Analysis of *S. pombe* SIN protein SPB-association reveals two genetically separable states of the SIN

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Abstract

The *S. pombe* Septation Initiation Network (SIN) regulates cytokinesis, and asymmetric association of SIN proteins with the mitotic spindle poles (SPBs) is important for its regulation. We have used semi-automated image analysis to study SIN proteins in large numbers of wild-type and mutant cells. Our principal conclusions are; First, the association of Cdc7p with the SPBs in early mitosis is asymmetric, with a bias in favour of the new SPB. Second, the early SPB association of Cdc7p-GFP depends on Plo1p but not Spg1p, and is unaffected by mutations that influence its asymmetry in anaphase. Third Cdc7p asymmetry in anaphase B is delayed by Pom1p and by activation of the spindle assembly checkpoint, and is promoted by Rad24p. Fourth, the length of the spindle, expressed as a fraction of the length of the cell, at which Cdc7p becomes asymmetric is similar in cells dividing at different sizes. These data reveal that multiple regulatory mechanisms control the SIN in mitosis and lead us to propose a two-state model to describe the SIN.
Introduction

Asymmetric events are of fundamental importance in biology. Asymmetry of centrosome behaviour and inheritance is also implicated in an increasing number of cellular and developmental processes (Knoblich, 2010; Tajbakhsh et al., 2009). Schizosaccharomyces pombe is an excellent model for the analysis of the conserved basic mechanisms of cell division. S. pombe cells are rod-shaped, grow by tip-elongation and divide by binary fission. As in higher eukaryotes, a contractile actomyosin ring (CAR) is important for cytokinesis; reviewed by (Ishiguro, 1998; Pollard and Wu, 2010), acting as a “guide” for synthesis of the division septum (Proctor et al., 2012). The position of the division plane is determined by signalling from the nucleus and the cell tips; reviewed by (Goyal et al., 2011; Oliferenko et al., 2009). The coordination of cytokinesis with other mitotic events is assured by a conserved network of protein kinases known as the Septation Initiation Network (SIN). The SIN is one of two conserved NDR-kinase signalling pathways in S. pombe, the other being the Morphology Network (MOR). As in other eukaryotes, they control cell proliferation and growth; reviewed by (Gupta and McCollum, 2011). Loss of SIN signalling causes failure of cytokinesis generating multinucleated cells (Nurse et al., 1976). In contrast, continuous SIN signalling results in multisepetated cells (Minet et al., 1979). Ectopic activation of SIN signalling induces CAR and septum formation from any stage of the cell cycle (Schmidt et al., 1997), emphasising the importance of coordinating cytokinesis with mitosis.

The SIN plays multiple roles during cytokinesis; reviewed by (Goyal et al., 2011; Johnson et al., 2012; Roberts-Galbraith and Gould, 2008) and mitotic commitment (Grallert et al., 2012). The spindle pole body (SPB) serves as a microtubule organising centre, and coordination point for cell cycle regulators (Grallert et al., 2012; Grallert et al., 2013; Hagan, 2008). SPB-association of SIN proteins plays an important part in regulation of the SIN; reviewed by (Johnson et al., 2012; Simanis, 2003). SIN proteins associate with a tripartite scaffold (Cdc11p-Sid4p-Ppc89p), which is essential for signalling (Chang and Gould, 2000; Krapp et al., 2001; Morrell et al., 2004; Rosenberg et al., 2006; Tomlin et al., 2002). The core SIN components are three protein kinases (Cdc7p, Sid1p and Sid2p), and their regulatory subunits (Spg1p, Cdc14p and Mob1p, respectively) (Fankhauser and Simanis, 1993; Fankhauser and Simanis, 1994; Guertin et al., 2000; Hou et al., 2000; Salimova et al., 2000; Schmidt et al., 1997; Sparks et al., 1999). Signalling is mediated by the small GTPase Spg1p, which is regulated by a GAP (Cdc16p) that interacts with Spg1p via a scaffold, Byr4p, (Fankhauser et al., 1993; Furge et al., 1998; Minet et al., 1979; Song et al., 1996), Etd1p (Daga et al., 2005; Garcia-Cortes and McCollum, 2009; Lahoz et al., 2010) and the conserved kinase Plo1p (Rachfall et al., 2014; Tanaka et al., 2001).
Immuno-electron microscopy has localised Ppc89p, Sid4p (Rosenberg et al., 2006) and Sid2p (Sparks et al., 1999) to the cytoplasmic side of the SPB, suggesting that SIN signalling is activated in the cytoplasm.

The intensity of some SIN protein SPB-associated signals changes during mitosis (Cerutti and Simanis, 1999; Feoktistova et al., 2012; Garcia-Cortes and McCollum, 2009; Simanis, 2003; Sohrmann et al., 1998; Wu and Pollard, 2005). In anaphase B, Cdc7p (Grallert et al., 2004; Sohrmann et al., 1998) and Sid1p (Guertin et al., 2000) associate with the new SPB (nSPB), while Byr4p and Cdc16p associate with the old SPB (oSPB) (Cerutti and Simanis, 1999; Li et al., 2000). Mutants that compromise asymmetric distribution of SIN proteins on the SPBs during mitosis deregulate septation (Garcia-Cortes and McCollum, 2009; Singh et al., 2011; Sohrmann et al., 1998). If the SIN fails to signal, then SIN protein asymmetry in anaphase is not established. Therefore, it has been proposed that there is a feedback loop within the SIN, mediated by Sid2p; phosphorylation of the scaffold protein Cdc11p by Sid2p contributes to this regulation (Bajpai et al., 2013; Feoktistova et al., 2012; Johnson et al., 2012).

The data presented in this study suggest there are at least two, genetically separable, states of the SIN; an early state that is dependent upon Plo1p for its establishment, and a late state, which is dependent upon Spg1p.
Results

The RodCellJ ImageJ plugin (Schmitter et al., 2013) was used to examine large numbers of mitotic S. pombe cells. The cells express a tagged SIN protein together with pcp1-mCherry (Pcp1p-CHY hereafter) to localise SPBs, and the kinetochore marker cnp1-mCherry as required (Cnp1p-CHY hereafter) (Alvarez-Tabares et al., 2007); the different intensity of the Cnp1p-CHY and Pcp1p-CHY signals allows them to be differentiated in early mitosis. To facilitate comparison between cells of different size, the data are presented as the ratio of the SPB intensities plotted against the separation of the SPBs, expressed as a fraction of the cell length (length fraction; LF; figure 1C). The raw data were divided into 10 bins with step size LF0.1. To facilitate the analysis and presentation of the data, three states were defined; symmetry as ≤2-fold difference between the GFP signals on the two SPBs; transition as >2-fold, but <4-fold; asymmetric as ≥4-fold difference.

