Distinct Phosphatases Mediate the Deactivation of the DNA Damage Checkpoint Kinase Rad53*

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The DNA damage checkpoint regulates DNA replication and arrests cell cycle progression in response to genotoxic stress. In Saccharomyces cerevisiae, the protein kinase Rad53 plays a central role in preventing genomic instability and maintaining viability in the presence of replication stress and DNA damage. Activation of Rad53 depends on phosphorylation by the upstream kinase Mec1, followed by autophosphorylation on multiple residues. Also critical for cell viability, the molecular mechanism of Rad53 deactivation remains incompletely understood. Rad53 dephosphorylation after repair of a persistent double strand break in G2/M has been shown to depend on the presence of the PP2C-type phosphatases Ptc2 and Ptc3. More recently, the PP2A-like protein phosphatase Pph3 has been shown to be required to dephosphorylate Rad53 after DNA methylation damage in S phase. However, we show here that Ptc2/3 are dispensable for Rad53 deactivation after replication stress or DNA methylation damage. Pph3 is also dispensable for the deactivation of Rad53 after replication stress. In addition, Rad53 kinase activity is still deactivated in pph3 null cells after DNA methylation damage, despite persistent Rad53 hyperphosphorylation. Finally, a strain in which the three phosphatases are deleted shows a severe defect in Rad53 kinase deactivation after DNA methylation damage but not after replication stress. In all, our results suggest that distinct phosphatases operate to return Rad53 to its basal state after different genotoxic stresses and that a yet unidentified phosphatase may be responsible for the deactivation of Rad53 after replication stress.

The DNA damage checkpoint monitors and responds to genotoxic stress that threatens genomic integrity in eukaryotic cells (1–3). In humans it constitutes a barrier to cancer transformation (4, 5). The elements that constitute this surveillance mechanism are highly conserved in eukaryotic cells and follow the typical structure of signal transduction pathways (6). In the budding yeast Saccharomyces cerevisiae, genotoxic stress leads to the activation of central transducer protein kinases Mec1 and Tel1 (7), the orthologs of human ATM and ATR. Mec1, in turn, activates the effector protein kinases Chk1 and Rad53 (8), the orthologs of human Chk1 and Chk2, respectively. In S. cerevisiae, Rad53 is the main checkpoint effector (9), and rad53 mutants present a strong sensitivity to all types of DNA damage and to replication stress (10).

In response to genotoxic stress, Mec1 phosphorylates Rad53, priming it for activatory autophosphorylation in trans (7, 11). Activated Rad53 targets a number of substrate proteins, resulting in stabilization of stalled replisomes, suppression of recombination at arrested replication forks, block of yet unfired chromosomal origins of replication, and block of progression to mitosis (10, 12–15). Also, to increase the pools of dNTP and counteract replication stress, Rad53 mediates the transcriptional induction of ribonucleotide reductase genes (10, 16) and targets the ribonucleotide reductase inhibitor Sm1 for destruction (17, 18).

Whereas we have a fair understanding of the activation process, much less is known about how cells deactivate the damage response once genotoxic stress is overcome. This process, also termed checkpoint recovery, is required for the essential resumption of cell cycle progression. Significantly, destruction of Rad53 is sufficient to allow cells to resume cell cycle progression in the continued presence of DNA damage (19). Because deactivation of Rad53 kinase activity correlates with a return to its hypophosphorylated form and does not involve proteolysis (20, 21), it is expected that a phosphatase activity is responsible for Rad53 deactivation. PP2C-type phosphatases Ptc2 and Ptc3 have been shown to be necessary for checkpoint recovery once DNA double strand break (DSB)2 damage is repaired in G2/M (22, 23). Although direct in vitro dephosphorylation of Rad53 by Ptc2/3 has not been demonstrated, and therefore an indirect effect cannot be ruled out, a human Ptc2/3 homolog, Wip1/Ppm1d, has been shown to directly dephosphorylate the Rad53 homolog Chk2 (24, 25). Wip1/Ppm1d is an oncogenic phosphatase, therefore fulfilling the prediction that overexpression of phosphatases responsible for checkpoint deactivation may have a similar effect as the loss-of-function mutations. In any case, it is not known whether Ptc2 and Ptc3 are also required for Rad53 deactivation following other types of genotoxic insults, in particular those that are sensed and dealt with in S phase, such as replication stress or DNA methylation damage. Cells are particularly vulnerable during S phase, when the presence of

