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RESEARCH ARTICLE

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

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ABSTRACT

The *Schizosaccharomyces pombe* septation initiation network (SIN) regulates cytokinesis, and asymmetric association of SIN proteins with the mitotic spindle pole bodies (SPBs) is important for its regulation. Here, 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, that the association of Cdc7p with the SPBs in early mitosis is frequently asymmetric, with a bias in favour of the new SPB; second, that the early association of Cdc7p–GFP to the SPB depends on Plo1p but not Spg1p, and is unaffected by mutations that influence its asymmetry in anaphase; third, that Cdc7p asymmetry in anaphase B is delayed by Pom1p and by activation of the spindle assembly checkpoint, and is promoted by Rad24p; and fourth, that 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.

KEY WORDS: *S. pombe*, Cytokinesis, 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) and acts 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 nuclear Dbf2-related (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 multiseptated cells (Minet et al., 1979). Ectopic activation of SIN signalling induces CAR and septum formation during 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). Association of SIN proteins to the SPB plays an important part in the 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 the GTPase-activating protein (GAP) Cdc16p that interacts with Spg1p through the scaffold Byr4p, (Fankhauser et al., 1993; Furge et al., 1998; Minet et al., 1979; Song et al., 1996), Etd1p (Daga et al., 2005; García-Cortés and McCollum, 2009; Lahoz et al., 2010) and the conserved kinase Plo1p (Rachfall et al., 2014; Tanaka et al., 2001). Immunoelectron microscopy has shown that Ppc89p, Sid4p (Rosenberg et al., 2006) and Sid2p (Sparks et al., 1999) are localized at 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; García-Cortés 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), whereas 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 (García-Cortés and McCollum, 2009; Singh et al., 2011; Sohrmann et al., 1998). If

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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 and that 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 mCherry-tagged Pcp1p (Pcp1p–CHY hereafter) to visualise SPBs, and the kinetochore marker mCherry-tagged Cnp1p as required (Cnp1p–CHY hereafter) (Alvarez-Tabarés 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; Fig. 1C). The raw data were divided into ten bins with a step size of LF0.1. To facilitate the analysis and presentation of the data, three states were defined; symmetry as $\leq$2-fold difference between the GFP signals on the two SPBs; transition as $>$2-fold but $<$4-fold; asymmetric as $\geq$4-fold difference.

