Yeast PP4 Interacts with ATR Homolog Ddc2-Mec1 and Regulates Checkpoint Signaling

Graphical Abstract

Highlights

- S phase functions of Mec1 kinase are balanced by phosphatase Pph3-Psy2
- Mec1-Ddc2 binds PP4 (Pph3-Psy2) through a Ddc2-Psy2 interaction site
- The two complexes colocalize at foci of stalled replication forks
- Most mec1-100-sensitive phosphorylation events on HU are PP4 targets

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In Brief

The central checkpoint kinase Mec1-Ddc2 kinase (HsATR-ATRIP) is essential for survival of acute replication stress. Hustedt et al. show that the conserved yeast PP4 phosphatase (Psy2-Pph3) binds Mec1-Ddc2 and dephosphorylates S phase targets of Mec1-Ddc2 including Mec1.

Hustedt et al., 2015, Molecular Cell 57, 273–289

January 22, 2015 © 2015 Elsevier Inc.

http://dx.doi.org/10.1016/j.molcel.2014.11.016
Yeast PP4 Interacts with ATR Homolog Ddc2-Mec1 and Regulates Checkpoint Signaling

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SUMMARY

Mec1-Ddc2 (ATR-ATRIP) controls the DNA damage checkpoint and shows differential cell-cycle regulation in yeast. To find regulators of Mec1-Ddc2, we exploited a mec1 mutant that retains catalytic activity in G2 and recruitment to stalled replication forks, but which is compromised for the intra-S phase checkpoint. Two screens, one for spontaneous survivors and an E-MAP screen for synthetic growth effects, identified loss of PP4 phosphatase, pph3Δ and psy2Δ, as the strongest suppressors of mec1-100 lethality on HU. Restored Rad53 phosphorylation accounts for part, but not all, of the pph3Δ-mediated survival. Phosphoproteomic analysis confirmed that 94% of the mec1-100-compromised targets on HU are PP4 regulated, including a phosphoaceptor site within Mec1 itself, mutation of which confers damage sensitivity. Physical interaction between Pph3 and Mec1, mediated by cofactors Psy2 and Ddc2, is shown biochemically and through FRET in subnuclear repair foci. This establishes a physical and functional Mec1-PP4 unit for regulating the checkpoint response.

INTRODUCTION

Cells are constantly exposed to DNA damage. Lesions can arise either from exogenous agents (e.g., DNA damaging drugs) or endogenous events (e.g., replication forks encountering barriers) (Aguilera and García-Muse, 2013). DNA damage checkpoints sense damage, stop the cell cycle, and induce DNA repair events in order to preserve genome integrity (Friedel et al., 2009). Key to these signaling cascades are the PI3K-like kinases (PI3K) ATM and ATR, or Tel1 and Mec1 in budding yeast (Cimprich and Cortez, 2008).

Whereas ATM is primarily activated in response to DNA double strand breaks (DSBs), ATR can sense a variety of lesions (Cimprich and Cortez, 2008). Most ATR activation appears to involve single-stranded (ss)DNA coated by the ssDNA binding protein replication protein A (RPA). The ATR interacting protein, ATRIP (Ddc2 in yeast), is needed to bind ssDNA (Zou and Elledge, 2003), whereas the Rad17-RFC2-5 clamp loading complex (Rad24-Rfc2-5 in S. cerevisiae) recognizes a double-stranded (ds)DNA adjacent to ssDNA structure and indirectly recruits TopBP1 (S. Dpb11) to further activate ATR/Mec1 (Mordes et al., 2008).

Once activated, the yeast Mec1 kinase phosphorylates the downstream kinases Rad53 and Chk1 in a manner dependent on mediator proteins. In the case of Mec1 activation in response to DSB or DNA adducts (methyl methanesulfonate [MMS] treatment), the checkpoint protein Rad9 (SBP1 in mammals) recruits Rad53 and facilitates its phosphorylation, while in response to hydroxyurea (HU)-induced replication stress, the fork components Mrc1 and Sgs1 promote Rad53 activation by Mec1 (Hustedt et al., 2013). In S phase cells, higher levels of damage are required to activate the Mec1-dependent checkpoint, suggesting an activation threshold for the intra-S checkpoint (Shimada et al., 2002; Tercero et al., 2003). This threshold may ensure that the ssDNA found at normal replication forks does not trigger the checkpoint response.

Whereas Mec1 activation has been studied extensively, how the replication checkpoint is downregulated and/or modulated to prevent unwarranted checkpoint induction is not well understood. A number of phosphatases have been shown to dephosphorylate Rad53, and it is proposed that the phosphatase used depends on the type of lesion that provokes Rad53 activation (Heideker et al., 2007). For instance, the PP1 phosphatase Glc7 was reported to promote Rad53 dephosphorylation after exposure to HU (Bazzi et al., 2010), while the PP2C phosphatases Ptc2 and Ptc3 appear to dephosphorylate Rad53 after MMS treatment (O’Neill et al., 2007; Szyjka et al., 2008), although Ptc2/3 may compensate for loss Pph3 and vice versa during...
recovery from MMS treatment or DSBs (Kim et al., 2011; Travesa et al., 2006). Finally, PP4 was also implicated in the dephosphorylation of Mec1 substrates Zip1 (Falk et al., 2010), Cdc13 (Zhang and Durocher, 2010), Cbf1 (Bandyopadhyay et al., 2010), and histone H2A (Keogh et al., 2006).

In human cells, the data on phosphatases and checkpoints are no less complicated: both downstream kinases CHK1 and CHK2 are counteracted by both the PP2C (Wip1) and PP2A phosphatases, while PP4 was shown to dephosphorylate γH2AX (phosphorylated H2AX) (Chowdhury et al., 2008; Freeman and Monteiro, 2010; Nakada et al., 2008). PP4 was also implicated in dephosphorylation of RPA2 in C. albicans and mammals (Lee et al., 2010; Wang et al., 2013), as well as mammalian 53BP1, KAP1, and CHD4 (Lee et al., 2012, 2014). Other mechanisms that downregulate the checkpoint act by degrading Mrc1 or human CLASPIN (Fong et al., 2013; Malland et al., 2006; Peschiaroli et al., 2006), or by sequestering Rad9 by Rtt107-Slx4 in yeast (Ohouo et al., 2013). To date, however, no study has examined whether Mec1-Ddc2 activity itself is under negative control.

Here, we describe an interaction between the Mec1-Ddc2 checkpoint kinase and the yeast PP4 phosphatase Pph3-Psy2. A strong genetic relationship between mutants in the two complexes was identified in forward and reverse genome-wide genetic screens. We find that Mec1-Ddc2 and PP4 coregulate many Mec1-dependent phosphorylation targets in response to HU stress, including Rad53 and H2A, suggesting that this interaction maintains a balance of phosphorylation that is important for surviving fork-associated stress. We also identify a phosphoacceptor site within Mec1 that is regulated in a Pph3-dependent manner, mutation of which compromises survival of Zeocin-induced damage.

RESULTS

Spontaneous mec1-100 Suppressor Mutations Map to PSY2 and PPH3 Genes

To study how the replication checkpoint is controlled, we used a mutant allele of the checkpoint kinase Mec1, mec1-100, which shows a delayed activation of Rad53 in S phase cells, but robust Rad53 phosphorylation in G2 (Paciotti et al., 2001). This allele carries two mutations outside of the catalytic domain, N1700S is within and F1179S is flanking the FAT domain (Paciotti et al., 2001). The kinase activity of the mec1-100 kinase is intact: the mutant kinase recovered from cell lysates by coprecipitation with Ddc2-GFP, phosphorylates a target peptide (Sgs1 amino acids [aa] 404–604) (Hegnauer et al., 2012) as efficiently as wild-type Mec1 (Figure S1A available online). Mec1-100-Ddc2 recruitment to stalled forks is equivalent to that of wild-type Mec1-Ddc2, yet the mutation compromises the recovery of engaged polymerases near stalled forks and fails to prevent late origin firing on HU (Cobb et al., 2005). Its synthetic defects in combination with sgs1Δ are not mimicked by rad53Δ, which argues that the Mec1-100 kinase fails to phosphorylate a select set of S phase specific targets that ensure survival of replicative stress.

When plated on HU, spontaneous suppressor mutations arise quite frequently in mec1-100 cells, but not in mec1Δ strains (Figure 1A, full list of yeast strains in Tables S1 and S2). Since suppression could stem from either loss of negative regulators or upregulation of downstream Mec1 targets, we analyzed 31 suppressor colonies by sequencing, after backcrossing 2–3 times with the parental wild-type strain. The suppressors fell into two classes: those that cosegregated with the MEC1 locus (“intragenic”), and those that segregated independently (“extragenic”). All intragenic suppressors had acquired one additional mutation in mec1-100, rendering the cells HU-resistant (Figures 1B and S1B). Remarkably, genome-wide sequencing showed that all 12 extragenic suppressor mutations were in one of two genes, PSY2 or PPH3, which encode subunits of the PP4 phosphatase (Figures 1B and S1C). In the catalytic subunit, PPH3, mutations occurred throughout the coding region; while in PSY2 we detected premature STOP codons at aa 40 or aa 183 (Figure 1B). All alleles were recessive, as HU sensitivity was restored to psy2 or pph3 double mutants with mec1-100, after transformation with wild-type PSY2 or PPH3 genes (data not shown).

Epistatic Miniarray Profiling Groups mec1-100 with Replication Checkpoint Deficient Mutations

To better characterize mec1-100 effects, we performed a high-throughput genetic interaction screen based on the previously described Epistatic Miniarray profiling (E-MAP) method (Collins et al., 2006, 2007). We combined 35 query strains bearing mutations in 35 genes implicated in DNA replication fork or checkpoint function, with an array of 1,525 deletions and a few decreased abundance by mRNA perturbation (DAmp) mutants, all representing functions that are required for chromatin-based processes (Guénolé et al., 2013) (Table S3). The resulting double mutants were scored for their growth in the presence of 0, 20, and 100 mM HU, and quantitative genetic interaction scores were calculated (Collins et al., 2006) (Figure 1C). A positive score indicates suppression (or potentially, epistasis), while a negative score shows synthetic sickness or synthetic lethality. Quality control of the data led to the exclusion of 214 mutants (see Supplemental Experimental Procedures), yielding a network of 45,885 (35 × 1,311) genetic interactions (Table S4).