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2 The abbreviations used are: DSB, double strand break; MMS, methyl methanesulfonate.
DNA lesions or replication stress results in replication fork stalling. In the absence of an adequate checkpoint response, essential replisome components fall apart (26), replication forks collapse (14, 27, 28), and undesirable recombination events take place (29, 30).

More recently, it has been reported that the PP2A-like protein phosphatase Pph3 is required for Rad53 dephosphorylation after DNA methylation damage (31). In addition, Pph3 can directly dephosphorylate Rad53 in vitro (31). However, pph3 null cells present normal viability in the presence of hydroxyurea in two different screens (32, 33), suggesting that Pph3 may be dispensable for Rad53 deactivation after replication stress.

We therefore wished to explore whether Ptc2/3 and Pph3 are also involved in Rad53 recovery after replication stress. The double deletion of Ptc2 and Ptc3 has no effect on the timely deactivation of Rad53 after replication stress or DNA methylation damage in S phase. Deletion of Pph3 has no effect on Rad53 deactivation after replication stress either. Surprisingly, the reported defect in Rad53 dephosphorylation after DNA methylation damage (31) does not prevent the deactivation of Rad53 kinase activity. This result indicates that a distinct phosphatase is able to dephosphorylate one or more key kinase activating residues. This result also indicates that Rad53 hyperphosphorylation can be separated from its kinase activity, which is likely to depend on phosphorylation of a limited number of activatory sites. To this respect, cells carrying a triple pph3 ptc2 ptc3 deletion fail to deactivate Rad53 kinase activity after DNA methylation damage, suggesting that, in the absence of Pph3, Ptc2/3 may dephosphorylate one or more sites critical for kinase activation. However, the triple null mutant strain efficiently deactivates Rad53 after replication stress, indicating that at least another yet unidentified phosphatase is sufficient to dephosphorylate and deactivate Rad53 after this type of genotoxic stress.

**EXPERIMENTAL PROCEDURES**

**Strains, Culture Medium, and Cell Synchronization—**Strains used in this study are listed in supplemental Table 1. All strains generated in our laboratory are derived from *S. cerevisiae* W303–1a (34). The two BY4741-derived strains (35, 36) used for comparison were obtained from Euroscarf. Cultures were grown at 30 °C, under orbital rotation, in YPD medium (1% yeast extract [BD Biosciences, catalog no. 212750], 2% peptone [BD Biosciences, catalog no. 211677], and 2% dextrose [Merck, catalog no. 1.04074]). To synchronize cells in G1 phase, the mating pheromone α-factor was added at 5 μg/ml to cultures in mid-exponential growth phase. After one doubling time, cells were inspected under the microscope to confirm that the budding index was <5%. To release cells into S phase, synchronized cultures were washed three times in the corresponding release medium.

**Generation of Replication Stress and DNA Damage—**Replication stress was generated with hydroxyurea (Sigma, catalog no. H8627), a reversible inhibitor of the ribonucleotide reductase activity that catalyzes the rate-limiting step in dNTP synthesis. DNA methylation damage was generated with methylmethane sulfonate (MMS) (Aldrich, catalog no. 129925). For sensitivity assays, cells were spotted in serial 10-fold dilutions onto YPD plates containing 0.2 M hydroxyurea or 0.022% MMS. For genotoxic stress experiments in liquid culture, cells synchronized in G1 were released into YPD containing either 0.2 M hydroxyurea or 0.033% MMS. Hydroxyurea was eliminated after a 1-h incubation by washing cells three times in YPD without hydroxyurea. To eliminate MMS, after 15–30 min, incubation cells were washed three times in YPD supplemented with 5% sodium thiosulfate, followed by two washes in YPD.

