Multiple protein kinases influence the redistribution of fission yeast Clp1/Cdc14 phosphatase upon genotoxic stress

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ABSTRACT **The Cdc14 phosphatase family antagonizes Cdk1 phosphorylation and is important for mitotic exit. To access their substrates, Cdc14 phosphatases are released from nucleolar sequestration during mitosis. Clp1/Flp1, the *Schizosaccharomyces pombe* Cdc14 orthologue, and Cdc14B, a mammalian orthologue, also exit the nucleolus during interphase upon DNA replication stress or damage, respectively, implicating Cdc14 phosphatases in the response to genotoxic insults. However, a mechanistic understanding of Cdc14 phosphatase nucleolar release under these conditions is incomplete. We show here that relocalization of Clp1 during genotoxic stress is governed by complex phosphoregulation. Specifically, the Rad3 checkpoint effector kinases Cds1 and/or Chk1, the cell wall integrity mitogen-activated protein kinase Pmk1, and the cell cycle kinase Cdk1 directly phosphorylate Clp1 to promote genotoxic stress–induced nucleoplasmic accumulation. However, Cds1 and/or Chk1 phosphorylate RxxS sites preferentially upon hydroxyurea treatment, whereas Pmk1 and Cdk1 preferentially phosphorylate Clp1 TP sites upon H_2O_2 treatment. Abolishing both Clp1 RxxS and TP phosphosites eliminates any genotoxic stress–induced redistribution. Reciprocally, preventing dephosphorylation of Clp1 TP sites shifts the distribution of the enzyme to the nucleoplasm constitutively. This work advances our understanding of pathways influencing Clp1 localization and may provide insight into mechanisms controlling Cdc14B phosphatases in higher eukaryotes.**

Monitoring Editor
Rong Li
Stowers Institute

Received: Jun 22, 2012
Revised: Aug 3, 2012
Accepted: Aug 14, 2012

INTRODUCTION

The eukaryotic cell cycle is driven by the activity of cyclin-dependent kinases (Cdks). Cdk1, bound to its cyclin B partner, controls entry into and progression through mitosis (Morgan, 1997). For proper mitotic exit and cytokinesis, a reduction in Cdk1 activity, as well as dephosphorylation of its substrates, must occur. The conserved Cdc14 phosphatase family contributes to the reversal of Cdk1 substrate phosphorylation during anaphase, at least in yeasts (Stegmeier and Amon, 2004; Queralt and Uhlmann, 2008; Mocciaro and Schiebel, 2010).

The founding family member, Cdc14, was identified in *Saccharomyces cerevisiae* as an essential cell cycle phosphatase necessary for Cdk1 inactivation (Stegmeier and Amon, 2004). Further studies on Cdc14 orthologues from yeast to humans have characterized additional roles for this enzyme family in cytokinesis (Clifford *et al.*, 2008a), chromosome segregation (Stegmeier and Amon, 2004; Mocciaro and Schiebel, 2010; Clemente-Blanco *et al.*, 2011), transcription (Clemente-Blanco *et al.*, 2009, 2011; Papadopoulou *et al.*, 2010; Guillamot *et al.*, 2011), centrosome duplication (Mocciaro and Schiebel, 2010), and ciliogenesis (Clement *et al.*, 2011).

In line with their multiple roles during anaphase, Cdc14 phosphatases undergo dynamic subcellular localization changes that are dependent on the cell cycle (Stegmeier and Amon, 2004; Mocciaro and Schiebel, 2010). During interphase, Cdc14 phosphatases are sequestered within the nucleolus and at the spindle pole body/centrosome and are released from these locales during mitosis. The release from sequestration during mitosis is considered an activation step allowing Cdc14 phosphatases access to their substrates.

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E12-06-0475) on August 23, 2012.

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Abbreviations used: APC/C, anaphase promoting complex/cyclosome; CB, Coomassie blue; Cdk,cyclin-dependent kinase; FEAR, Cdc Fourteen Early Anaphase Release; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; MBP, maltose-binding protein; MEN, Mitotic Exit Network; 1NM-PP1, 4-amino-1-tert-butyl-3-(1′-naphthylmethyl) pyrazolo(3,4-d) pyrimidine; SIN, septation initiation network.

Nucleolar release of Cdc14 in *S. cerevisiae* during anaphase involves two networks, the Cdc Fourteen Early Anaphase Release (FEAR) network and the Mitotic Exit Network (MEN; Stegmeier and Amon, 2004; Liang *et al.*, 2009; Mohl *et al.*, 2009; Rock and Amon, 2009; Tomson *et al.*, 2009; Waples *et al.*, 2009; Manzoni *et al.*, 2010).

Unlike budding yeast Cdc14, the *Schizosaccharomyces pombe* orthologue, Clp1/Flp1 (hereafter referred to as Clp1), and mammalian Cdc14B exit the nucleolus before anaphase (Cueille *et al.*, 2001; Trautmann *et al.*, 2001; Kaiser *et al.*, 2002; Nalepa and Harper, 2004; Cho *et al.*, 2005; Berdougo *et al.*, 2008). In addition, *S. pombe* orthologues of the FEAR network or MEN (known as the Septation Initiation Network [SIN] in *S. pombe*) components are not required for Clp1 nucleolar release (Chen *et al.*, 2006). These observations suggest that the mechanisms controlling Clp1 and Cdc14B nucleolar release may differ from those controlling nucleolar release of *S. cerevisiae* Cdc14. However, the SIN does prohibit return of Clp1 to the nucleolus until the completion of cytokinesis (Mishra *et al.*, 2005; Chen *et al.*, 2008). This is due to direct phosphorylation on RxxS (x can be any amino acid) sites by the SIN kinase Sid2 and consequent association between Clp1 and the *S. pombe* 14-3-3 proteins, Rad24 and Rad25 (Mishra *et al.*, 2005; Chen *et al.*, 2008). A Clp1 mutant that cannot be phosphorylated by Sid2 returns to the nucleolus before the completion of cytokinesis, which results in increased cytokinetic failure (Chen *et al.*, 2008).

In addition to roles in mitosis, one mammalian orthologue, Cdc14B, is believed to participate in the DNA damage checkpoint by dephosphorylating Cdh1, an activator of the anaphase-promoting complex/cyclosome (APC/C); premature APC/C activation promotes a cell cycle delay at the G2/M transition (Bassermann *et al.*, 2008; Takahashi *et al.*, 2012). Other reports indicate that when Cdc14A, a second mammalian orthologue, or Cdc14B genes are deleted, repair of DNA double-strand breaks is prolonged, suggesting that Cdc14 phosphatases play a role in DNA damage repair but not checkpoint arrest (Mocciaro *et al.*, 2010; Wei *et al.*, 2011b). Despite the lack of clarity regarding Cdc14 phosphatase function in response to genotoxic stress, there is agreement that this stimulus results in nucleolar release of Cdc14B during interphase (Bassermann *et al.*, 2008; Mocciaro *et al.*, 2010; Takahashi *et al.*, 2012). Similarly, Clp1 moves out of the nucleolus during interphase when DNA replication is blocked by treatment with hydroxyurea (HU), and Clp1 release is required for a normal checkpoint response to HU (Diaz-Cuervo and Bueno, 2008).

Interphase nucleolar release of Clp1 and Cdc14B in cells treated with HU or DNA-damaging agents, respectively, depends on checkpoint effector kinases (Diaz-Cuervo and Bueno, 2008; Peddibhotla *et al.*, 2011), suggesting that relocalization of these two phosphatases may occur through a conserved mechanism. In *S. pombe*, the ATR orthologue Rad3 is the major sensor of genotoxic insults and is essential for arresting the cell cycle in response to stalled replication forks or DNA damage (Langerak and Russell, 2011). Rad3 activates two downstream effector kinases—Cds1 in response to genotoxic insults during DNA replication, and Chk1 in response to genotoxic insults during G2 (Walworth and Bernards, 1996; Lindsay *et al.*, 1998; Martinho *et al.*, 1998; Brondello *et al.*, 1999). Of interest, in response to HU treatment, Cds1 phosphorylates Clp1 on the same sites as does Sid2 during mitosis, and a Clp1 phosphomutant that abolishes these RxxS phosphosites prevents HU-induced nucleoplasmic relocalization (Diaz-Cuervo and Bueno, 2008). However, the RxxS phosphomutant does exit the nucleolus normally during mitosis (Chen *et al.*, 2008; Diaz-Cuervo and Bueno, 2008).

In this work, we examined whether interphase nucleolar release of *S. pombe* Clp1 is a general response to cellular stress and found that it occurs in response to peroxide, as well as to hydroxyurea, suggesting that it is a specific response to genotoxic stress. We investigated the pathways triggering interphase nucleolar release after these treatments and found that in addition to Chk1 and Cds1, the cell wall integrity mitogen-activated protein kinase (MAPK) Pmk1 and the cell cycle kinase Cdk1 are involved, although the relative effect of these kinases on Clp1 localization is dependent on the type of genotoxic insult. Accordingly, a Clp1 phosphomutant that abolishes Chk1, Cds1, Pmk1, and Cdk1 phosphosites prevented interphase nucleoplasmic accumulation of Clp1 upon either type of genotoxic stress. Reciprocally, a Clp1 mutant that is constitutively phosphorylated on TP sites cannot be retained in the nucleolus. This study advances our understanding of Clp1 phosphatase regulation and may provide insight into the mechanisms controlling Cdc14B localization in higher eukaryotes.

RESULTS

Clp1 relocalizes to the nucleoplasm after genotoxic stress

Because Clp1 relocalizes from the nucleolus to the nucleoplasm during interphase when cells encounter a block to DNA replication (Diaz-Cuervo and Bueno, 2008), we explored whether other cellular stresses would have the same effect. To answer this question, we treated asynchronously growing *clp1-GFP gar2-mCherry* cells (Gar2 is a nucleolar marker; Sicard *et al.*, 1998) with various cellular stresses for 1 h. As previously reported, HU induced relocalization of Clp1-GFP to the nucleoplasm (Figure 1, A and B; Diaz-Cuervo and Bueno, 2008). This could be readily seen in line scans measuring the relative fluorescence intensity of Clp1-GFP and Gar2-mCherry through the nucleus. Cells treated with HU had a lower, broader peak of Clp1-GFP than those of untreated cells, where Clp1-GFP intensity mirrored that of Gar2-mCherry (Figure 1C). In contrast, Clp1-GFP did not relocalize to the nucleoplasm in either thermally or osmotically stressed cells (Figure 1, A–C) suggesting that nucleolar release is not a general response to cellular stress. Although not the topic of this study, we note that thermal stress caused release of Clp1 from spindle pole bodies (Figure 1A). The addition of 1 mM H_2O_2, however, did cause Clp1-GFP to accumulate within the nucleoplasm at the expense of nucleolar localization (Figure 1, A–C). Because H_2O_2 can cause DNA damage (Cadet *et al.*, 2010), Clp1 nucleoplasmic accumulation may be a common response to different forms of genotoxic stress.

