Dma1 ubiquitinates the SIN scaffold, Sid4, to impede the mitotic localization of Plo1 kinase

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Proper cell division requires strict coordination between mitotic exit and cytokinesis. In the event of a mitotic error, cytokinesis must be inhibited to ensure equal partitioning of genetic material. In the fission yeast, *Schizosaccharomyces pombe*, the checkpoint protein and E3 ubiquitin ligase, Dma1, delays cytokinesis by inhibiting the septation initiation network (SIN) when chromosomes are not attached to the mitotic spindle. To elucidate the mechanism by which Dma1 inhibits the SIN, we screened all SIN components as potential Dma1 substrates and found that the SIN scaffold protein, Sid4, is ubiquitinated *in vivo* in a Dma1-dependent manner. To investigate the role of Sid4 ubiquitination in checkpoint function, a ubiquitination deficient *sid4* allele was generated and our data indicate that Sid4 ubiquitination by Dma1 is required to prevent cytokinesis during a mitotic checkpoint arrest. Furthermore, Sid4 ubiquitination delays recruitment of the Polo-like kinase and SIN activator, Plo1, to spindle pole bodies (SPBs), while at the same time prolonging residence of the SIN inhibitor, Byr4, providing a mechanistic link between Dma1 activity and cytokinesis inhibition.

The EMBO Journal (2011) 30, 341–354. doi:10.1038/emboj.2010.317; Published online 3 December 2010

Subject Categories: proteins; cell cycle

Keywords: cytokinesis; Dma1; Plo1; septation initiation network; Sid4

Introduction

At the end of each cell division cycle, chromosomes segregate to opposite sides of the cell and a cytokinetic ring (CR) assembles and constricts between them to physically separate the two new cells. Clearly, it is critical that chromosome segregation occurs before ring constriction and, thus, mitosis and cytokinesis must be coupled to ensure that each new cell inherits the proper genetic complement. In the fission yeast, *Schizosaccharomyces pombe*, the septation initiation network (SIN) confers proper coordination by triggering contractile ring constriction once mitosis is complete (for review see McCollum and Gould, 2001; Krapp *et al.*, 2004b). Thus,

precise activation of the SIN is required for the fidelity of each cell division.

SIN signalling is restricted to the spindle pole bodies (SPBs) and is initiated by the GTPase, Spg1 (Schmidt *et al.*, 1997). Upon conversion to its GTP-bound form during metaphase, its effector kinase Cdc7 (Sohrmann *et al.*, 1998) is recruited, followed by Sid1–Cdc14 (Guertin *et al.*, 2000). In anaphase B, the downstream SIN kinase, Sid2–Mob1, which localizes constitutively to SPBs, concentrates at the CR before constriction and is thought to transduce the signal to constrict (Sparks *et al.*, 1999; Hou *et al.*, 2000; Salimova *et al.*, 2000). To prevent premature septation in interphase, a bipartite GAP complex comprising Cdc16 and Byr4 binds and inhibits Spg1 at SPBs (Song *et al.*, 1996; Furge *et al.*, 1998; Jwa and Song, 1998; Krapp *et al.*, 2008). The GAP complex also regulates asymmetric distribution of SIN activity at the two SPBs throughout mitosis (Cerutti and Simanis, 1999; Li *et al.*, 2000). In metaphase, Byr4–Cdc16 are absent from both SPBs, but are then recruited to the old SPB during anaphase B, thus permitting SIN activity only on the new SPB (Sohrmann *et al.*, 1998; Grallert *et al.*, 2004). Asymmetric distribution of SIN activity is critically important for the SIN to trigger septation precisely and also to silence the SIN after the completion of cytokinesis (Garcia-Cortes and McCollum, 2009).

Two scaffold proteins, Sid4 and Cdc11, provide the spatial cues for assembly of the SIN and its regulators at SPBs (Krapp *et al.*, 2001, 2003, 2004a; Tomlin *et al.*, 2002; Morrell *et al.*, 2004), while the conserved Polo-like kinase, Plo1, temporally regulates SIN signalling (Mulvihill *et al.*, 1999; Tanaka *et al.*, 2001). Among its other activities, Plo1 has a key role in forming the contractile ring, predicting the site of division, driving septum formation (Ohkura *et al.*, 1995; Bahler *et al.*, 1998), and *plo1*⁺ overexpression activates the SIN pathway (Ohkura *et al.*, 1995; Mulvihill *et al.*, 1999). To execute these events faithfully, Plo1 localization within the cell is controlled precisely. Plo1 concentrates on the mitotic, but not the interphase SPB (Mulvihill *et al.*, 1999), partly through association with the SIN scaffold, Sid4 (Morrell *et al.*, 2004), suggesting that Plo1 might directly target one or more SIN components to drive septum formation. A pathway homologous to the SIN, called the mitotic exit network (MEN), exists in *Saccharomyces cerevisiae* (for reviews see Bardin and Amon, 2001; McCollum and Gould, 2001; de Bettignies and Johnston, 2003; Seshan and Amon, 2004). While the Plo1 target at the SIN has not yet been identified in *S. pombe*, the *S. cerevisiae* Plo1 homologue, Cdc5, phosphorylates and inhibits the GAP complex, Bub2–Bfa1, allowing MEN activation (Hu *et al.*, 2001; Geymonat *et al.*, 2003).

In addition to the SIN/MEN pathways that function during every cell cycle, multiple checkpoint pathways also control mitotic progression. For instance, in the event that chromosomes are not properly attached to the mitotic spindle during metaphase, the spindle assembly checkpoint (SAC) inhibits the anaphase-promoting complex/cyclosome to prevent

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Received: 18 August 2010; accepted: 12 November 2010; published online: 3 December 2010

anaphase onset and mitotic exit (for reviews see Malmanche *et al.*, 2006; Varetto and Musacchio, 2008; Zich and Hardwick, 2010). In addition to the SAC, studies in yeast have identified a SAC-independent pathway required to inhibit cytokinesis when chromosomes are not properly attached to the mitotic spindle (Alexandru *et al.*, 1999; Beltraminelli *et al.*, 1999; Gardner and Burke, 2000). In *S. pombe*, one effector of the SAC-independent pathway is the checkpoint protein, Dma1 (Murone and Simanis, 1996; Guertin *et al.*, 2002). Dma1 localizes to SPBs and the division site, and *dma1*⁺ over-expression prevents the SIN kinases from assembling at SPBs (Guertin *et al.*, 2002). Furthermore, Dma1 binds the SIN scaffold, Sid4, and delays recruitment of Plo1 to SPBs during a checkpoint response (Guertin *et al.*, 2002). However, the mechanism by which this occurs is unknown.