SIN protein association with the SPB in anaphase in wild-type cells

_Sid4p-GFP, Cdc11p-GFP, Spg1p-GFP and Cdc7p-GFP_

The SPB-associated signals of Cdc11p-GFP (figure S1A) and Sid4p-GFP (figure S1B) were symmetric in the vast majority of cells throughout mitosis, as described previously (Chang and Gould, 2000; Krapp et al., 2001). Spg1p-GFP is symmetrical in the majority of cells throughout mitosis (figure S1C); its intensity increased 1.5-fold at both SPBs during mitosis (figure S1D, S1E). Cdc11p-GFP showed a similar increase, while Sid4p-GFP remained constant (data not shown). This is consistent with the slow turnover of Sid4p-GFP (Feoktistova et al., 2012) and indicates that the asymmetry of SIN proteins is not due to changes in the level of scaffold proteins at the SPBs.

The essential effector of Spg1p, Cdc7p (Schmidt et al., 1997), was chosen as the primary reporter of SIN protein behaviour. It associates with both SPBs in early mitosis, then only with the nSPB during anaphase B (Grallert et al., 2004; Sohrmann et al., 1998); (figure 1A).

As cells progressed through mitosis, the Cdc7p-GFP signal at the oSPB decreased, while the nSPB brightened (figure 1B), consistent with previous studies (Cerutti and Simanis, 1999; Garcia-Cortes and McCollum, 2009). The rate was similar at both SPBs (3.6 +/- 0.5 AU min\(^{-1}\) for the nSPB and -4.1 +/-0.6 AU min\(^{-1}\) at the oSPB). The signal at the oSPB began to decline 2 minutes before the nSPB brightened, suggesting that asymmetry initiates at the oSPB.

In wild-type cells, the fraction of asymmetric Cdc7p-GFP signals increased with SPB separation (Figure 1C). At LF0.6 (=8.5 μm spindle in a 14μm wild-type cell) >95% of cells showed an asymmetric signal, rising to >99% at LF0.8 (=11.5 μm spindle in wild-type). These data are consistent with previous analysis of wild-type cells (Feoktistova et al., 2012).
SPB-association of Cdc7p-GFP in early mitosis is asymmetric.

Filming revealed an initial asymmetric SPB-association of Cdc7p-GFP in 25 of 33 cells (76%); 2 cells (6%) had signals on both SPBs, with asymmetric intensity, and 6 cells (18%) showed a symmetric signal. Cdc7p-GFP associated with the nSPB first in 21/27 cells (78%); (figure 1D). Examples of Cdc7p-GFP loading first to the nSPB (figure S1F) and oSPB (figure 1A) are presented. Thus, the initial association of Cdc7p-GFP with SPBs is asymmetric in ≈80% of cells, with a ≈3:1 bias towards the nSPB. These films also revealed transient fluctuations in the relative intensity of the SPB-associated Cdc7p-GFP signal in some (figure 1A) but not all cells (figure S1F), which may explain why approximately 40% of cells in single timepoint images showed a significantly asymmetric signal in early mitosis (figure 1C; LF0.2 to 0.3, corresponding to SPB separation of approximately 3-4 μm in a 14 μm wild-type cell). This may not have been observed previously because they were performed by indirect immunofluorescence (Sohrmann et al., 1998).

Since Cdc7p-GFP asymmetry occurs in anaphase, a conditionally expressed allele of slp1/CDC20 (Petrova et al., 2013) was used to investigate whether SPB-association of Cdc7p-GFP requires APC/C<sup>slp1p/cdc20p</sup>. Expression of slp1 was silenced in the strains atb2-CHY cdc7-GFP (hereafter referred to as slp1-OFF). Cdc7p-GFP associated with both SPBs in 95% of the arrested cells (240 of 253 cells; figure 1E); 85% of these showed a symmetric signal (LF0.1 and 0.2; not shown). Since the initial SPB-association of Cdc7p-GFP is often asymmetric, the difference between these data and figure 1C may arise because the slp1-OFF cells are arrested for an extended period in metaphase, giving sufficient time for the Cdc7p-GFP to equalise on the SPBs.

The initial SPB-association of Cdc7p-GFP is not influenced by mutants affecting its anaphase B asymmetry.

The STRIPAK-related SIN-inhibitory phosphatase complex (SIP) is required to establish Cdc7p-GFP and GFP-Sid1p asymmetry in anaphase (Singh et al., 2011). The SPB-association of Cdc7p-GFP in early mitosis was asymmetric in 7 of 8 csc1-D cells examined (figure 1D). Cdc7p-GFP remained symmetric in the majority of cells, in the later stages of mitosis as expected (figure 2C), though a minority of cells displayed a Cdc7p-GFP signal that differed by >4-fold. Consistent with earlier studies (Singh et al., 2011), the signal intensity increased at both SPBs as cells progressed through mitosis (not shown) As described previously, (Schmidt et al., 1997; Sohrmann et al., 1998), association of Cdc7p-GFP with the SPBs in anaphase was mostly symmetric in cdc16-116 (compare figure 2B with 2D). As cells entered mitosis 17 of 21 cdc16-116 cells (79%) showed an asymmetric SPB association of Cdc7p-GFP with the SPBs, 1 (5%) showed association with both SPBs, with a stronger signal on one pole than the other, and 3 (14%) showed a symmetric signal (figure 1D). Therefore, despite the reduction of GAP
function, the initial association of Cdc7p-GFP with the SPBs was asymmetric in >80% of the cells. No Cdc7p-GFP signal was observed prior to SPB separation (not shown). Since the signal is symmetric in the majority of cells in anaphase compared to Cdc7p-GFP at 36°C, it was not possible to determine which SPB initially loaded Cdc7p-GFP. The Cdc7p-GFP signal reached maximum intensity more rapidly in cdc16-116 than in wild-type cells (Figure 2G; LF0.1 – 0.4).

The early loading of Cdc7p-GFP was still asymmetric in sid2-250 at 36°C (8 of 14 cells), indicating that Sid2p is not obligatory for the asymmetric early loading of Cdc7p-GFP (figure 1D). Consistent with previous studies (Feoktistova et al., 2012; Mishra et al., 2005), Cdc7p-GFP remained largely symmetric in sid2-250 anaphase cells both at 25°C (figure 2E) and 36°C (figure 2F). The intensity increased at both SPBs until LF0.3 and then plateaued (figure 2H). Together, these data are consistent with the idea that the association of Cdc7p with the SPB early in mitosis is not affected by the regulators of its asymmetric distribution in anaphase. However, though the csc1-D mutant is a null allele, sid2-250 and cdc16-116 are “tight” conditional alleles. Thus, we cannot formally exclude the possibility that the latter mutants have residual activity that still permits normal loading of Cdc7p-GFP at mitotic onset. Future analysis of null mutants will examine this.