**Cell Sorting, Western Blotting, and in Situ Kinase Assay—**Samples for propidium iodide fluorescence-activated cell sorting were processed as described (37) and analyzed using a BD Biosciences FACScan. Whole cell extracts for Western blot analysis and in situ kinase assay were prepared by glass bead beating in trichloroacetic acid as described (38). To resolve the different forms of Rad53, extracts were overrun in a 7.5% SDS-polyacrylamide gel and probed with polyclonal anti-Rad53 yC-19 antibody (Santa Cruz Biotechnology, catalog no. sc-6749). In situ kinase assays were carried out as described (21).

**RESULTS**

**Ptc2 and Ptc3 Are dispensable for Rad53 Deactivation Following Genotoxic Stress in S Phase—**If Ptc2 and Ptc3 were required for the deactivation of Rad53 after replication stress, persistent cell cycle progression block in the phosphatase mutants should result in limited growth in the presence of hydroxyurea. However, a *ptc2Δ ptc3Δ* mutant strain grows at the same rate and to the same extent as wild-type cells in the presence of hydroxyurea (Fig. 1), arguing against an abnormally sustained Rad53 response.

However, cells deleted for the *PTC2* and *PTC3* genes could be defective in Rad53 dephosphorylation but yet proficient in the deactivation of downstream effectors and targets, hence able to override persistent activation of Rad53. To directly analyze Rad53 deactivation following replication stress, we studied recovery in synchronous cultures transiently exposed to hydroxyurea. Exponentially growing cells were synchronized in G1 with the mating pheromone α-factor and then released into S phase in the presence of hydroxyurea. After a 1-h incubation, cells were transferred to medium without hydroxyurea, and
forms correlate with the presence of forms corresponding to hyperphosphorylated Rad53. These in the presence of hydroxyurea present the slow migrating forms of wild-type, ptc2Δ and ptc3Δ when hydroxyurea is removed, both wild-type and ptc2Δ ptc3Δ cells quickly deactivate Rad53, as assessed by return to its faster migrating form, absence of detectable in situ kinase activity, and resumption of DNA synthesis. Taken together, these results indicate that Ptc2 and Ptc3 are dispensable for Rad53 deactivation and checkpoint recovery after replication stress.

Ptc2 and Ptc3 might be dispensable for Rad53 deactivation after replication stress but still required to dephosphorylate Rad53 after DNA damage. To this respect, the methylating agent MMS and hydroxyurea result in sensibly distinct electrophoretic mobility shifts (7). In addition, different patterns of phosphorylated residues result from different genotoxic insults such as the UV light-mimetic drug 4-nitroquinoline oxide (39) and MMS (40). Dephosphorylation of the different activated forms of Rad53 might involve different phosphatases.

To explore whether Ptc2 and Ptc3 are required for Rad53 recovery following DNA methylation damage, serial dilutions of wild-type, ptc2Δ ptc3Δ, and mec1Δ cells were made on YPD plates containing MMS. As shown in Fig. 1C, ptc2Δ ptc3Δ cells remain viable and grow at the same rate as wild-type cells, indicating that they can respond to and recover from DNA methylation damage normally.

To more directly study whether Ptc2 and Ptc3 play a role in Rad53 deactivation following DNA methylation damage in S phase, exponentially growing cultures were synchronized in G1 with the mating pheromone α-factor and then released into S phase in the presence of MMS. After incubation in the presence of the DNA methylating agent, cells have started replication, and Rad53 is activated, presenting its slow migrating hyperphosphorylated forms and in situ kinase activity (Fig. 2B). Upon removal of MMS, Rad53 deactivates with the same kinetics in ptc2Δ ptc3Δ mutant cells as in wild-type cells, and DNA synthesis and the cell division cycle progress in parallel. Therefore, Ptc2 and Ptc3 are also dispensable for the dephosphorylation of Rad53 that has been activated in S phase in response to DNA methylation.