At its N terminus, Dma1 has a Forkhead-associated (FHA) domain, which is predicted to interact with phosphothreonine residues (Durocher *et al.*, 2000; Durocher and Jackson, 2002; Mahajan *et al.*, 2008). At its C terminus, Dma1 contains a ring-finger (RF) domain, which most likely confers E3 ubiquitin ligase activity to the protein (for reviews see Joazeiro and Weissman, 2000; Deshaies and Joazeiro, 2009). The FHA domain is required for Dma1 localization to SPBs and the cell division site, and both domains are required for proper checkpoint function (Guertin *et al.*, 2002). Dma1 belongs to a small class of proteins that encode both FHA and RF domains, of which there are two in humans, CHFR (Scolnick and Halazonetis, 2000) and RNF8 (Kolas *et al.*, 2007). CHFR (CHeckpoint protein with FHA and RF domains) is a tumour suppressor protein, which has been implicated in a mitotic checkpoint termed the antephasal checkpoint (Scolnick and Halazonetis, 2000; Matsusaka and Pines, 2004). RNF8 is also a checkpoint protein, which has a role in the DNA damage response pathway (Huen *et al.*, 2007; Kolas *et al.*, 2007; Wang and Elledge, 2007). Two homologues exist in *S. cerevisiae*, Dma1 and Dma2, which are functionally redundant and are required for the spindle position checkpoint (Fraschini *et al.*, 2004). While all of these proteins share little sequence similarity outside of their FHA and RF domains, all participate in cell cycle checkpoints (for review see Brooks *et al.*, 2008), which might imply a conserved mode of action.

To elucidate the mechanism of Dma1 inhibition of the SIN, we screened all SIN components as potential Dma1 substrates and found that the SIN scaffold, Sid4, is ubiquitinated in a Dma1-dependent manner and that Sid4 ubiquitination is required to prevent cytokinesis during a mitotic checkpoint arrest. Furthermore, when the spindle checkpoint is activated in the absence of Sid4 ubiquitination, Plo1 prematurely accumulates at SPBs and the GAP component, Byr4, is driven off SPBs earlier compared with wild type cells. Our data indicate that Dma1 ubiquitinates the SIN scaffold protein, Sid4, to antagonize Plo1 localization and access to SIN substrates in order to delay cytokinesis.

Results

The SIN scaffold, Sid4, is ubiquitinated *in vivo*

dma1⁺ encodes a RF domain, which is predicted to have E3 ubiquitin ligase activity (Figure 1A). To determine whether Dma1 is in fact a functional E3 ubiquitin ligase, Dma1 was tagged at its endogenous C terminus with HA₃-TAP and

purified from *S. pombe* lysates. When the TAP eluate was incubated with an E1-activating enzyme and the E2-conjugating enzyme, Ubc13-Uev1a, Dma1 catalysed formation of polyubiquitin chains *in vitro* (Figure 1B, left panel). To be sure that the polyubiquitin chains were formed in a Dma1-specific manner and were not a product of another E3 contaminant present in the TAP eluate, a conserved hydrophobic residue within the RF domain (I194) that is expected to disrupt interaction with its cognate E2 enzyme (Katoh *et al.*, 2003) was mutated to alanine (Figure 1A). When the Dma1(I194A)-HA₃-TAP eluate was incubated with the E1 and E2 enzymes, polyubiquitin chains were not formed (Figure 1B, right panel). Taken together, these data indicate that the predicted RF domain of Dma1 confers ubiquitin ligase activity to the protein.

Given that the Dma1 RF domain is required to maintain a spindle checkpoint arrest and that *dma1*⁺ antagonizes SIN signalling by perturbing Plo1 SPB localization (Guertin *et al.*, 2002), we reasoned that Dma1 performed its checkpoint function by targeting Plo1 or other SIN component(s) for ubiquitination. Therefore, the *in vivo* ubiquitination status of Plo1 and every SIN component (Figure 1C) was examined in checkpoint-activated cells (Figure 1D). We also tested the ubiquitination status of the SPB component Ppc89, which is required for Sid4 association with the SPB, and Cut12, with which Plo1 also interacts at the SPB (Flory *et al.*, 2002; MacIver *et al.*, 2003) (Figure 1C). Each protein was tagged at its endogenous C terminus with a His₆-BIO-His₆ (HBH) epitope and purified from denatured lysates using Ni²⁺-NTA and streptavidin resin (Tagwerker *et al.*, 2006). Proteins were purified from cells in which the spindle checkpoint had been activated using a reversible cold-sensitive mutation in the β -tubulin gene (*nda3*-KM311) (Hiraoka *et al.*, 1984) and the ubiquitination status was determined by immunoblotting for ubiquitin. To validate that each protein was indeed purified, we also blotted with streptavidin, which recognizes the biotinylated epitope. Through this approach, we found that the SIN scaffold, Sid4, was the only protein tested to be robustly ubiquitinated *in vivo* (Figure 1D).

Sid4 is ubiquitinated in a Dma1-dependent manner

The finding that Sid4 is ubiquitinated *in vivo* during a checkpoint arrest suggests that it might be a Dma1 substrate. In this regard, it is noteworthy that Dma1 and Sid4 were shown previously to interact with each other by yeast two-hybrid analysis (Guertin *et al.*, 2002). We therefore examined whether Sid4 ubiquitination required Dma1. Mutants were generated in which either the entire coding region of *dma1*⁺ was deleted or single mutations within the *dma1*⁺ coding region (R64 or I194) were mutated to alanine and integrated at the endogenous *dma1*⁺ locus (Figure 2A). Mutating R64 to alanine is predicted to disrupt interaction with phosphothreonine residues (Durocher and Jackson, 2002) and impedes localization of Dma1 to SPBs and the cell division site (Figure 2B, compare panels I and II), while the I194A mutation eliminates Dma1 E3 ligase activity (Figure 1B), but does not disrupt its localization to SPBs or the division site (Figure 2B, compare panels I and III).