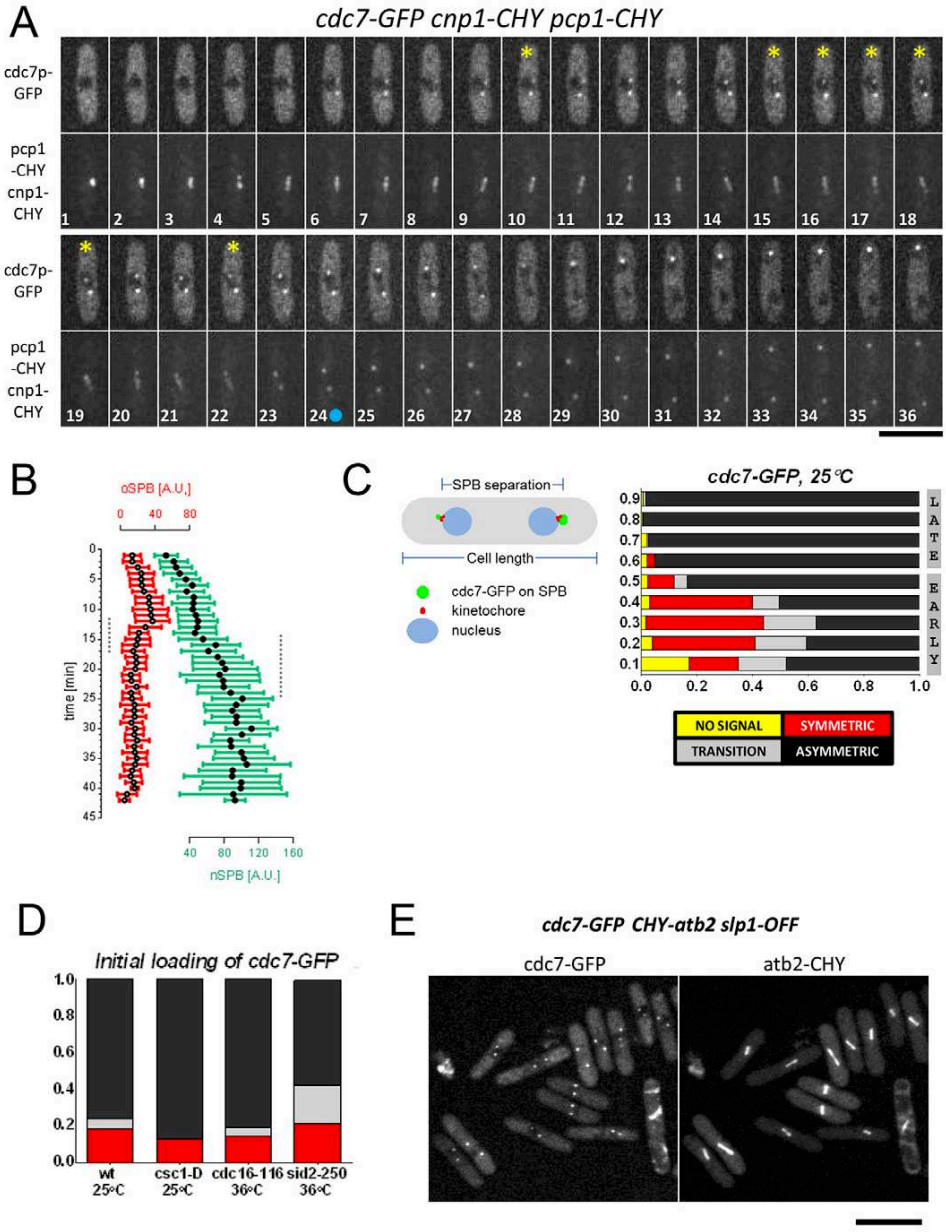

Fig. 1. Analysis of the distribution of Cdc7p–GFP in wild-type and mutant cells. (A) Images of the indicated strain were captured at 1-min intervals. The yellow asterisks indicate early mitotic cells in which the intensity ratio of Cdc7p–GFP at the SPBs differs significantly. The blue dot indicates anaphase B onset. (B) *cdc7-GFP pcp1-CHY* cells were filmed through mitosis after synchronisation. The intensity of the Cdc7p–GFP signal at the oSPB (red) and nSPB (green) 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. (C) Left, cartoon representation of 'length fraction' (LF). 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 the 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$<$0.2. The bars labelled 'early' and 'late' indicate the SIN states proposed in the discussion. (D) Histogram representing the initial association of Cdc7p–GFP with the SPB (LF$<$0.1) in wild-type and selected mutant cells. The colour code is the same as B. (E) *Nmt41-slp1* cells expressing mCherry-tagged Atb2 (hereafter Atb2-CHY) and Cdc7–GFP were arrested as described in the Materials and Methods. Scale bars: 10 μm.

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

Sid4p–GFP, Cdc11p–GFP, Spg1p–GFP and Cdc7p–GFP

The SPB-associated signals of Cdc11p–GFP (supplementary material Fig. S1A) and Sid4p–GFP (supplementary material Fig. 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 (supplementary material Fig. S1C); its intensity increased 1.5-fold at both SPBs during mitosis (supplementary material Fig. S1D,E). Cdc11p–GFP showed a similar increase, whereas 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) (Fig. 1A).

As cells progressed through mitosis, the Cdc7p–GFP signal at the oSPB decreased, whereas the nSPB brightened (Fig. 1B), consistent with previous studies (Cerutti and Simanis, 1999; García-Cortés 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 min 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 (Fig. 1C). At LF0.6 (≈8.5 µm spindle in a 14 µm 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%); two cells (6%) had signals on both SPBs, with asymmetric intensity, and six cells (18%) showed a symmetric signal. Cdc7p–GFP associated with the nSPB first in 21/27 cells (78%) (Fig. 1D). Examples of Cdc7p–GFP loading first to the nSPB (supplementary material Fig. S1F) and oSPB (Fig. 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 (Fig. 1A) but not all cells (supplementary material Fig. S1F), which might explain why ∼40% of cells in single timepoint images showed a significantly asymmetric signal in early mitosis (Fig. 1C; LF0.2 to 0.3, corresponding to SPB separation of ∼3–4 µm in a 14 µm wild-type cell). This might not have been observed previously because they were performed by indirect immunofluorescence (Sohrmann et al., 1998).

Given that Cdc7p–GFP asymmetry occurs in anaphase, a conditionally expressed allele of *slp1* (also known as *CDC20*) (Petrova et al., 2013) was used to investigate whether SPB association of Cdc7p–GFP requires the anaphase-promoting complex/cyclosome containing Slp1p (APC/C^{Slp1p}). Expression of *slp1* was silenced in the strain *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; Fig. 1E); 85% of these showed a symmetric signal (LF0.1 and 0.2; data not shown). Given that the initial SPB association of Cdc7p–GFP is often asymmetric, the difference between these data and those in Fig. 1C might have arisen 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 seven of eight *csc1-D* cells examined (Fig. 1D). Cdc7p–GFP remained symmetric in the majority of cells in the later stages of mitosis, as expected (Fig. 2C), although 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 (data 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 Fig. 2B,D). As cells entered mitosis 17 of 21 *cdc16-116* cells (79%) showed an asymmetric SPB association of Cdc7p–GFP with the SPBs, one cell (5%) showed association with both SPBs, with a stronger signal on one pole than the other, and three cells (14%) showed a symmetric signal (Fig. 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 (data not shown). Given that the Cdc7p–GFP signal is symmetric in the majority of *cdc16-116* cells in anaphase compared to Cdc7p–GFP in wild-type cells at 36℃, 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 (Fig. 2G; LF0.1–0.4).

The early loading of Cdc7p–GFP was still asymmetric in *sid2-250* cells at 36℃ (eight of 14 cells), indicating that Sid2p is not obligatory for the asymmetric early loading of Cdc7p–GFP (Fig. 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℃ (Fig. 2E) and 36℃ (Fig. 2F). The intensity increased at both SPBs until LF0.3 and then plateaued (Fig. 2H). Taken 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, although 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 be necessary to 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℃ (compare Fig. 2I with Fig. 2B). In late mitosis, the intensity of the signal at the nSPB became fainter, rather than brighter (Fig. 2H), and the signal remained symmetrical in >30% of cells (Fig. 2I). Cytokinesis failed in the mutant cells, indicating that