Pph3 Is Dispensable for Rad53 Dephosphorylation and Deactivation Following Replication Stress—It has been recently reported that the type 2A-like protein phosphatase Pph3 is required for Rad53 dephosphorylation after DNA methylation damage generated with MMS in S phase (31). We therefore wished to explore whether Pph3 is also required for the deactivation of Rad53 after replication stress. As shown in Fig. 1B, pph3Δ cells remain viable and grow at the same rate as wild-type cells in the presence of hydroxyurea, indicating that they can respond to, and recover from, replication stress normally.

To determine whether deletion of Pph3 results in a defective recovery of Rad53 after replication stress, we analyzed the kinetics of Rad53 dephosphorylation and kinase activity after exposure to hydroxyurea. As seen in Fig. 3A, Rad53 is dephosphorylated and deactivated with identical efficiency in pph3Δ as in wild-type cells. Accordingly, replication resumes and progresses normally, and cells divide at the same time, regardless of the presence or absence of Pph3. Therefore, in contrast with the requirement of Pph3 for Rad53 dephosphorylation after DNA methylation damage (31), Pph3 is dispensable for recovery of Rad53 after replication stress, indicating that a different phosphatase may be responsible for Rad53 deactivation after this type of genotoxic stress.
**Rad53-deactivating Phosphatases**

**A** wild type  
pph3Δ

![Diagram A](image)

**B** wild type  
pph3Δ

![Diagram B](image)

**FIGURE 3.** Pph3 is dispensable for Rad53 recovery after replication stress and also for Rad53 kinase deactivation after DNA methylation damage. See Fig. 2 for experimental details, except for this figure we compared a wild-type strain and a pph3Δ mutant strain. HU, hydroxyurea.

Pph3 Is Dispensable for the Deactivation of Rad53 Kinase Activity after DNA Damage in S Phase—**pph3** null cells cannot dephosphorylate Rad53 after DNA methylation damage (31). Intriguingly, such a hyperphosphorylated form of Rad53 fails to inhibit the firing of origins of replication (31). This observation is suggestive of the existence of a different phosphatase responsible for the dephosphorylation of one or more Rad53 sites, affecting its ability to block origin firing. Those sites may be critical for this particular downstream pathway, for instance resulting in loss of affinity for specific substrates targeted by Rad53 to block origin firing. Alternatively, the site or sites may, for instance, be critical kinase activation residues. To distinguish between these two options, we measured the kinase activity of Rad53 in **pph3** cells after DNA damage generated with MMS. As reported (31), upon removal of MMS, Rad53 remains hyperphosphorylated in **pph3** cells long after Rad53 has returned to its basal phosphorylation state in wild-type cells (Fig. 3B). Surprisingly, rather than paralleling the hyperphosphorylated form, Rad53 kinase is deactivated in **pph3** cells with only a minor delay compared with wild-type cells. This result indicates that Rad53 hyperphosphorylation and kinase activity can be separated and also that different phosphatases may regulate Rad53 function after DNA methylation damage in S phase.

A Triple **pph3 ptc2 ptc3** Deletion Mutant Fails to Deactivate Rad53 after DNA Damage but Is Still Proficient in Rad53 Recovery after Replication Stress—We have just shown that despite Pph3 being required for Rad53 dephosphorylation after DNA methylation damage in S phase, Rad53 kinase activity is still switched off in the **pph3** mutant. One possibility is that Ptc2/3, which are required for the recovery of Rad53 after a persistent DSB (22), are able to turn off Rad53 kinase activity. Whereas Pph3 and Ptc2/3 may specifically recognize and dephosphorylate the distinct Rad53 hyperphosphorylation patterns that result from different genotoxic stresses (39, 40), they may share redundant activity on one or more sites that are determinant for Rad53 kinase activity. To explore this possibility, we generated a **pph3Δ ptc2Δ ptc3Δ** strain. The triple deletion strain is viable and grows with no apparent defect compared with a wild-type strain. However, this strain presents sensitivity to hydroxyurea and to MMS (Fig. 1).

