A does have a residual activity to activate Dun1 for Sml1 hyperphosphorylation. As expected, Rad53-KD failed to activate Dun1 for Sml1 hyperphosphorylation completely.

FIGURE 5. GFP-Sml1 abundance in rad9Δ, mrc1Δ, and dun1-T380A mutants. A, Sml1 abundance is analyzed in rad9Δ and mrc1Δ cells. B, GFP-Sml1 abundance in dun1-T380A cells is similar to dun1-KD in response to MMS treatment. C, anti-green fluorescent protein Western blot analysis of GFP-Sml1.

hypophosphorylated form, which is not shifted slower in the gel compared with the hyperphosphorylated Sml1 by WT Dun1. Therefore, phosphorylation of Thr-380 of Dun1 enables Dun1 to hyperphosphorylate Sml1.

Dun1 Activity Is Inversely Correlated with Sml1 Abundance in Cells and It Is Regulated by Thr-380 Phosphorylation—Because Dun1 is important for DNA damage-induced degradation of Sml1 in vivo, we asked whether the activity of Dun1 for Sml1 hyperphosphorylation is correlated with the abundance of Sml1 in cells. As shown in Fig. 5A, Sml1 abundance is reduced in mrc1Δ cells compared with that in WT. In contrast, Sml1 appears to be more abundant in rad9Δ cells in the absence of MMS treatment. After MMS treatment, the abundance of Sml1 is reduced in both cases. We next examined the abundance of GFP-Sml1 in dun1-T380A cells because dun1-T380A is hypersensitive to genotoxic stresses and Dun1-T380A fails to hyperphosphorylate Sml1 (see Fig. 4C). T380A mutation of Dun1 led to a greatly elevated Sml1 abundance in cells with or without MMS treatment, which is similar to that in dun1Δ and dun1-KD cells (see Fig. 5B).

To quantitatively compare the abundance of Sml1 in various mutants, anti-green fluorescent protein Western blot was used.
We next examined the sensitivity of rad53-T354A cells to various genotoxic stresses. First, rad53-T354A cells have a similar growth rate as WT cells, whereas rad53-KD and rad53/H9004 cells grow slower. This might be related to the fact that Rad53-T354A is not completely inactive. Second, compared with WT cells, rad53-T354A cells are hypersensitive to UV and HU treatments (see Fig. 6C). Taken together, phosphorylation of Thr-354 of Rad53 is important for Rad53 activity and functions in vivo. Because phosphorylation of Thr-354 was found in recombinant Rad53 and it is followed by a phenylalanine, phosphorylation of Thr-354 of Rad53 appears to be a result of its autophosphorylation.

**DISCUSSION**

The Dun1-Sml1 Assay Is a Sensitive and Reliable Assay to Dissect the Pathway of Dun1 Activation—Dun1 was known to function downstream in the kinase cascade in the DNA damage checkpoint, which has been extensively studied previously (1, 3, 7).
Here we sought to establish an in vitro activity assay to monitor Dun1 activity, using its physiological substrate Sml1. Once established, this assay will be used to reconstitute the activation of Dun1 and its upstream kinases. To validate the Dun1-Sml1 assay, we used it to examine the roles of various known DNA damage checkpoint proteins in Dun1 activation. Our observations are summarized in Fig. 7, which essentially recapitulate existing knowledge of this pathway. As expected, Mec1 is primarily responsible for DNA damage-induced Dun1 activation, whereas Tel1 plays a much less important role. Interestingly, the observation that Dun1 activity is completely abolished in mec1Δ cells, but not mec1Δ cells (see Fig. 2D), indicated that Tel1 does play a role in controlling Dun1 activity.

Rad9 and Mrc1 are adaptor proteins that were known to interact with Rad53 (5–7, 10, 28). As expected, Rad9 is important for Dun1 activation. Interestingly, a novel dual function of Mrc1 in Dun1 activation was identified, including a role in promoting MMS-induced Dun1 activation and preventing Dun1 activation in the absence of exogenous DNA damage treatment. Mrc1 was known to function in the maintenance of the DNA replication fork (4, 5, 10). Recently, it was shown that deletion of MRC1 causes slowing of DNA replication fork progression, possibly defects in replication fork maintenance and thus damaged DNA in the cells (29). Up-regulation of RNR gene transcription was also observed in mrc1Δ cells (30). These observations are consistent with our observations that Dun1 is activated in unperturbed mrc1Δ cells (see Fig. 2B) and Sml1 abundance is diminished in mrc1Δ cells (see Fig. 5A). On the other hand, Mrcl is also important for the activation of Dun1 in response to DNA damage (see Fig. 2, B and D).