To validate that the *dma1* mutants compromise Dma1 function, each *dma1* mutant was combined with the *nda3*-KM311 mutation and tested for checkpoint function. Cells were synchronized in G2 by centrifugal elutriation, shifted to

Figure 1 The SIN scaffold, Sid4, is ubiquitinated *in vivo*. (A) Schematic diagram of Dma1 protein with relative positions of Dma1 FHA and RF domains and the I194A point mutation indicated. (B) *In vitro* ubiquitination assay using an E1-activating enzyme, the human E2-conjugating enzyme, Ubc13/Uev1a and either *dma1*-HA₃-TAP or *dma1*(I194A)-HA₃-TAP purified from *S. pombe* lysates arrested by the *nda3*-KM311 mutation. (C) List of SIN and SPB proteins screened for *in vivo* ubiquitination. (D) *In vivo* ubiquitination assay of proteins listed in C. Each protein was purified from checkpoint-activated cells (*nda3*-KM311) and visualized by immunoblot using fluorescently labelled streptavidin (bottom panels) and a Ubiquitin antibody (top panels).

the restrictive temperature (18°C) to activate the spindle checkpoint, and septation indices were measured at 30 min intervals for 9 h. While the *nda3*-KM311 *dma1*⁺ strain maintained a checkpoint arrest for ~7 h, the *nda3*-KM311 *dma1*(R64A) and *nda3*-KM311 *dma1*(I194A) mutant strains could not maintain an arrest and formed aberrant septa at ~5 h, which is comparable with *nda3*-KM311 *dma1*Δ cells (Figure 2C). Thus, the R64A and I194A mutations compromise Dma1-dependent checkpoint function.

We next examined Sid4 ubiquitination in checkpoint activated (*nda3*-KM311) *dma1*Δ, *dma1*(R64A) and *dma1*(I194A) mutants. Cells were shifted to 18°C for 5 h to activate the spindle checkpoint and Sid4 ubiquitination was examined. Strikingly, in the absence of Dma1 protein, activity or localization, Sid4 ubiquitination was abolished (Figure 2D).

These data indicate that Sid4 is ubiquitinated in a Dma1-dependent manner.

Sid4 ubiquitination is required for Dma1-dependent checkpoint function

To determine if Sid4 ubiquitination is required for the Dma1-dependent checkpoint arrest, a ubiquitination deficient *sid4* allele was generated. Ubiquitin transfer often occurs in a sequence-independent manner and can occur on multiple substrate lysines, making site identification challenging (for reviews see Laney and Hochstrasser, 1999; Pickart, 2001). Sid4 contains 49 lysines (Figure 3A, top diagram) and mutating all 49 sites simultaneously would likely disrupt protein function. Thus, four *sid4* mutants were made, in which clusters of lysine residues were mutated that, collectively,

Figure 2 Sid4 ubiquitination requires Dma1 function. **(A)** Schematic diagram of Dma1 domains and positions of the R64A and I194A mutations. The R64A mutation prevents interaction with phosphothreonine motifs and I196A inactivates ubiquitin ligase activity. **(B)** Localization of *dma1*-GFP (panel I), *dma1*(R64A)-GFP (panel II) and *dma1*(I194A)-GFP (panel III) in cells growing in log phase. Scale bar, 5 μm. **(C)** Spindle checkpoint assay. Cells of the indicated strains were synchronized at 32°C in G2 by centrifugal elutriation, shifted to 18°C, and the septation index of each strain determined every 30 min for 9 h. **(D)** *In vivo* ubiquitination status of Sid4-HBH in *nda3*-KM311 *dma1Δ* or *nda3*-KM311 *dma1* mutants.

cover every lysine within Sid4 (Supplementary Figure 1A). As *sid4*⁺ is essential for viability, we first tested whether the four mutants could rescue the temperature-sensitive *sid4*-SA1 mutant at the restrictive temperature (data not shown) and as they all could, each was then integrated at the endogenous *sid4*⁺ locus to examine its *in vivo* ubiquitination status. Surprisingly, all four mutants were still ubiquitinated *in vivo* (Supplementary Figure 1B). Therefore, in order to create a ubiquitin-deficient *sid4* allele, we needed to generate a mutant that would eliminate more lysine residues simultaneously. However, all four mutants generated above were severely cold sensitive (data not shown), indicating that Sid4 function was already compromised and adding more mutations would likely exacerbate these phenotypes.

Thus, as an alternative means of eliminating relevant Sid4 lysine residues without disrupting protein function, we made use of previous structure and function analyses of Sid4 and the core SPB protein, Ppc89. The N-terminal 300 amino acids of Sid4 are required for direct binding to Plo1 (Morrell *et al*, 2004), Cdc11 (Tomlin *et al*, 2002) and Dma1 (Guertin *et al*,

2002), indicating that this region contains the essential SIN scaffolding activity of Sid4. The C termini of both Sid4 and Ppc89 contain several predicted coiled-coil regions (Figure 3A, top and middle diagram, respectively), which are only required for their SPB localization (Rosenberg *et al*, 2006). In fact, replacing the Sid4 C terminus with the SPB targeting region of Ppc89 (Figure 3A, bottom diagram) rescues both the temperature-sensitive *sid4*-SA1 allele at 36°C and the *sid4Δ* (Rosenberg *et al*, 2006). Thus, the *sid4N*-*ppc89C* fusion mutant, which eliminates ~76% of Sid4 lysines on the protein, was integrated at the endogenous *sid4*⁺ locus and tested for *in vivo* ubiquitination. While Sid4-HBH was robustly ubiquitinated, ubiquitination of the Sid4N-Ppc89C-HBH mutant was essentially eliminated (Figure 3B). Importantly, *sid4N*-*ppc89C* mutant cells were wild type for morphology and were not temperature sensitive. These data indicate that Dma1 targets the Sid4 C terminus for ubiquitination *in vivo*.

As expected, Sid4N-Ppc89C-GFP localized to SPBs properly (Figure 3C, panel I) and Cdc11-GFP, whose localization depends on Sid4, also localized to SPBs normally (Figure 3C, panel II).

Figure 3 Sid4 ubiquitination is required to maintain a checkpoint arrest. **(A)** Schematic diagrams of Sid4 with relative positions of all 49 lysines (top), Ppc89 (middle) and the Sid4N-Ppc89C fusion mutant (bottom). Predicted coiled-coil regions are shown in black. **(B)** *In vivo* ubiquitination of Sid4-HBH and Sid4N-Ppc89C-HBH. **(C)** Localization of Sid4N-Ppc89C-GFP (panel I), Cdc11-GFP (panel II) and Dma1-GFP (panel III) in *sid4N-ppc89C*-HBH mutant cells. Scale bar, 5 μ m. **(D)** Spindle checkpoint assay. Cells of the indicated strains were blocked at 32°C in S phase with hydroxyurea, released into hydroxyurea-free media at 18°C, and the septation index of each strain was determined every 30 min for 9 h. **(E)** Cells from each of the strains examined in 3D at the 7 h time point stained with methyl blue, which stains the septa, and DAPI, which stains DNA. (v) indicate septated cells that have bypassed the checkpoint. Scale bar, 5 μ m.