In addition, Rad53 kinase deactivation after DNA methylation damage is now severely impaired (Fig. 4A), and an increased Rad53 kinase activity is detected. This result is compatible with Ptc2/3 being able to deactivate Rad53 in the absence of Pph3, although other possibilities cannot be discarded, as discussed below.

On the other hand, when the triple deletion strain is allowed to recover from replication stress (Fig. 4B), Rad53 is dephosphorylated, and the kinase activity is turned off, albeit with a minor delay with respect to the wild-type control. This result indicates that deactivation of Rad53 after replication stress involves one or more yet unidentified phosphatases and reinforces the idea that different phosphatases may be responsible for dephosphorylation of Rad53 activated in response to different types of genotoxic stress. Despite Rad53 being dephosphorylated and deactivated after replication stress in the triple deletion mutant, this strain presents a growth defect in hydroxyurea plates (Fig. 1). Viability assays show that the mutant cells remain as viable as wild-type cells (data not shown), indicating that the growth defect is the result of a delay in cell cycle progression. Analysis of cell cycle progression by flow cytometry (Fig. 4B) shows that whereas DNA replication progresses and ends timely compared with the wild-type control, cell division is delayed in the **pph3Δ ptc2Δ ptc3Δ** mutant. Such delay might result from the observed residual Rad53 kinase activity at later time points. However, a similar delay in Rad53 kinase deactivation in **pph3Δ** cells after MMS does not result in a significant delay in cell cycle progression. We therefore cannot exclude that the **pph3Δ ptc2Δ ptc3Δ** mutant fails to dephosphorylate a checkpoint downstream target that limits cell cycle progression.
DISCUSSION

The protein kinase Rad53 is the main effector of the DNA damage checkpoint in *S. cerevisiae* (9). Checkpoint activation is crucial to preserve genomic integrity in the face of genotoxic insults. Once the lesions are repaired, checkpoint deactivation is required for cell cycle progression to resume.

Rad53 activation correlates with hyperphosphorylation, whereas deactivation correlates with a return to its hypophosphorylated form (21). Because new protein synthesis is not required for the reappearance of hypophosphorylated Rad53 (20), it is expected that one or more phosphatases are responsible for Rad53 deactivation. Identification of such phosphatases presents an additional interest based on the possibility that their human counterparts may be oncogenic. Overexpression of phosphatases responsible for checkpoint deactivation may have a similar effect as checkpoint loss-of-function mutations. This has been shown to be the case for Wip1/Ppm1d, a phosphatase responsible for dephosphorylation of the human Rad53 homolog Chk2 (24, 25).

Previous work in budding yeast identified the PP2C-type phosphatases Ptc2 and Ptc3 as necessary for Rad53 recovery after a DNA DSB is repaired (22, 23). More recently, the PP2A-like phosphatase Pph3 has been shown to be required for Rad53 dephosphorylation after DNA methylation damage (31).

We have explored here whether the role of these phosphatases in Rad53 recovery can be generalized to other types of genotoxic stress. Whereas Rad53 recovery after a DSB requires Ptc2/3 (22, 23), our results show that they are dispensable after other types of genotoxic insult, such as DNA methylation damage and replication stress. Likewise, Pph3, required for Rad53 dephosphorylation after DNA methylation damage (31), is not required for Rad53 recovery after replication stress. We have also shown that Rad53 is efficiently dephosphorylated and deactivates after replication stress in a triple *pph3 ptc2 ptc3* deletion mutant. Therefore, the phosphatase activity responsible for Rad53 recovery after replication stress remains unidentified. In all, our results confirm and extend the model recently put forward (41) that distinct phosphatases specifically recover the different active forms of Rad53 that result in response to different genotoxic insults.