We first compared the overall genetic interaction profile of mec1-100 with the profiles of the other query mutants. Mutants with similar genetic interaction profiles often indicate shared function (Collins et al., 2007). As expected, the mec1-100 profile was highly correlated with mutants that compromise the S phase checkpoint (i.e., sgs1Δ, rad24Δ, rad17Δ, ddc1Δ, mrc1Δ, and ddc1Δ; Figure 1D). The replication checkpoint mediator, mrc1Δ, showed the strongest correlation with mec1-100 in the presence of HU, yet did not correlate in its absence, arguing that the proteins cooperate on HU, but function distinctly in an unperturbed S phase (Figure 1D). These S phase checkpoint mutants also show negative genetic interactions with mec1-100 (Figures 1C, S1D, and S1E). Thus, they most likely act on parallel pathways that achieve the same function as mec1-100, or else on the same pathway in a redundant fashion (Figure 1C).

Interestingly, there are two groups of mutants whose genetic correlation patterns change dramatically upon HU treatment. Profiles of mutants that compromise break-induced replication or translesion synthesis (i.e., rad18Δ, six5Δ, six8Δ, mre11Δ, rad52Δ, and bre1Δ) correlate with mec1-100 only in the
Figure 1. Mutations in PSY2 and PPH3 Genes Suppress mec1-100 HU Sensitivity

(A) The indicated strains (see Tables S1 and S2) were plated on YPAD + 50 mM HU for 3 days at 30°C. Colonies appear white on dark background.
(B) Mec1, Ddc2, Psy2, and Pph3 domain architecture with mec1-100 mutations in black and mec1-100 suppressor mutations in red. Bold, mutations found more than once independently. Asterisks, STOP codon at indicated residue or frameshift (aa 181) resulting in STOP at aa 183 (GA-6610).
(C) Upper panel, overview of genetic interaction screen (E-MAP; full data in Table S4), 35 mutant ''query'' strains combined with 1,525 mutant strains (1,311 after quality control), see Table S3. Double mutant growth was scored on 0, 20, and 100 mM HU. Genetic interaction scoring is at right. Hatching indicates ''no data'' in E-MAP, but confirmed negative interaction by drop assay (see Figure S1E). Lower panel, selected mec1-100 genetic interactions, including phosphatase mutants (significant positive interaction with mec1-100 are in bold). DAmP allele = D. Complete mec1-100 genetic interactions are in Figure S1.
(D) Heat map of Pearson correlation coefficients for mec1-100 genetic interaction profile with those of the other strains on 0, 20, and 100 mM HU. Correlation coding is at right.
Figure 2. Validation of psy2Δ and pph3Δ as Suppressors of mec1-100 HU Sensitivity

(A) Scheme of yeast phosphatases and relationships with mec1-100 or checkpoint downregulation roles, see text.

(B) pph3Δ or pph3Δ mec1-100 cells with TRP1-based control plasmid or plasmids expressing PPH3 or pph3-H112N from PPH3 promoter. Cells grown in synthetic complete medium (lacking tryptophan) (SC-TRP) in a 5-fold dilution series on SC-TRP ±100 mM HU.

(legend continued on next page)
presence of HU, while the opposite was observed for rad9Δ, dot1Δ, sae2Δ, yku70Δ, and sgs1-r1Δ (Hegnauer et al., 2012), which confer selective sensitivity to Zeocin. These genetic interaction profiles suggest that in the absence of HU, mec1-100 cells are somewhat compromised for DSB repair, while on HU, replication fork stabilization and recovery are lost. Indeed, even on HU, the specific mutants that interact with mec1-100 (based on threshold scores ≥ 2 for suppressive and ≤ –2 for negative interactions) fall into distinct functional groups (Figures 1C and S1D, discussed in legend). However, of the 1,311 mutants scored, psy2Δ and pph3Δ showed the highest suppressive genetic interaction with mec1-100, and clearly promoted survival on HU (Figures 1C and S1D; Table S4).

PP4 Subunits Psy2 and Pph3 Counteract mec1-100 Sensitivities

Given that two independent screens show that loss of Psy2 or Pph3 robustly suppresses mec1-100 lethality on HU, we studied these factors in depth. Psy2 and Pph3 form the PP4 phosphatase, with Pph3 as the catalytic subunit (Gingras et al., 2005). We first showed that the suppression of mec1-100 by pph3Δ indeed reflects loss of phosphatase activity, since the catalytically inactive mutant, pph3-H112N (O’Neill et al., 2007), supports mec1-100 growth on HU to the same extent as pph3Δ (Figure 2B).

Because both the E-MAP and past experiments had implicated multiple phosphatases in yeast checkpoint control (Figure 2A), we created double mutants in a second yeast background (W303) of mec1-100 with other phosphatase genes and scored for survival of HU stress (Figure 2C). The E-MAP suggested that the loss of PP5 phosphatase (ppt1Δ), like those of the PP2A phosphatase (pph21Δ, pph22Δ, sap155Δ, sap185Δ, sap190Δ, and sap33Δ), had no genetic interaction with mec1-100 (Figure 1C). On the other hand, loss of Rdr2, which interacts with and regulates PP2A, or Rrd1, a binding partner of Pph3 (PP4), like Ppg1 (related to PP4 and PP6) and Sut4 (PP6) (Van Hoof et al., 2005), did show low suppression level by E-MAP (Figure 1C). These latter effects, however, were extremely weak when deletions were recreated in W303 (Figure 2D), as was loss of Ptc2 (one of seven PP2C proteins; Figure 2C). We also could rule out robust effects of other PP2C mutants (ptc1Δ and ptc4Δ) and of a phosphotyrosyl phosphatase mutant oca1Δ, which showed little or no suppression of mec1-100 in W303 on HU (Figure 2C).

Deletions of PPH3 or PSY2 Counteract Failed Replication Fork Recovery in mec1-100 Cells

Previous work suggested that PP4 dephosphorylates the checkpoint effector kinase Rad53 in a manner that is redundant with Ptc2 and Ptc3 (PP2C-type phosphatases) and the PP1-type phosphatase, Glc7, depending on the type of damage that activated the checkpoint (Bazzi et al., 2010; Heideker et al., 2007; Leroy et al., 2003; O’Neill et al., 2007; Travesa et al., 2008) (Figure 2A). Indeed, it was reported that Pph3 was dispensable for Rad53 dephosphorylation after HU arrest, while Glc7 was not. However, in the context of the HU-induced checkpoint in mec1-100, neither the partial loss of function allele glc7-132 (Bazzi et al., 2010), nor ptc2 or ptc3 deletions, showed significant suppression of HU-induced lethality (Figure 2C). Thus, Glc7 and Ptc2/Ptc3 probably counteract responses stimulated by conditions other than HU.

Previous work suggested that Rrd1 and Pph3 act on the same pathway at DSBs to dephosphorylate the Mec1 target Cdc13 (Zhang and Durocher, 2010). Therefore, we tested rdr1Δ epistasis with pph3Δ in triple mutants. Surprisingly, the coupling of rdr1Δ with psy2Δ or pph3Δ reduced the suppression of mec1-100 sensitivity to HU (Figure 2D), arguing that rdr1Δ interferes with suppression by psy2Δ or pph3Δ, while both the pph3Δ ptc2Δ and pph3Δ psy2Δ double mutants suppressed in an additive fashion. We conclude that Rdr1 counteracts mec1-100 lethality on HU in a manner distinct from PP4 (Figure 2D).

To shed more light on how PP4 suppresses mec1-100 lethality on HU, we checked whether stalled replication forks remain engaged in the phosphatase mutants, allowing fork restart upon HU removal. When phenome synchronized cultures are released into S phase on HU, mec1-100 cells suffer a partial loss of replicative polymerase engagement at sites of early replication and show reduced recovery upon removal of HU (Cobb et al., 2005). We tested single and double mutants of PP4 and PP2C with mec1-100, and scored for recovery after release from a synchronous arrest in S phase, both in the presence and absence of Tel1 (Figures 2E and 2F). The combination of pph3Δ with mec1-100 robustly rescued the defect, particularly at early time points (1–2 hr, Figure 2E), while ptc2Δ had a weaker effect, particularly in the absence of Tel1 (Figure 2F). We conclude that loss of Pph3 efficiently suppresses both the HU sensitivity and fork recovery defects of mec1-100, without recourse to the Tel1/DSB checkpoint response.

Rad53 Activation Correlates with High Levels of Suppression

In the checkpoint cascade, Rad53 is activated by Mec1-mediated phosphorylation, which is compromised in mec1-100 strains (Paciotti et al., 2001). Given that Ptc2 and Pph3 were both implicated in Rad53 phosphorylation under other conditions (Travesa et al., 2008), we tested whether the reduced S phase level of Rad53 phosphorylation found in mec1-100 cells is counteracted by loss of Pph3 or Ptc2. Cells bearing mec1-100 in combination with pph3Δ or ptc2Δ were phenome synchronized in G1 and released into S phase in the presence of HU. Western blots showed the characteristic delay in Rad53 activation in the mec1-100 background; this was compensated by pph3Δ or more weakly, by ptc2Δ (Figure 3A). By performing all assays in a tel1Δ background, we could exclude that the
observed suppression stems from compensation by Tel1 (Figures 3B and S2A). Neither ptc2Δ nor pph3Δ influenced Rad53 activation kinetics in MEC1+ cells (Figure 3A), although in both MEC1+ and mec1-100 backgrounds, Rad53 remained more robustly phosphorylated at 90 min when Pph3 was ablated, than with loss of Ptc2, with very pronounced differences by
120 min after HU removal (Figures 3A, 3B, S2A, and S2B). Nonetheless, this delay in Rad53 dephosphorylation does not compromise survival in the recovery assay, while the efficiency of activation does (Figure 2E).

In these assays, ptcΔ had effects similar to pph3Δ, although generally less pronounced (Figures 2 and 3). Consistent with the notion that PP4 acts by dephosphorylating targets of checkpoint kinases, we found that Tel1 was necessary in a mec1 Δ null for pph3Δ to exert its suppressor effect, although it was not necessary in mec1-100, which retains residual Mec1 kinase activity (Figures 3C, 3D, and S1A). In conclusion, the correlation between Rad53 activation kinetics and the suppression of HU sensitivity argues that pph3Δ suppresses mec1-100, at least partly by regulating the efficiency of Rad53 activation.