**Spg1 is not required for Cdc7p association with the SPB in early mitosis**

Inactivation of Spg1p revealed that Cdc7p-GFP was still associated with the SPB in early mitosis in spg1-B8 at 36°C (compare figure 2I with 2B). In late mitosis, the intensity of the signal at the nSPB became fainter, rather than brighter (figure 2H), and the signal remained symmetrical in 30% of cells (figure 2I). Cytokinesis failed in the mutant cells, indicating that Spg1p had been inactivated (not shown). If cells were incubated for 5h at 36°C before analysis, we did not observe any SPB-associated Cdc7p-GFP signal (not shown), consistent with previous studies (Sohrmann et al., 1998). Thus, in the first cycle after inactivation of Spg1p, the early mitotic association of Cdc7p-GFP with the SPB is independent of Spg1p, and the late mitotic association and transition to asymmetry requires Spg1p function.

**POLO is required for Cdc7p-GFP SPB-association in early mitosis, but not in anaphase.**

The conserved kinase Plo1p has multiple roles during mitosis (Bahler et al., 1998; Ohkura et al., 1995). Epistasis analysis places plo1 upstream of the SIN (Tanaka et al., 2001); Plo1-ts4 is a hypomorphic allele whose primary defect is in SIN regulation. There was no detectable Cdc7p-GFP signal at the SPBs in a large fraction of early mitotic plo1-ts4 cells at 36°C (figure 3A, 3B; 79% of LF0.1 cells (n=279), and 73% of LF0.2 (n=193)); when a signal was observed, it was asymmetric. This was recapitulated in the plo1-as3 mutant (Grallert et al., 2012); not shown. Mixing of early mitotic cells of
cdc7-GFP and cdc7-GFP plo1-ts4 at 36°C showed that Cdc7p-GFP was detectable in wild-type cells at 36°C (figure 3C). However, by LF0.6, more than 80% of cells had an asymmetric signal of Cdc7p-GFP on one SPB in anaphase B; (compare figure 3B with 2B). The signal intensity in anaphase was comparable to wild-type (figure 3D). Thus, Plo1p is required for the SPB-recruitment of Cdc7p-GFP at the onset of mitosis. Furthermore, the appearance of Cdc7p at one SPB in anaphase B does not require SPB-association of Cdc7p earlier in mitosis.

**Symmetric SPB-association of Mob1p-GFP in early mitosis requires Plo1p**

Mob1p-Sid2p was examined using mob1-GFP (Salimova et al., 2000), since sid2-GFP (Sparks et al., 1999) is functionally compromised (Grallert et al., 2012; Salimova et al., 2000). Mob1p-GFP associated with both SPBs in most cells, exceeding 98% by LF0.7 (figure 3F, 3G), consistent with earlier observations (Hou et al., 2000; Salimova et al., 2000). Filming of Mob1-GFP revealed some fluctuations in the relative intensity of the two SPBs throughout mitosis (figure 3E). In the early stages of mitosis, the Mob1p-GFP signal was seen on both SPBs in ≈75% of cells and on one SPB in ≈25% cells. This was not noted previously (Hou et al., 2000; Salimova et al., 2000), and is most likely due to improvements in detection. Reduction of Plo1p activity increased the fraction of cells with asymmetric and transition Mob1p-GFP early in mitosis (LF0.1 – 0.4) compared to wild-type (compare figure 3H to 3G). Furthermore, no signal was detected in approximately 25% of LF0.1 cells, compared to 2% in wild-type. However, in late anaphase cells (LF>0.8), the fraction of cells with a symmetrical signal was similar to wild-type. Thus, decreased plo1 activity affects the early, but not the late mitotic SPB association of Mob1p-GFP, as observed for Cdc7p-GFP.

**The association of GFP-Sid1p with the SPB is spatially unstable in early mitosis**

Approximately 90% of cells had an asymmetric GFP-Sid1p signal at the SPB at LF0.6, rising to >98% at LF0.7 (figure 4A, 4B, 4C), consistent with previous studies (Guertin et al., 2000). However, >50% of cells with short spindles (LF0.1 – 0.3) displayed an SPB-associated GFP-Sid1p signal (figure 4B, 4C). Filming revealed that the association of GFP-Sid1p with the SPB is spatially unstable in early mitosis, with a signal seen at one SPB, both SPBs, or absent, in sequential frames (figure 4A), before stabilising at the nSPB in anaphase B (note that “stable” in this context indicates a fixed location for the protein). Faint, symmetric signals in early mitotic cells, though not an unstable SPB association, were noted previously (Guertin et al., 2000). Inactivation of Plo1p decreased the number of cells with a GFP-Sid1p signal in early mitosis (LF<0.4), and increased the number of cells without a detectable signal in anaphase (figure 4D; S2A). GFP-Sid1p was absent from the SPBs in most slp1-OFF cells (figure 4E; no signal in 93% of cells (n=269); signal on one SPB in 2% of cells; signal on both SPBs in
5% of cells). This demonstrates that the APC/C^{Slp1p/Cdc20p} must function for stable recruitment of GFP-Sid1p to the SPB.

**The GAP Byr4p-Cdc16p**

The signal of Cdc16p-GFP became very faint or undetectable on both SPBs shortly after SPB separation (figure 4G), reappearing on the oSPB in anaphase, as described previously (Cerutti and Simanis, 1999). A significant fraction of cells in early mitosis had no detectable signal. The fraction of cells with an asymmetric signal increased as cells progressed through mitosis (figure 4F).

Filming of GFP-byr4 cells revealed that it remained associated with the SPBs early in mitosis, became faint or undetectable, and subsequently reappeared at the oSPB (figure 5A). Some spatial instability of the GFP-Byr4p signal was seen as the signal declined. In LF<0.1 cells, 52% of signals were symmetric, 19% were transition and 26% were asymmetric; no signal was observed in 3% of cells. No signal was detected in 18 and 15% of LF0.2 and LF0.3 cells, respectively (figure 5B). Cells with longer spindles (figure 5B; LF≥0.6) showed predominantly asymmetric signals, as described previously (Cerutti and Simanis, 1999; Li et al., 2000), and the fraction of cells with no detectable signal decreased to <2%.