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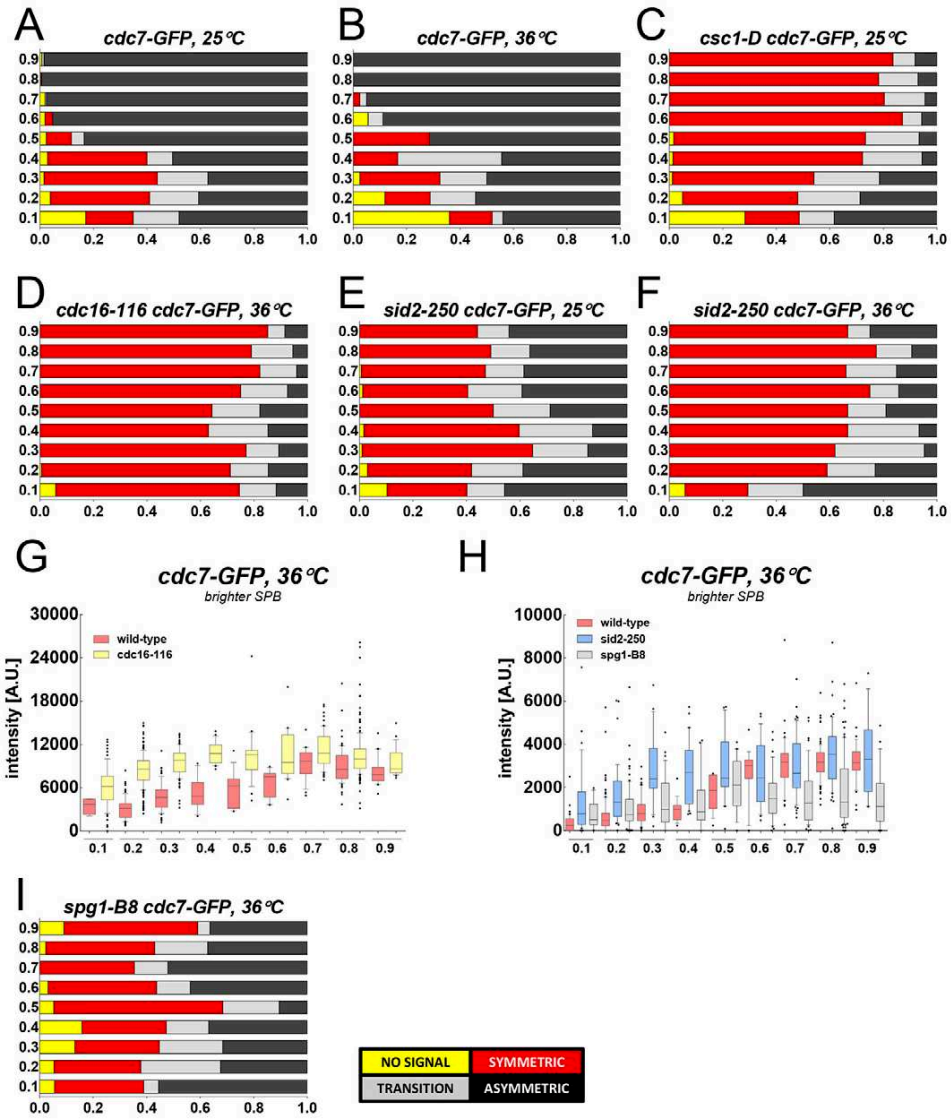

Fig. 2. Analysis of Cdc7p–GFP distribution in wild-type and mutant cells. Cells of the indicated strains were synchronised and analysed at the indicated temperatures as described in the Materials and Methods. (A–F, I) Analysis of Cdc7p–GFP SPB localisation presented as described for Fig. 1C. A is identical to Fig. 1B, and is re-presented here for convenience. (G,H) Box plots 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 (A.U., arbitrary units) 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.

Spg1p had been inactivated (data not shown). If cells were incubated for 5 h at 36°C before analysis, we did not observe any SPB-associated Cdc7p–GFP signal (data 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.

The kinase POLO is required for Cdc7p–GFP SPB association in early mitosis but not in anaphase

The conserved POLO-like kinase Plo1p has multiple roles during mitosis (Bähler 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 [Fig. 3A,B; 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) (data not shown). Mixing of early mitotic *cdc7-GFP* and *cdc7-GFP plo1-ts4* cells at 36°C showed that Cdc7p–GFP was detectable in wild-type cells at 36°C (Fig. 3C). However, by LF0.6, more than 80% of cells had an asymmetric signal with Cdc7p–GFP on one SPB in anaphase B (compare Fig. 3B with Fig. 2B). The signal intensity in anaphase was comparable to wild-type (Fig. 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

The Mob1p–Sid2p complex (Hou et al., 2000; Salimova et al., 2000) was examined using *mob1-GFP* (Salimova et al., 2000), because *sid2-GFP* (Sparks et al., 1999) is functionally compromised