PP4 Targets Rad53 and Other Factors to Mediate mec1-100 Suppression

Rad53 initiates many of the downstream checkpoint responses on HU (e.g., cell cycle arrest and late origin firing), yet there is extensive evidence that Mec1 has unique roles in the replication checkpoint that are independent of Rad53 (Hustedt et al., 2013). To see if the suppression of mec1-100 by pph3Δ involves targets beyond Rad53, we asked whether pph3Δ can suppress mec1-100 in the absence of Rad53. To avoid rad53Δ lethality, we coupled it with a bypass mutation, sml1Δ (Zhao et al., 1998), generating a strain that is extremely sensitive to HU. Nonetheless, serial dilution of the mec1-100 rad53Δ sml1Δ mutant on plates with low HU concentrations revealed a mild, but reproducible increase in survival upon deletion of PPH3 (Figure 3E). This was also true in the absence of Chk1 (Figure 3F). Thus, while Rad53 plays an important role, the phosphorylation status of proteins other than Rad53 and Chk1 also help rescue the mec1-100 lethality on HU. This residual suppression was not observed in rad53Δ mec1Δ sml1Δ cells, indicating again that the remaining kinase activity of mec1-100 is required for pph3Δ suppression. This underscores the crucial role of Mec1, and not Tel1, in HU survival. In conclusion, activation of the downstream kinase Rad53 is important, but is not the only Mec1-mediated phosphorylation event enhanced by loss of PP4, ensuring mec1-100 growth on HU.

Restoration of H2A Phosphorylation Does Not Correlate with Suppression

A known target of Pph3 (PP4) during the DNA damage response in both yeast and mammals is histone H2A/H2AX (Chowdhury et al., 2008; Keogh et al., 2006; Nakada et al., 2008). Yeast H2A is phosphorylated on Serine 129 by Mec1 and/or Tel1 at DSBs and stalled replication forks (Cobb et al., 2005; Downs et al., 2000; van Attikum et al., 2004). We confirmed that H2A phosphorylation levels were increased by PPH3 or PSY2 deletion in both wild-type (WT) and mec1-100 backgrounds after treating S phase cells with HU (Figure S2D). However, unlike Rad53, phospho-H2A regulation is not only dependent on Pph3 and Psy4, but also on Psy4, a variable third subunit of the complex (Keogh et al., 2005; O’Neill et al., 2007). Given that loss of Psy4 did not rescue mec1-100 HU sensitivity in WT or rad53Δ sml1Δ backgrounds (Figures 1C, 2C, and S2C), we conclude that enhanced H2A phosphorylation cannot be responsible for the rescue of mec1-100 cells on HU.

Phosphopeptides Downregulated in mec1-100 Cells Are Upregulated by pph3 Deletion

To find the Mec1 targets that are responsible for mec1-100 suppression on HU, we performed a quantitative phosphoproteomic study. Specifically, we screened for modifications that are downregulated in mec1-100, compensated by pph3Δ, and left unaffected by rad53Δ (Figure 4A). To eliminate contributions from Tel1 (Figures 2E, 2F, 3A, and 3B), we used a tel1Δ mec1-100 double mutant in the screen. Prior to extraction of proteins, the cultures were arrested in G1 by α factor and released into S phase in the presence of HU (Figure 4B).

There were 2,368 phosphopeptides that could be quantified (Table S5), of which 47 were specifically reduced in mec1-100 tel1Δ, but not in rad53Δ sml1Δ cells (Figure 4C; Table S6). Among them were the repair factor Rdh54, chromatin remodeler INO80 subunit 1es4, the mismatch repair protein Msh6, and transcription regulators like Swi3 and Leo1, the latter being a component of the PAF1 complex (Figure 4C). We did not find any proteins known to control DNA replication. Remarkably, however, when the abundance of these phosphopeptides was scored after deletion of PPH3, almost the entire set was upregulated (i.e., 94% showed restored phosphop phosphorylation in mec1-100 tel1Δ pph3Δ versus mec1-100 tel1Δ; Figure 4D). This effect, averaged over all mec1-100-dependent targets, is both highly significant (Figure 4D, inlay) and specific, because it was not observed when the entire population of quantified phosphopeptides was compared ±Pph3 (Figure S3A). Remarkably, the loss of Pph3 balances out almost all of the phosphorylation defects that we detect in mec1-100 cells on HU. This supports our genetic results, which showed opposing functions for these two mutations (Figure 1).

Serine/Threonine followed by Glutamine (Q) Phosphopeptides Are Upregulated in rad53Δ, but Are Unaffected by mec1-100

Among the 47 phosphopeptides that were specifically downregulated in the mec1-100 tel1Δ mutant, only a few (ten phosphopeptides) fit the generally assumed ATR/ATM consensus p[S/T]Q (Kim et al., 1999). This could reflect technical problems in our detection of serine/threonine followed by glutamine (Q) (S/T)Q sites, or simply arise because S/TQ-containing peptides did not match the stringent criteria we applied to identify mec1-100 tel1Δ-dependent phosphopeptides. We therefore triaged for phosphopeptides that were less abundant in mec1-100 tel1Δ versus rad53Δ sml1Δ cells (log2 ratio ≤ -1, p value ≤ 0.05), regardless of their abundance in WT cells, and screened independently for known Mec1/Tel1 targets (Chen et al., 2010). Using this approach, we identified many p[S/T]Q phosphopeptides in our phosphoproteomic data set, including known fork-associated Mec1/Tel1 targets such as Rfa2 (Brush et al., 1996) and H2A (Downs et al., 2000) (Figures S3B and S3C; Table S7). These hits were eliminated in our earlier analysis because they were not downregulated in mec1-100 tel1Δ cells versus WT. Thus, mec1-100 kinase is actually proficient for phosphorylating many S/TQ Mec1 targets on HU, consistent with the robust kinase...
activity we detect in the pull-down assay (Figure S1A). These S/TQ acceptor sites are, therefore, unlikely to be responsible for the mec1-100 lethality on HU.

Intriguingly, the majority of p[S/T]Q phosphopeptides that we recovered in the second analysis were more abundant in the rad53Δ sml1Δ mutant than in WT cells, and they were not further affected by loss of Pph3 (Figures S3C–S3E). This suggests that in rad53Δ sml1Δ cells, Mec1 and/or Tel1 may be hyperactivated on HU, either because the cells accumulate additional DNA damage at the fork, or because they lose a negative feedback loop through which Rad53 normally downregulates Mec1 activity.

**Mec1-Ddc2 and Pph3-Psy2 Physically Interact in a DNA Damage-Independent Manner**

From our phosphoproteome analysis, we conclude that almost every phosphopeptide (94%) that was reduced due to the mec1-100 mutation was restored by further restoration of Pph3, in the absence of Tel1. How could this robust coordination be guaranteed? We speculated that the Mec1 kinase and PP4 phosphatase might bind each other to ensure coordinated action. To test this, we created yeast strains that expressed epitope-tagged versions of the kinase or phosphatase subunits from their native genomic loci. Whereas the tagged Psy2 and Ddc2 forms were fully functional, tags on Mec1 or Pph3 restored cells slightly sensitive to MMS or HU (Figures S4A–S4D). Since Ddc2-Mec1 and Psy2-Pph3 are both stable complexes (Gingras et al., 2005; Paciotti et al., 2000), we used the functional tagged versions of Psy2 or Ddc2 in subsequent assays. As positive and negative controls, we tested for interaction with Rfa1 and Ptc2. Consistent with our hypothesis, immunoprecipitation (IP) of Ddc2-GFP efficiently recovered Psy2-MYC, but not Ptc2 (Ptc2-PK; Figures 5A and S4E). The Psy2-Ddc2 interaction is not compromised by removal of nucleic acids with RNaseA (Figure 5A), whereas Ddc2-GFP-Rfa1 signals were sensitive to this treatment (Figure 5A). The Ddc2-GFP/Psy2-MYC interaction was also independent of HU, being scored both in untreated G1- and in treated S phase cells, and the IP worked reciprocally (Figures 5B and S5B). Finally, the robust Ddc2-Psy2 interaction did not require Pph3 or Mec1 and was not affected by the mec1-100 mutant (Figures 5B and S5A).

The interaction was further mapped by yeast-two hybrid, through which we could define the minimal Psy2 domain (aa 130–350) that robustly binds Ddc2 (Figures 5C and S5C). Although a smaller Psy2 fragment (aa 130–350) only weakly binds Ddc2, its deletion fully abolished the interaction. Another robustly expressed Psy2 fragment (aa 25–129; Figure S5D) failed to interact significantly, although its deletion reduced the interaction by about 50%.

**Ddc2-Psy2 Homologs Interact in Mammalian Cell Extracts**

Psy2 has two human homologs, PP4R3A and PP4R3B, which share an overall sequence similarity with Psy2 of 37% and 44% and an identity of 24% and 22%, respectively. Intriguingly, the Ddc2 binding domain within Psy2 (aa 130–350) is highly conserved in PP4R3A (50% identity) and PP4R3B (51% identity) (Figure 5D). We therefore tested whether these regulatory phosphatase subunits bind ATRIP in human cells, following transient transfection of human embryonic kidney cells (HEK)293T cells with plasmids encoding for MYC-tagged ATRIP and either PP4R3A-GFP, PP4R3B-GFP, or GFP alone. MYC-ATRIP bound efficiently to PP4R3B-GFP, but not to GFP alone and only weakly to PP4R3A-GFP (Figure 5E), even though PP4R3A expression levels were much higher. Thus, the PP4R3B-ATRIP interaction is strongly preferred. We conclude that in budding yeast and human cells Ddc2/ATRIP binds the phosphatase subunit Psy2/PP4R3B, and thus kinase and phosphatase appear capable of forming a complex. In yeast, the interaction involves a conserved N-terminal region of Psy2, and is HU-, Mec1-, and Pph3-independent.