Release of Clp1 from the nucleolus upon replication stress depends on the checkpoint effector kinases Chk1 and Cds1 (Diaz-Cuervo and Bueno, 2008), which are activated by Rad3 (Walworth and Bernards, 1996; Lindsay *et al.*, 1998; Martinho *et al.*, 1998; Brondello *et al.*, 1999). To determine whether the same mechanism contributed to H_2O_2-induced Clp1-GFP relocalization, we examined *clp1-GFP gar2-mCherry* or *clp1-GFP gar2-mCherry rad3*Δ cells after the addition of 12 mM HU or 1 mM H_2O_2 over time (Figure 2, A and B). Because asynchronous cells were used in this analysis, some cells have Clp1 released from the nucleolus before treatment because they are in mitosis (Cueille *et al.*, 2001; Trautmann *et al.*, 2001). Late mitotic cells (e.g., cells with Clp1 on the mitotic spindle) were excluded from our analysis; however, residual cells with Clp1 released from the nucleolus at time 0 are likely early mitotic cells (Supplemental Figure S1). As expected, Clp1-GFP accumulated throughout the nucleoplasm of cells after HU treatment. Nearly 100% of these cells had nucleoplasmic Clp1 by 105 min, whereas mock-treated cells did not relocalize Clp1-GFP (Figure 2A). HU-induced Clp1 nucleoplasmic accumulation was not observed in cells lacking *rad3*$^+$, consistent with previously reported results (Diaz-Cuervo and Bueno, 2008; Figure 2A). In response to H_2O_2, Clp1-GFP also accumulated within the nucleoplasm and to the same extent per cell

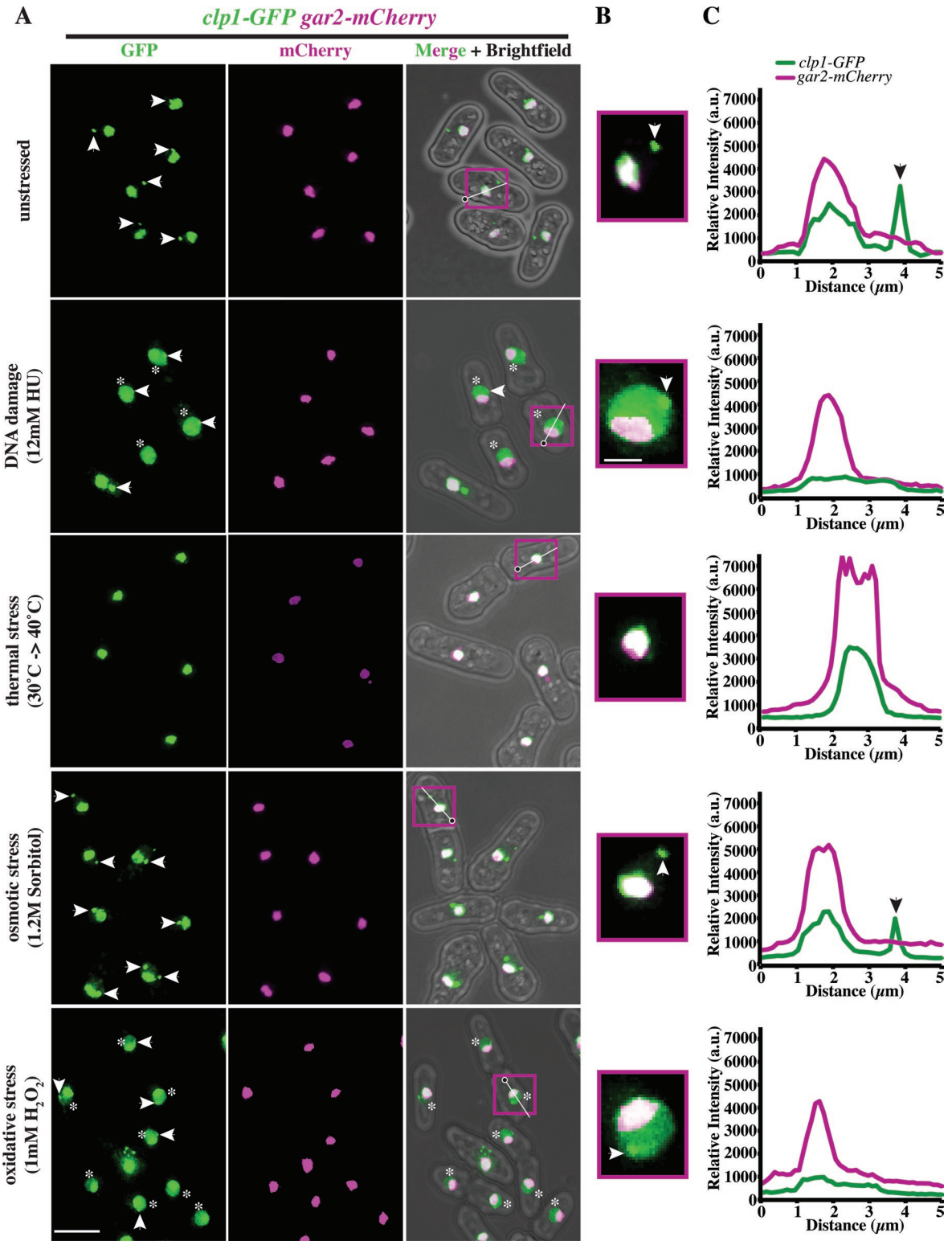

FIGURE 1: Genotoxic stress induces Clp1-GFP nucleoplasmic accumulation. (A) Live-cell images of *clp1-GFP gar2-mCherry* cells after treatment with the specified stress for 1 h. Scale bar, 5 μm. Asterisks indicate nuclei with Clp1-GFP relocalized from the nucleolus to the nucleoplasm. Arrowheads point to spindle pole bodies. (B) Zoomed views of the boxed nuclei from the merged images in A. Scale bar, 1.5 μm. Arrowheads point to spindle pole bodies. (C) Line scans of Clp1-GFP and Gar2-mCherry fluorescence, each spanning a distance of 5 μm, in which the paths are represented in A by a white line with a black circle to indicate the origin of the line scan. Arrowheads point to spindle pole bodies.

(Figure 1 and unpublished data); however, the response was faster (Figure 2B). The largest percentage of cells showing nucleoplasmic Clp1 occurred at 20 min after H_2O_2 addition (Figure 2B). Unlike HU-treated cells, H_2O_2-induced relocalization of Clp1-GFP was not abolished in the absence of *rad3*$^+$, although the response was altered in terms of both timing and the percentage of cells responding at any

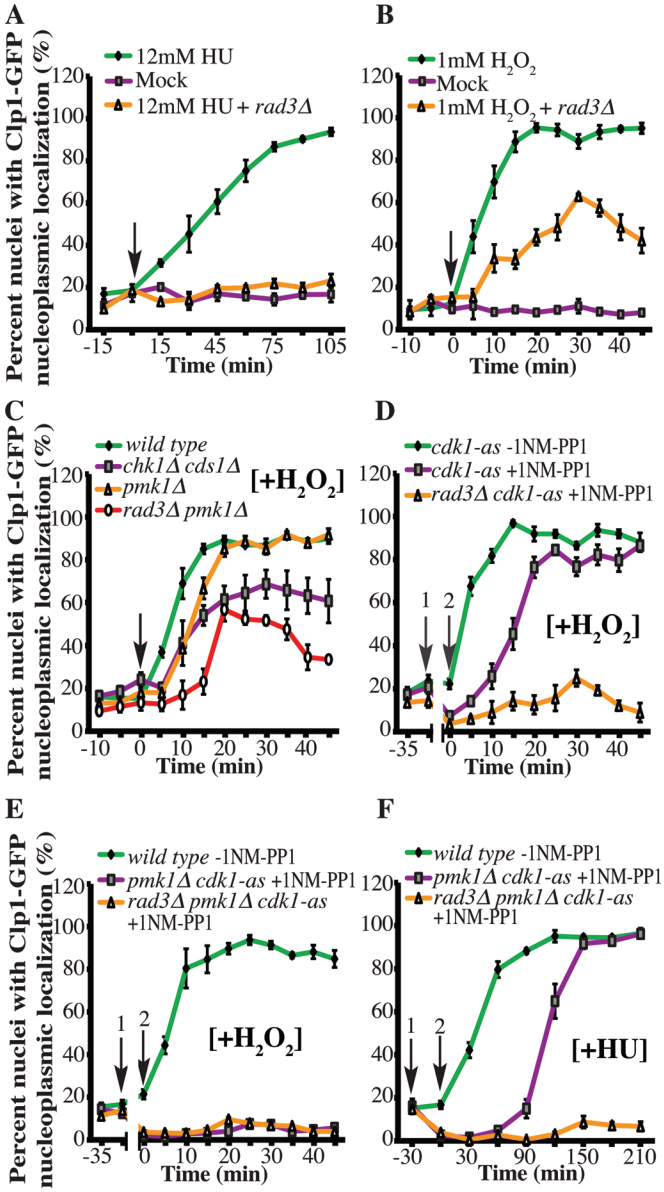

FIGURE 2: Multiple kinases affect genotoxic stress–induced Clp1-GFP nucleoplasmic accumulation. (A) The graphs show the percentage of nuclei with Clp1-GFP detected in the nucleoplasm of *clp1-GFP gar2-mCherry* or *clp1-GFP gar2-mCherry rad3*Δ cells as judged by live-cell microscopy after the addition of 12 mM HU or H_2O (mock treatment) at $t = 0$ min, indicated by the arrow. (B) Same as A, except that 1 mM H_2O_2 was used to induce cellular stress. (C) Similar to B; the graph shows the percentage of nuclei with Clp1-GFP detected in the nucleoplasm after oxidative stress (1 mM H_2O_2) in the indicated strains. (D, E) Same as in B and C, except that 5 μM 1NM-PP1 was added at $t = -30$ min, indicated by arrow 1, to asynchronous cultures before H_2O_2 addition at $t = 0$ min, indicated by arrow 2. Dimethyl sulfoxide was added instead of 1NM-PP1 for mock-treated cells. (F) As in D and E, except that 12 mM HU was used to induce stress at $t = 0$ min. In A–F, each curve represents the average of four experiments, and each time point represents ≥132 cells. Standard error of the mean is shown for each time point.

given time point (Figure 2B). Of interest, after the peak accumulation of nucleoplasmic Clp1-GFP, the percentage of cells with nucleoplasmic Clp1-GFP decreased, suggesting that the maintenance of Clp1 nucleoplasmic distribution was affected (Figure 2B). To examine whether these changes in the response to H_2O_2 were due to loss of checkpoint effector kinase activation, we examined Clp1 localization in a *chk1*Δ *cds1*Δ strain. As in *rad3*Δ cells, the appearance of Clp1-GFP in the nucleoplasm was delayed relative to wild-type cells; only 68% of the nuclei simultaneously accumulated Clp1-GFP in the nucleoplasm during the time course (Figure 2, B and C). In addition, after the peak, the percentage of nuclei containing nucleoplasmic Clp1-GFP decreased (Figure 2, B and C). We therefore conclude that the relocalization defects seen in *rad3*Δ cells are due to loss of Cds1 and/or Chk1 activities. Of note, these results indicate that whereas the Rad3 pathway contributes to Clp1-GFP relocalization induced by oxidative stress, additional signaling pathways must contribute.