Furthermore, Cdc11-GFP intensities at SPBs are not significantly altered in *sid4N-ppc89C* mutant cells compared with wild-type cells (Supplementary Figure 2A and B). Thus, as predicted by its wild type morphology, the Sid4N-Ppc89C mutant does not disrupt the SIN scaffold complex. To ensure that the loss of Sid4N-Ppc89C ubiquitination was not due to a failure to recruit Dma1, we examined Dma1-GFP localization and found that it was present at SPBs in *sid4N-ppc89C*

mutant cells (Figure 3C, panel III), consistent with the previous observation that Dma1 interacts with the Sid4 N-terminal 300 amino acids (Guertin *et al*, 2002). Thus, while the Sid4 N terminus binds Dma1, its C terminus is required for ubiquitination. Collectively, these data indicate that the *sid4N-ppc89C* mutant retains full scaffolding and essential SIN functions of *sid4*⁺, but is unable to be ubiquitinated *in vivo* even in the presence of Dma1.

We then assessed the checkpoint function of the *sid4N-ppc89C* mutant. Cells were arrested in S phase with hydroxyurea (HU), released synchronously at 18°C to activate the spindle checkpoint, and septation indices were measured at 30 min intervals for 9 h. The *nda3-KM311* strain maintained a checkpoint arrest for ~7 h (Figure 3D and E, top left panel); however, the *nda3-KM311 sid4N-ppc89C* mutant formed aberrant septa (marked with ^) in Figure 3E, bottom left panel) at ~5 h, which phenocopied the *dma1*-RF mutant (*nda3-KM311 dma1(I194A)*) (Figure 3D and E, top right panel). Importantly, a double *dma1(I194A) sid4N-ppc89C* mutant septated with similar kinetics as either mutant alone and did not display any other additive effects (Figure 3D and E, bottom right panel), suggesting that these mutants bypass a checkpoint arrest via the same mechanism. Thus, Sid4 ubiquitination is necessary to inhibit cytokinesis during a *dma1*-dependent checkpoint arrest.

Sid4 ubiquitination antagonizes Plo1 recruitment to SPBs during a checkpoint response

When the spindle checkpoint is activated in the absence of *dma1*⁺, Plo1 is recruited to SPBs earlier (Guertin *et al*, 2002). Because our data suggest that Dma1 ubiquitinates Sid4 when a mitotic checkpoint is activated, we tested if Sid4 ubiquitination was the biochemical signal that perturbs Plo1 recruitment to SPBs by measuring the timing of Plo1 recruitment to SPBs in checkpoint-activated *sid4N-ppc89C* cells. Endogenously expressed Plo1 fused to a single GFP is difficult to visualize *in vivo*. Thus, to improve visualization three tandem copies of GFP were fused to the C terminus of Plo1 (Plo1-GFP₃) and used in the subsequent experiments.

nda3-KM311, *nda3-KM311 dma1(I194A)* and *nda3-KM311 sid4N-ppc89C* cells were synchronized in G2 by lactose gradient sedimentation, shifted to 18°C to activate the spindle checkpoint, and Plo1-GFP₃ was visualized at 30 min intervals for 9 h. In *dma1*⁺ cells, Plo1-GFP₃ was not visible on SPBs until ~4 to 5 h (Figure 4A and D). However, in the *dma1(I194A)* mutant, Plo1-GFP₃ was detected at SPBs ~2 h earlier compared with *dma1*⁺ cells and cells failed to arrest in mitosis (Figure 4B and D), which is similar to the premature recruitment observed previously for *dma1Δ* cells (Guertin *et al*, 2002). Similarly, Plo1-GFP₃ was recruited to SPBs earlier in *sid4N-ppc89C* mutant cells (Figure 4C and D). It should be noted that when cells are arrested in prometaphase by the *nda3-KM311* mutation, Plo1 localizes to both SPBs; however, because the mitotic spindle does not form and SPBs do not separate in this arrest, Plo1's signal in the later time points is slightly obscured by the fact that it is localizing on two SPBs that are sometimes overlapping in the Z axis. To be sure that we were quantitating SPB-localized Plo1, Plo1-GFP₃ was colocalized with the constitutive SPB marker, Sad1-mCherry (Hagan and Yanagida, 1995; Figure 4A–C, right panels). These data suggest that when the spindle checkpoint is activated, Sid4 ubiquitination antagonizes Plo1 recruitment to SPBs and thereby prevents it from reaching its substrates and activating the SIN.

Sid4 ubiquitination antagonizes Plo1 recruitment to SPBs during interphase

As Dma1 can be detected at SPBs in the absence of checkpoint induction, we examined a potential role for Sid4 ubiquitination during normal cell cycle progression. While

Sid4 was most robustly ubiquitinated during a mitotic arrest, as expected, it was also ubiquitinated in G2 cells, but significantly less ubiquitination was detected during S phase (Figure 5A). As Sid4 ubiquitination levels fluctuate throughout the cell cycle, we tested if Dma1 concentration at SPBs was also cell cycle dependent by measuring Dma1-GFP intensities at SPBs in different cell cycle stages and comparing these intensities with the constitutive SPB marker Sid4-RFP (Morrell *et al*, 2004). Dma1-GFP intensity was detected at low levels in prometaphase cells grown to log phase under permissible conditions (Figure 5B and C) and was significantly increased during a mitotic checkpoint arrest (*nda3-KM311* arrest) (Figure 5B and C), suggesting that Dma1 concentrates at SPBs in response to mitotic stress. Dma1-GFP was also detected in cells arrested in G2 (*cdc25-22*) (Figures 5B and C), although with significantly decreased intensity compared with *nda3-KM311*-arrested cells, and it was not detected on SPBs in cells arrested in S phase (Figure 5B and C). Thus, the levels of Sid4 ubiquitination correlate with the concentration of SPB-localized Dma1.

Plo1 localization to SPBs is also cell cycle regulated, accumulating at SPBs upon commitment to mitosis (Mulvihill *et al*, 1999). As Sid4 ubiquitination antagonizes Plo1 localization at SPBs and Sid4 is ubiquitinated in interphase cells, we wondered if the absence of Sid4 ubiquitination would allow Plo1 to concentrate at SPBs in interphase. Thus, *dma1*⁺, *dma1Δ* or *sid4N-ppc89C* cells were arrested in G2 using the temperature-sensitive *cdc25-22* mutation, and Plo1-GFP₃ intensities at SPBs were measured relative to Sad1-mCherry. While Plo1-GFP₃ was only detected at low levels in *dma1*⁺ cells (Figure 5D and E), Plo1-GFP₃ intensities at SPBs were significantly increased in *dma1Δ* cells (Figure 5D and E). A similar increase in Plo1-GFP₃ intensities was observed in *sid4N-ppc89C* cells, in which Sid4 ubiquitination is abolished (Figure 5D and E). These data suggest that Sid4 ubiquitination antagonizes Plo1 localization to SPBs during interphase and during a mitotic checkpoint arrest.