Rad53 Recovery after DNA Methylation Damage—We have confirmed the reported observation that deletion of *Pph3* results in persistence of Rad53 hyperphosphorylation after DNA methylation damage (31). However, using a specific kinase assay, we have shown that the activity of this hyperphosphorylated form is still turned off. We have thus separated for the first time Rad53 kinase deactivation from bulk dephosphorylation. Rad53 activation correlates with the hyperphosphorylated forms that may be resolved electrophoretically (7, 11, 21). Yet, it is reasonable to expect that only a subset of the phosphorylated sites (39, 40) are required for Rad53 kinase activation, whereas the rest of the sites likely determine the specific set of interactions implicated in the response to a particular type of genotoxic stress.

Our finding reconciles the reported observation that *pph3* mutants fail to keep the firing of origins of replication inhibited, despite the persistence of hyperphosphorylated Rad53 after DNA methylation damage (31). It may also explain the normal growth we observe for *pph3* mutant cells in MMS plates, which would be difficult to reconcile with a sustained checkpoint activation. Because *pph3* mutants have been previously reported to be sensitive to MMS (31), we explored whether differences in background may account for the different behaviors. Indeed, contrary to the W303 background used in our study, the *pph3* mutant in the BY4741 background (36) shows a growth defect in MMS (Fig. 5). However, the mutant remains
Rad53-deactivating Phosphatases

FIGURE 5. Sensitivity to MMS of a pph3Δ deletion strain in the BY4741 genetic background. 10-fold serial dilutions of the indicated cell cultures, growing exponentially in YPD, were spotted onto plates containing YPD (A) or YPD + 0.022% MMS (B and C). Pictures of the MMS plate were taken after incubation at 30 °C for 3 days (B) and 5 days (C).

viable and continues growing at longer incubation times (Fig. 5C), indicating that sensitivity to MMS is the result of delayed growth rather than loss of viability. At the moment we do not know why deletion of Pph3 results in slower growth in MMS in the BY4741 background but not in the W303 background. One possibility worth exploring is whether Ptc2/3 activity, which is likely responsible for deactivation of Rad53 kinase in the absence of Pph3 (see below), is lower in the BY4741 background but not in the W303 background, resulting in slower Rad53 deactivation and a sensible growth delay in MMS plates.

If hyperphosphorylated Rad53 is deactivated in the pph3Δ mutant after DNA methylation damage, which phosphatase is responsible for this deactivation? Whereas we have shown that Pph3 and Ptc2/3 are separately dispensable for Rad53 kinase deactivation after DNA methylation damage, the triple pph3 ptc2 ptc3 deletion mutant shows a severe defect. Rad53 remains hyperphosphorylated and kinase hyperactive long after wild-type cells have deactivated Rad53 and resumed cell cycle progression. Therefore, Ptc2/3, which have been shown to be required for Rad53 recovery after a DSB in G2/M (22), may be sufficient to deactivate Rad53 kinase activity in the absence of Pph3. One attractive explanatory model is that Ptc2/3 and Pph3 specifically dephosphorylate the distinct Rad53 phosphorylation patterns that result from checkpoint activation in response to their respective genotoxic stresses (see below). It is expected, though, that the different patterns share in common one or more sites critical for Rad53 kinase activation, e.g. the evolutionarily conserved Thr354 in the kinase activating loop (39, 42).

Thus, in the absence of Pph3, Ptc2/3 cannot dephosphorylate the bulk of Rad53 sites phosphorylated in response to MMS, and Rad53 remains hyperphosphorylated after DNA methylation damage. However, Ptc2/3 might still dephosphorylate one or more sites required for Rad53 kinase activity and therefore deactivate Rad53 in the absence of Pph3. Only when Pph3 and Ptc2/3 activities are suppressed, Rad53 kinase remains active after the DNA damage has been repaired.