Multiple protein kinases contribute to Clp1 nucleoplasmic relocalization

Clp1 is a highly phosphoregulated protein (Wolfe *et al.*, 2006; Chen *et al.*, 2008; Diaz-Cuervo and Bueno, 2008). Because H_2O_2-induced Clp1-GFP relocalization was still observed in the absence of *rad3*$^+$ (Figure 2, B and C), we reasoned that additional protein kinases might contribute to oxidative stress–induced relocalization of Clp1-GFP.

First, we looked at the possible influence of MAPKs, which become activated by various environmental stresses (Roux and Blenis, 2004). In *S. pombe*, there are three MAPKs: Spk1, Sty1, and Pmk1. Spk1 and Sty1 are involved in the mating response and stress sensing, respectively, whereas Pmk1 functions in the cell wall integrity pathway (Toda *et al.*, 1996). Of the three MAPKs, only *pmk1*$^+$ loss affected H_2O_2-induced Clp1-GFP relocalization, delaying nucleolar release by 5 min compared with *pmk1*$^+$ cells (Figure 2C and unpublished data). However, *pmk1*Δ cells did reach the same high level of Clp1 release as wild-type cells, suggesting that Pmk1 likely has a role in timing Clp1-GFP nucleoplasmic accumulation during oxidative stress but not its maintenance within the nucleoplasm (Figure 2C). To test whether the Rad3 and cell wall integrity pathways act in concert to influence Clp1 localization, we examined Clp1-GFP in *rad3*Δ *pmk1*Δ cells. Clp1 redistribution was delayed by 10–15 min, with a decreased percentage of Clp1-GFP nucleoplasmic-localized nuclei in *rad3*Δ *pmk1*Δ cells, although the peak was still reached at 20 min after H_2O_2 addition (Figure 2C). In addition, similar to other *rad3*Δ cells examined, the percentage of nuclei that contained nucleoplasmic Clp1-GFP was not maintained. These results suggest that whereas both Rad3 and Pmk1 contribute to Clp1 relocalization from the nucleolus to the nucleoplasm during oxidative stress, they do so independently of each other, and still additional pathways or factors contribute.

We then examined whether Cdk activity had a role in Clp1 relocalization, since Cdk1 had previously been shown to directly phosphorylate Clp1 to modulate the phosphatase's activity, but the effect on Clp1 localization was not examined (Wolfe *et al.*, 2006). Because Cdk1 (Cdc2 in *S. pombe*) is an essential kinase, we used an analogue-sensitive mutant, *cdc2-F84G* (*cdk1-as*), which is inhibited by the ATP analogue 4-amino-1-*tert*-butyl-3-(1′-naphthylmethyl) pyrazolo(3,4-d)pyrimidine (1NM-PP1; Dischinger *et al.*, 2008). Inactivation of Cdk1-as by 1NM-PP1 caused a drop in the number of cells with Clp1-GFP localized to the nucleoplasm at $t = 0$ min due to the cells' inability to enter mitosis (Figure 2D). After the addition of 1 mM H_2O_2 there was a 5-min delay in Clp1-GFP nucleoplasmic accumulation, similar to *pmk1*Δ cells (Figure 2, C and D), indicating a potential role for Cdk1 in regulating Clp1 relocalization.

To assess the collective roles of the Rad3 pathway, the cell wall integrity pathway, and Cdk1, we examined Clp1-GFP nucleoplasmic relocalization in strains with combinations of the kinase mutants.

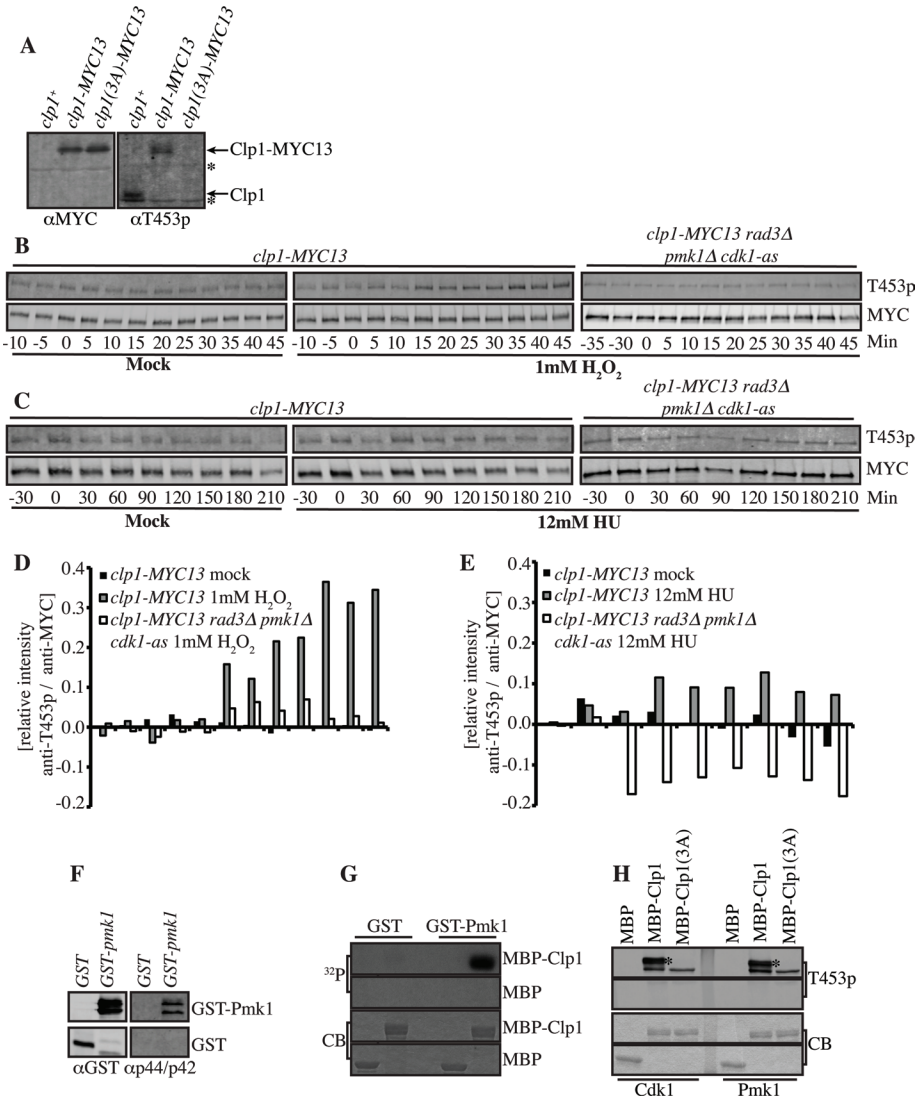

FIGURE 3: Clp1 is phosphorylated at TP sites upon genotoxic stress. (A) Immunoblots of lysates from the indicated strains arrested in prometaphase with the cold-sensitive β-tubulin mutant *nda3-km311*. Anti-MYC or anti-T453p, a Clp1 phospho-specific antibody, were used for immunoblotting. Asterisks indicate nonspecific bands. (B) Immunoblots of immunoprecipitated Clp1-MYC13 from the indicated strains over time treated at $t = 0$ min with 1 mM H_2O_2 or H_2O (mock treatment). Immunoblots were probed with anti-MYC or anti-T453p. To inhibit Cdk1-as, cells were treated with 5 μM 1NM-PP1 at $t = -30$ min, and then at $t = 0$ min with either 1 mM H_2O_2 or H_2O (mock treatment). (C) Same as B, except that 12 mM HU was used to stress cells. (D, E) The ratios of the relative intensities of anti-T453p to that of anti-MYC signals normalized to the prestress time points. Intensities were calculated using an Odyssey instrument from the immunoblots in B and C. (F) Immunoblot analysis of GST or GST-Pmk1 purified from cells treated with 1 mM H_2O_2 for 30 min with anti-GST or anti-P44/P42. (G) MBP or MBP-Clp1 was incubated with GST or GST-Pmk1 in an in vitro kinase reaction. The inputs, detected by Coomassie blue (CB) staining, and the corresponding autoradiographs are shown. (H) Recombinantly produced MBP, MBP-Clp1, or MBP-Clp1(3A) were incubated with either recombinantly produced Cdk1-Cdc13/cyclin B or activated and immunoprecipitated GST-Pmk1. The inputs, detected by CB staining, and the corresponding autoradiographs are shown. The asterisks indicate a phosphoshifted form of MBP-Clp1.

Although combining *rad3*Δ and *cdk1-as* had a significant effect on Clp1-GFP relocalization to the nucleoplasm, it did not completely eliminate it (Figure 2D). In both *pmk1*Δ *cdk1-as* and *rad3*Δ *pmk1*Δ *cdk1-as* cells, however, the ability of Clp1-GFP to relocalize from the nucleolus to the nucleoplasm upon oxidative stress was abolished (Figure 2E). These results indicate that Pmk1 and Cdk1 cooperate to promote Clp1-GFP relocalization during oxidative stress and suggest that the Rad3 pathway is important for maintenance of the nucleoplasmic Clp1 pool.