Byr4 is a potential Plo1 target

While the direct SIN target(s) of Plo1 have not yet been identified in *S. pombe*, the *S. cerevisiae* Plo1 homologue, Cdc5, is known to phosphorylate and inhibit the Byr4 ortholog and GAP component Bfa1, resulting in MEN activation (Hu *et al*, 2001; Geymonat *et al*, 2003). Bfa1 phosphorylation by Cdc5 inhibits its GAP activity *in vitro* and also ejects it from SPBs (Hu *et al*, 2001; Geymonat *et al*, 2003). *S. pombe* Byr4 is also a phosphoprotein (Song *et al*, 1996) and is hyperphosphorylated just before septation (Krapp *et al*, 2008). Thus, the potential of Plo1 SPB recruitment influencing Byr4 phosphorylation status and SPB localization was examined.

First, Byr4 was tested as a Plo1 substrate *in vitro*. MBP and MBP-Byr4 were produced in *E. coli*, and purified on amylose resin. When purified proteins were incubated with Plo1 purified from baculovirus-infected insect cells and ³²γ-ATP, we found that Plo1 could directly phosphorylate full-length Byr4 (Figure 6A). Next, Byr4 phosphorylation *in vivo* was examined. As previously reported (Song *et al*, 1996), Byr4 was hyperphosphorylated in a mitotic arrest (Figure 6B). However, in a temperature-sensitive *plo1* mutant (*plo1-25*) that had been synchronized and shifted to the restrictive temperature, the extent of Byr4 phosphorylation was

Figure 4 Sid4 ubiquitination delays Plo1 recruitment to the SPBs when the spindle checkpoint is activated. (A–C) *nda3*-KM311 (A), *nda3*-KM311 *dma1*(I194A) (B) or *nda3*-KM311 *sid4N-ppc89C* (C) cells were synchronized at 32°C in G2 by lactose gradient sedimentation, released to 18°C to activate the spindle checkpoint, and Plo1-GFP₃ and Sad1-mCherry localization at the SPBs were imaged periodically for 9 h. In each panel, the images on the left show Plo1-GFP₃ localization alone and the images on the right show merged images of Plo1-GFP₃ colocalized with Sad1-mCherry at each of the times indicated. Scale bar, 10 μm. (D) The kinetics of Plo1 recruitment to SPBs was measured for each of the strains shown by calculating the percentage of cells with Plo1-GFP₃ on SPBs at each time point.

drastically reduced (Figure 6B). Significantly, the degree of Byr4 phosphorylation in the *plo1*-25 mutant was comparable with Byr4 phosphorylation in *sid4*-SA1 mutant cells at the restrictive temperature (Figure 6B), indicating that Byr4 must be associated with the SPBs to become phosphorylated. Taken together, these data suggest that Plo1 contributes to the majority of Byr4 phosphorylation at the SPB.

We next examined the timing of Byr4 and Plo1 localization at SPBs relative to each other via time-lapse microscopy. To visualize Plo1 and Byr4 in the same cells, Byr4 was tagged at its C terminus with three tandem copies of GFP (Byr4-GFP₃) and Plo1 was tagged at its C terminus with three tandem

copies of mCherry (Plo1-mCherry₃). *byr4*-GFP₃ *plo1*-mCherry₃ cells were morphologically wild type, were not temperature sensitive and did not display any observable cell cycle defects, suggesting that Byr4 and Plo1 functions were not significantly compromised. In a representative movie, Byr4-GFP₃ was detected until the 10 min time point, when Plo1-mCherry₃ was first detected on SPBs, and continued to decrease until it was undetectable at 20 min, just before SPB separation (Figure 6C). We also quantitated the relative intensities of Byr4-GFP₃ and Plo1-mCherry₃ at SPBs in an asynchronous population of cells (Figure 6D). In the few cells in which both proteins were detected at SPBs, Byr4

Figure 5 Sid4 ubiquitination prevents Plo1 recruitment to SPBs during interphase. **(A)** *In vivo* ubiquitination of Sid4-HBH in asynchronous cells or cells arrested in G2 (*cdc25-22*), prometaphase (*nda3-KM311*) or S phase (hydroxyurea; HU). **(B)** Representative images showing Dma1-GFP and Sid4-RFP localization in a G2 arrest (*cdc25-22* arrest), an S-phase arrest (HU arrest), prometaphase cell growing in log phase, and a mitotic arrest when the checkpoint is active (*nda3-KM311* arrest). Scale bar, 5 μ m. **(C)** Quantitation of relative Dma1-GFP/Sid4-RFP intensity ratios for each of the cell cycle stages shown in **B** plotted as arbitrary units. For each cell cycle stage, Dma1-GFP and Sid4-RFP intensities were measured for at least 20 cells and averaged; error bars represent standard error of the mean, $*P < 0.05$. **(D)** Representative images showing Plo1-GFP₃ and Sad1-mCherry localization at SPBs during a *cdc25-22* arrest in wild type (left panels), *dma1Δ* (middle panels) and *sid4N-ppc89C* (right panels) cells. Scale bar, 5 μ m. **(E)** Quantitation of relative Plo1-GFP₃/Sad1-mCherry intensity ratios at SPBs for each of the strains shown in **D** plotted in arbitrary units. For each strain, Plo1-GFP₃ and Sad1-mCherry intensities were measured for at least 20 cells and averaged; error bars represent standard error of the mean, $*P < 0.05$.

and Plo1 intensities showed a strong negative correlation (Figure 6D and E). Collectively, these data suggest that Plo1 phosphorylation of Byr4 at SPBs promotes Byr4 dissociation from SPBs.

Sid4 ubiquitination is required to prolong Byr4 residence on SPBs during a checkpoint arrest

We next examined the kinetics of Byr4 and Plo1 localization during a checkpoint response in *nda3-KM311* and *nda3-KM311 sid4N-ppc89C* mutant cells. Cells were synchronized in G2, shifted to 18°C to activate the checkpoint and Byr4-GFP₃ and Plo1-mCherry₃ were visualized periodically for 9 h. In *nda3-KM311 sid4⁺* cells, Byr4-GFP₃ was

maintained on SPBs for ~3 h (Figure 7A and C). However, in the absence of Sid4 ubiquitination (*nda3-KM311 sid4N-ppc89C* mutant), Byr4-GFP₃ began to disappear from SPBs after just 1 h and was absent from almost 100% of the cells by 5 h (Figure 7B and C). In both strains, the time in which SPB-localized Byr4 was absent in 50% of the cells (~5 h in *sid4⁺* cells and ~2 h in the *sid4N-ppc89C* mutant) corresponds to the same time in which SPB-localized Plo1 was detected in 50% of the cells (Figure 7C, intersections marked by dashed lines). These data suggest that when a mitotic checkpoint is activated, Sid4 ubiquitination antagonizes Plo1 SPB recruitment in order to retain Byr4 on SPBs and inhibit SIN signalling.