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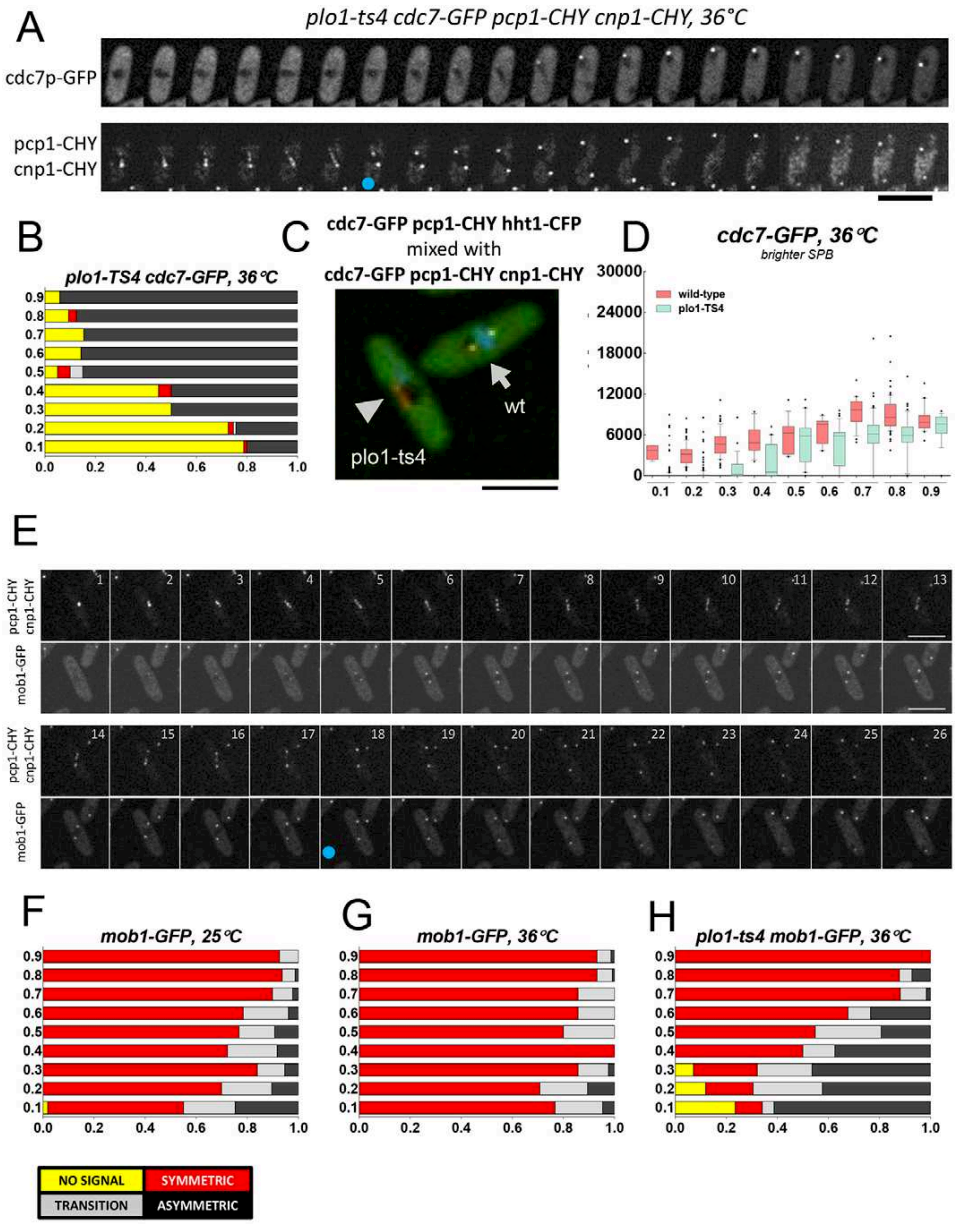

Fig. 3. Analysis of SIN proteins in mitosis and effect of loss of *plo1* function. The marked strains were analysed at the indicated temperature. The graphs in B and F–H are presented as described for Fig. 1C. (A) Localisation of Cdc7p–GFP in *plo1-ts4* cells. Cells were synchronised and images were 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. (B) Analysis of Cdc7p–GFP in *plo1-ts4* at 36˚C; the wild-type control is shown in Fig. 2B. (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. (D) Box plot of the Cdc7p–GFP signal in the indicated mutants presented as described in Fig. 2G. (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. (F–H) The distribution of Mob1p–GFP was analysed in the indicated strains. Scale bars: 10 μm.

(Grallert et al., 2012; Salimova et al., 2000). Mob1p–GFP associated with both SPBs in most cells, exceeding 98% by LF0.7 (Fig. 3F,G), consistent with previous 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 (Fig. 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 Fig. 3H to Fig. 3G). Furthermore, no signal was detected in ~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 (Fig. 4A–C), 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 (Fig. 4B,C). Filming revealed that the association of GFP–Sid1p with the SPB was spatially unstable in early mitosis, with a signal seen at one SPB, both SPBs or not at all, in sequential frames (Fig. 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

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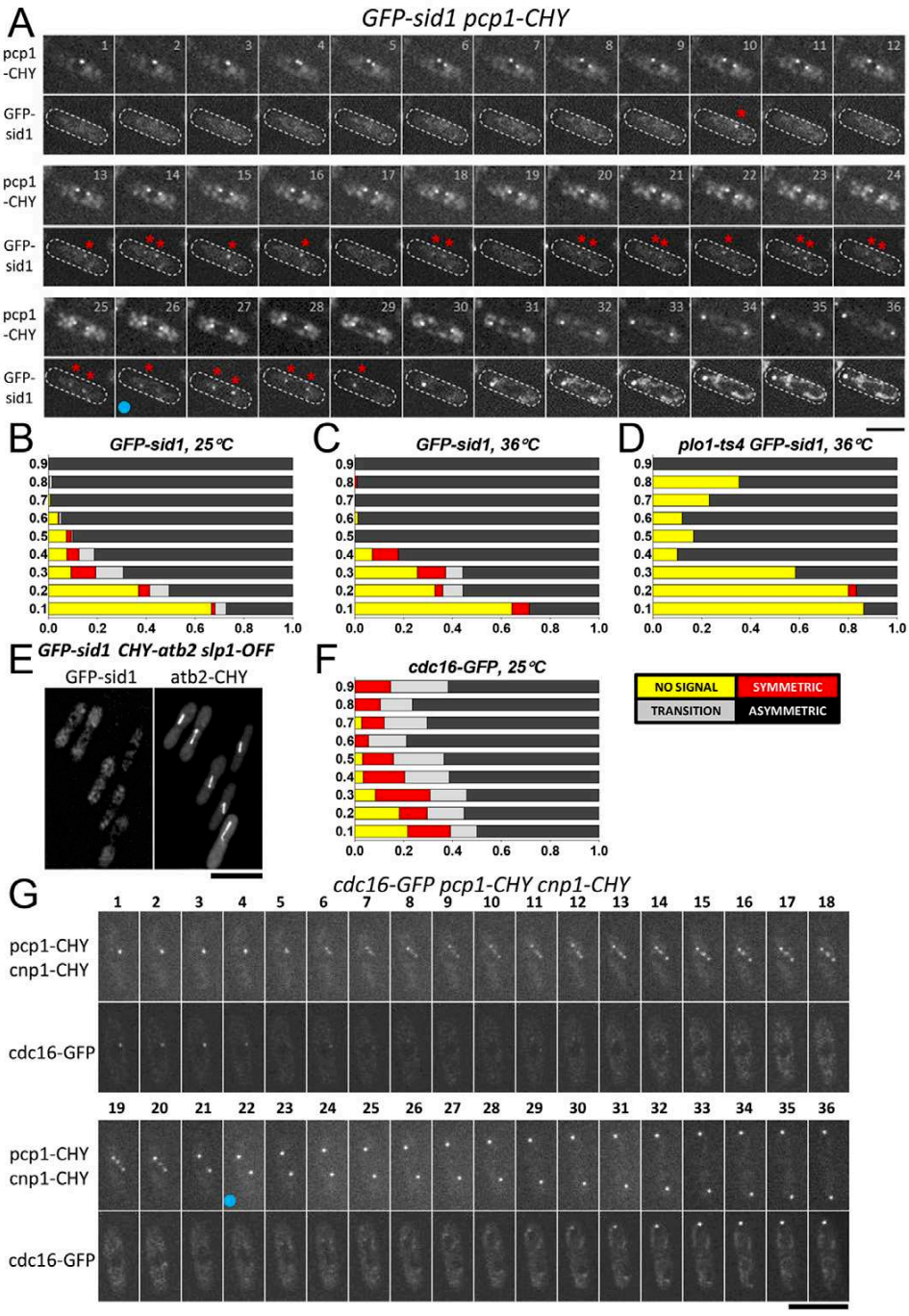