Surprisingly, since Byr4p and Cdc16p are interdependent for localisation (Cerutti and Simanis, 1999), the numbers of asymmetric cells did not match those observed for GFP-Cdc16p in anaphase. The discrepancy may arise because the cdc16-GFP allele is partially compromised (the cdc16-GFP cdc16-D strain becomes multiseptated at >34°C; not shown).

GFP-Byr4p was associated with both SPBs in 95% of metaphase-arrested slp1-OFF cells (figure 5C; n=111), which is consistent with localisation of Byr4p by indirect immunofluorescence in nda3-KM311 arrested cells (Krapp et al., 2003). The intensity of the GFP-Byr4p signal in the arrested cells was significantly lower than that seen in late anaphase (figure 5D; P<0.001). Together, these data indicate that the removal of Byr4p from the SPBs requires APC/C^{Slp1p/Cdc20p} function. Comparison with Cdc7p-GFP showed that GFP-Byr4p becomes asymmetric significantly before Cdc7p-GFP (compare figure 1C with 5B; LF0.4, P=0.045), confirming earlier analysis by indirect immunofluorescence (Cerutti and Simanis, 1999). Since the steady state level of Cdc16p at the SPBs is very low from the onset of mitosis, while Byr4p persists until metaphase, these data also show that the interdependence of localisation of the GAP proteins does not apply in early mitosis in wild-type cells.

The behaviour of GFP-Byr4p was significantly altered in the plo1-ts4 mutant. Over 50% of cells of LF≥0.6 still showed symmetric GFP-Byr4p (compare figure 5E with 5G). Quantification of GFP-Byr4p at the SPBs confirmed the decrease in signal intensity at constant spindle length in wild-type (LF0.2 and 0.3) followed by an increase at one SPB (figure 5F). In contrast, in plo1-ts4 the decrease at LF0.2
and LF0.3 and the subsequent asymmetry was less apparent (figure 5H; Figure S2B). Cdc16p-GFP could not be analysed in plo1-ts4 as the tagged protein is thermosensitive. Thus, Plo1p is required to establish the asymmetric localisation of Byr4p in anaphase, consistent with recent studies (Rachfall et al., 2014).

**Pom1p inhibits, and Rad24p promotes, the transition to SIN asymmetry**

Phosphorylation of the Sid2p consensus creates a 14-3-3 protein binding site (Gupta et al., 2013; Mah et al., 2005). Phosphorylation of Cdc11p by Sid2p helps promote Cdc7p-GFP asymmetry in anaphase (Feoktistova et al., 2012), and SPB-localisation of the 14-3-3 protein Rad24p in mitosis is Cdc11p-dependent (Mishra et al., 2005). In rad24-D, more cells with long spindles showed a symmetrical distribution of Cdc7p-GFP (figure 6B), implicating Rad24p in promoting Cdc7p-GFP asymmetry during anaphase.

The conserved DYRK-family protein kinase Pom1p is required for bipolar growth, septum positioning and is also implicated in mitotic commitment (Bahler and Pringle, 1998; Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). The onset of Cdc7p-GFP asymmetry occurred earlier in pom1-D cells (Figure 6C; P=0.01 for LF0.4, P= 0.05 for LF0.5). Pom1p associates with the plasma membrane to form a cortical gradient that emanates from the cell tips (Bahler and Pringle, 1998; Hachet et al., 2011), and gradient formation requires the scaffold protein Wsh3p/Tea4p (Hachet et al., 2011). Analysis of Cdc7p-GFP in wsh3-D cells revealed no significant difference from wild-type (figure 6D) indicating that the effect of Pom1p upon the SIN is not mediated via its cortical gradient.

**The effect of cell size and regulators of mitotic commitment upon Cdc7p-GFP asymmetry.**

Since plo1 affects SIN protein behaviour during mitosis, we studied the effect of the gain-of-function cut12-s11 mutant, which promotes premature recruitment of Plo1p to the SPB in interphase (Bridge et al., 1998; Grallert and Hagan, 2002). The distribution of Cdc7p-GFP was not altered significantly in cut12-s11 (figure 6E), no association of Cdc7p-GFP was seen with interphase SPBs, and the initial asymmetric loading of Cdc7p was also unaffected (not shown).

Fin1p regulates SIN signalling (Grallert et al., 2004), and Sid2p phosphorylates and activates Fin1p at the G2-M transition (Grallert et al., 2012). However, analysis of Cdc7p in a fin1 mutant that cannot be phosphorylated by Sid2p revealed no significant difference in the distribution of Cdc7p (figure 6F). This indicates that the proposed feedback loop promoting SIN protein asymmetry in anaphase does not require Sid2p to phosphorylate Fin1p.

Previous studies have implicated PP2A in SIN regulation (Alcaide-Gavilan et al., 2013; Goyal and Simanis, 2012; Jiang and Hallberg, 2001; Le Goff et al., 2001; Singh et al., 2011); Loss of Ypa2p or
Ppa2p function delay the onset of Cdc7p-GFP asymmetry (Goyal and Simanis, 2012). Mutation of the B’ subunit par1 rescues some SIN mutants (Jiang and Hallberg, 2001; Le Goff et al., 2001). 50% of par1-D cells showed symmetric Cdc7p-GFP late in anaphase (compare figure 6H with 6G), implicating PP2A-Par1p in the establishment of Cdc7p-GFP asymmetry in anaphase B. Loss of the other B’ subunit Par2p had no significant effect (figure 6I). The CDC14-family phosphatase Flp1p/Clp1p influences mitotic commitment and mitotic exit and affects SIN signalling (Chen et al., 2013; Cueille et al., 2001; Esteban et al., 2004; Mishra et al., 2004; Trautmann et al., 2001; Wolfe and Gould, 2004). However, loss of Clp1p/Flp1p did not alter the Cdc7p-GFP distribution significantly (figure 6J).

Previous studies have revealed an intramitotic control in S. pombe (Hagan et al., 1990). To investigate whether cell size affected the asymmetric transition of the SIN, Cdc7p-GFP was analysed in wee1-6 and cdc25-22 (Russell and Nurse, 1986; Russell and Nurse, 1987). Plotting the intensity of Cdc7p-GFP against spindle length revealed that >95% of 9 μm spindles showed an asymmetric signal in wild-type cells (figure S3A). This threshold was reached at 6 μm (figure S3B) in the loss-of-function wee1-6 mutant, which divides at a reduced size (figure S3E). The cdc25-22 mutant, is partly defective at 25°C (Fantes, 1979), increasing its cell length at division; in these cells, the >95% threshold was not reached until spindle lengths of >18 μm (figure S3C). When the length fraction at which asymmetry was established was considered, in order to account for the length of the cell in which the spindle was present, the profile of Cdc7p-GFP distribution in wee1-6 and cdc25-22 did not differ significantly from wild-type (compare figure 6K and 6L with 6A). Together, these data suggest that the control mechanism over Cdc7p-GFP asymmetry in anaphase scales with cell size.