In agreement with previous studies (13, 14), we found that Rad53 is directly responsible for Dun1 activation. Because Rad53 was known to function downstream of Mec1, Tel1, Rad9, and Mrc1, our results thus recapitulated the known genetic pathway of Dun1 activation (see Fig. 7A). Taken together, these observations indicated that the Dun1-Sml1 assay is a sensitive and reliable assay to study DNA damage checkpoint activation in yeast.

**Consensus Phosphorylation Site of Rad53 and Autophosphorylation Site of Dun1**—Quantitative analysis of the phosphorylation sites of Dun1 using quantitative MS revealed new insights into the preferred phosphorylation sites of Rad53 and Dun1. Using quantitative MS, several strongly phosphorylated sites of Dun1 by Rad53 were determined. Interestingly all of them appear to have a bulky hydrophobic residue at the +1 position relative to the phosphorylated Ser/Thr residues, including Thr-380 of Dun1 (see Fig. 3D). Phosphorylations of both the Thr-380 of Dun1 and Thr-354 of Rad53 are critical for their respective activities. Importantly, both are followed by a hydrophobic residue at the +1 position. Thus, Rad53 appears to prefer to phosphorylate Ser/Thr followed by a bulky hydrophobic residue. We further identified the autophosphorylation sites of Dun1, and they include Ser-10 and Ser-139 (see Fig. 3D). Interestingly, the SSSST sequence of Dun1 (residue 139–143) was found to be hyperphosphorylated. It was known that Dun1 hyperphosphorylates a serine cluster in Sml1 (31). Therefore, Dun1 may prefer to phosphorylate Ser/Thr clusters. Characterization of additional Dun1 substrates should further help to establish the consensus phosphorylation motif for Dun1. In summary, the preferred phosphorylation sites of Rad53 and Dun1 identified here should facilitate functional studies of additional Rad53 and Dun1 substrates in the future.
Phosphorylation Regulation of Dun1 and Rad53—Hyperphosphorylations of Rad53 and Dun1 were known to occur in response to DNA damage treatment (3, 15, 27). However, the key phosphorylation site that regulates their activities was not determined. We have established an in vitro assay to activate Dun1 by recombinant Rad53 (see Fig. 3B). Using this assay, we showed that the interaction between the N-terminal TQ phosphorylation cluster of Rad53 and the FHA domain of Dun1 promotes Dun1 activation by Rad53. Mutation to either the FHA domain of Dun1 or the 4TQ cluster in the N-terminal region of Rad53 diminished the ability of Rad53 to activate Dun1 for Smo1 hyperphosphorylation (see Fig. 3B).

Rad53 phosphorylates Dun1 on many serine and threonine residues (see Fig. 3C). Because phosphorylation of Thr-380 of Dun1-KD purified from MMS-treated cells is similar to that of WT Dun1 and Thr-380 is strongly phosphorylated by Rad53 in vitro (see Fig. 3D), Rad53 appears to be directly responsible for phosphorylation of Thr-380 of Dun1. Importantly, this residue resides in the activation loop of the Dun1 kinase domain, and it is conserved in various Chk2 family kinases, including Rad53. Despite the numerous autophosphorylation sites of Rad53 that exist, phosphorylation of a single conserved Thr-354 of Rad53 was found to play a crucial role in controlling Rad53 activity. Importantly, Thr-354 of Rad53 agrees with our proposed consensus phosphorylation site of Rad53, and it is an autophosphorylation site. In summary, a model of Dun1 activation by Rad53 is proposed (see Fig. 7B) (13, 14). We suggest that autophosphorylation of Thr-354 activates Rad53, which then phosphorylates the Thr-380 of Dun1 to activate Dun1.

Further study is needed to elucidate the molecular mechanism of Rad53 activation. Rad9 and Mrcl1 likely play a direct role, although direct Mec1 phosphorylation of Rad53 may also contribute to its activation (26, 28, 32). This Dun1-Smo1 assay could be used to examine these possibilities and reconstitute the activation of the kinase cascade in the DNA damage checkpoint. It is interesting to also note that phosphorylation of the conserved Thr-383 in the activation loop of human Chk2 was also found to be important for Chk2 activity (33). Although phosphorylation of the N-terminal TQ site of Chk2 family kinases is widely believed to be important for their activities, our results strongly suggest that phosphorylation of this conserved threonine residue in the activation loop appears to be a conserved and key mechanism of the activation of Chk2 family kinases.

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