Fig. 4. Analysis of GFP–Sid1p and Cdc16p–GFP. (A) Localisation of GFP–Sid1p during mitosis. Cells were synchronised and images were taken every minute as cells progressed through mitosis. The blue dot indicates anaphase B onset. In frames 10–29, the red asterisks indicate the presence of GFP–Sid1p on the SPB. (B–D) Cells were synchronised and the intensity of GFP–Sid1p was analysed. The graphs are presented as described for Fig. 1C. (E) The indicated strain was arrested as described in the Materials and Methods. Note the absence of GFP–Sid1p from the SPB. (F) The indicated strain was synchronised and the intensity of Cdc16p–GFP was analysed. The graph is presented as described for Fig. 1C. (G) Localisation of Cdc16–GFP. Cells were synchronised and images were taken every minute as cells progressed through mitosis. The blue dot indicates anaphase B onset. Scale bars: 10 μm.

mitotic cells, although not an unstable SPB association, have been 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 (Fig. 4D; supplementary material Fig. S2A). GFP–Sid1p was absent from the SPBs in most *slp1-OFF* cells [Fig. 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} 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 (Fig. 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 Cdc16p–GFP signal. The fraction of cells with an asymmetric signal increased as cells progressed through mitosis (Fig. 4F).

Filming of *GFP-byr4* cells revealed that it remained associated with the SPBs early in mitosis, then became faint or undetectable

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and subsequently reappeared at the oSPB (Fig. 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 (Fig. 5B). Cells with longer spindles (Fig. 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, given that 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. This discrepancy might arise because the *cdc16-GFP* allele is partially compromised (the *cdc16-GFP cdc16-D* strain becomes multiseptated at >34℃; data not shown).

GFP–Byr4p was associated with both SPBs in 95% of metaphase-arrested *slp1-OFF* cells (Fig. 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 (Fig. 5D; $P<0.001$). Taken together, these data indicate that the removal of Byr4p from the SPBs requires APC/C^{Slp1p} function. Comparison with Cdc7p–GFP showed that GFP–Byr4p becomes asymmetric significantly before Cdc7p–GFP (compare Fig. 1C with Fig. 5B; LF0.4, $P=0.045$), confirming earlier analysis by indirect immunofluorescence (Cerutti and Simanis, 1999). Given that the steady state level of Cdc16p at the SPBs is very low from the onset of mitosis, whereas 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 Fig. 5E with Fig. 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 (Fig. 5F). In contrast, in *plo1-ts4* the decrease at LF0.2 and LF0.3 and the subsequent asymmetry was less apparent (Fig. 5H; supplementary material Fig. 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 dependent on Cdc11p (Mishra et al., 2005). In *rad24-D*, more cells with long spindles showed a symmetrical distribution of Cdc7p–GFP (Fig. 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 (Bähler and Pringle, 1998; Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). The onset of Cdc7p–GFP asymmetry occurred earlier in *pom1-D* cells (Fig. 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 (Bähler 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 (Fig. 6D) indicating that the effect of Pom1p upon the SIN is not mediated by its cortical gradient.

The effect of cell size and regulators of mitotic commitment upon Cdc7p–GFP asymmetry

Given that *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* cells (Fig. 6E), no association of Cdc7p–GFP was seen with interphase SPBs and the initial asymmetric loading of Cdc7p was also unaffected (data 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 (Fig. 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 PP2A 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 Fig. 6H with Fig. 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 (Fig. 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 (Fig. 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* cells (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 (supplementary material Fig. S3A). This threshold was reached at 6 µm (supplementary material Fig. S3B) in the loss-of-function *wee1-6* mutant, which divides at a reduced size (supplementary material Fig. S3E). The *cdc25-22* mutant, is partly defective at 25℃ (Fantes, 1979), increasing its cell length at division; in these cells, the >95% threshold was not reached until spindle lengths of >18 µm (supplementary material Fig. 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