**Ddc2 and Psy2 Interact and Colocalize in Nuclear Foci In Vivo**

Although the genetic and biochemical evidence for interaction was strong, it was unclear whether the Mec1/Ddc2-PP4 interaction occurs at the right place and the right time, i.e., at stalled replication forks or sites of damage. To localize the putative complex in living cells, we fused Psy2, Ddc2, and Rfa1 with distinct fluorescent proteins (RFP, GFP, and CFP, respectively). All fusions were expressed under their endogenous promoters from their genomic loci and were fully functional (Figures S4A, S4D, and S4F). As expected, Rfa1-CFP has a punctate nuclear signal in S phase cells, consistent with the existence of replication foci (Pasero et al., 1997) (Figures 6A and 6B). Whereas the abundance of Rfa1 renders Rfa1-CFP replication foci difficult to resolve in yeast, both Ddc2-GFP and Psy2-RFP formed foci that were larger and less numerous, even in untreated S phase cells (Figures 6A and S6A), most likely indicating repair foci. Indeed, consistent with previous reports, Rfa1/Ddc2 foci were also detected in G1-phase cells, albeit rarely (Figure S6A) (Lisby et al., 2004).
Figure 5. Ddc2 and Psy2 Interact Physically

(A) Native extracts from cycling cultures of indicated strains (see Tables S1 and S2) ± RNaseA and benzonase treatment were subjected to anti-GFP IP and western blotting with indicated antibodies. Nucleic acid digestion in GFP-depleted extracts after IP was analyzed by agarose gel and SYBR Safe.

(B) Cells of indicated genotypes (see Tables S1 and S2) were arrested in G1 by a-factor and held or released into 0.2 M HU for 30 min. Extracts were subjected to anti-GFP IP and western blotting with indicated antibodies.

(legend continued on next page)
Following incubation with HU, Rfa1, Ddc2, and Psy2 concentrated in intense nuclear foci (Figure 6B), allowing us to score both their number and colocalization (Figures 6C, 6D, and S6C–S6F). Approximately 15% of untreated S phase cells contained a single bright focus of Rfa1 and/or Ddc2, likely indicative of spontaneous damage, while Psy2-RFP occasionally formed two (Figure 6C). On HU, on the other hand, we frequently scored >2 Ddc2 or Psy2 foci per cell (Figure 6C). We quantified the degree of colocalization of the tagged proteins on HU and found that 70% of the Rfa1 foci coincided with both Ddc2 and Psy2, while an additional 20% contained only Rfa1 and Ddc2 (Figure 6D). There were about 70% of the Ddc2 foci that also contained Psy2 (Figure 6D). In cells treated with 400 μg/ml Zeocin, a radiomimetic drug that induces both ssDNA lesions and DSBs, extensive spontaneous damage, while Psy2-Rfa1 FRET signals were less strong (Figure 6F, Psy2-Rfa2 data not shown), whereas there is no observable FRET between nonfocal nuclear fractions of any of these reporter pairs. This does not exclude interactions outside the Mec1 phosphatase, could dephosphorylate both Cdc13 and Mec1 peptides (Figure 7E) (Piston and Kremers, 2007). This suggests that Ser1991 requires both Mec1 and Rad53 for its phosphorylation is elevated on HU, is not detected in an unperturbed S phase, shows a slight enhancement on Zeocin, and was virtually absent in the mec1-100 strain (Figures 7B and 7C). Importantly, Ser1991 phosphorylation could be restored in mec1-100 cells by deleting PPH3, indicating that PP4 indeed regulates Mec1 phosphorylation, compensating for the mec1-100-associated loss of S1991 modification (Figure 7D).

Mec1 Ser1991-phosphorylation was absent in a catalytically dead Mec1 protein, yet also in a strain lacking Rad53 (Figure 7D). This suggests that Ser1991 requires both Mec1 and Rad53 for its phosphorylation, although it remains unclear whether either acts directly. We attempted to demonstrate direct dephosphorylation of Ser1991 in vitro using Pph3-Psy2 isolated from cells expressing Psy2-Halo (Figure S7A), but the precipitated Pph3-Psy2 could not dephosphorylate the appropriate Mec1 peptide, even though a nonspecific enzyme, calf intestinal alkaline phosphatase, could dephosphorylate both Cdc13 and Mec1 peptides in vitro and Pph3-Psy2 was able to dephosphorylate Cdc13 (Zhang and Durocher, 2010) (Figure S7B). We cannot exclude that our conditions were inappropriate to monitor dephosphorylation of Ser1991, yet it appears that it is not a preferred substrate of Pph3-Psy2 in vitro.

Surprisingly, the mec1-S1991A mutant showed impaired growth in the presence of Zeocin, but not on HU, MMS, UV, or γ-irradiation (Figures 7E and 7C). This effect is independent of Ser38. We further confirmed the sensitivity of mec1-S1991A cells to induced DSBs by ectopically expressing EcoRl in all strains. Indeed, the mec1-S1991A mutant showed EcoRI-sensitivity (Figure 7F), while mec1-S1991D had a slight resistance. Consistently, the mec1-S1991A mutant shows a strong synergetic genetic interaction with deletion of the DNA damage checkpoint protein Rad9 and additive interactions with other checkpoint mutants rad24Δ, ddc1Δ, and mer1-c1-AQ (Osborn and Elledge, 2003), and DSB repair mutants rad51Δ and dnl4Δ (Figure S7D). If Mec1 S1991 were the only target site through
which Pph3 regulated Mec1 function, the nonphosphorylatable mec1-S1991A mutant should suppress pph3 defects. This was not the case, indicating that there are other sites in Mec1 or Ddc2 through which Pph3 might regulate Mec1-Ddc2 activity (Figure S7E). Future studies should clarify the molecular details of this regulation pathway.

DISCUSSION

We show that the central checkpoint kinase Mec1-Ddc2 (human ATR-ATRIP) forms a stable complex with the PP4 (Pph3-Psy2) phosphatase. The two enzymes act in a coordinated, yet opposing, manner on a large number of substrates (Figure 7G).
The modification of this target set is compromised in the S phase-specific Mec1 mutant, mec1-100, which confers hypersensitivity to replication stress. The sensitivity of mec1-100 to HU is, however, efficiently depressed by pph3Δ or psy2Δ. PP4 also counteracts a phosphoacceptor site on Mec1 itself, which is sensitive to the mec1-100 mutation (Figure 1H). In vivo FRET studies then confirmed that Mec1-Ddc2 and PP4 interact at sites of replication fork-induced damage and at DSBs (Figure 6). Although the majority (70%) of the Ddc2 foci colocalize with Psy2, this does not allow us to draw conclusions about the fraction of Mec1-Ddc2 in the cell that is bound to PP4. We do not exclude that Mec1-Ddc2 is in complex with Pph3-Psy2 in undamaged conditions, whereby it might regulate noncheckpoint functions of the kinase.

The complex of phosphatase and kinase may allow the fine-tuning of ATR-ATRIP (Mec1-Ddc2) for different functions through the cell cycle. The fact that there seems to be a higher threshold for damage-induced checkpoint activation in S, as opposed to G2, phase, suggests that Mec1/ATR modulation is an important regulatory event in the cell cycle (Shimada et al., 2002; Tercero et al., 2003). Indeed, Mec1 not only has to react appropriately to ssDNA, but must also be switched off rapidly to allow efficient replication and cell cycle resumption. This role may be ensured by the closely associated Pph3-Psy2 complex. We show that Rad53 dephosphorylation during recovery from HU treatment is delayed in pph3Δ cells (Figures 3A, 3B, S2A, and S2B), although loss of PP4 did not compromise recovery from arrest, as scored by colony formation (Figures 2E and 2F). Presumably other phosphatases (Ptc2, Ptc3, or Glc7) compensate over time for the loss of PP4, as reported for recovery from MMS treatment and DSB repair (Kim et al., 2011; Szyjka et al., 2008). We and others find that pph3Δ is synthetic sick with dia2Δ, ptc2Δ, and sae2Δ on HU, which also impair recovery from checkpoint-induced arrest (Figure S1D) (Guénolé et al., 2013; Keogh et al., 2006; Kim et al., 2011; O’Neill et al., 2007; Szyjka et al., 2008).

These proteins may target Rad53 (Guillermain et al., 2007; Leroy et al., 2003; O’Neill et al., 2007; Travesa et al., 2008), limit Tel1 signaling (Clerici et al., 2006), or promote Mrc1 degradation (Fong et al., 2013). In either case, loss of Ptc2 showed significantly milder effects than pph3Δ and does not physically interact with Mec1-Ddc2 (Figures 2, 3, and 5).

The Yin/Yang of Mec1/PP4 Complexes

The copurification of opposing enzymatic activities is not unique. We note that the human RAP80 complex contains both ubiquitin ligase and deubiquitinase enzymes (Sobhian et al., 2007), and histone acetyltransferases and deacetylases have been shown to not only create a dynamic balance of acetylation of target proteins, but also be physically associated with each other, sometimes even regulating each other’s activity (Yamagoe et al., 2003). Intriguingly, Pph3-Psy2 regulates a phosphorylation site within the Mec1 kinase, Ser1991, possibly indirectly through Rad53 (Figure 7H), mutation of which compromises survival in face of DSBs, and not on HU. We have no clear explanation for this sensitivity, but note that the mec1-100 E-MAP profile in the absence of HU shows similarity to those of genes involved in DSB repair (Figure 1D). In fact, Ser1991 phosphorylation may alter the specificity of Mec1 and/or trigger its downregulation, rather than its induction. Intriguingly, Rad53 itself seems to be required for Mec1 Ser1991 phosphorylation, and given that we detected a large set of S/TQ phosphoacceptor peptides (Mec1 targets) that are upregulated in a rad53 deletion strain (Figures S3C–S3E), it is possible that Rad53 controls Mec1-Ddc2 in a negative fashion. An alternative interpretation, however, is that there is more damage in rad53Δ cells, which indirectly triggers Mec1/Tel1 activation.

How does checkpoint activation occur if an antagonizing activity is stably associated with the activating kinase? The phosphorylation of any given protein at any given time is almost the result of competing kinase and phosphatase activities. Obviously, upon checkpoint activation, the catalytic rate of phosphorylation becomes stronger, and phosphorylated proteins accumulate. It appears that once Mec1 activation is triggered, it does not matter how much stronger it is (i.e., whether Pph3 is present or not). This would explain why Pph3 does not affect phosphorylation in mec1Δ cells, but does in mec1-100, which may have lower catalytic rates toward a subset of substrates in vivo.