Various forms of genotoxic stress including HU activate the *S. cerevisiae* orthologue of Pmk1, Slt2, and the closely related Erk1/2 MAPKs of higher eukaryotes; this activation is important for coping with genotoxic insults (Queralt and Igual, 2005; Wei *et al.*, 2011a; Soriano-Carot *et al.*, 2012). In addition, *pmk1*Δ cells are sensitive to HU (Han *et al.*, 2010). Because Pmk1 and Cdk1 both modulate Clp1's behavior in response to H_2O_2, we investigated the potential role of Pmk1 and Cdk1 in HU-induced Clp1-GFP relocalization. To examine whether Pmk1 and/or Cdk1 affected HU-induced Clp1 nucleoplasmic accumulation, we used *pmk1*Δ *cdk1-as* and *rad3*Δ *pmk1*Δ *cdk1-as* strains. Compared to wild-type cells, in which Clp1-GFP began to rapidly relocalize in some cells, *pmk1*Δ *cdk1-as* cells did not start to relocalize Clp1-GFP to the nucleoplasm until 90 min after HU addition. Once relocalization began, however, it quickly reached the same penetrance as in wild-type cells (Figure 2F). As in *rad3*Δ cells, Clp1-GFP did not accumulate in the nucleoplasm in *rad3*Δ *pmk1*Δ *cdk1-as* cells under replicative stress, suggesting that Pmk1, Cdk1, or both kinases contribute to release under these circumstances, but Rad3 plays a more dominant role (Figure 2, E and F). Collectively, these results suggest that HU and H_2O_2 activate the Rad3 pathway, cell wall integrity pathway, and Cdk1, although to different extents and with possibly different kinetics, to redistribute Clp1 from the nucleolus into the nucleoplasm.

Cds1 and/or Chk1, Pmk1, and Cdk1 phosphorylate Clp1 upon HU and H_2O_2 stress treatment

As introduced earlier, Cds1 and Sid2 directly phosphorylate Clp1 on its multiple RxxS sites; this promotes Clp1's association with the two *S. pombe* 14-3-3 proteins, Rad24 and Rad25, which, at least in the case of Sid2, prevents the return of Clp1 to the nucleolus until the completion of cytokinesis (Mishra *et al.*, 2005; Chen *et al.*, 2008; Diaz-Cuervo and Bueno, 2008). In addition, Cdk1 directly phosphorylates Clp1 on TP sites primarily in early mitosis and inhibits Clp1 catalytic activity (Wolfe *et al.*, 2006). To determine whether phosphorylation on Clp1 RxxS and/or TP sites occurred during genotoxic stress, we first generated a phosphospecific antibody to one Cdk1 phosphosite, T453. To test the specificity of anti-T453p, we analyzed lysates of *clp1*$^+$, *clp1-MYC13*, or *clp1(3A)-MYC13* cells arrested in mitosis, when Clp1 is highly phosphorylated by Cdk1 (Figure 3A; Wolfe *et al.*, 2006). The Clp1(3A) mutant abolishes three Cdk1 phosphorylation sites, including T453 (see later discussion of Figure 5A; Wolfe *et al.*,

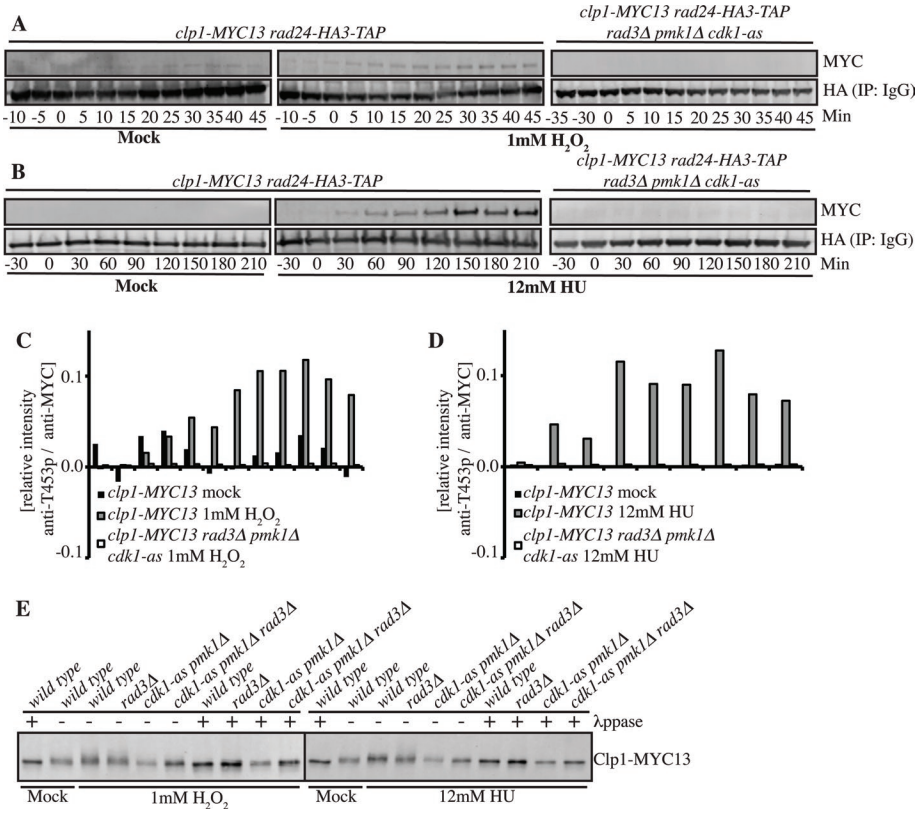

FIGURE 4: Clp1 is phosphorylated at RxxS sites upon genotoxic stress. (A) Clp1-MYC13 was captured by Rad24-HA3-TAP at different times after stress induction by the addition of 1 mM H_2O_2 or H_2O (mock treatment) at $t = 0$ min to asynchronous cultures of the indicated genotypes. Anti-MYC or anti-HA antibodies were used to detect Clp1-MYC13 or Rad24-HA3-TAP, respectively. Cdk1-as was inhibited by first incubating cells with 5 μM 1NM-PP1 at $t = -30$ min before the addition of H_2O_2 at $t = 0$ min. (B) Same as in A, except that 12 mM HU was used to induce stress. (C, D) The ratios of the relative intensities of anti-MYC to that of anti-HA signals, normalized to the prestress time points, calculated using an Odyssey instrument from the immunoblots in A and B, respectively. (E) Cells were treated with 5 μM 1NM-PP1 for 30 min to inhibit Cdk1-as and then with 1 mM H_2O_2 for 45 min or 12 mM HU for 210 min. Clp1-MYC13 immunoprecipitates from the indicated strains were treated with (+) or without (–) λ-phosphatase and then examined by anti-MYC immunoblotting.

2006). Clp1 and Clp1-MYC13 were detected by anti-T453p; however, Clp1(3A)-MYC13 was not (Figure 3A), indicating that anti-T453p is specific for Clp1 phosphorylated at T453.

Next we used anti-T453p to probe immunoblots of immunoprecipitated Clp1-MYC13 protein from asynchronous cultures of either wild-type or *rad3Δ pmk1Δ cdk1-as* cells treated with 1 mM H_2O_2, 12 mM HU, or H_2O (mock). Clp1-MYC13 phosphorylation at T453 in wild-type cells increased over time after the induction of stress by either H_2O_2 or HU compared with mock-treated cells; however, the H_2O_2-treated cells show a more robust phosphorylation at T453 than HU-treated cells (Figure 3, B–E). In contrast, Clp1-MYC13 immunoprecipitated from *rad3Δ pmk1Δ cdk1-as* cells first treated with 5 μM 1NM-PP1 and then with H_2O_2 or HU did not show an increase in T453 phosphorylation (Figure 3, B–E). These results suggest a correlation between genotoxic stress–mediated Clp1 nucleoplasmic relocalization and accumulation of Clp1 T453 phosphorylation.

MAPKs, like Cdk1, are proline-directed kinases (Roux and Blenis, 2004). Because deleting *pmk1*$^+$ affected Clp1-GFP nucleoplasmic relocalization (Figure 2C), we investigated whether Pmk1 could phosphorylate Clp1 and whether it did so at sites overlapping with Cdk1. Glutathione *S*-transferase (GST) or GST-Pmk1 was produced in *S. pombe* cells, activated by treatment with 1 mM H_2O_2, and then purified. Activation of the kinase was confirmed by immunoblotting with anti-p44/p42 (Figure 3F; Barba *et al.*, 2008). GST-Pmk1, but not GST, phosphorylated MBP-Clp1, but not maltose-binding protein (MBP) alone, indicating that Pmk1 can directly phosphorylate Clp1 in vitro (Figure 3G). To test whether Pmk1 may phosphorylate the same residues as Cdk1, we incubated recombinant MBP, MBP-Clp1, or MBP-Clp1(3A) in vitro with recombinant Cdk1-Cdc13/cyclin B or activated GST-Pmk1 (Figure 3H). Whereas anti-T453p did not recognize MBP, it detected MBP-Clp1 and MBP-Clp1(3A) (likely due to the high concentration of recombinant protein used and the fact that the serum was not affinity purified); however, the phospho-shifted form was only recognized in MBP-Clp1 and not MBP-Clp1(3A) for both kinases (Figure 3H). These results indicate that both Pmk1 and Cdk1 can phosphorylate Clp1 at T453 in vitro.

To observe Clp1 RxxS phosphorylation, we followed the RxxS phospho-dependent association between Rad24 and Clp1 as an indirect measure of Clp1 phosphorylation at RxxS sites (Chen *et al.*, 2008; Diaz-Cuervo and Bueno, 2008). In accord with Clp1 phosphorylation correlating with nucleoplasmic accumulation, we saw an increase in the Rad24–Clp1 association after the addition of 1 mM H_2O_2 or 12 mM HU, suggesting that Clp1 is phosphorylated at RxxS sites after induction of genotoxic stress (Figure 4, A–D). Of interest, HU treatment promoted a more robust association between Rad24 and Clp1 than did H_2O_2 treatment, the converse of what we observed with Clp1 T453 phosphorylation (Figures 3, B–E, and 4, A–D). The H_2O_2- and/or HU-induced Rad24–Clp1 association was disrupted in *rad3Δ pmk1Δ cdk1-as* cells (Figure 4, A–D). These results suggest a correlation between genotoxic stress–induced Clp1 relocalization, and the accumulation of RxxS phosphorylation. Furthermore, the timing in which T453 phosphorylation and Clp1–Rad24 interactions accumulate directly parallels Clp1 nucleoplasmic accumulation upon H_2O_2 or HU stress, suggesting a direct relationship.