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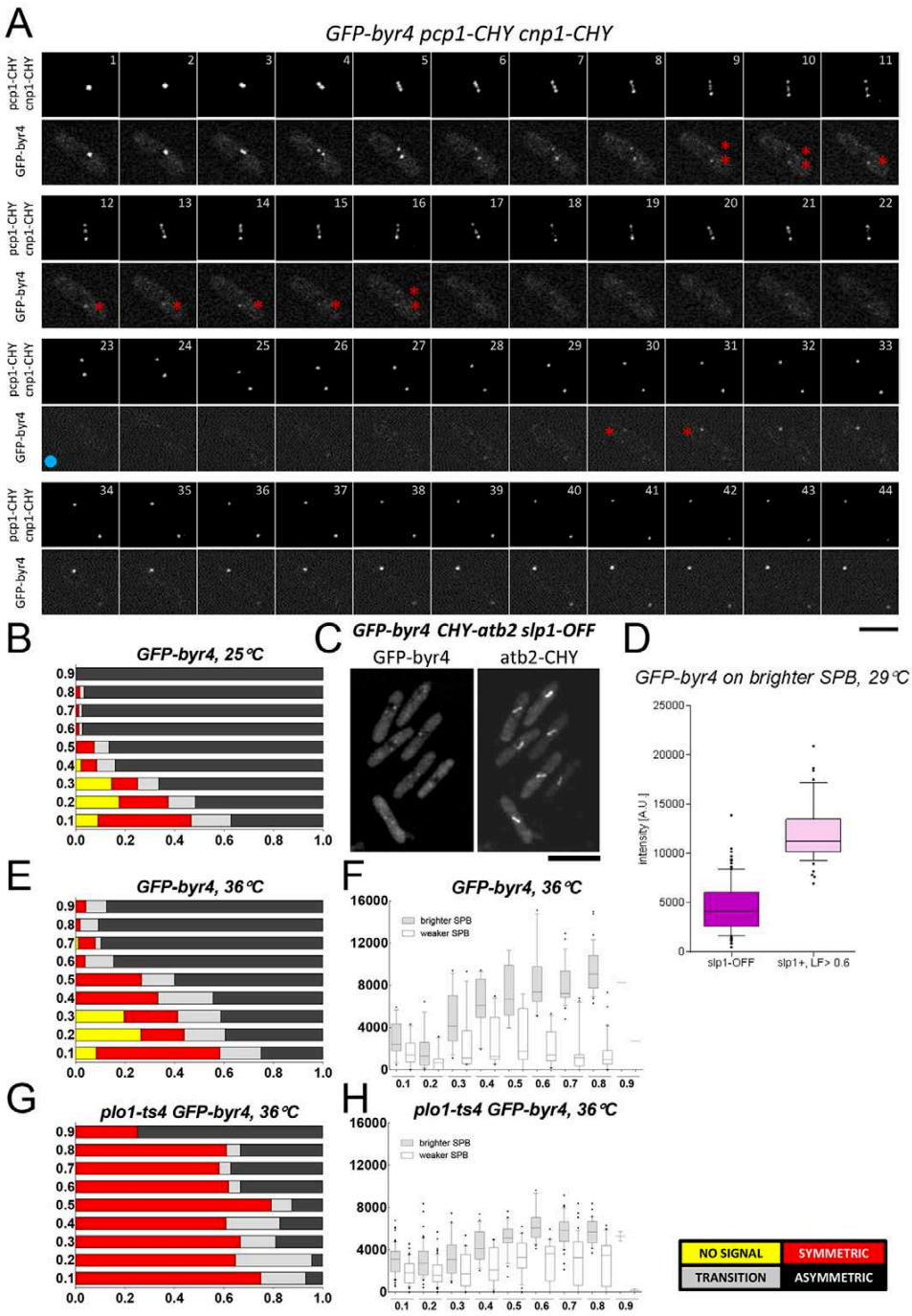

Fig. 5. Analysis of GFP–Byr4p in wild-type and mutant cells. (A) The indicated strains were imaged at 1-min intervals. The blue dot represents anaphase B onset. Note that the GFP–Byr4p signal is initially present on both SPBs, and then decreases in intensity. From frame 9 onwards, the red asterisks indicate 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 29; it reappears thereafter on one SPB. Note that the other SPB reacquires a signal from frame 36 onwards. See text for details. (B,E,G) The indicated strains were synchronised and the distribution of GFP–Byr4p on the SPBs was analysed. The graphs are presented as described for Fig. 1C. (C,D) The indicated strain was arrested as described in the 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 and presented as described in Fig. 2G (D). (F,H) The intensity of SPB-associated GFP–Byr4p is plotted and presented as described in Fig. 2G. Scale bars: 10 µm.

cdc25-22 did not differ significantly from wild-type (compare Fig. 6K and Fig. 6L with Fig. 6A). Taken 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 hydroxyurea. 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 (supplementary material Fig. S3D). Expressing the data in terms of the length fraction, revealed a slight but significant delay in establishment of asymmetry (Fig. 6M LF0.6; $P=0.02$). Previous studies have noted mitotic delays after release from hydroxyurea-mediated arrest (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.