The balance between the opposing activities can only be flipped by altering their specific activities. For instance, the recruitment of Rad53 by Sgs1 or Mrc1 (Alcasabas et al., 2001; Hegnauer et al., 2012) may induce a change in the specific activity of Mec1 toward Rad53, triggering checkpoint activation. Alternatively, the kinase may alter activity of the phosphatase, inhibiting it once a specific level of damage has occurred, and releasing it once damage is repaired. Finally, although we did not detect phosphopeptides from either Psy2 or Pph3, we cannot exclude that PP4 is a target of Rad53 or Mec1.

Other studies have identified DNA damage-related PP4 targets, both in yeast and in mammals, among them mammalian RPA2, Kap1, 53BP1, Chd4, and yeast Cbf1, even though these studies were partially performed under nondamaging conditions (Bandypadhyay et al., 2010; Lee et al., 2010, 2012). Those additional targets, and the 47 targets hit by both Ptc2 and Psy2 identified here, are most likely a nonexhaustive list, given that phosphopeptide coverage is rarely complete. Nonetheless, it is remarkable that 94% of the 47 phosphopeptides showing reduced phosphorylation levels in mec1-100 responded to a deletion of PPH3. This corroborates our genetic data, which showed suppression of mec1-100 by pph3Δ and a strong anticorrelation of their genetic interaction profiles.

Among many other interesting genetic interactions scored for mec1-100, we found several subunits of the chromatin remodeling complexes INO80, SWI/SNF (Switch or Sucrose nonfermentable), ISW (imitation SWI/SNF), and Chd1, which show HU-induced synthetic lethality with mec1-100 (Figures 1C and S1D). Relevant to the phenotypes attributed to chromatin remodelers for stalled replication forks recovery (Papamichalopoulos-Chronakis and Peterson, 2008; Shimada et al., 2008), subunits of these chromatin remodelers were also among the mec1-100 downregulated phosphopeptides (e.g., Ies4 and Swi3). This reflects the close relationship of remodelers such as INO80 with DNA fork-associated damage, as well as their recruitment to DSBs (van Attikum et al., 2004).
in response to damage (M. Smolka, personal communication). It is tempting to speculate that the association of Mec1-Ddc2 with Pph3-Psy2 is involved in regulating the switch between these two functions. Further work will delineate the underlying molecular mechanisms of such a switch.

**EXPERIMENTAL PROCEDURES**

Yeast Materials, Microscopy, Phosphoproteomics, Phosphatase Assay, and E-MAP

Yeast strains and plasmids are described in Tables S1, S2, and S3. Details of yeast two hybrid assay, growth conditions, antibodies, microscopy, phosphoproteomics, and the E-MAP assay are found in Supplemental Experimental Procedures. In general, the conditional E-MAP analysis was performed as described in Guenolé et al., 2013.

Mammalian Cell Culture

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Transfections were carried out using jetPEI (Polyplus) transfection reagent according to manufacturer’s instructions.

Spontaneous Suppressor Screening

mec1-100 (GA-4978) and mec1-100 exo1Δ (GA-6356) cells were plated on yeast extract, peptone, adenine, and dextrose (YPD) + 50 mM HU and incubated for three days. Colonies were picked and backcrossed 2–3 times with WT (GA-1982) cells. Strains yielding no HU sensitive LEU + HIS + (mec1-100) spores were considered to have intragenic suppressors and the MEC1 locus was sequenced. Strains yielding both HU sensitive and insensitive LEU + HIS + (mec1-100) spores were deep sequenced to find extragenic mutations. Details are in Supplemental Experimental Procedures.

Kinase and Phosphatase Assays, Recovery and Drop Assay, Rad53 and H2A Phosphorylation, Fluorescence Activated Cell Sorting, and IP

Enzymatic assays, recovery and drop assays, fluorescence activated cell sorting (FACS) analysis, and Rad53 and H2A phosphorylation analysis were done as described previously (Hustedt and Shimada, 2014) or as detailed in Supplemental Experimental Procedures. AntiGFP IP was carried out as described for kinase assays, except that the lysis buffer was supplemented with protease and phosphatase inhibitors (see Supplemental Experimental Procedures) and bead-bound protein complexes were washed three times with lysis buffer prior elution with 0.2 M glycine. IP for mammalian cells was essentially the same, except that cells were harvested 48 hr post transfection by scraping off the plate into PBS, and washed once in PBS before snap-freezing pellets in liquid nitrogen. Nuclease treatment and nucleic acid monitoring is described in online Supplemental Experimental Procedures.

**Figure 7. Mec1 Phosphoserine 1991 Is Regulated by Rad53 and Pph3**

(A) Mec1 phosphosites in blue, black lines = mec1-100 mutations, and red lines = suppressor mutations (Figure 1), and interaction domains and structural domains indicated below.

(B) Ddc2-GFP and Ddc2-GFP mec1-S1991A cells were treated with 0.2 M HU for 1 hr or arrested in G1 and released into YPAD at 25°C for indicated times. FACS was performed on samples to confirm cell cycle stages. After IP with α-GFP, western blots were performed with indicated antibodies, e.g., α-pMec1 (Mec1 phosphoserine 1991).

(C) Exponential cultures of Ddc2-GFP and Ddc2-GFP mec1-100 ±0.2 M HU or 400 μg/ml Zeocin for 1 hr were extracted and subjected to IP by α-GFP. Western blots were probed with indicated antibodies, and input samples were probed with α-Rad53 to monitor checkpoint activation.

(D) Native extracts were prepared from Ddc2-GFP strains with indicated genotypes (see Tables S1 and S2) after 1 hr incubation + 0.2 M HU. α-GFP IP and western blotting with indicated antibodies was performed.

(E) 10-fold dilution series on YPAD ±100 μg/ml Zeocin of isogenic strains of indicated genotypes (Tables S1 and S2).

(F) Cells transformed with pGAL-EcoRI (♯2,745) and grown in selective medium to ensure plasmid retention were plated on 2% glucose or galactose supplemented with 2% raffinose, in 10-fold dilution series.

(G) Model of Ddc2-Psy2 interaction and coordinated interplay of Mec1-Ddc2 and Pph3-Psy2. Both target Rad53, H2A, and other targets. Most mec1-100/Te11-specific phosphosites are regulated by Pph3-Psy2 (“B”), while a few are not (“A”).

(H) Mec1 phosphoserine 1991 requires Rad53 and Mec1, is compromised in mec1-100 cells, and rescued by loss of Pph3-Psy2. Mec1 regulation of Mec1 may be indirect. See Figure S7.

**ACCESSION NUMBERS**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomexchange.org) via the PRIDE partner repository (http://www.ebi.ac.uk/pride) with the data set identifier PXD001492.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.11.016.

**AUTHOR CONTRIBUTIONS**

N.H. planned, performed, and evaluated most experiments; made all figures; and wrote the paper. A.S. performed mass spectroscopy; M.T.-P. performed β-galactosidase assays; B.B. sequenced yeast mutants; H.V., F.v.L., A.G., H.v.A, T.I., and R. Srivas helped with E-MAP studies; K.S. and S.M.G. supervised and helped write the paper.

**ACKNOWLEDGMENTS**

N.H. thanks the European Union Innovative Training Networks “Image DDR” and the Swiss Cancer League for support. The Gasser laboratory is further supported by the Swiss National Science Foundation, Novartis Research Foundation, and the Human Frontier Science Program. F.v.L. and H.V. were supported by the Dutch Cancer Society and H.v.A. by the Netherlands Organization for Scientific Research (TOP-GO grant). R. Srivas is supported by the Damon Runyon Cancer Research Foundation (DRO-2187-14). We thank M.P. Longhese for strains; F.E. Rromberg for plasmids; D. Durocher for reagents; D. Houpfer (Novartis) for E-MAP library analysis; E. Oakeley, S. Schuierer, T. Roloff, S. Dessus-Babus, and M. Stadler for deep sequencing and analysis; H. Gut and I. Deshpande for construct design; and Y. Murata, S. Nohara, M. Kawai, S. Ishikawa, and K. Doi for assistance.

Received: June 20, 2014

Accepted: November 14, 2014

Published: December 18, 2014

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Nicole Hustedt, Andrew Seeber, Ragna Sack, Monika Tsai-Pflugfelder, Bhupinder Bhullar, Hanneke Vlaming, Fred van Leeuwen, Aude Guénolé, Haico van Attikum, Rohith Srivas, Trey Ideker, Kenji Shimada, and Susan M. Gasser
A

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B

**Cycling G1 arrest** 0.2M HU

**wash**

**recovery**

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D

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Hustedt et al. Figure S2
A

![Graph showing log2 ratio (mutant / WT) for mec1-100 tel1 Δ and pph3 Δ](image)

B

![Graph showing log2 ratio (rad53 Δ / WT) and quantified phosphopeptides](image)

C

![Heatmap and scatter plot showing selected p[S/T]Q (n=48)](image)

D

![Box plot showing log2 ratio (mutant / WT) for rad53 Δ sm1 Δ mec1-100 tel1 Δ mec1-100 tel1 Δ pph3 Δ](image)

E

![Western blots for α-pH2A, α-Mcm2, Rad53-P, and Rad53](image)

Hustedt et al. Figure S3
Hustedt et al. Figure S5
A)

HALO-Link Resin

covalent interaction

TEV cleavage site

Pph3

Psy2

P

Mec1 peptide

Cdc13 peptide

control peptide

Pph3-Psy2

P

P

P

P

P

P

Mec1-P

Cdc13-P

S1991

S306

dephosphorylation reaction

free phosphate measured

B)

Phosphatase activity (A.U.)