To examine Clp1's overall phosphostate, we analyzed the SDS–PAGE mobility of Clp1-MYC13 in wild-type, *rad3Δ*, *pmk1Δ cdk1-as*, and *rad3Δ pmk1Δ cdk1-as* cells first treated with 5 μM 1NM-PP1 and then with 1 mM H_2O_2, 12 mM HU, or H_2O (mock). Clp1-MYC13 immunoprecipitated from mock-treated cells had a slower mobility than its λ-phosphatase–collapsed counterpart (Figure 4E). This is likely due to the mitotic fraction of cells within the asynchronous culture in which Clp1 is known to be phosphorylated (Wolfe *et al.*, 2006). Clp1-MYC13 immunoprecipitated from either H_2O_2- or HU-treated cells had an even slower mobility than Clp1-MYC13 from mock-treated cells (Figure 4E), indicating that the population of Clp1 is more heavily phosphorylated in genotoxically stressed cells. In addition, whereas Clp1 from genotoxically stressed *rad3Δ* cells

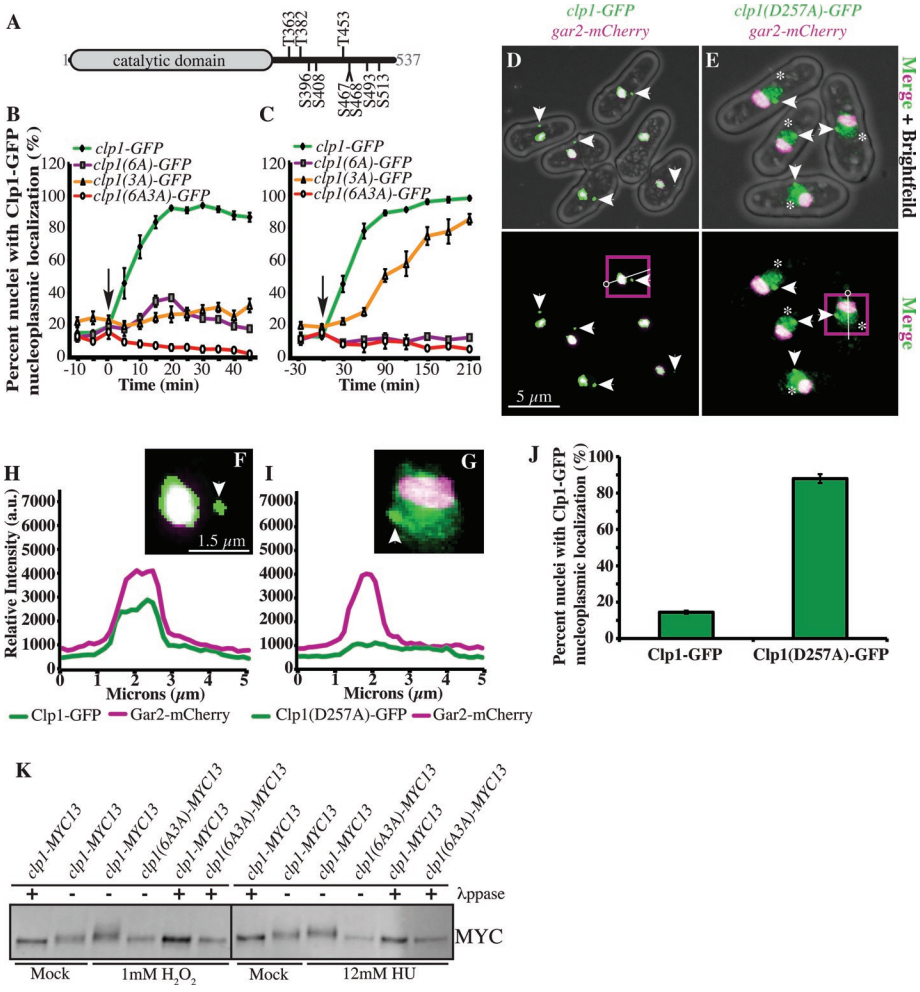

FIGURE 5: Mutation of RxxS and TP phosphosites abolishes Clp1 nucleoplasmic accumulation due to genotoxic stress. (A) A schematic of Clp1 with its TP (above) and RxxS (below) phosphosites indicated. (B) Same as in Figure 2, B and C, except that the indicated Clp1 phosphomutants were examined for their ability to accumulate within the nucleoplasm after genotoxic stress induced at t = 0 min (indicated by arrow) by the addition of 1 mM H_2O_2. (C) Same as in B, except that 12 mM HU was used to induce stress at t = 0 min (indicated by arrow). (D, E) A live-cell image of unstressed *clp1-GFP gar2-mCherry* (D) or *clp1(D257A)-GFP gar2-mCherry* (E) cells. Scale bar, 5 μm. Asterisks indicate nuclei with Clp1-GFP relocalized from the nucleolus to the nucleoplasm. Arrowheads point to spindle pole bodies. (F, G) Zoomed views of the boxed nuclei from the merged images in D and E, respectively. Scale bar, 1.5 μm. Arrowheads point to SPBs. (H, I) Line scans of either Clp1-GFP (H) or Clp1(D257A)-GFP (I) and Gar2-mCherry fluorescence, each spanning a distance of 5 μm, in which the paths are represented in D and E, respectively, by a white line with a black circle to indicate the origin of the line scan. (J) The percentage of nuclei with Clp1-GFP or Clp1(D257A)-GFP localized to the nucleoplasm was determined in asynchronous cultures of the indicated strains. The experiment was repeated three times, and SE of the mean is shown. $n \geq 429$. (K) Immunoblot of Clp1-MYC13 and Clp1(6A3A)-MYC13 immunoprecipitates from cells treated with 1 mM H_2O_2, 12 mM HU, or H_2O (mock). Immunoprecipitates were treated with (+) or without (–) λ-phosphatase.

had a similar mobility as stressed wild-type cells, Clp1-MYC13 immunoprecipitated from either *pmk1Δ cdk1-as* or *rad3Δ pmk1Δ cdk1-as* cells migrated significantly faster than that from wild-type cells (Figure 4E). Together with the Rad24-binding experiments, these results indicate that inhibiting the Rad3 pathway, the cell wall integrity pathway, and Cdk1 affects the phosphostate of Clp1-MYC13 during genotoxic stress and indicates a correlation between genotoxic stress–mediated Clp1 nucleoplasmic relocalization and the accumulation of Clp1 phosphomodifications mediated by Cds1 and/or Chk1, Pmk1, and Cdk1 kinases.

Clp1 phosphosite mutants do not relocalize properly in response to genotoxic stress

Given the foregoing results, we expected that abolishing Clp1 TP and/or RxxS phosphosites would prevent Clp1 relocalization during genotoxic stress. To test this, we first used previously characterized Clp1 phosphomutants that were integrated at the *clp1*$^+$ endogenous locus and tagged at their C-terminus with GFP (Figure 5A; Wolfe *et al.*, 2006; Chen *et al.*, 2008).

Because Cdk1 and Pmk1 likely phosphorylate the same Clp1 phosphosites, we anticipated that Clp1(3A)-GFP (Figure 5A) would act similarly to Clp1-GFP in *pmk1Δ cdk1-as* cells after stress induction. Indeed, although Clp1(3A)-GFP still relocalized to the nucleoplasm upon H_2O_2 treatment, its ability to relocalize was severely hindered (Figure 5B). In addition, in HU-stressed cells, Clp1(3A)-GFP was delayed between 60 and 90 min in the ability to accumulate within the nucleoplasm (Figure 5C). These results support the idea that Pmk1 and Cdk1 cooperate to phosphorylate the same TP sites to promote Clp1 relocalization to the nucleoplasm upon genotoxic stress.

Given that Clp1 autodephosphorylates its Cdk1 phosphorylations (Wolfe *et al.*, 2006), we next considered whether Clp1 activity contributed to its nucleolar sequestration. To test this idea, we examined the distribution of a catalytically compromised Clp1 mutant, *clp1(D257A)*, which is constitutively phosphorylated on multiple Cdk1 sites due to its inability to autodephosphorylate (Wolfe *et al.*, 2006). On examining unstressed *clp1(D257A)-GFP gar2-mCherry* cells, we observed that Clp1(D257A)-GFP was present in the nucleoplasm in a much higher percentage of cells compared with unstressed *clp1*$^+$ cells (Figure 5, D–J). This is consistent with Clp1 hyperphosphorylation on Cdk1 sites promoting nucleoplasmic accumulation.

A second mutant, *clp1(6A)-GFP*, has six Ser residues essential for the phospho-dependent association of Rad24 with Clp1 mutated to Ala (Figure 5A; Chen *et al.*, 2008). After H_2O_2 treatment, much like Clp1-GFP in *rad3Δ* cells, Clp1(6A)-GFP showed a delay in release, fewer nuclei contained nucleoplasmic Clp1 at the peak of release, and there was a decrease in the percentage of nuclei with nucleoplasmic Clp1 after the peak (Figure 5B). However, the decrease in percentage of nuclei accumulating Clp1(6A)-GFP was greater than that for Clp1-GFP in *rad3Δ* cells treated with H_2O_2. Among other possibilities, this difference may reflect modulation of Clp1 phosphorylation at TP sites and/or Clp1 conformation resulting from the six mutations. In HU-treated cells, Clp1(6A)-GFP never accumulated in the nucleoplasm, a result similar to that for Clp1-GFP in *rad3Δ* cells (Figures 2A and 5C).

Although mutating the six RxxS sites abolished HU-induced Clp1 nucleoplasmic accumulation, Clp1(6A)-GFP and Clp1(3A)-GFP still relocalized in response to peroxide treatment in a population of cells. Thus we reasoned that combining the phosphosite mutants to make *clp1(6A3A)* might affect this response more significantly. Indeed, Clp1(6A3A)-GFP did not relocalize to the nucleoplasm in response to either H_2O_2 or HU (Figure 5, B and C). In addition, Clp1(6A3A)-MYC13 immunoprecipitated from cells treated with 1 mM H_2O_2 for 45 min or 12 mM HU for 210 min had a faster mobility than did wild-type Clp1-MYC13 (Figure 5K). These data further support the model that direct phosphorylation of Clp1 RxxS and TP sites by Cds1 and/or Chk1, Pmk1, and Cdk1 upon genotoxic stress facilitates the relocalization of Clp1 from the nucleolus to the nucleoplasm during interphase.