Figure 6 Byr4 is a potential Plo1 target. (A) Left, autoradiograph of recombinant MBP and MBP-Byr4 phosphorylated *in vitro* by Plo1 kinase. Right, Coomassie blue (CB) gel of purified MBP and MBP-Byr4 proteins. (B) Gel shifts of endogenous Byr4 immunoprecipitated from asynchronous, *nda3*-KM311, *plo1*-25 or *sid4*-SA1 temperature-sensitive cells, which were synchronized in S phase by hydroxyurea and released at the restrictive temperature. Immunoprecipitates were treated with (+) or without (–) λ-phosphatase and detected by immunoblotting using an anti-Byr4 serum. (C) A Byr4-GFP₃ Plo1-mCherry₃ strain was imaged via time-lapse microscopy and a representative montage is depicted. (D) A Byr4-GFP₃ Plo1-mCherry₃ strain was grown to log phase and imaged. Top and bottom panels show representative images of cells in which Byr4-GFP₃ or Plo1-mCherry₃, respectively, localization to the SPB predominates. (E) Byr4-GFP₃ and Plo1-mCherry₃ fluorescence intensities were measured and plotted against each other. A linear regression analysis was performed to calculate the best-fit line, $r^2 = 0.687$. The data points boxed in yellow and blue represent the intensity calculations for the top and bottom panels shown in D, respectively.

Discussion

Ubiquitin-mediated inhibition of cytokinesis

Mitotic exit and cytokinesis must be coupled for proper partitioning of genetic material. In *S. pombe*, this entrainment is achieved by the SIN. Here, we have presented new evidence regarding how Dma1 influences SIN signalling. Our data indicate that when chromosomes are not attached properly to the mitotic spindle, Dma1 concentrates at SPBs and ubiquitinates the SIN scaffold, Sid4 (Figure 8A, step 1). We propose that Sid4 ubiquitination antagonizes Plo1 localization at SPBs (Figure 8A, step 2) to restrict its ability to activate the SIN and cytokinesis (Figure 8A, steps 3 and 4).

The SIN pathway consists of several protein kinases, which assemble sequentially at the SPBs. The first kinase on the scene is Plo1, which directly binds the SIN scaffold, Sid4 (Morrell *et al*, 2004). Once recruited, Plo1 initiates the SIN pathway presumably by phosphorylating one or more SIN components directly; however, its direct target(s) have

remained unknown to date. Here, we find that the GAP component, Byr4, is a likely Plo1 target (Figure 8B, step 1). Additionally, the observation that Plo1 and Byr4 localization to SPBs are negatively correlated suggests a model wherein Plo1 ejects Byr4 from SPBs (Figure 8B, step 2), highlighting yet another conserved mechanism between the *S. pombe* SIN and the *S. cerevisiae* MEN. Subsequently, expulsion of the GAP complex from SPBs relieves the inhibition on the GTPase, Spg1, which subsequently facilitates recruitment of the SIN kinases, Cdc7 and Sid1-Cdc14 (Figure 8B, steps 3 and 4, respectively), and finally allows Sid2-Mob1 to accumulate at the division site to trigger cytokinesis (Figure 8B, step 5). During a checkpoint response, the delayed Plo1-mediated phosphorylation of Byr4 would detain Byr4 on SPBs and thereby prevent cytokinesis from occurring before chromosome segregation (Figure 8A, steps 3 and 4).

We have also uncovered a potential role for Dma1 during normal cell cycle progression. Here, we find that Dma1's SPB concentration varies according to cell cycle stage and,

Figure 7 Sid4 ubiquitination is required to prolong Byr4 residence on SPBs when a mitotic checkpoint is activated. (A, B) *nda3-KM311* (A) and *nda3-KM311 sid4N-ppc89C* (B) cells were synchronized in G2 by lactose gradient sedimentation, shifted to 18°C to activate the spindle checkpoint, and Byr4-GFP₃ and Plo1-mCherry₃ localizations at the SPBs were imaged periodically for 9 h. Representative images of Byr4-GFP₃, Plo1-mCherry₃ and the merged images are shown for the times indicated. Scale bar, 10 μm. (C) At each time point, the percentage of cells with Byr4-GFP₃ and Plo1-mCherry₃ were calculated and plotted over time. Dashed lines represent the time in which the plots for Byr4 and Plo1 intersect (~5 h for *nda3-KM311* cells and ~2 h for *nda3-KM311 sid4N-ppc89C* cells).

consistent with our model, its concentration correlates with the degree of Sid4 ubiquitination. It is possible that in addition to its role as a mitotic checkpoint protein, Dma1 cooperates with other SIN inhibitors to minimize SIN activity during interphase. However, these basal levels of Dma1 and Sid4 ubiquitination are likely not sufficient when SIN activity must be kept low for longer periods, such as during a checkpoint arrest. Thus, Dma1 might be 'activated' during a checkpoint response, at least in part, through additional SPB recruitment. This is supported by the fact that Dma1 intensities increase significantly at SPBs and Sid4 ubiquitination is observed more robustly when a mitotic checkpoint is

activated. In order to understand how Dma1 responds to a mitotic checkpoint, it will be pertinent to identify upstream factors that regulate the extent of Dma1 recruitment to SPBs during normal cell cycle progression and in response to a mitotic checkpoint.

Distinct roles of SPB-localized Plo1 kinase in mitosis and cytokinesis

Plo1 accumulates at the mitotic, but not the interphase SPB, through association with Sid4 (Morrell *et al*, 2004) and at least two other SPB components, Cut12 (Mulvihill *et al*, 1999) and Pcp1 (Fong *et al*, 2010). Its recruitment upon

Figure 8 Model of Dma1 inhibition of the SIN during a mitotic checkpoint. (A) Proposed mechanism of Dma1 inhibition of the SIN when the mitotic checkpoint is active. (B) Mechanism of SIN activation when chromosomes are properly attached to the mitotic spindle and the checkpoint is satisfied.

commitment to mitosis is dependent on cyclin-dependent kinase activity; however, a hypermorphic Cut12 mutant (*stf1-1*) can bypass a *cdc25-22* arrest by increasing Plo1 recruitment to SPBs and increasing its kinase activity suggesting that the Plo1–Cut12 interaction promotes mitotic entry (Mulvihill *et al.*, 1999). Similarly, Plo1's association with Pcp1 also seems to have a role in promoting mitotic entry as the mitotic defects observed in a temperature-sensitive *pcp1* mutant that exhibits reduced Plo1 localization at SPBs can be rescued by a *wee1* mutant that causes premature mitotic entry (Fong *et al.*, 2010). Here, we find that the absence of Sid4 ubiquitination also allows Plo1 to accumulate at SPBs in interphase; however, this on its own did not affect normal cell cycle progression and cells did not bypass a *cdc25-22* arrest. Given that Cut12 (Bridge *et al.*, 1998) and Pcp1 (Flory *et al.*, 2002) associate with the nuclear side of the SPB, while Sid4 resides on the cytoplasmic face (our unpublished data), suggests that the role of Plo1 in promoting mitotic entry is spatially restricted to the nuclear SPB surface, while its association with Sid4 on the cytoplasmic surface may have a distinct role in regulating cytokinesis.