[Bar chart showing phosphatase activity levels for different conditions]

C)

WT

mec1-S1991A

mec1-S38A-S1991A

mec1-S1991D

mec1-S38D-S1991D

mec1Δ

control 100 mM HU UV (240 J/m²)

0.03% MMS

100 ug/ml Zeocin

D)

WT

rad51Δ

dnl4Δ

rad24Δ

ddc1Δ

tel1Δ

mrc1-AQ

rad9Δ

MEC1 mec1-S1991A

100 ug/ml Zeocin

E)

WT

pph3Δ

mec1-S1991A

mec1-S1991A pph3Δ

mec1-S1991D

mec1-S1991D pph3Δ

control 100 ug/ml Zeocin 0.03% MMS 100 mM HU

Hustedt et al. Figure S7
Supplemental Material

Supplemental Figures S1 – S7
Supplemental Tables S1 – S7
Supplemental Experimental Procedures
Supplemental References

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. The mec1-100/Ddc2 kinase shows robust activity, yet genetic interactions implicate its deficiency in replication checkpoint and fork recovery; related to Figure 1

(A) Kinase assay using anti-GFP IP from DDC2-GFP (GA-7268), DDC2-GFP mec1-100 (GA-7327) or WT (GA-1981) cell extracts. A Sgs1 peptide (aa 404–604, Hegnauer et al., 2010) was used as a substrate in the presence of γ-32P-ATP and was analysed by gel electrophoresis and autoradiography. Where indicated, 30mM caffeine was added to inhibit Mec1. 32P autoradiography and Coomassie Brilliant Blue (CBB) staining are shown. Lower panel shows quantification of 32P signal over CBB signal. Error bars indicate standard deviation of three independent experiments. (B) Drop assay of intragenic suppressors. Serial ten-fold dilutions of cultures were plated on YPAD ± 50 mM HU. Relevant genotypes are indicated in the figure. Isogenic strains used were: GA-1981, GA-6583, GA-6769, GA-6410, GA-6412, GA-6416, GA-6418, GA-6422, GA-6466, GA-6468, GA-6470, GA-6472, GA-6480, GA-6510, GA-6514, GA-6518, GA-6522, GA-6529, GA-6533, GA-6537 and GA-6539. (C) Drop assay of extragenic suppressors. Cells were treated as in (B). Isogenic strains used were: GA-1981, GA-6583, GA-6575, GA-6596, GA-6577, GA-6598, GA-6598, GA-6600, GA-6601, GA-6603, GA-6605, GA-6606, GA-6610, GA-6608 and GA-6572. Genotypes indicated in figure and in Table S2 Asterisks: STOP codon at indicated residue. (D) Genetic interactions with mec1-100 (genetic interaction score ≤2 or ≥+2) are shown. Interactions of mrc1Δ and pph3Δ with the same mutants are shown for comparison. Besides some phosphatase mutants
(pph3Δ, ptc2Δ, rrd1Δ, rrd2Δ, oca1Δ), mec1-100 showed positive genetic interactions (yellow) with mutations that dampen the checkpoint response (rtt107Δ, sae2Δ, dia2Δ, cdc53Δ, irc21Δ), with some of which pph3Δ shows synthetic sickness. Negative genetic interactions (blue) of mec1-100 include chromatin remodelers, nuclear envelope and various transcription regulators. Mutations of additional subunits of multisubunit complexes were included. Hatched areas indicate “no data” in E-MAP, but confirmed negative interaction by Drop Assay (see (E)). D = DAmP allele. 

(E) Serial ten-fold dilutions of cultures were plated on YPAD ± 10 mM HU. Relevant genotypes are indicated. Isogenic strains were: GA-1981, GA-6826, GA-7907, GA-5321, GA-6582, GA-6828 GA-7964 and GA-7209.

Figure S2. Psy4 dephosphorylates H2A, but does not suppress mec1-100 HU sensitivity; related to Figure 3

(A) Rad53 activation was monitored in WT (GA-1981) and tel1Δ (GA-6912) cells. Cells were synchronized in G1 by α-factor arrest and released for indicated times into 0.2M HU before denatured extract preparation and Western blot analysis. (B) Cells were treated as in (A) except that after 60 minutes cells were washed and resuspended in medium without HU. Relevant genotypes are indicated. Samples were taken at indicated timepoints and analysed for Rad53 phosphorylation. Relevant genotypes are indicated. Isogenic strains were: GA-1981, GA-7049, GA-7273, GA-6582, GA-7086 and GA-7329. (C) Serial five-fold dilutions of saturated rad53Δ sml1Δ mec1-100 (GA-7401), rad53Δ, sml1Δ mec1-100 pph3Δ (GA-7377), rad53Δ sml1Δ mec1-100 psy4Δ (GA-8581) cultures (2 cultures derived from 2 single colonies each) were plated on YPAD ± 2 mM HU. (D) Rad53 and H2A serine 129 phosphorylation was monitored by treating cells as in (A) and blotting with indicated antibodies. Relevant genotypes are indicated. Isogenic strains were GA-1981, GA-7049, GA-7391, GA-7383, GA-6582, GA-7086, GA-7393 and GA-7385 cells.
Figure S3. Most S/TQ phosphopeptides are not reduced in mecl-100 cells on HU; related to Figure 4

(A) Tukey boxplot of indicated abundance ratios of all quantified phosphopeptides. P-value was calculated by one-tailed Wilcoxon signed rank test. (B) Abundances (log₂ ratio (mutant/WT)) of all quantified phosphopeptides in mecl-100 tellΔ cells were plotted against abundances in rad53Δ sml1Δ. Blue marked peptides are phosphorylated on [pS/pT]Q consenses and are (1) specifically downregulated in mecl-100/tel1Δ cells (see Figure 4) and/or (2) were at least 2-fold more abundant in rad53Δ compared to mecl-100 tellΔ samples (log₂ ratio ≥ 1, p≤0.05, Student’s paired t-test) regardless of the levels in WT sample and/or (3) were previously described as Mec1/Tel1-dependent (Chen et al., 2010) (full list in Table S7). Dotted lines indicate area enlarged in (C). (C) Enlarged area of plot shown in (B). Displayed are selected phosphopeptides marked blue in (A). Labels indicated protein names. Bold: phosphopeptides previously described as Mec1/Tel1 specific (Chen et al., 2010). Dotted lines indicate thresholds for up/downregulation. n= number of phosphopeptides that fall between dotted lines. (D) Tukey boxplot of indicated ratios of phosphopeptides that match the [pS/pT]Q consensus and were marked blue in (A). (E) Samples taken before synchronization (C) and prior protein extraction (HU) (Fig.4B) were analyzed by Western blotting with indicated antibodies.

Figure S4. Functionality of tagged strains used in this study; Related to Figure 5

(A) Serial 5-fold dilutions of cultures were plated on YPAD ± 100 mM HU. Relevant genotypes are indicated. Isogenic strains were GA-1981, GA-7268, GA-7337 and GA-6582. (B) Serial 5-fold dilutions of cultures were plated on YPAD, YPAD + 5 mM HU and YPAD + 100 mM HU. Isogenic strains were GA-1981, GA-7353, GA-7354, GA-7355, GA-6582, GA-7366, GA-7367 and GA7368. (C) Serial 10-fold dilutions of cultures were plated on YPAD, YPAD + 100 mM HU and YPAD + 0.03% methyl methanesulfonate (MMS).
Isogenic trains were GA-1981, GA-7049, GA-7852, GA-7853, GA-7865, GA-7966 and GA-7967. (D) Cells were treated as in (C). Isogenic strains were GA-1981, GA-7391, GA-7798, GA-7799, GA-8033, GA-8021, GA-8659 and GA-8179 (E) Cells were treated as in (C). Isogenic strains were GA-1981 and GA-7923. (F) Cells were treated as in (C). Isogenic strains were GA-1981, GA-8396, GA-8687, GA-8688, GA-8694, GA-8695 and GA-8676.

Relevant genotypes are indicated.

**Figure S5. Ddc2-GFP and Psy2-MYC interaction does not require Pph3, and is detected when tags are swapped and in yeast two-hybrid (Y2H) experiments; Related to Figure 5**

(A) Native extracts were prepared from cycling *Psy2-13MYC* (GA-7798), *Psy2-13MYC DDC2-GFP* (GA-7824) and *Psy2-13MYC DDC2-GFP pph3Δ* (GA-7939) cells, subjected to IP with α-GFP and analysis by Western blotting with indicated antibodies. (B) Cells were treated as in (A). Strains used were *PSY2-13MYC* (GA-7972), *PSY2-13MYC DDC2-GFP* (GA-7975), DDC2-13MYC (GA-7337) and *DDC2-13MYC PSY2-GFP* (GA-8034). (C) Y2H analysis between full length Ddc2 fused with the B42 transcription activation domain (B42 AD) and an HA-tag and Psy2 fragments fused with the lexA DNA binding domain (DBD) was performed by observing blue color formation on X-GAL plates. negative control: empty lexA-DBD plasmid. positive control: LexA-DBD directly fused with B42-AD. (D) Y2H construct expression: Cells were grown in 2% raffinose-containing selective medium to ensure plasmid retention and expression was induced with 2% galactose for 4h prior to denatured extract preparation and Western blot analysis with indicated antibodies.

**Figure S6. Ddc2 and Psy2 colocalize and show FRET at spontaneous and Zeocin-induced foci; related to Figure 6**

White dashed line: outline of cell nucleus. (B) Example images of Zeocin-treated cells, showing a few bright foci per cell. Displayed are examples of cell in which foci of all three fluorophores colocalize. The same strain was used as in (A). Arrowheads indicate foci. (C) Cells were treated +/- 0.2M HU or 400 ug/ml Zeocin for 1h prior to fixation. Bright spots per cell were quantified for S-phase cells. The same strain was used as in (A). (D) Cells were treated the same as in (C). Colocalization of Ddc2-GFP and/or Psy2-RFP spots with Rfa1-CFP spots was quantified. The same strain was used as in (A). (E) Colocalization of Rfa1-CFP and/or Psy2-RFP spots with Ddc2-GFP spots was quantified in the same experiment as (D). (F) Colocalization of Rfa1-CFP and/or Ddc2-GFP spots with Psy2-RFP spots was quantified in the same experiment as (D).