DISCUSSION

Nucleolar release of the Cdc14 phosphatases is a major activation step allowing access to substrates. The redistribution of *S. pombe* Clp1 from an exclusively nucleolar to nucleoplasmic localization pattern after HU treatment of interphase cells was previously described (Diaz-Cuervo and Bueno, 2008). Here we extended this observation by showing that Clp1 also accumulates in the nucleoplasm upon treatment of interphase cells with H_2O_2. Using these treatments as assays for Clp1 redistribution, we were able to dissect the signaling networks capable of controlling Clp1 localization during interphase. We identified multiple kinase pathways that directly phosphorylate Clp1 to regulate the nucleolar-to-nucleoplasmic transition and showed that the type of genotoxic stress influences the contribution to Clp1 regulation provided by each network. Furthermore, we found that Clp1 regulates its own nucleolar sequestration by antagonizing a subset of these networks. Collectively, our results reveal unexpected complexities in the regulation of Clp1 nucleolar localization.

At the single-cell level, the response of Clp1 to genotoxic stress appeared to be all-or-none; Clp1-GFP was either obviously restricted to the nucleolus or clearly diffuse within the nucleus in each cell (Figure 1 and Supplemental Figure S2). All-or-none responses are typical outputs produced by phosphoregulated systems and have been extensively studied within the context of MAPK networks (Ferrell, 2002; Ferrell *et al.*, 2009; Kholodenko *et al.*, 2010). In multiple eukaryotic systems, MAPK signaling networks turn graded inputs into switch-like outputs (Bagowski *et al.*, 2003; Mackeigan *et al.*, 2005; Melen *et al.*, 2005; Malleshaiah *et al.*, 2010). This setup allows MAPKs to filter out signaling noise and generate decisive cellular responses. The Raf/MAPK kinase (MEK)/extracellular signal-regulated kinase (ERK) network, which is most closely related to the cell wall integrity pathway, also exhibits negative feedback control and has been proposed to act similarly to a negative feedback amplifier (Sturm *et al.*, 2010) to limit the duration of responses and produce more graded responses to a broad range of inputs (Birtwistle and Kolch, 2011). When Clp1 was released from the nucleolus in a Pmk1-dependent manner, an increasing number of cells responded to H_2O_2 for 30 min; however, afterward Clp1 began to transition back to the nucleolus. This indicates that Pmk1 signaling may be turned off in cells after 30 min, even though H_2O_2 is still present. Indeed, Pmk1 activation, as determined by activating phosphorylation on Pmk1, decreases after 30 min even though an environmental stress remains (Madrid *et al.*, 2006). In addition, examples of negative feedback loops within Pmk1 signaling have been described (Madrid *et al.*, 2007; Takada *et al.*, 2010). These observations suggest that the cell wall integrity pathway likely functions similarly to the Raf/MEK/ERK pathway in terms of limiting its effect on Clp1 localization.

Cdk1-dependent redistribution of Clp1 upon H_2O_2 addition also stops responding after 20 min. However, when Cds1/Chk1 signaling is functional along with either Pmk1 or Cdk1, cells accumulated Clp1 within the nucleoplasm to levels similar to those for wild-type cells, and this response was maintained in the presence of the genotoxic agent. Therefore it is likely that Pmk1 and Cdk1 induce the initial nucleolar release of Clp1, whereas activity of the checkpoint effector kinases are necessary to maintain Clp1 nucleoplasmic localization and provide robustness during persistent genotoxic stress. This is similar to the role of Sid2 in mitosis (Mishra *et al.*, 2005; Chen *et al.*, 2008) and indicates that cells may use overlapping strategies to localize Cdc14 phosphatases regardless of the situation.

That H_2O_2 among the tested stresses was the only one to influence Clp1 nucleolar release like HU treatment is not entirely surprising, given that reactive oxygen species also cause DNA damage (Cadet *et al.*, 2010). However, the difference in overall time to accumulate Clp1 within the nucleoplasm may reflect the distinct mechanisms and time frames by which HU and H_2O_2 cause DNA damage. HU-treated cells may respond more slowly because the initial stalled replication forks do not signal Clp1 redistribution, but DNA double-strand breaks that result from unmaintained replication forks after prolonged HU exposure may trigger Clp1 relocalization. More interesting was that Rad3 was not required for Clp1 nucleoplasmic accumulation upon H_2O_2 treatment as in HU treatment. This finding led us to identify Pmk1 and Cdk1 as additional kinases that function separately from Chk1 and Cds1 to facilitate Clp1 nucleoplasmic accumulation. In accord with the involvement of Cdk1 in phosphorylating Clp1 upon genotoxic insults and in promoting Clp1 redistribution, Cdk activity is involved in the DNA replication/damage checkpoint and promotes homologous recombination over nonhomologous end joining repair in response to DNA double-strand breaks postreplication (Ira *et al.*, 2004; Cerqueira *et al.*, 2009; Enserink *et al.*, 2009; Enserink and Kolodner, 2010; Chen *et al.*, 2011; Langerak and Russell, 2011). In addition, Cdk1 phosphorylates the *S. cerevisiae*–specific Cdc14 nucleolar tether Net1 (Azzam *et al.*, 2004). This event assists FEAR network–dependent nucleolar release of Cdc14 during mitosis, and the loss of this phosphorylation event modulates early-anaphase release of Cdc14 (Azzam *et al.*, 2004). Thus the role of Cdk1 in promoting nucleolar release of Cdc14 phosphatases appears conserved between these two yeasts. Taken together, our results support a model in which, upon genotoxic stress during interphase, Cds1 and/or Chk1 together with Pmk1 and Cdk1 ensure the redistribution of Clp1 throughout the nucleoplasm (Figure 6).

Clp1 adopts both nuclear and cytoplasmic localizations during mitosis. However, we observed that Clp1 did not accumulate in the cytoplasm when genotoxic stress was applied during interphase (see Figure 1). In addition, although Clp1(6A3A)-GFP did not relocalize from the nucleolus to the nucleoplasm in HU- or H_2O_2-treated cells, Clp1(6A3A)-GFP still left the nucleolus and localized to kinetochores, mitotic spindle, and actomyosin ring during mitosis (Supplemental Figure S3). These results suggest that redistribution of Clp1 that occurs during mitosis requires additional modification to Clp1 and/or its regulators and interacting proteins.

Although strict regulation of Clp1 nucleolar release during mitosis is important for the fidelity and timing of mitotic events (Cueille *et al.*, 2001; Trautmann *et al.*, 2001, 2004; Trautmann and McCollum, 2005; Mishra *et al.*, 2004; Chen *et al.*, 2008; Clifford *et al.*, 2008b), it is unclear why such complex regulation of genotoxic stress–induced Clp1 nucleolar release exists. Clp1 contributes modestly to Cds1 activation (Diaz-Cuervo and Bueno, 2008); however, Clp1 is not important for cell viability in response to genotoxic stress (Supplemental Figure S4). Given that Cdk1 phosphorylation

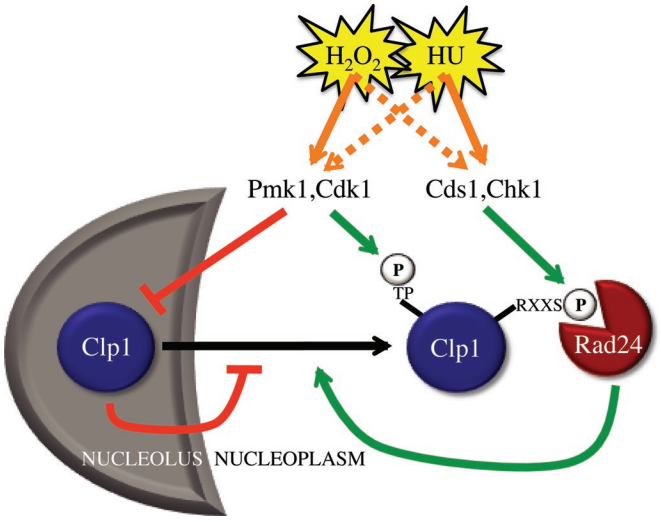

FIGURE 6: Model of the phosphoregulatory networks that control interphase genotoxic stress–induced redistribution of Clp1. Solid orange lines represent the more dominant network response relative to the dotted orange lines. Green lines indicate positive regulation, and negative relationships are in red.

promotes Clp1 nucleoplasmic accumulation upon genotoxic stress and Cdk1 phosphorylation inhibits Clp1 activity (Wolfe *et al.*, 2006), Clp1 may be only primed by its nucleolar release but not actually active under these circumstances. Alternatively, Clp1 may be involved in resetting the phosphostate of DNA replication/damage checkpoint substrates after resolution of a genotoxic insult in combination with other phosphatases, and its role may be masked by their presence. Given that mammalian Cdc14B is also mobilized from the nucleolus by direct DNA damage (Bassermann *et al.*, 2008; Diaz-Cuervo and Bueno, 2008; Mocciaro *et al.*, 2010; Guillamot *et al.*, 2011; Wei *et al.*, 2011b) or H_2O_2 treatment (D. McCollum, unpublished observations), our results may provide insight toward understanding its relocalization upon genotoxic stress and contribute to a better understanding of Cdc14-family phosphatase regulation.

MATERIALS AND METHODS

Strains and general yeast methods

S. pombe strains used in this study (Supplemental Table S1) were grown in yeast extract (YE) or Edinburgh minimal media (EMM) plus supplements. For induction from the *nmt41* promoter (Maundrell, 1993), cell were first grown overnight in media containing 5 μg/ml thiamine, then washed three times with media lacking thiamine and allowed to grow for 18 h in thiamine-free media. *clp1*$^+$, *clp1(D257A)*, *clp1(6A)*, *clp1(3A)*, and *clp1(6A3A)* were tagged endogenously at the 3′ end of the open reading frame with *GFP:kan*^{R} or *MYC13:kan*^{R} cassettes as previously described (Bahler *et al.*, 1998). The *clp1(D257A)*, *clp1(6A)*, *clp1(3A)*, and *clp1(6A3A)* mutants were constructed by site-directed mutagenesis of *clp1*$^+$ cDNA flanked by *clp1*$^+$ 5′ and 3′ genomic sequences in pIRT2 vector. Correct mutations were verified by sequencing (GenHunter, Nashville, TN). Integration of phosphosite mutants at the *clp1*$^+$ endogenous locus and subsequent tag insertions were done by lithium acetate transformation (Keeney and Boeke, 1994), and correct integrations were confirmed by whole-cell PCR and immunoblot or fluorescence microscopy as appropriate. Introduction of various *clp1* alleles into other genetic backgrounds was accomplished using standard *S. pombe* mating, sporulation, and tetrad dissection techniques. For spot assays, cells were grown to mid-log phase at 32°C, 8 million cells were resuspended in 1 ml of water, and 1:10 serial dilutions were made. Then 2.5 μl of each dilution was plated on YE, YE plus 1.2 M sorbitol, 1 mM H_2O_2, or 5 mM HU or EMM+ supplements, EMM+ supplements plus 1.2 M sorbitol, 1 mM H_2O_2, or 5 mM HU. Plates were incubated at 32°C for 3 d.