How does Sid4 ubiquitination antagonize Plo1?

The fact that Sid4 is ubiquitinated might suggest that Sid4 polyubiquitination signals it for degradation by the proteasome, thereby preventing access of Plo1 to core SIN components. However, mimicking a checkpoint response by overexpressing *dma1*⁺ does not alter Sid4 protein levels or disrupt its localization at SPBs (Guertin *et al.*, 2002), and fluorescence recovery after photobleaching experiments indicate that Sid4 is stably bound to the SPB (Morrell *et al.*, 2004). Furthermore, the other major SIN scaffold protein, Cdc11, whose localization to the SPBs depends on Sid4, remains localized to SPBs during *dma1*⁺ overexpression

(Guertin *et al.*, 2002), and its intensity at SPBs is not affected in the *sid4N-ppc89C* mutant, indicating that Dma1 does not disrupt the Cdc11–Sid4 scaffold complex. An alternative possibility is that ubiquitination physically masks the Plo1 binding site. However, both Plo1 (Morrell *et al.*, 2004) and Dma1 (Guertin *et al.*, 2002) physically interact with the N terminus of Sid4, while Sid4 ubiquitination appears to occur on the C terminus. We do not rule out this possibility, however, due to the lack of information about Sid4's three-dimensional conformation. Sid4 is predicted to contain several intrinsically unstructured regions and it has been proposed that some scaffold proteins are intrinsically unstructured to increase their flexibility and versatility for the proteins that they bind (reviewed in Cortese *et al.*, 2008). Potentially, ubiquitination might induce a structural change within Sid4 that alters the Plo1 binding site, reducing Sid4 affinity for Plo1. To address these outstanding questions, structural studies of Sid4 will be required.

From our studies, it is clear that Sid4 is ubiquitinated *in vivo*. However, the type of ubiquitin modification formed on Sid4 remains to be characterized. Because Sid4 is not targeted for degradation, it is unlikely that it is polyubiquitinated with K48-linked chains. Our *in vitro* studies indicate that Dma1 forms polyubiquitin chains with the E2 enzyme complex, Ubc13–Uev1a, which specifically forms K63-linked chains (Hofmann and Pickart, 1999). Also, the Dma1-related proteins, CHFR (Bothos *et al.*, 2003), RNF8 (Plans *et al.*, 2006) and *S. cerevisiae* Dma1 and Dma2 (Loring *et al.*, 2008), have all been shown to function with Ubc13 *in vitro* and/or *in vivo*. K63-linked polyubiquitin chains are not typically associated with proteasome-mediated degradation, but regulate proteins by other mechanisms (for reviews see Ikeda and Dikic, 2008; Pickart and Fushman, 2004). Recent studies indicate that the linear architecture of K63-linked chains can provide a scaffold

to recruit proteins with ubiquitin-binding domains in a spatially and temporally regulated manner (Kim *et al.*, 2007; Komander *et al.*, 2009; Sims and Cohen, 2009). Potentially, K63-linked chains could recruit an unidentified factor to Sid4 that antagonizes either Plo1 binding or its kinase activity.

Yet another possibility is that Sid4 is not polyubiquitinated, but multiubiquitinated. Our observation that mutating all endogenous lysines within Sid4 in large clusters has no impact on the extent of Sid4 ubiquitination supports this idea and indicates that Dma1 has loose specificity towards its target lysine(s). We have attempted to examine Sid4 ubiquitination *in vitro* to address this issue and to validate Sid4 as a direct Dma1 substrate. However, given that Dma1 binds Sid4 through its FHA domain, it is likely that Sid4 must first be phosphorylated on a threonine residue in order to interact with Dma1; establishing the proper *in vitro* conditions will require phospho-characterization of Sid4. Thus, characterizing the type of ubiquitin modification on Sid4 will be a challenging, yet important future endeavour necessary to understand the detailed mechanism by which it antagonizes Plo1 SPB recruitment.

Conservation of mechanism

Although previously assumed based on its domain architecture, we have shown here for the first time that Dma1 is in fact a *bona fide* ubiquitin ligase. Four other proteins with similar architecture and activity, the human tumour suppressor protein, CHFR (Scolnick and Halazonetis, 2000), human RNF8 (Tuttle *et al.*, 2007) and *S. cerevisiae* proteins, Dma1 and Dma2 (Fraschini *et al.*, 2004), have also been implicated in mitotic checkpoints for which the pertinent substrates are unknown. It has been reported that CHFR can directly ubiquitinate the human Polo-like kinase, Plk1, in *Xenopus laevis* extracts (Kang *et al.*, 2002) and downregulates Plk1 protein levels in human cells (Shtivelman, 2003). These studies suggest that CHFR might directly ubiquitinate Polo-like kinases, targeting them for degradation. However, other reports indicate that a CHFR-dependent checkpoint arrest does not require the function of the proteasome at all, as cells can arrest when treated with proteasomal inhibitors (Matsusaka and Pines, 2004). Here, we find that in *S. pombe*, Plo1 localization to SPBs is at least in part regulated by ubiquitination of its scaffold rather than ubiquitination of itself. Indeed, we obtained no evidence that Plo1 or Dma1 was ubiquitinated during a mitotic checkpoint response. Whether similar mechanisms operate in other organisms to control Polo-like kinase activity during mitotic checkpoints will be important future studies.

Materials and methods

Yeast methods

Yeast strains (Supplementary Table 1) were grown in yeast extract media supplemented with appropriate amino acids (Moreno *et al.*, 1991). For *in vivo* ubiquitination assays, strains were grown in 100 ml of 4 × YE media, with the exception of *cdc11*-linkerHBH and *cut12*-linkerHBH, which were grown in 2L 4 × YE media. For *nda3*-KM311 arrests, cultures were shifted to 18°C for 6.5 h before harvesting. For *cdc25-22* arrests, cultures were shifted to 36°C for 3.5 h before analysis.