Elongated cells were also generated by treating wild-type cells with HU. Analysis of Cdc7p-GFP after release from the arrest revealed that the threshold for 95% asymmetry was not reached until a spindle length of 21 μm (figure S3D). Expressing the data in terms of the length fraction, revealed a slight but significant delay in establishment of asymmetry (figure 6M LF0.6; P=0.02). Previous studies have noted mitotic delays after HU-arrest release (Tange and Niwa, 2007), which are likely to result from chromosomal rearrangements following homologous-recombination mediated replication restart; reviewed by (Carr and Lambert, 2013). Whether the DNA structure checkpoint affects SIN protein localisation will be the subject of future studies.

The spindle checkpoint (SAC) affects Cdc7p-GFP SPB-association in anaphase

Previous studies have suggested that the onset of asymmetry of Cdc7p-GFP in anaphase B is delayed by the presence of unsegregated DNA (Mayer et al., 2006). To investigate this further, we examined mutants in which lagging chromosomes are observed on an extended anaphase spindle.
We first analysed pcs1-D, which ablates the monopolin complex, and generates merotelically attached chromosomes (Gregan et al., 2007). Examination of pcs1-D cdc7-GFP cnp1-CHY cells revealed that the timing with which Cdc7p-GFP became asymmetric was similar, whether the cells had a lagging kinetochore or not (figure 7A, 7B).

The kinesin8 Klp5p is required for microtubule–kinetochore attachment, chromosome congression in metaphase (Garcia et al., 2002a; Garcia et al., 2002b; West et al., 2002) and SAC silencing (Meadows et al., 2011). In klp5-D cdc7-GFP cnp1-CHY cells (LF>0.5), the Cdc7p-GFP signal was symmetric in cells with long spindles (LF>0.5) that had lagging kinetochores, and asymmetric in those that did not (figure 7C, 7D).

The apparent contradiction between these mutants may lie in the fact that the SAC is activated in klp5-D (Garcia et al., 2002b), but not in pcs1-D (Gregan et al., 2007). Analysis of klp5-D cdc7-GFP cnp1-CHY pcp1-CHY mad2-D showed that the percentage of cells with asymmetric Cdc7p-GFP in LF>0.5 was similar whether lagging kinetochores were present or not (figure 7E, 7F), and deletion of mad2 did not affect the onset of asymmetry (figure 7G, 7H). These data indicate that the delay in establishing the asymmetry of Cdc7p-GFP in cells that have lagging kinetochores is due to activation of the SAC.

**Discussion**

**A “two state” representation of the SIN**

This study has revealed several novel aspects of SIN regulation. The data point to the existence of two states of the SIN during mitosis, which are referred to herein as “early” and “late”.

The early state is typified by a mixture of symmetric, transition and asymmetric signals and corresponds to LF<0.5. Many SIN protein signals are positionally mutable, meaning that the signal intensity varies at a given location during continuous observation.

The late state is characterised by the asymmetric association of Cdc7p, Sid1p, Byr4p and Cdc16p with one SPB in >80% of cells, and a positionally stable signal, meaning that it is seen at one principal location; this corresponds to LF≥0.6 in wild-type cells. The intensity of the SPB-signal is greater in the late than early state. This is summarised in figure 7I.

**What regulates the early-late transition?**

The data presented here show that the control governing the transition between the states of the SIN scales with cell size, and can be altered. The transition is inhibited by active SAC. Previous studies have shown that Cdc2p inactivation promotes the transition to the asymmetric state of the SIN.
(Chang et al., 2001; Dischinger et al., 2008; Yamano et al., 1996), suggesting that the SAC effect results from delayed inactivation of Cdc2p.

Deletion of the DYRK family protein kinase pom1, accelerated the transition to the late state of the SIN, implying the Pom1p inhibits it. It is interesting to note that mutation of Sid2p sites on Cdc11p also result in an earlier transition to Cdc7p-GFP asymmetry (Feoktistova et al., 2012); it will be of interest to determine whether Pom1p regulates SIN asymmetry via Cdc11p.

Ablation of the 14-3-3 protein Rad24p delayed the onset of asymmetry, though not as significantly as loss of Sid2p, SIP, or GAP functions; the other 14-3-3 protein Rad25p may compensate for the loss of Rad24p. A non-exclusive alternative is that not all the effects of Sid2p are mediated via the creation of 14-3-3 binding sites. After reduction of GAP function (cdc16-116), the intensity of the Cdc7p-GFP signal reached late-anaphase levels earlier than in wild-type cells, indicating that the GAP proteins also inhibit the transition from the early to the late state.

Deletion of the principal B’ regulatory subunit Par1p delayed the onset of Cdc7p asymmetry, consistent with previous studies conducted by indirect immunofluorescence (Jiang and Hallberg, 2001). Previous studies have shown that mutation of the regulatory B-subunit Pab1p does not affect the appearance of Cdc7p-GFP at one SPB in anaphase (Lahoz et al., 2010), suggesting that that PP2A-Par1p is involved in promoting the asymmetry of Cdc7p-GFP in anaphase. Previous studies have implicated other PP2A complexes and regulators in promoting the asymmetry of Cdc7p (Goyal and Simanis, 2012; Singh et al., 2011).

A minor population of cells showed asymmetric Cdc7p signals in the absence of GAP or SIP function. This may indicate that the individual proteins that influence asymmetry cooperate to bring about asymmetry. In this context, it is noteworthy that there is a negative genetic interaction between a SIP complex null mutant and cdc16-116 (Singh et al., 2011), and PP2A subunits and cdc16-116 (Goyal and Simanis, 2012; Le Goff et al., 2001).

Previous studies have indicated that Etd1p is required both for maintenance of Cdc7p-GFP at the SPBs in anaphase and for the intensity increase of Cdc7p-GFP in anaphase (Alcaide-Gavilan et al., 2013; Daga et al., 2005; Lahoz et al., 2010). Though these studies differ qualitatively in their conclusions, they both implicate Etd1p in establishing the late state of the SIN. Etd1p interacts with Spg1p in vitro, (Garcia-Cortes and McCollum, 2009), suggesting that they may cooperate to establish this state of the SIN (figure 7I).