It is unlikely that the persistent Rad53 activity in the triple pph3 ptc2 ptc3 deletion mutant after DNA methylation damage results from a defect in deactivation of an upstream signaling element. Thus, deletion of Ptc2 has been shown to result in a defect in Rad9 dephosphorylation after a DSB that parallels the defect in Rad53 recovery (22). Rad9, required for the activation of Rad53 (8, 43), is phosphorylated in response to DNA damage in a Mec1-dependent (44–46), Rad53-independent (45) manner. However, we have shown above that the kinetics of Rad53 recovery in ptc2Δ ptc3Δ mutant cells is undistinguishable from wild-type cells, indicating either that Rad9 deactivation is not affected in ptc2Δ ptc3Δ cells after DNA methylation damage or that it does not affect Rad53 deactivation. Also, Pph3 has been shown to be required for the dephosphorylation of Ser129 in histone H2A (47). In response to DNA damage, H2A is phosphorylated at Ser129 in a Mec1-dependent manner (48, 49). Continued Rad53 kinase activation in pph3Δ cells, long after a DSB has been repaired, has been shown to result from persistent H2A signaling, as mutation of H2A Ser129 to Ala allows the recovery of Rad53 with normal kinetics (47). However, deletion of Psy4, a Pph3 regulatory subunit also essential for dephosphorylation of H2A (47), results in no Rad53 dephosphorylation defect after DNA methylation damage, despite continued H2A phosphorylation at Ser129 (31). Therefore, persistent Rad53 activity because of continued H2A signaling may be specific to some types of genotoxic stress, such as DSBs, but not in the case of DNA methylation damage.

After DNA methylation damage, the triple mutant strain ends replication at a slower pace than wild-type cells. In this case, full Rad53 kinase activity should preclude the firing of origins of replication (13). Therefore, completion of replication would depend on passive completion of replication by forks established early in S phase, prior to checkpoint activation. Passive replication would in principle be in contradiction with reports suggesting that continued Rad53 activity after DNA methylation damage in pph3Δ mutants prevents the restart of early forks (31). However, we know now that Rad53 kinase activity is still switched off in pph3 Δ mutants. In addition, the analysis of fork progression in wild-type cells by means of dense isotope substitution has shown that despite the continued presence of MMS-activated Rad53, the fork progression rate remains constant as the replisome encounters DNA lesions (14), indicating that checkpoint-dependent fork stabilization is short-lived. If checkpoint-stabilized forks required Rad53 deactivation to restart, the fork progression rate in MMS should decay with time, mirroring the effect of fork collapse in checkpoint mutants (14), which is not the case. The reported block of forks in pph3Δ mutants exposed to MMS involved incorporation of the thymidine analog bromodeoxyuridine. Cells die after incorporation may perturb fork progression (50). In any case, we cannot discard an effect specific to the hyperphosphorylated yet kinase inactive Rad53 form in pph3Δ cells after DNA methylation damage. This form might, for instance, remain stably bound to its replisome targets, obstructing interaction with factors required to resume fork progression. Alternatively, Pph3 may be required to dephosphorylate some of the replisome proteins that are targeted by Mec1 and Rad53 to stabilize stalled forks (26, 51). If stabilized forks are indeed unable to restart after DNA methylation damage, the slow progression of replication in the pph3Δ ptc2Δ ptc3Δ mutant should result from leaky origin firing despite hyperactivated Rad53.
Rad53 Recovery after Replication Stress—In contrast to the defect in Rad53 deactivation after DNA methylation damage, the triple phosphatase deletion has only a marginal effect on the dephosphorylation and kinase deactivation of Rad53 after replication stress, again reinforcing the view that different phosphatases operate in the recovery of Rad53 after different types of genotoxic stress. Which, then, is the phosphatase responsible for Rad53 deactivation after replication stress? Because Rad53 arrests cell cycle progression, deletion of the phosphatase responsible for Rad53 deactivation after replication stress should result in a growth defect in hydroxyurea plates. To this respect, the PP2C phosphatase Ptc1 was identified in two independent drug sensitivity deletion screens as the only non-essential phosphatase catalytic subunit for which deletion results in a moderate hydroxyurea sensitivity when 




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