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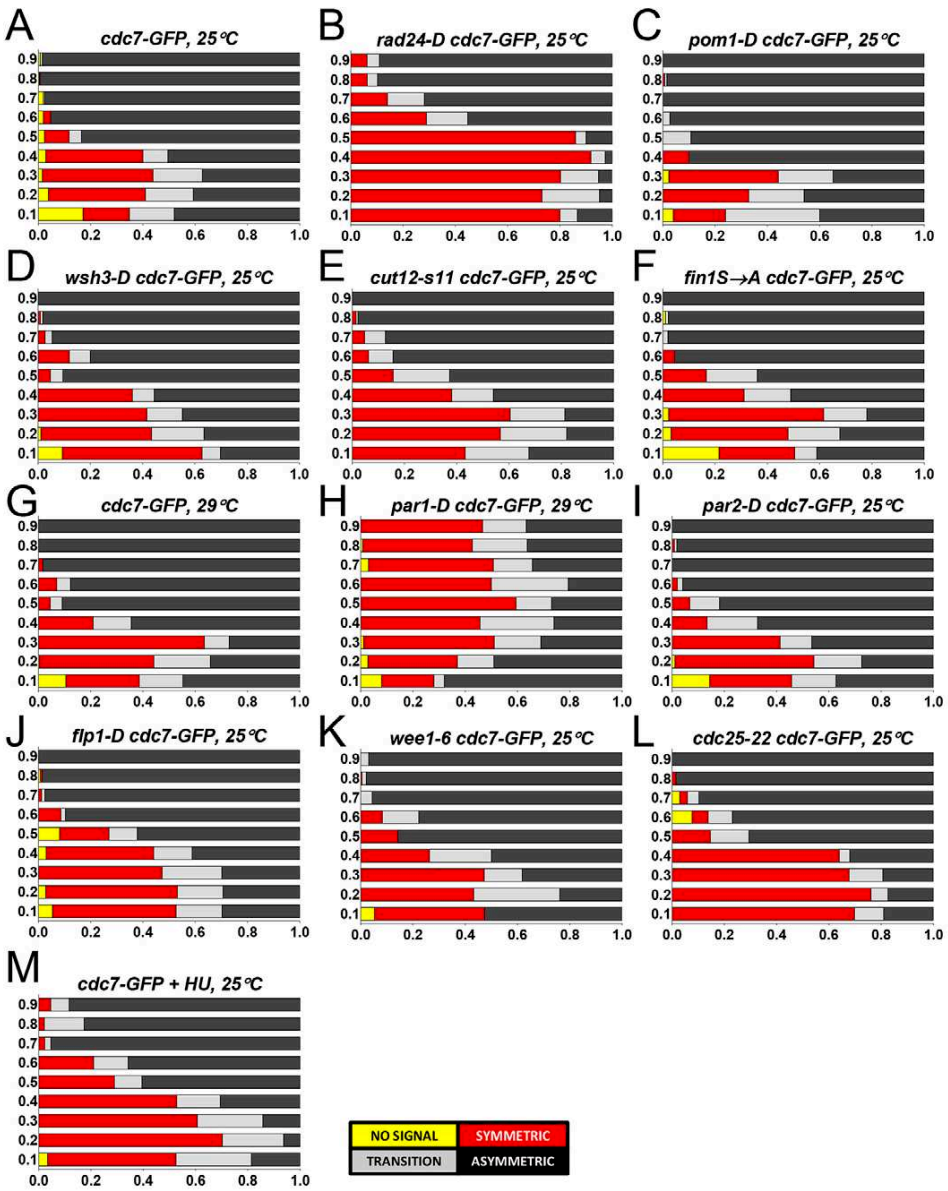

Fig. 6. Analysis of Cdc7p-GFP in wild-type and mutant cells. The intensity of Cdc7p–GFP was analysed in the indicated strains. The graphs are presented as described for Fig. 1C. The 25°C wild-type control (A) is duplicated from Fig. 1, for convenience. *Par1-D* was analysed using asynchronous cultures, as its abnormal shape and asymmetric division preclude efficient synchronisation by elutriation. Hydroxyurea (HU) indicates that cells were arrested by addition of 12 mM HU to for 5 h. Cells were analysed in the first mitosis after removal of HU.

The spindle assembly checkpoint 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 (Fig. 7A,B).

The kinesin-8 protein Klp5p is required for microtubule–kinetochore attachment, chromosome congression in metaphase (Garcia et al., 2002a; Garcia et al., 2002b; West et al., 2002) and spindle assembly checkpoint (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 (Fig. 7C,D).

The apparent contradiction between these mutants might be because the SAC is activated in *klp5-D* (Garcia et al., 2002b) but not in *pcs1-D* cells (Gregan et al., 2007). Analysis of *klp5-D cdc7-GFP cnp1-CHY pcp1-CHY mad2-D* cells showed that the percentage of cells with asymmetric Cdc7p–GFP at a LF>0.5 was similar whether lagging kinetochores were present or not (Fig. 7E,F), and deletion of *mad2* did not affect the onset of asymmetry (Fig. 7G,H). 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.

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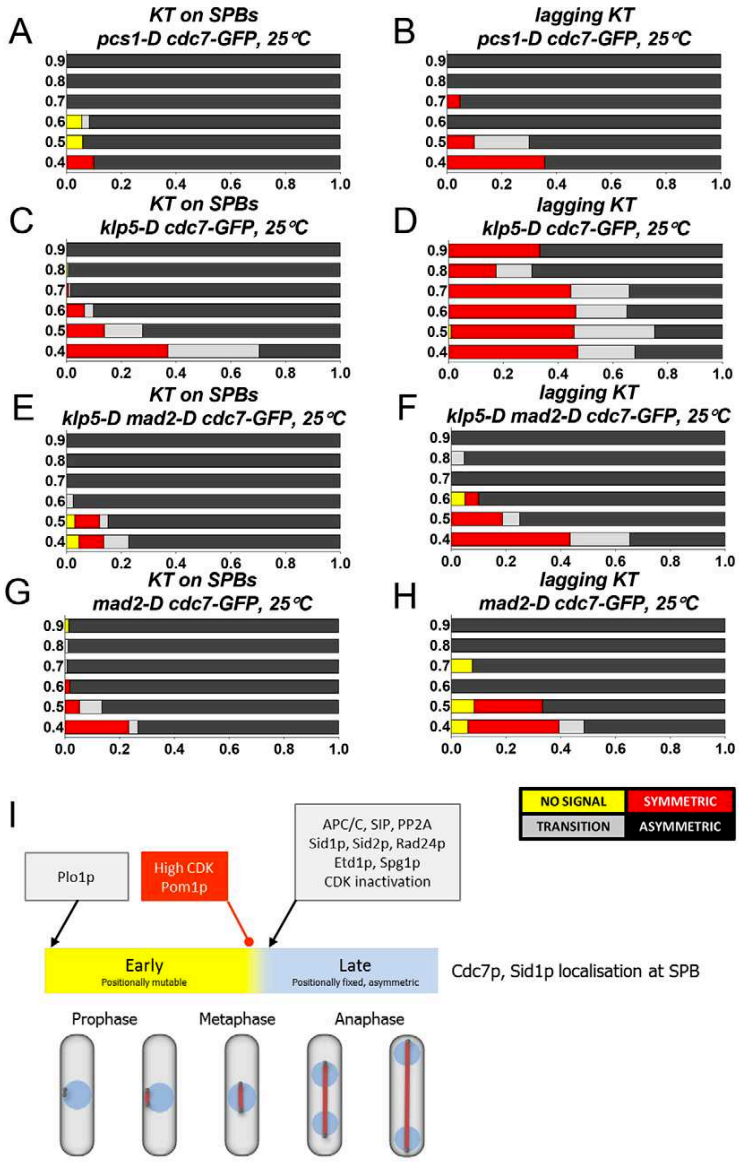