Compromising plo1 function has multiple effects upon the behaviour of SIN proteins. Association of Cdc7p-GFP with the SPB is abolished in early mitosis, but it then appears at one SPB in anaphase. These data imply that association of Cdc7p-GFP with the SPB in early mitosis is not required for its asymmetric SPB-association in anaphase, suggesting that these events may be controlled differently, and perhaps independently. Reduced Plo1p activity resulted in a more asymmetric SPB-association of
Mob1p-GFP in the early state of the SIN. The penetrance was incomplete, which may reflect differences in SPB age, or the hypomorphic \textit{plo1-ts4} allele. The different effects of \textit{plo1-ts4} upon Mob1p-GFP and Cdc7p-GFP may reflect differences in how SPB-association of these two proteins is mediated.

Cdc7p-GFP did not show a significant increase in asymmetric signals in LF\textgreater0.6, in \textit{spg1-B8} at 36°C, retaining a distribution similar to early mitosis. The intensity of the Cdc7p-GFP signal was similar with and without Spg1p until LF0.5, after which the signal became brighter in the \textit{spg1}+ cells, and fainter in the \textit{spg1-B8} cells. This is consistent with the idea that establishment and/or maintenance of the late state requires Spg1p.

Earlier studies showed that there is no SPB-associated Cdc7p signal in \textit{spg1-B8} cells in the second mitosis at the restrictive temperature (Sohrmann et al., 1998). It was therefore surprising to find that inactivation of Spg1p did not compromise the association of Cdc7p-GFP in the early stages of mitosis in the first cycle after shift to the restrictive temperature.

Since Cdc7p associates directly with the scaffold protein Cdc11p (Feoktistova et al., 2012), as well as Spg1p (Mehta and Gould, 2006; Schmidt et al., 1997), these data could be explained if the association of Cdc7p with the SPB before anaphase depends principally upon binding to Cdc11p. The subsequent absence of Cdc7p from the SPBs in the second mitosis could be explained by postulating that Cdc7p cannot reload to the SPB unless the SIN has signalled during the first mitosis. This view is supported by previous studies showing that Byr4p is not seen on all interphase SPBs in tetranucleate SIN mutant cells (Li et al., 2000), and that functional Sid2p is required to establish SIN asymmetry in anaphase (Feoktistova et al., 2012). The first-cycle block to septation in \textit{spg1-B8}, and the fact that Spg1-B8p-GFP is not SPB-associated (AK and VS, unpublished data, and (Krapp et al., 2008)), argue that SIN signalling is not functional. Nonetheless, we cannot formally exclude that \textit{spg1-B8} is a hypomorphic allele and that the presence of the Cdc7p of the SPB in the first mitosis reflects residual Spg1-B8p function. Future analysis of \textit{spg1-D} will resolve this.

In summary, the “early” state of the SIN requires Plo1p, but not Spg1p for establishment. In contrast, Spg1p becomes important after LF0.5, when it is required to generate Cdc7p-GFP asymmetry and to maintain a robust asymmetric Cdc7p-GFP signal at the SPB.

Analysis of the \textit{S. cerevisiae} Mitotic Exit Network (MEN) has shown that CDC15p (Cdc7p) requires inputs from both CDC5p (Plo1p) and TEM1p (Spg1p) for SPB-association in anaphase (Rock and Amon, 2011). Though the SIN and MEN is differ in their “wiring” (reviewed by (Simanis, 2003; Weiss, 2012), this points to a conserved role for Spg1p/TEM1p and Plo1p/CDC5p in regulating SPB-association of Cdc7p-family kinases.
**Spatial instability of SIN proteins in the early state.**

A defining characteristic of the early state is spatial instability of the signals of some SIN proteins. The GFP-Sid1p was unstable in the early stages of mitosis, in the sense that its location varied between images, appearing at one, two or no SPBs, before finally stabilising at the nSPB in anaphase. No significant signal was seen in most slp1-deficient cells, indicating that the APC/C\(^{Slp1p/Cdc20p}\) must function for GFP-Sid1p to associate stably with the SPB.

Spatial instability of the Cdc7p signal was also observed in early mitosis, and has recently been observed in anaphase in a byr4 mutant that cannot be phosphorylated by Cdc2p (Rachfall et al., 2014). Interestingly, Mob1p-GFP showed much less variability in the early state than Cdc7p-GFP, GFP-Sid1p or GFP-Byr4p, suggesting that it may be regulated differently.

The reason for the instability of the early SIN is presently unclear; several non-exclusive possibilities may be considered. In *S. cerevisiae*, the MEN-effector CDC14p is activated in a two-step process; the FEAR network promotes transient release from the nucleolar RENT complex, priming it for activation. The release becomes definitive following activation of the MEN, and promotes mitotic exit. If the MEN is not activated, CDC14p release is only transient; reviewed by (Weiss, 2012). This bears some similarity with the unstable SPB association of SIN proteins, particularly GFP-Sid1p. It is therefore possible that the early state of the SIN reflects “preparatory” events prior to its full activation at the end of anaphase B. Since the activity of all the core SIN proteins and scaffolds is required for CAR assembly, the early state may be a manifestation of this function.

Laser ablation of the nSPB in anaphase results in increased Cdc7p-GFP association with the oSPB, and it has been proposed that this indicates feedback between the SPBs in anaphase, so that each maintains its character with respect to SIN proteins (Magidson et al., 2006). The flickering of the SIN signals earlier in mitosis may reflect the initiation of these feedback loops. It is also possible that the identity of the SPBs with regard to SIN protein association is less well defined (or less respected) in metaphase than anaphase.

The spatial instability might also reflects vetoing of premature attempts to initiate SIN signalling by mitotic checkpoints. The SAC can be reactivated even after proteolysis of cyclin B begins (Clute and Pines, 1999), so the unstable SIN-protein signals may reflect SAC reactivation following transient chromosome detachment from the spindle. The changes may also be produced by the early mitotic casein kinase I/dma1p-dependent checkpoint (Johnson et al., 2013).

Regulators of the GTPase Cdc42p show periodic oscillations between the cell’s tips. This is governed by a combination of feedback loops (Das et al., 2012). Since there is no clear periodicity in the fluctuations GFP-Sid1p signal, it seems unlikely that these mechanisms govern the appearance of GFP-Sid1p at the SPBs in early mitosis.
Asymmetric SPB-association of Cdc7p at mitotic entry.

This study has revealed that the initial SPB-association of Cdc7p-GFP is asymmetric in ~80% of cells, biased 3:1 to the nSPB. SPB duplication is conservative, and analysis of Fin1p has suggested that SPBs mature fully over two cell cycles (Grallert et al., 2004). Since Cdc7p-GFP associates asymmetrically with the SPBs during spindle formation, the choice of SPB may reflect the SPB’s age.