Microscopy

All images were taken using a personal DeltaVision microscopy system (Applied Precision, Issaquah, WA). This system includes an IX71 microscope (Olympus, Center Valley, PA), 60×/numerical aperture 1.42 PlanApo objective, a CoolSnap HQ2 camera (Photometrics, Tucson, AZ), and SoftWoRx imaging software (Applied Precision). *Z*-series optical sections were taken at 0.5-μm steps.

Time-point analyses of *clp1-GFP* strains were performed on asynchronous cultures grown in YE at 30°C with constant shaking. To stress cells, we either shifted cultures from 30 to 40°C for thermal stress, added sorbitol (Sigma-Aldrich, St. Louis, MO) to a final concentration of 1.2 M for osmotic stress, added H_2O_2 (Sigma-Aldrich) to a final concentration of 1 mM for oxidative stress, or added HU (Sigma-Aldrich) to a final concentration of 12 mM. Mock treatment was the addition of the same volume of H_2O. Images were taken at 5-, 15-. or 30-min intervals, and nuclei with nucleoplasmic-localized Clp1-GFP were determined by comparing Clp1-GFP to Gar2-mCherry, a known nucleolar marker. Cells with Clp1 localized to mitotic spindles were not considered. Line scans 5 μm in length were made using SoftWoRx imaging software. Experiments were repeated at least four times per strain and done on at least two separate days. In addition, at least one experiment for each mutant strain was done at the same time as the wild-type *clp1-GFP gar2-mCherry* strain (Supplemental Figure S5).

To inhibit Cdk1 kinase activity, strains containing an analogue-sensitive allele of Cdk1 (*cdc2-F84G*) were treated with 5 μM 1NM-PP1) (EMD Biosciences, San Diego, CA) for 30 min prior to the addition of stress agents, and dimethyl sulfoxide (Sigma-Aldrich) addition served as mock treatment.

Protein methods

Whole-cell lysates were prepared in NP-40 buffer as previously described (Gould *et al.*, 1991). Lysates were subjected to immunoprecipitation with anti-MYC (9E10), Novagen GST-Bind Resin (EMD Millipore, Billerica, MA), or immunoglobulin G–Sepharose (GE Healthcare, Uppsala, Sweden). Recombinant MBP-Clp1 protein was purified from bacterial cell lysates in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 10 mM PMSF, and 0.1% Triton with Amylose Resin (New England Biolabs, Ipswich, MA) according to manufacturer's suggestions. Phospho-p44/42 XP rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) was used to detect activated Pmk1. Polyclonal antibodies for the Clp1 T453 phosphorylated residue, anti-T453p, were raised against a phosphorylated peptide (DETRTVGpTPTETISV) coupled to keyhole limpet hemocyanin in rabbits using the premium protocol by Invitrogen (Carlsbad, CA). Immunoblot analysis and λ-phosphatase assays were performed as previously described (Tasto *et al.*, 2003), except that primary antibodies were detected by secondary antibodies coupled to IRDye 680LT or IRDye 800CW (LI-COR Biosciences, Lincoln, NE), visualized and quantitated using an Odyssey scanner (LI-COR Biosciences).

Protein kinase assays

In vitro protein kinase assays were preformed as previously described (Yoon *et al.*, 2006), except that the kinase buffer consisted of 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, and 2 mM dithiothreitol supplemented with 100 μM cold ATP and 5 μCi [γ-^{32}P]

ATP (GE Healthcare, Piscataway, NJ). For Pmk1 kinase assays, GST-Pmk1 on GST-Bind Resin was washed three times in 1 ml of NP-40 buffer and then three times in 1 ml of kinase buffer. For Cdk1, ~50 ng of recombinant Cdk1-Cdc13 purified from baculovirus-infected insect cells was used as previously described (Yoon *et al.*, 2002). We used 1 μg of recombinant MBP-fusion substrates in all experiments, and reactions were for 30 min at 30°C and were terminated by the addition of 5× SDS–PAGE sample buffer and boiling. Phosphorylation was analyzed by separation on SDS–PAGE and visualization by Coomassie blue staining, autoradiography, and/or immunoblotting.

ACKNOWLEDGMENTS

We thank Reiko Sugiura for providing pREP41 GST-pmk1. We also thank members of the Gould lab, especially Anna Feoktistova and Liping Ren, for their technical advice. We are grateful to Adam Bohnert, Alyssa Johnson, Janel McLean, Lucy X. Lu, Jeanne Jodoin, and Dannel McCollum for critical reading of the manuscript and helpful discussions. M.R.B. was supported by the Biochemical and Chemical Training for Cancer Research Program, National Institutes of Health Grant T32 CA009582. This work was supported by National Institutes of health Grant GM068786. K.L.G. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

Azzam R, Chen SL, Shou W, Mah AS, Alexandru G, Nasmyth K, Annan RS, Carr SA, Deshaies RJ (2004). Phosphorylation by cyclin B-Cdk underlies release of mitotic exit activator Cdc14 from the nucleolus. Science 305, 516–519.

Bagowski CP, Besser J, Frey CR, Ferrell JE Jr (2003). The JNK cascade as a biochemical switch in mammalian cells: ultrasensitive and all-or-none responses. Curr Biol 13, 315–320.

Bahler J, Wu JQ, Longtine MS, Shah NG, McKenzie A 3rd, Steever AB, Wach A, Philippsen P, Pringle JR (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast 14, 943–951.

Barba G, Soto T, Madrid M, Nunez A, Vicente J, Gacto M, Cansado J (2008). Activation of the cell integrity pathway is channelled through diverse signalling elements in fission yeast. Cell Signal 20, 748–757.

Bassermann F, Frescas D, Guardavaccaro D, Busino L, Peschiaroli A, Pagano M (2008). The Cdc14B-Cdh1-Plk1 axis controls the G2 DNA-damage-response checkpoint. Cell 134, 256–267.

Berdougo E, Nachury MV, Jackson PK, Jallepalli PV (2008). The nucleolar phosphatase Cdc14B is dispensable for chromosome segregation and mitotic exit in human cells. Cell Cycle 7, 1184–1190.

Birtwistle MR, Kolch W (2011). Biology using engineering tools: the negative feedback amplifier. Cell Cycle 10, 2069–2076.

Brondello JM, Boddy MN, Furnari B, Russell P (1999). Basis for the checkpoint signal specificity that regulates Chk1 and Cds1 protein kinases. Mol Cell Biol 19, 4262–4269.

Cadet J, Douki T, Ravanat JL (2010). Oxidatively generated base damage to cellular DNA. Free Radic Biol Med 49, 9–21.

Cerqueira A, Santamaria D, Martinez-Pastor B, Cuadrado M, Fernandez-Capetillo O, Barbacid M (2009). Overall Cdk activity modulates the DNA damage response in mammalian cells. J Cell Biol 187, 773–780.

Chen CT, Feoktistova A, Chen JS, Shim YS, Clifford DM, Gould KL, McCollum D (2008). The SIN kinase Sid2 regulates cytoplasmic retention of the *S. pombe* Cdc14-like phosphatase Clp1. Curr Biol 18, 1594–1599.

Chen CT, Peli-Gulli MP, Simanis V, McCollum D (2006). *S. pombe* FEAR protein orthologs are not required for release of Clp1/Flp1 phosphatase from the nucleolus during mitosis. J Cell Sci 119, 4462–4466.

Chen X, Niu H, Chung WH, Zhu Z, Papusha A, Shim EY, Lee SE, Sung P, Ira G (2011). Cell cycle regulation of DNA double-strand break end resection by Cdk1-dependent Dna2 phosphorylation. Nat Struct Mol Biol 18, 1015–1019.

Cho HP, Liu Y, Gomez M, Dunlap J, Tyers M, Wang Y (2005). The dual-specificity phosphatase CDC14B bundles and stabilizes microtubules. Mol Cell Biol 25, 4541–4551.

Clement A, Solnica-Krezel L, Gould KL (2011). The Cdc14B phosphatase contributes to ciliogenesis in zebrafish. Development 138, 291–302.

Clemente-Blanco A, Mayan-Santos M, Schneider DA, Machin F, Jarmuz A, Tschochner H, Aragon L (2009). Cdc14 inhibits transcription by RNA polymerase I during anaphase. Nature 458, 219–222.

Clemente-Blanco A *et al.* (2011). Cdc14 phosphatase promotes segregation of telomeres through repression of RNA polymerase II transcription. Nat Cell Biol 13, 1450–1456.

Clifford DM, Chen CT, Roberts RH, Feoktistova A, Wolfe BA, Chen JS, McCollum D, Gould KL (2008a). The role of Cdc14 phosphatases in the control of cell division. Biochem Soc Trans 36, 436–438.

Clifford DM, Wolfe BA, Roberts-Galbraith RH, McDonald WH, Yates JR 3rd, Gould KL (2008b). The Clp1/Cdc14 phosphatase contributes to the robustness of cytokinesis by association with anillin-related Mid1. J Cell Biol 181, 79–88.

Cueille N, Salimova E, Esteban V, Blanco M, Moreno S, Bueno A, Simanis V (2001). Flp1, a fission yeast orthologue of the *S. cerevisiae* CDC14 gene, is not required for cyclin degradation or rum1p stabilisation at the end of mitosis. J Cell Sci 114, 2649–2664.

Diaz-Cuervo H, Bueno A (2008). Cds1 controls the release of Cdc14-like phosphatase Flp1 from the nucleolus to drive full activation of the checkpoint response to replication stress in fission yeast. Mol Biol Cell 19, 2488–2499.

Dischinger S, Krapp A, Xie L, Paulson JR, Simanis V (2008). Chemical genetic analysis of the regulatory role of Cdc2p in the *S. pombe* septation initiation network. J Cell Sci 121, 843–853.

Enserink JM, Kolodner RD (2010). An overview of Cdk1-controlled targets and processes. Cell Div 5, 11.

Enserink JM, Hombauer H, Huang ME, Kolodner RD (2009). Cdc28/Cdk1 positively and negatively affects genome stability in *S. cerevisiae*. J Cell Biol 185, 423–437.

Ferrell JE Jr (2002). Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. Curr Opin Cell Biol 14, 140–148.

Ferrell JE Jr, Pomerening JR, Kim SY, Trunnell NB, Xiong W, Huang CY, Machleder EM (2009). Simple, realistic models of complex biological processes: positive feedback and bistability in a cell fate switch and a cell cycle oscillator. FEBS Lett 583, 3999–4005.