Figure S7. Damage sensitivity of the non-phosphorylatable mutant mec1-S1991A; related to Figure 7

(A) Setup of Pph3-Psy2 purification using HaloTag (Promega) and Phosphatase Assay (B) Phosphatase Assay to test dephosphorylation of Mec1 serine 1991 and Cdc13 serine 306 by Psy2-Pph3. Psy2-Pph3 was purified from PSY2-HALO cells (GA-8179) and WT cells (GA-1981, “no tag”) served as a negative control. Purifications were incubated with indicated phosphopeptides for 60 min at 30°C prior measurement of phosphatase activity by release of phosphate. Results are representative of two independent experiments. (C) Serial ten-fold dilutions of cultures were plated on YPAD, YPAD + 100 μg/ml Zeocin, YPAD + 100 mM HU, YPAD + 0.03% MMS and on YPAD plates and subsequently treated with γ-irradiation (100 Gy) or UV light (240 J/m²). Relevant genotypes are indicated in the figure. Isogenic strains (see Table S2) used were: GA-4533, GA-8242, GA-8246, GA-8243, GA-8247 and GA-5286. (D) Serial ten-fold dilutions of cultures were plated on YPAD ± 100 μg/ml Zeocin. Relevant genotypes are indicated. Isogenic strains were GA-1981, GA-5919, GA-6096, GA-5321, GA-7907, GA-6912, GA-6671, GA-6625, GA-8338, GA-8829, GA-8838, GA-8890,
GA-8867, GA-8340, GA-8835 and GA-8841(E) Serial ten-fold dilutions of cultures were plated on YPAD, + 100 μg/ml Zeocin, YPAD + 0.03% MMS and YPAD + 100 mM HU. Relevant genotypes are indicated. Isogenic strains were GA-1981, GA-7049, GA-8338, GA-8873, GA-8342 and GA-8869.

**SUPPLEMENTAL TABLES**

Table S1: Yeast strains sorted by use in main figures

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Table S2: Yeast strains and plasmids used in this study

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Table S3: Query strains and library used for E-MAP

separate Excel file

Table S4: Genetic interaction data

separate Excel file

Table S5: All quantified phosphopeptides

separate Excel file

Table S6: Mec1-100 dependent phosphopeptides

separate Excel file

Table S7: Selected SQ/TQ phosphopeptides

separate Excel file
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Yeast materials, microscopy, phosphoproteomics, phosphatase assay, and E-MAP

Yeast strains and plasmids are described in Tables S1/S2. If not stated otherwise cells were cultured at 30°C in YPAD medium using standard procedures. For Y2H analysis, fragments of \textit{PSY2} were fused to the lexA DNA binding domain (#3616 - #3623, #3629,#3630) and \textit{DDC2} was fused to the B42 transcription activation domain of pJG4-6 (#993), resulting in pJG4-6-DDC2 (#3308). WT cells (GA-338/EGY48) containing the lacZ reporter pSH18-34 (#359), the bait and the prey were streaked on X-GAL plates (Golemis et al., 2011) and incubated 1-2 days at 30°C. β-galactosidase assays were performed as previously described (Hegnauer et al., 2012) using pGAL-lexA Psy2 constructs (#3307, #3592-#3595, #3649-#3651, #3653).

Enzyme assays, recovery and drop assay, Rad53 and H2A phosphorylation, FACS

Mec1 kinase assay was performed as described (Hustedt and Shimada, 2014). A recombinant domain of Sgs1 (Sgs1 aa404-604) was used as substrate (Hegnauer et al., 2012). Recovery and drop assays, FACS analysis and Rad53 and H2A phosphorylation analysis were done as described previously (Hustedt and Shimada, 2014). Phosphatase assays are described below.

Immunoprecipitation (IP)

Anti-GFP IP was carried out as described for kinase assays, except that the lysis buffer was supplemented with protease and phosphatase inhibitors (Complete protease inhibitors (Roche), 1mM phenylmethylsulfonyl fluoride, PhosSTOP phosphatase inhibitors (Roche), 0.1 mM Na3VO4, 1 mM NaF and 10 mM NaPP) and bead-bound protein complexes were washed three times with lysis buffer prior elution with 0.2 M glycine. Eluates were neutralized with Tris/HCl and analysed by Western blotting.

Immunoprecipitation for mammalian cells was essentially performed the same, except that cells were harvested 48h post transfection by scraping off the plate into PBS, and washed once with PBS before snap-freezing pellets in liquid nitrogen.

Nuclease treatment was performed in a modified lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 1 mM MgCl2 and 0.5% NP-40) supplemented with protease and phosphatase inhibitors. Cleared lysates were incubated with 5 μl RNaseA (Sigma) and 5 μl benzonase
(Invitrogen) for 30 min on ice prior to immunoprecipitation. After 1h incubation at 4°C with antibody-coupled beads, lysates were recovered, DNA was isolated by phenol/chloroform extraction and analyzed by agarose gel electrophoresis and SYBR SAFE (Invitrogen) staining. Bead-bound protein complexes were analyzed as described above.

**Antibodies for Western blotting**

Antibodies used were: Monoclonal α-Rad53 antibody (custom made by Genscript), goat αMcm2 antibody (Santa Cruz) and rabbit αH2A phospho-serine 129 antibody (custom made by Sigma Genosys), mouse α-GFP (Roche), rabbit α-GFP, (Invitrogen), rabbit α-MYC (Santa Cruz), rabbit α-Rfa1 (Agrisera), rabbit α-PK (Novus Biologicals), rabbit α-Mec1 (custom made by SDIX), rabbit α Mec1 phospho-serine 1991 (custom made by Genscript), rat α-HA (Roche), rabbit α–lexA (Santa Cruz)

**Sequencing of extragenic suppressors**

Genomic DNA was isolated using Qiagen Genomic Tip100 (Qiagen) or phenol chloroform extraction and NucleoSpin PCR clean-up kit (Macherey-Nagel) in case of GA-6610 and quantified using a Nanodrop 3000 (Thermo Scientific) PicoGreen Assay. 50 ng of each individual sample were processed for library generation using Illumina’s Nextera DNA sample preparation protocol, and barcoded as described for Illumina’s TruSeq Dual Index Sequencing primers (Illumina). The samples were pooled at equimolar concentrations and sequenced using a single-end 75-base reads (50-base reads for GA-6610) on the Illumina’s HiSeq2000 platform. The sequence data had >95% alignment, using BWA, to a reference S288C genome (genome build S288C_reference_genome_R62-1-1_20090218, obtained from Saccharomyces Genome Database, www.yeastgenome.org). A sequence of progenitor strains used in the experiment was done for mecl-100 (GA-4978 and GA-6336) and mecl-100 exo1Δ (GA-6356 and GA-6335). By comparison to the progenitor strain sequence, the SNP and indel calls were made in the sequence of the various derived sub-strains. In addition, large regions (>0.1 Megabase) of chromosome amplifications and deletions were assessed in each strain by comparison across all of the strains using read depth information.

**High throughput genetic interaction screening**

All E-MAP query and array strains are described in Table S3. For mecl-100, sgs1-r1 and rfa1-t11 query strains a NAT resistance cassette was integrated approximately 150 bp
downstream of the gene. Subsequently, *mec1-100* and *sgs1-r1* mutations were introduced by pop-in/pop-out (Reid et al., 2002) with linear DNA fragments engineered by PCR using genomic DNA from already mutated strains (Hegnauer et al., 2012; Paciotti et al., 2001) as a template. *rfa1-t11* was created by transforming *NheI*-linearized plasmid #2221.

Double mutants of query strains and library strains were created as described (Tong et al., 2004). Colony sizes were quantified using HT Colony Grid Analyzer (version 1.1) and genetic interaction scores were computed using the E-MAP toolkit (version 2.0) as previously described (Collins et al., 2006). A stringent QA/QC pipeline was employed to identify and remove (i) strains with a high error of measurement, and (ii) incorrectly deleted strains as identified through linkage analysis (Collins et al., 2010; Collins et al., 2006). In addition, all 1525 array mutants were pooled, genomic DNA extracted, and finally hybridized to a microarray containing probes covering tag sequences from the yeast deletion collection as described (Hegnauer et al., 2012). Strains with an intensity of <800 arbitrary fluorescent units were excluded from further analysis. In total, 214 array strains were dropped for further analysis. This included *ufo1Δ*, for which a genetic interaction with *mec1-100* could not be confirmed despite recreating the mutation, most likely indicating mislabeling of this strain.

**Mec1 phosphorylation**

Immunoprecipitation to map phosphorylation sites in Ddc2-Mec1 was carried out starting from 1 liter cultures treated with 0.2M HU for 1h. Cells were harvested and washed once with PBS. Pellets were weighed, resuspended in 1ml/g PBS and dropped into liquid nitrogen. Droplets were subjected to three rounds of bead-milling using Mixer Mill MM 400 (Retsch). The frozen cell powder was resuspended in an equal volume of cold lysis buffer and Anti-GFP IP was performed as described above.

Eluted protein samples were treated with trypsin overnight at 37°C after reduction and alkylation with tris(2-carboxyethyl)phosphine (TCEP) and iodoacetic acetic acid. The TFA acidified tryptic peptides (final concentration 0.1%) were separated on an Agilent 1100 nanoLC system (Agilent Technologies) coupled to an LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Scientific). The LC system was equipped with a Peptide CapTrap column (Michrom BioResources, Inc.) and a capillary column with integrated nanospray tip (75 μm i.d. x 100 mm, Swiss BioAnalytics AG) filled with MagicC18 (5 μm, Michrom Bioresources, Inc.). Elution was performed with a gradient starting with 2% solvent B and
continued with 2 to 10% solvent B in 3 min and 10 to 40% solvent B in 80 min at a flow rate of 400 nL/ min. Solvent A consisted of 0.1% formic acid/ 2% acetonitrile, solvent B was composed of 0.1% formic acid/ 80% acetonitrile. The mass spectrometer operated in positive mode using the top 15 DDA method. MS scans were acquired at a resolution of 60,000 over a range of m/z 350 to 1200. Singly charged ions were rejected from MSMS fragmentation.

Peptides were identified searching SwissProt (version 2011-08) using Mascot Distiller 2.3 and Mascot 2.3.0.2 (Matrix Science) considering acetylation at protein N-terms, deamidation at asparagine and glutamine, oxidation at methionine and phosphorylation at serine as well as at threonine. Two missed cleavage sites were allowed. Results were compiled in Scaffold 3.0 (Proteome Software).

**Microscopy**

Fluorescence microscopy used an Olympus IX81 microscope equipped with a Yokogawa CSU-X1 scan head, a Evolve 512 Delta EMCCD camera, a ASI MS-2000 Z-piezo stage and a PlanApo x1000 NA 1.45 total internal fluorescence microscope oil objective. Cells where grown from an overnight culture in synthetic complete media complemented with all amino acids until they reached approximately 5 x 10^6 cells/ml. They were treated for 1 hour + 400 µg/ml Zeocin, + 0.2 M HU or left untreated. Cells where then fixed in 4% PFA for 30 seconds washed three times with PBS and finally resuspended in PBS. Images were acquired on 2% agarose pads. The lasers used to excite the different fluorophores are as follows: 445 nm for CFP (eCFP), 491 nm for GFP and 561 nm for RFP.