Asymmetric association of Cdc7p/Sid1p with the nSPB is widely considered to represent “active” SIN signalling. However, our data indicate that association of Cdc7p-GFP in anaphase is insufficient to promote cytokinesis if Plo1p activity is reduced, even though ectopic SIN activation places spg1/cdc7 downstream of plo1 (Krapp et al., 2003; Tanaka et al., 2001). This may be because the level of signalling that can be achieved is low compared to constitutive activation of Spg1p in cdc16-116. Alternatively, since the SIN cooperates with Mid1p in CAR assembly (Hachet and Simanis, 2008; Huang et al., 2008), failure of an early mitotic Plo1p/Cdc7p-dependent event may prevent cytokinesis.

Is Cdc7p asymmetry mediated by regulation of Byr4p?

The data presented here also show that GFP-Byr4p disappears transiently from both SPBs as cells progress into anaphase, and then reappears on the oSPB. This may account for the failure to detect Byr4p at SPBs in cells with metaphase-length spindles in some studies (Johnson and Gould, 2011; Li et al., 2000; Rachfall et al., 2014). However, if the APC/C<sup>Slp1p/Cdc20p</sup> is blocked, then GFP-Byr4p is still seen at the SPBs, though the signal much less intense than in anaphase, indicating that its removal from the SPBs requires APC/C<sup>Slp1p/Cdc20p</sup> function. This is also consistent with SPB-localisation of Byr4p by indirect immunofluorescence in nda3-KM311 arrested cells (Krapp et al., 2003).

Previous studies of SIN proteins in fixed cells revealed the presence of early mitotic cells in which Cdc7p was detected at both SPBs, while Byr4p was present on one SPB (Cerutti and Simanis, 1999). Consistent with this, the GFP-Byr4p signal becomes asymmetric before Cdc7p-GFP. The decrease in intensity at the oSPB initiated before any increase was seen at the nSPB. This is consistent with a model in which the initiation of asymmetry in wild-type cells is mediated by regulation of Byr4p, and begins at the oSPB. The transition between the two SIN states requires the action of the APC/C<sup>Slp1p/Cdc20p</sup>. It is presently unclear whether this is due to the direct action of the APC/C<sup>Slp1p/Cdc20p</sup>, or whether it is due to a downstream event.

GFP-Byr4p remained associated with both SPBs throughout mitosis in plo1-ts4 cells, indicating that the asymmetric distribution of Byr4p to the oSPB requires Plo1p function. This is consistent with data showing that Cdc2p and Plo1p cooperate to regulate Byr4p localisation (Rachfall et al., 2014), and poses an interesting conundrum; if removal of Byr4p from the SPB is required for association of
Cdc7p with the SPB in anaphase (Rachfall et al., 2014), then how does Cdc7p associate with the SPBs in \textit{plo1-ts4}? It is possible that removal of Byr4p from the SPB is not required for Cdc7p to associate with it if Plo1p has not made modifications at the SPB earlier in mitosis. Alternatively, since there are \(\approx 400\) molecules of Cdc7p-GFP at the nSPB in late mitosis (Wu and Pollard, 2005), it is likely that there are a similar number of signalling scaffold complexes at the SPB. The presence of both Cdc7p-GFP and Byr4p-GFP in these cell populations may therefore reflect heterogeneity of the complexes at the SPBs in the \textit{plo1-ts4} mutant.

**Summary**

The data presented in this study are consistent with the notion of two, genetically separable, states of the SIN; state one which is dependent upon Plo1p for its establishment, and state two, which is dependent upon Etd1p and Spg1p. Future studies will investigate how the transition occurs.

**Legends**

**Figure 1: Analysis of the distribution of Cdc7p-GFP in wild-type and mutant cells**

**Panel A:** Images of the indicated strain were captured at 1min intervals. The yellow asterisks indicate early mitotic cells in which the intensity ratio of Cdc7p-GFP at the SPBs differs significantly. The blue dot indicates anaphase B onset. The scale bar represents \(10\) \(\mu\)m.

**Panel B:** \textit{cdc7-GFP pcp1-CHY} cells were filmed through mitosis after synchronisation. The intensity of the Cdc7p-GFP signal at the oSPB and nSPB was determined, and plotted against time. The plots for multiple cells were aligned using the increase of intensity at the nSPB. For clarity, the data for oSPB and nSPB are plotted on separate axes. Note that the “wishbone” as the SPBs separate is not symmetrical. The dotted lines indicate the region of the graph used to calculate the rate of change.

**Panel C:** Left: Cartoon representation of “length fraction”. To facilitate comparison between cells of different length, spindle length is expressed as a fraction of cell length. Right: The indicated strain was analysed as described in materials and methods. The ordinate indicates the length fraction and the abscissa the fraction of cells in which the ratio of the SPB-associated signals are symmetric, asymmetric, transition, or lacking a detectable signal. The colour key is shown underneath. LF0.1 indicates \(0.1 \leq LF < 0.2\). The bars labelled “early” and “late” indicate the SIN states proposed in the discussion.

**Panel D:** Histogram representing the initial association of Cdc7p-GFP with the SPB (LF<0.1) in wild-type and selected mutant cells. The colour code is the same as panel B.

**Panel E:** \textit{Nmt41-slp1} cells expressing \textit{atb2-mcherry} (hereafter \textit{atb2-CHY}) and \textit{cdc7-GFP} were arrested as described in materials and methods. The scale bar represents \(10\) \(\mu\)m.

**Figure 2: Analysis of Cdc7p-GFP distribution in wild-type and mutant cells.**
Cells of the indicated strains were synchronised and analysed at the indicated temperatures as described in materials and methods.

Panels A-F, Panel I: analysis of Cdc7p-GFP. Panel A is identical to figure 1B, and is re-presented here for convenience.

Panels G, H: Plot of the intensity of Cdc7p-GFP at the brighter SPB.

Cells were synchronised and samples were taken for analysis as they progressed through mitosis. The intensity (arbitrary units ; AU) is plotted on the ordinate, LF on the abscissa. The box shows 25%-75% range for the population, the line indicates the median. The bars indicate 10% and 90% range for the population, and dots indicate more extreme individual values.

Figure 3: Analysis of SIN proteins in mitosis and effect of loss of plo1 function.

The marked strains were analysed at the indicated temperature. The graphs are displayed according to the legend in figure 1.


Cells were synchronised and images were taken every minute as cells progressed through mitosis. The blue dot indicates anaphase onset. Note the absence of Cdc7p-GFP in early mitosis, followed by the asymmetric appearance of Cdc7p-GFP in anaphase B. Scale bar represents 10μm.