Gould KL, Moreno S, Owen DJ, Sazer S, Nurse P (1991). Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* p34cdc2 function. EMBO J 10, 3297–3309.

Guillamot M, Manchado E, Chiesa M, Gomez-Lopez G, Pisano DG, Sacristan MP, Malumbres M (2011). Cdc14b regulates mammalian RNA polymerase II and represses cell cycle transcription. Sci Rep 1, 189.

Han TX, Xu XY, Zhang MJ, Peng X, Du LL (2010). Global fitness profiling of fission yeast deletion strains by barcode sequencing. Genome Biol 11, R60.

Ira G *et al.* (2004). DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. Nature 431, 1011–1017.

Kaiser BK, Zimmerman ZA, Charbonneau H, Jackson PK (2002). Disruption of centrosome structure, chromosome segregation, and cytokinesis by misexpression of human Cdc14A phosphatase. Mol Biol Cell 13, 2289–2300.

Keeney JB, Boeke JD (1994). Efficient targeted integration at leu1-32 and ura4-294 in *Schizosaccharomyces pombe*. Genetics 136, 849–856.

Kholodenko BN, Hancock JF, Kolch W (2010). Signalling ballet in space and time. Nat Rev Mol Cell Biol 11, 414–426.

Langerak P, Russell P (2011). Regulatory networks integrating cell cycle control with DNA damage checkpoints and double-strand break repair. Philos Trans R Soc Lond B Biol Sci 366, 3562–3571.

Liang F, Jin F, Liu H, Wang Y (2009). The molecular function of the yeast polo-like kinase Cdc5 in Cdc14 release during early anaphase. Mol Biol Cell 20, 3671–3679.

Lindsay HD, Griffiths DJ, Edwards RJ, Christensen PU, Murray JM, Osman F, Walworth N, Carr AM (1998). S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in *Schizosaccharomyces pombe*. Genes Dev 12, 382–395.

Mackeigan JP, Murphy LO, Dimitri CA, Blenis J (2005). Graded mitogen-activated protein kinase activity precedes switch-like c-Fos induction in mammalian cells. Mol Cell Biol 25, 4676–4682.

Madrid M, Nunez A, Soto T, Vicente-Soler J, Gacto M, Cansado J (2007). Stress-activated protein kinase-mediated down-regulation of the cell integrity pathway mitogen-activated protein kinase Pmk1p by protein phosphatases. Mol Biol Cell 18, 4405–4419.

Madrid M, Soto T, Khong HK, Franco A, Vicente J, Perez P, Gacto M, Cansado J (2006). Stress-induced response, localization, and regulation of the Pmk1 cell integrity pathway in *Schizosaccharomyces pombe*. J Biol Chem 281, 2033–2043.

Malleshaiah MK, Shahrezaei V, Swain PS, Michnick SW (2010). The scaffold protein Ste5 directly controls a switch-like mating decision in yeast. Nature 465, 101–105.

Manzoni R, Montani F, Visintin C, Caudron F, Ciliberto A, Visintin R (2010). Oscillations in Cdc14 release and sequestration reveal a circuit underlying mitotic exit. J Cell Biol 190, 209–222.

Martinho RG, Lindsay HD, Flaggs G, DeMaggio AJ, Hoekstra MF, Carr AM, Bentley NJ (1998). Analysis of Rad3 and Chk1 protein kinases defines different checkpoint responses. EMBO J 17, 7239–7249.

Maundrell K (1993). Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene 123, 127–130.

Melen GJ, Levy S, Barkai N, Shilo BZ (2005). Threshold responses to morphogen gradients by zero-order ultrasensitivity. Mol Syst Biol 1, 2005.0028.

Mishra M, Karagiannis J, Sevugan M, Singh P, Balasubramanian MK (2005). The 14-3-3 protein rad24p modulates function of the cdc14p family phosphatase clp1p/flp1p in fission yeast. Curr Biol 15, 1376–1383.

Mishra M, Karagiannis J, Trautmann S, Wang H, McCollum D, Balasubramanian MK (2004). The Clp1p/Flp1p phosphatase ensures completion of cytokinesis in response to minor perturbation of the cell division machinery in *Schizosaccharomyces pombe*. J Cell Sci 117, 3897–3910.

Mocciaro A, Schiebel E (2010). Cdc14: a highly conserved family of phosphatases with non-conserved functions? J Cell Sci 123, 2867–2876.

Mocciaro A, Berdougo E, Zeng K, Black E, Vagnarelli P, Earnshaw W, Gillespie D, Jallepalli P, Schiebel E (2010). Vertebrate cells genetically deficient for Cdc14A or Cdc14B retain DNA damage checkpoint proficiency but are impaired in DNA repair. J Cell Biol 189, 631–639.

Mohl DA, Huddleston MJ, Collingwood TS, Annan RS, Deshaies RJ (2009). Dbf2-Mob1 drives relocalization of protein phosphatase Cdc14 to the cytoplasm during exit from mitosis. J Cell Biol 184, 527–539.

Morgan DO (1997). Cyclin-dependent kinases: engines, clocks, microprocessors. Annu Rev Cell Dev Biol 13, 261–291.

Nalepa G, Harper JW (2004). Visualization of a highly organized intranuclear network of filaments in living mammalian cells. Cell Motil Cytoskeleton 59, 94–108.

Papadopoulou K *et al*. (2010). Regulation of cell cycle-specific gene expression in fission yeast by the Cdc14p-like phosphatase Clp1p. J Cell Sci 123, 4374–4381.

Peddibhotla S, Wei Z, Papineni R, Lam MH, Rosen JM, Zhang P (2011). The DNA damage effector Chk1 kinase regulates Cdc14B nucleolar shuttling during cell cycle progression. Cell Cycle 10, 671–679.

Queralt E, Igual JC (2005). Functional connection between the Clb5 cyclin, the protein kinase C pathway and the Swi4 transcription factor in *Saccharomyces cerevisiae*. Genetics 171, 1485–1498.

Queralt E, Uhlmann F (2008). Cdk-counteracting phosphatases unlock mitotic exit. Curr Opin Cell Biol 20, 661–668.

Rock JM, Amon A (2009). The FEAR network. Curr Biol 19, R1063–R1068.

Roux PP, Blenis J (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68, 320–344.

Sicard H, Faubladier M, Noaillac-Depeyre J, Leger-Silvestre I, Gas N, Caizergues-Ferrer M (1998). The role of the *Schizosaccharomyces pombe* gar2 protein in nucleolar structure and function depends on the concerted action of its highly charged N terminus and its RNA-binding domains. Mol Biol Cell 9, 2011–2023.

Soriano-Carot M, Bano MC, Igual JC (2012). The yeast mitogen-activated protein kinase Slt2 is involved in the cellular response to genotoxic stress. Cell Div 7, 1.

Stegmeier F, Amon A (2004). Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. Annu Rev Genet 38, 203–232.

Sturm OE, Orton R, Grindlay J, Birtwistle M, Vyshemirsky V, Gilbert D, Calder M, Pitt A, Kholodenko B, Kolch W (2010). The mammalian MAPK/ERK pathway exhibits properties of a negative feedback amplifier. Sci Signal 3, ra90.

Takada H *et al*. (2010). The cell surface protein gene ecm33+ is a target of the two transcription factors Atf1 and Mbx1 and negatively regulates Pmk1 MAPK cell integrity signaling in fission yeast. Mol Biol Cell 21, 674–685.

Takahashi A *et al*. (2012). DNA damage signaling triggers degradation of histone methyltransferases through APC/C(Cdh1) in senescent cells. Mol Cell 45, 123–131.

Tasto JJ, Morrell JL, Gould KL (2003). An anillin homologue, Mid2p, acts during fission yeast cytokinesis to organize the septin ring and promote cell separation. J Cell Biol 160, 1093–1103.

Toda T, Dhut S, Superti-Furga G, Gotoh Y, Nishida E, Sugiura R, Kuno T (1996). The fission yeast pmk1+ gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the protein kinase C pathway. Mol Cell Biol 16, 6752–6764.

Tomson BN, Rahal R, Reiser V, Monje-Casas F, Mekhail K, Moazed D, Amon A (2009). Regulation of Spo12 phosphorylation and its essential role in the FEAR network. Curr Biol 19, 449–460.

Trautmann S, McCollum D (2005). Distinct nuclear and cytoplasmic functions of the *S. pombe* Cdc14-like phosphatase Clp1p/Flp1p and a role for nuclear shuttling in its regulation. Curr Biol 15, 1384–1389.

Trautmann S, Rajagopalan S, McCollum D (2004). The *S. pombe* Cdc14-like phosphatase Clp1p regulates chromosome biorientation and interacts with Aurora kinase. Dev Cell 7, 755–762.

Trautmann S, Wolfe BA, Jorgensen P, Tyers M, Gould KL, McCollum D (2001). Fission yeast Clp1p phosphatase regulates G2/M transition and coordination of cytokinesis with cell cycle progression. Curr Biol 11, 931–940.

Walworth NC, Bernards R (1996). rad-dependent response of the chk1-encoded protein kinase at the DNA damage checkpoint. Science 271, 353–356.

Waples WG, Chahwan C, Ciechonska M, Lavoie BD (2009). Putting the brake on FEAR: Tof2 promotes the biphasic release of Cdc14 phosphatase during mitotic exit. Mol Biol Cell 20, 245–255.

Wei F, Xie Y, He L, Tao L, Tang D (2011a). ERK1 and ERK2 kinases activate hydroxyurea-induced S-phase checkpoint in MCF7 cells by mediating ATR activation. Cell Signal 23, 259–268.

Wei Z, Peddibhotla S, Lin H, Fang X, Li M, Rosen JM, Zhang P (2011b). Early-onset aging and defective DNA damage response in Cdc14b-deficient mice. Mol Cell Biol 31, 1470–1477.

Wolfe BA, McDonald WH, Yates JR 3rd, Gould KL (2006). Phospho-regulation of the Cdc14/Clp1 phosphatase delays late mitotic events in *S. pombe*. Dev Cell 11, 423–430.

Yoon HJ, Feoktistova A, Chen JS, Jennings JL, Link AJ, Gould KL (2006). Role of Hcn1 and its phosphorylation in fission yeast anaphase-promoting complex/cyclosome function. J Biol Chem 281, 32284–32293.

Yoon HJ, Feoktistova A, Wolfe BA, Jennings JL, Link AJ, Gould KL (2002). Proteomics analysis identifies new components of the fission and budding yeast anaphase-promoting complexes. Curr Biol 12, 2048–2054.