For gene replacements at endogenous loci, mutant open reading frames plus at least 500 bps of 5'- and 3'-flanking nucleotides were subcloned into the pIRT2 plasmid containing the *LEU2⁺* marker. For *dma1⁺* gene replacements, a haploid *dma1Δ*

strain was transformed with either pIRT2-*dma1*(R64A) or pIRT2-*dma1*(I194A) and stable integrants were selected by resistance to 5'-FOA. For *sid4⁺* gene replacements, a diploid *sid4⁺/sid4Δ* strain was transformed with pIRT2-*sid4* mutant constructs and grown on minimal media lacking leucine, adenine and uracil. Transformants were allowed to sporulate and stable haploid integrants were selected based on resistance to 5'-FOA. Mutants were validated by colony PCR with primers outside of the 5'- and 3'-flanking regions.

Cell synchronization methods

For HU block and release experiments, cells were grown to log phase at 32°C before adding HU to a final concentration of 12 mM. After 2 h, a second dose of HU (6 mM final concentration) was added to the cells and after 3.5 h, HU was washed out and cells were released at 18°C.

For synchronization by lactose gradient, cells were grown to log phase at 32°C and sedimented by centrifugation on a 7–30% lactose gradient. Small G2 cells were extracted from the gradient, washed with fresh media and inoculated in media pre-cooled to 18°C.

In vivo ubiquitination assay

Proteins of interest were tagged at their endogenous C termini with a HBH affinity tag with the exception of Cdc11 and Cut12, which were tagged with a linker-HBH affinity tag. Tagged proteins were purified using a modified version of the two-step tandem affinity purification under fully denatured conditions (Tagwerker *et al.*, 2006). Cell pellets were lysed by bead disruption into buffer 1 (8 M urea, 300 mM NaCl, 50 mM NaPO₄, 0.5% NP40 and 4 mM Imidazole, pH 8) and incubated with Ni²⁺-NTA agarose beads (Qiagen) for 3–4 h at room temperature. After incubation, beads were washed 4 × with buffer 3 (8 M urea, 300 mM NaCl, 50 mM NaPO₄, 0.5% NP40 and 20 mM Imidazole, pH 6.3) and eluted in buffer 4 (8 M urea, 200 mM NaCl, 50 mM NaPO₄, 0.5% NP40 and 2% SDS, 100 mM Tris and 10 mM EDTA, pH 4.3). The pH of the eluate was adjusted to 8 before adding streptavidin ultra-link resin (Pierce) and incubated overnight at room temperature. After the second incubation, streptavidin beads were washed 4 × with buffer 6 (8 M urea, 200 mM NaCl, 2% SDS and 100 mM Tris, pH 8) and 1 × with buffer 7 (8 M urea, 200 mM NaCl and 100 mM Tris, pH 8). Purified proteins were detected on a western blot using a ubiquitin antiserum (Sigma) and fluorescently labelled streptavidin (Licor).

In vitro ubiquitination assay

The E1-activating enzyme and E2 (Ubc13/Uev1a) were purchased from Boston Biochem. Dma1-HA₃-TAP and Dma1(I194A)-HA₃-TAP were purified from *S. pombe* lysates using a tandem affinity purification method (Gould *et al.*, 2004). All components were incubated in a reaction buffer containing 50 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂ and 0.5 mM DTT. Reactions were incubated at room temperature for 90 min before adding SDS sample buffer to quench the reaction. To assess Dma1 activity, samples were run on a 3–8% Tris-Acetate gel (Invitrogen) and immunoblotted with anti-Ub (Sigma).

In vitro kinase assays

MBP and MBP-Byr4 fusion proteins were purified on amylose beads (NEB) in column buffer (20 mM Tris (pH 7.0), 150 mM NaCl, 2 mM EDTA and 0.1% NP40) and eluted with maltose. One µg of recombinant protein was used as the substrate in each reaction. Kinase reactions were performed in protein kinase buffer (10 mM Tris pH7.4, 10 mM MgCl₂ and 1 mM DTT) at 30°C for 30 min. Reactions were quenched by adding SDS sample buffer, and proteins were separated by SDS-PAGE. Phosphorylated proteins were visualized by autoradiograph and relative protein quantities were assessed by Coomassie blue staining.

S. pombe protein methods

Cell pellets were lysed by bead disruption, and immunoprecipitations were performed in either NP40 buffer for native lysates, or in NP40 buffer containing SDS for denatured lysates as previously described (Gould *et al.*, 1991). For gel shifts, denatured lysates were treated with Lambda phosphatase (New England Biolabs) in 25 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, and 1 mM MnCl₂ and incubated at 30°C for 30 min with shaking. Proteins were separated on an 8% Tris-glycine gel and immunoblotted with a Byr4 antiserum (CoCalico).

Microscopy methods

All fluorescence microscopy was performed using a spinning disk confocal microscope (Ultraview LCI; PerkinElmer) with a 100 × NA 1.40 Plan-Apochromat oil immersion objective and either a 488-nm argon (GFP) ion or a 594 nm helium neon (RFP and mCherry) laser. Images were processed using a charge-coupled device camera (Orca-ER; Hamamatsu Photonics) and Metamorph 7.1 software (MDS Analytical Technologies). For imaging *cdc25-22*-arrested cells, slides, coverslips and immersion oil were pre-heated to 36°C, and cells were imaged on an objective heated to 36°C. For *nda3-KM311* strains, cells were fixed in 70% ethanol for 30 min before imaging.

DAPI and methyl blue images were obtained with a personal DeltaVision System equipped with an Olympus IX71 microscope using a 100 × NA 1.40 UPlansApo oil immersion objective. Images were processed using a Cool Snap HQ² camera and Softworx[®] software.

Quantitative microscopy was performed using ImageJ software available at: <http://rsbweb.nih.gov/ij/>. Average GFP fluorescence intensities at SPBs were measured for at least 20 cells with background correction for each. Average RFP or mCherry fluorescence intensities were measured similarly, and final values for each cell are expressed as Green/Red ratios. Measurements for the 20 cells in each group were averaged for statistical analysis.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Anna Feoktistova, Ping Liang and Liping Ren for their technical assistance and Dr Dannel McCollum for strains used in this study and helpful discussions. We also thank members of the Gould lab, especially Rachel Roberts-Galbraith, Dawn Clifford-Hart, Adam Bohnert, Matthew Broadus and Aurélie Clement for critically reading the manuscript and many helpful discussions. AEJ is supported by the Cellular, Biochemical and Molecular Sciences Training Program, NIH T32 GM08554. This work was supported by the Howard Hughes Medical Institute, of which KLG is an investigator.

Author contributions: AEJ performed all of the experiments; AEJ and KLG designed and interpreted the experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

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