Panel B: analysis of Cdc7p-GFP in plo1-ts4 at 36°C; the wild-type control is shown in figure 2B.

Panel C: plo1-ts4 cdc7-GFP pcp1-CHY cnp1-CHY cells were mixed with plo1+ cdc7-GFP pcp1-CHY hht1-CFP cells, synchronised, incubated at 36°C and images were taken in mitosis. Note the presence of a Cdc7p-GFP signal on the SPBs of the plo1+ cell (blue nuclear signal) and its absence from the plo1-ts4 cell.

Panel D: plot of the Cdc7p-GFP signal in the indicated mutants. For details see figure 1G.

Panel E: Analysis of Mob1p-GFP. Cells were synchronised and images were taken every minute as cells progressed through mitosis. Note the transient fluctuation of signal intensity in frames 11, 23 and 24. The blue dot indicates anaphase onset. The scale bar represents 10μm.

Panels F, G, H. The distribution of Mob1p-GFP was analysed in the indicated strains.

Figure 4: Analysis of GFP-Sid1p and Cdc16p-GFP

Panel A: Localisation of GFP-Sid1p during mitosis. Cells were synchronised and images were taken every minute as cells progressed through mitosis. The scale bar represents 10μm. The blue dot indicates anaphase B onset. In frames 10-29 the red asterisks indicate the presence of GFP-Sid1p on the SPB.

Panel B, C, and D: Cells were synchronised and the intensity of GFP-Sid1p was analysed.

Panel E: The indicated strain was arrested as described in materials and methods. Note the absence of GFP-Sid1p from the SPB. The scale bar represents 10μm.
Panel F: The indicated strain was synchronised and the intensity of Cdc16p-GFP was analysed.

Panel G: Localisation of Cdc16-GFP. Cells were synchronised and images were taken every minute as cells progressed through mitosis. The scale bar represents 10µm. The blue dot indicates anaphase B onset.

Figure 5: Analysis of GFP-Byr4p in wild-type and mutant cells.

Panel A: Cells were imaged at one minute intervals. The scale bar represents 10µm, the blue dot represents anaphase B onset. Note that the GFP-Byr4p signal is initially present on both SPBs, and then decreases in intensity. From frame 9 onwards, the red asterisks indicate SPBs with a GFP-Byr4p signal. A faint signal can also be detected at the other SPB in frames 11 to 15. The signal is very faint or undetectable from frame 17 to 30; it reappears thereafter on one SPB. Note that the other SPB reacquires a signal from frame 36 onwards. See text for details.

Panels B, E and G. The indicated strains were synchronised and the distribution of GFP-Byr4p on the SPBs was analysed.

Panels C and D. The indicated strain was arrested as described in materials and methods. Note the presence of a weak SPB-associated GFP-Byr4p signal (C); the signal intensity in the arrested cells is compared to that observed at the brightest SPB in anaphase (D).

Panels F and H. The intensity of SPB-associated GFP-Byr4p is plotted.

Figure 6: Analysis of Cdc7p-GFP in wild-type and mutant cells.

The intensity of Cdc7p-GFP was analysed in the indicated strains. The 25°C wild-type control (panel A) is duplicated from figure 1, for convenience. Par1-D was analysed using asynchronous cultures, as its abnormal shape and asymmetric division preclude efficient synchronisation by elutriation. HU indicates that cells were arrested by addition of HU to 12mM for 5h. Cells were analysed in the first mitosis after removal of HU.

Figure 7: Analysis of Cdc7p distribution in anaphase in cells with lagging chromosomes and data summary.

Panels A-H. The strains were analysed at the indicated temperature. For clarity only LF>0.4 are shown.

Panel I. Cartoon summarising the two state model of the SIN. In the early state (yellow), the proteins are positionally mutable, while in the late state, their position is fixed, and asymmetric. Factors which promote these states (arrow) or inhibit (ball) the transition between them are shown in boxes above. See text for additional details. The lower part shows cartoons of S. pombe cells at different stages of mitosis.
Materials and Methods

Standard techniques were used for the growth and manipulation of fission yeast (Moreno et al., 1991). Unless indicated otherwise, cells were grown in yeast extract (YE) medium. Briefly, cells were grown in liquid medium to a density of 3.5–6x10⁶ cells per milliliter and centrifugal elutriation (Beckman JS-5.0 system) was used to isolate G2 cells (Schmidt et al., 1997). Cells were concentrated by filtration to minimise stress. After 1h recovery, the first and second mitoses after elutriation were imaged. There was no significant difference whether the data were collected from cells in the first or second cycle. Data from biological repeats of a given cell type was also found to be statistically not significantly different, so data from multiple experiments have been pooled to generate the graphs presented for each marker or mutant. The majority of GFP-tagged strains used in this study have been described previously; see (Krapp and Simanis, 2014) and references therein. Strains expressing slp1⁺ from the nmt41 promoter were grown in EMM2 minimal medium without thiamine, supplemented with amino acids (100mg/l) as required. To repress slp1 expression, the cells were incubated for 5h in 29°C in YE medium (Petrova et al., 2013). For hydroxyurea arrest-release, HU was added to exponentially growing cells to 12mM. After 5h, cells were released from the arrest by washing twice in medium.

Microscopy

Living cells were imaged using a U-Plan-S-Apo 60× N.A. 1.42 objective lens mounted on a Perkin-Elmer or an Olympus IX-81 spinning disc confocal microscope. The temperature was maintained using a custom-built heating system. The presence of a cytoplasmic signal of Cdc7p-GFP limits the intensity difference estimations to approximately 10-fold, as the signal associated with the oSPB reaches the cytoplasmic background.

Image analysis and processing

Images analysed using RodCellJ (Schmitter et al., 2013). The background was set by averaging the cytoplasmic signal in >100 mitotic cells. Only signals that were ≥ the 90% confidence interval were considered significant. For analysis, these background values were subtracted from the intensity values of SPBs. Images were processed in ImageJ and Adobe Photoshop CS6, and assembled in Powerpoint.

Statistical analysis

The statistical analysis was performed using GraphPad Prism v6.03. For comparison between strains, data from at least 2 experiments (n>500 cells) were pooled and binned for the length fractions. The distribution of ratios of SPB intensities was compared for each length fraction using 2-tailed non-parametric Mann-Whitney test.
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References


A

$c\text{dc7-GFP}$ cnp1-CHY pcp1-CHY

B

$c\text{dc7-GFP}$, 25°C

C

D

Initial loading of $c\text{dc7-GFP}$

E

$c\text{dc7-GFP}$ CHY-atb2 slp1-OFF

cdc7-GFP

atb2-CHY