For foci number quantification 4 color Z-stacks were obtained taking 20 slices at 200 nm intervals. Exposure times were: 50 ms GFP, 100 ms CFP, 150 ms RFP, 10 ms brightfield. The EMCCD gain was set to approximately 600 in all cases except for the brightfield where it was set to 50. Images were deconvolved using Huygens Remote Manager v3.0.3. The deconvolution algorithm used was classic maximum likelihood estimate with a signal/noise ratio of 5, automatic background estimation and 30 iterations. “Bright foci” were counted and defined as foci that have clear borders. Thresholding was applied to help see foci over background nuclear signal and done with Fiji. We note that in the case of Dde2 many smaller less bright foci were present.

For colocalization analysis single slice 4 channel images were acquired and processed as above except the exposure of CFP was increased to 150 ms and RFP to 250 ms. CFP, GFP
and RFP were channel aligned using Huygens Pro software. Colocalization was scored when foci either completely overlapped or partially overlapped.

For FRET, the donor GFP was excited by light at 488 nm and the emission signals are collected using filters that allow selective detection of either donor or acceptor signals. Fluorescence bleed-through of the donor signal into the acceptor channel, or the reverse, is quantified by imaging strains that contain only donor or acceptor proteins. Automated subtraction of bleed-through signal, yields the significant FRET value.

Sensitized emission FRET images for each series were acquired on the same day. Four channel images were acquired: GFP channel, FRET channel, RFP channel as well as brightfield, even for donor only (GFP) and acceptor only strains. FRET was calculated using the PixFRET Fiji plugin (Feige et al., 2005). Spectral bleed through was calculated on using donor only (DDC2-GFP (GA-7268), PSY2-GFP (GA-8033), RFA2-GFP (GA-6022)) and acceptor only strains (PSY2-RFP (GA-8659), RFA1-RFP(GA-8687)). The PixFRET parameters used were 1.0 Gaussian blur, 1.0 Threshold and Output of FRET/sqrt(Donor*Acceptor). The donor only and acceptor only parameters for PixFRET are as follows and were acquired by a montage of 10 donor only or 10 acceptor only images: Ddc2-GFP, a = 2.328, b = 0.00047, linear; Psy2-RFP, a = 0.48385, -b = 0.00004 linear; rfa1-RFP, a= 1.27947, b = -0.00011, linear; Psy2-GFP, a = 0.03595, constant; Rfa2-GFP, a = 0.31318, constant. FRET values were calculated from the mean intensity of the NFRET image of each focus.

**Phosphoproteomics**

Cells were grown in synthetic medium. 50 ml cultures were grown to an OD<sub>600</sub>=0.75, arrested in G1 using α-factor, and released into 75 ml synthetic medium containing 0.2M HU until the appearance of small buds (45 min). Proteins were extracted as previously described (Bodenmiller and Aebersold). In brief, proteins were denatured and precipitated by adding a final 6% trichloracetic acid to the cultures and incubating on ice for 30 min. Precipitates were collected by centrifugation and washed three times with ice-cold acetone. Pellets were resuspended in 800 μl urea buffer (8M Urea, 50 mM ammonium bicarbonate, 5 mM EDTA and PhosSTOP phosphatase inhibitors (Roche)) and a corresponding volume of silica beads was added. Extracts were prepared by five round of bead beating. Supernatant was collected, another 800 μl urea buffer added to beads followed by five rounds of bead beating and pooling of supernatants.
Peptide generation and phosphopeptide enrichment

Extracts in 8 M urea, 50 mM ammonium bicarbonate, and 5 mM EDTA had a protein concentration of about 5.2 mg/ ml. 150 μl 200 mM HEPES were added to 1.5 ml of each of the twelve extracts. Reduction and alkylation of cysteines were performed by adding of 160 μl 45 mM DTT for 30 min followed by adding of 180 μl 100 mM iodoacetamide for another 30 min (in the dark), both at room temperature. Before adding of 20 μl of 1 mg/ ml LysC (Wako, Japan) the extracts were twofold diluted to keep a final HEPES concentration of 20 mM. First digest was performed overnight at 25°C. After diluting the extracts 2-fold, 100 μl of 0.5 mg/ ml trypsin were added and the second digest was performed at 37°C overnight. Before phosphopeptide enrichment the digests were desalted using SepPak C18 columns (Waters). The eluates were dried down in a SpeedVac (Thermo Scientific).

The digests were reconstituted in 150 μl 2.5% trifluoroacetic acid (TFA)/ 80% acetonitrile, saturated with phthalic acid and 30 min incubated with 1.5 mg TiO2 beads (Inertsil Titansphere 5μm, GL Science, Japan) using Mobitec tubes (MoBiTec, Germany). The beads were thoroughly washed 4 times with 200 μl 2.5% TFA/ 80% acetonitrile. Phospho-peptides were eluted with 100 μl 0.3 M NH4OH and 100 μl 0.3 M NH4OH/ 30% acetonitrile. The pH of the eluates was lowered to about 3 by adding 4 μl TFA before drying down in a SpeedVac. The final desalting step was performed on Oligo R3 media (Life technologies) immobilized on C18 GELoader pipette tips (Proxeon).

LC/MS/MS analyses of enriched phosphopeptides

The LC/MS/MS analyses were performed on an Easy-nLC 1000 pump coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) using a Digital PicoView ion source (New Objective). The peptides were separated on a New Objective analytical column (75 μm x 25 cm, Reprosil, 3 μm) with a gradient from 2 to 30% solvent B in 110 min, 30 to 50% solvent B in 30 min and 50 to 80% solvent B in 5 min. Solvent A consisted of 0.1% formic acid in water, solvent B of 0.1% formic acid in acetonitrile. The flow rate was 200 nL/ min. The dried TiO2 eluates were dissolved in 40 μl 0.1% TFA/ 2% acetonitrile and the peptide concentrations determined with a Qubit fluorimeter and the Qubit protein assay kit (Life technologies). The injection volumes were adapted accordingly for 1 μg peptides on column.

Data were acquired in a Top25 data dependent analysis mode using three different charge (z) rejection settings: positive charged ions are considered for MSMS scans with either z > 1,
z = 2 or with z > 2. A different charge selection mode was deployed for each of the replicates. MS scans were acquired at a resolution of 60,000 over a range of m/z 350 to 1200.

Data evaluation with Progenesis-LC

The twelve raw files were loaded into Progenesis-LC and automatically aligned. The alignments were manually corrected were needed. Finally, the alignment scores were 82.5% and better. Features with two charges and more than two isotopes, features with three to six charges having more than three isotopes and spectra with a limit fragment count of 150 were considered for a database search using MASCOT 2.3. Peptides were identified searching the Saccharomyces Genome Database (SGD), version Sept. 2011) considering the following settings: Carbamidomethylation at cysteines as fixed modification, deamidation at asparagine and glutamine, oxidation at methionine, acetylation at the protein N-terms and phosphorylation at serine, threonine and tyrosine as variable modifications, two missed cleavage sites, a peptide mass tolerance of 7 ppm and a fragment mass tolerance of 0.6 Da, trypsin as enzyme, allowing the cleavage after arginine and lysine also if followed by a proline. Finally, in Progenesis-LC features were kept if they had a MASCOT ion score greater than 15 and were identified as phosphopeptides without any other modifications except acetylation at protein N-terminals. The normalization was performed considering only those phosphopeptides. The final feature data list was exported into Excel. Ratios “mutant versus wild-type” were calculated from the average of the normalized abundances of the three replicates and the probability of a Student’s t-test was determined accordingly. Only phosphopeptides with a probability of 0.95 were considered for further evaluation. The phosphorylation localization probabilities were determined using phosphoRS within Proteome Discoverer (version 1.4.1.14, Thermo Fisher Scientific). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino et al., 2014) via the PRIDE partner repository with the dataset identifier PXD001492 and null (http://www.ebi.ac.uk/pride).

Phosphatase Assay

11 of late-log phase WT cells (GA-1981) or PSY2-HALO (GA-8179) cells were harvested and washed once with PBS. Pellets were weighed, resuspended in 1ml/g buffer 1 (50 mM HEPES pH7.5, 150 mM NaCl, 2mM EDTA) and dropped into liquid nitrogen. Droplets were subjected to three rounds of bead-milling using Mixer Mill MM 400 (Retsch). The frozen cell
powder was resuspended to 1ml/g of cold buffer 2 (50 mM HEPES pH7.5, 150 mM NaCl, 1mM DTT, 0.01% NP-40, and protease inhibitors: 1 mM PMSF, 300 μg/ml benzamidine, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin, 40μg/ml bestatin, 2μM E64 and 50 μg/ml TLCK), and cleared by centrifugation. Halo-Link resin (Promega) was washed five times with buffer 2, 1ml resin was added to 7 ml cleared lysates and incubated at 4°C overnight. Resin-bound protein complexes were washed once with buffer 3 (buffer 2 + 1mM EDTA), four times with buffer 3 lacking protease inhibitors and 2 times with phosphatase buffer (25mM TRIS pH7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% Brij-35). Resin-bound protein complexes were finally resuspended in 250 μl Phosphatase buffer + TEV protease and incubated at 4°C overnight. Eluates were separated from resin by centrifuging through micro spin columns (Biorad). 35 μl of eluates were incubated with 10 μM of peptide (positive control: RRA(pT)VA (provided with Ser/Thr phosphatase assay kit (Promega)), Cdc13-p: Biotin-GGGKSYIQ(pS)QTPERK-amide, Cdc13: Biotin-GGGKSYIQSQTPERK-amide (both gifts from D. Durocher), Mec1-p: VK(pS)ITSRGKSLEKC and Mec1: VKSITRSRGKSLEKC (both synthesized by Genscript)) in phosphatase buffer. Reactions also contained a final 5 mM manganese chloride. Release of phosphate was measured using a colorimetric Ser/Thr phosphatase assay kit (Promega). Phosphate leads to the proportional accumulation of a green dye, measured quantitatively as light absorption at 600 nm.
SUPPLEMENTAL REFERENCES