Fig. 7. Analysis of Cdc7p distribution in anaphase in cells with lagging chromosomes and data summary. (A–H) The strains were analysed at the indicated temperature. The graphs are presented as described for Fig. 1C. For clarity only LF>0.4 are shown. (I) Cartoon summarising the two-state model of the SIN. In the early state (yellow), the proteins are positionally mutable, whereas 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.

DISCUSSION

A 'two state' representation of the SIN

This study has revealed several new 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, which had a positionally stable signal, meaning that it was 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 Fig. 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 an 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 that Pom1p inhibits it. Interestingly, mutation of Sid2p sites on Cdc11p also results in an earlier transition to Cdc7p–GFP asymmetry (Feoktistova et al., 2012) and, hence, it will be of interest to determine whether Pom1p regulates SIN asymmetry through Cdc11p.

Ablation of the 14-3-3 protein Rad24p delayed the onset of asymmetry, although not as substantially as loss of Sid2p, SIP or GAP functions; it is possible that the other 14-3-3 protein Rad25p compensates for the loss of Rad24p. A non-exclusive alternative is that not all the effects of Sid2p are mediated by 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 PP2A 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 might 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). Although 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*, (García-Cortés and McCollum, 2009), suggesting that they cooperate to establish this state of the SIN (Fig. 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 are 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 might reflect differences in SPB age, or the hypomorphic *plo1-ts4* allele. The different effects of *plo1-ts4* upon Mob1p–GFP and Cdc7p–GFP might reflect differences in how SPB association of these two proteins is mediated.

Cdc7p–GFP did not show a significant increase in asymmetric signals at LF$\geq$0.6 in *spg1-B8* cells at 36℃, retaining a distribution similar to that in early mitosis. The intensity of the Cdc7p–GFP signal was similar with and without Spg1p until LF0.5, after which the signal became brighter in the *spg1*$^{+}$ cells, and fainter in the *spg1-B8* cells. This is consistent with the idea that establishment and/or maintenance of the late state requires Spg1p.

Previous studies have shown that there is no SPB-associated Cdc7p signal in *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 the shift to the restrictive temperature.

Given that 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 *spg1-B8*, and the fact that Spg1-B8p–GFP is not associated with the SPB (A.K. and V.S., unpublished data; Krapp et al., 2008), argue that SIN signalling is not functional. Nonetheless, we cannot formally exclude that *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 *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 strong asymmetric Cdc7p–GFP signal at the SPB.

Analysis of the *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). Although the SIN and MEN differ in their ‘wiring’ (reviewed by Simanis, 2003; Weiss, 2012), this points to a conserved role for Spg1p/TEM1p and Plo1p/CDC5p in regulating the 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} 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 might be regulated differently.

The reason for the instability of the early SIN is presently unclear; several non-exclusive possibilities can 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 has 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. Given that the activity of all the core SIN proteins and scaffolds is required for CAR

assembly, the early state might 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 might 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 in anaphase.

The spatial instability might also reflect '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 might 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 tips of the cell. This is governed by a combination of feedback loops (Das et al., 2012). Given that 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 at 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). Given that Cdc7p–GFP associates asymmetrically with the SPBs during spindle formation, the choice of SPB might reflect the age of the SPB.

Asymmetric association of Cdc7p and Sid1p with the nSPB is widely considered to represent 'active' SIN signalling. However, our data indicate that SPB association of Cdc7p–GFP in anaphase is insufficient to promote cytokinesis if Plo1p activity is reduced, even though ectopic SIN activation places *spg1* and *cdc7* downstream of *plo1* (Krapp et al., 2003; Tanaka et al., 2001). This might 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- and/or Cdc7p-dependent event might 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 might 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^{Slp1p} is blocked, then GFP–Byr4p is still seen at the SPBs, although the signal was much less intense than in anaphase, indicating that its removal from the SPBs requires APC/C^{Slp1p} function. This is also consistent with SPB localisation of Byr4p as determined 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, whereas 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^{Slp1p}, although it is presently unclear whether this is due to a direct action of APC/C^{Slp1p} 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 *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, because there are ≈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 might therefore reflect heterogeneity of the complexes at the SPBs in the *plo1-ts4* mutant.

Summary

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

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 3.5×10^6–6×10^6 cells/ml, 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 1 h recovery, the first and second mitoses after elutriation were imaged. There was no significant difference between whether the data were collected from cells in the first or second cycle. Data from biological repeats for a given cell type were also found to have no statistical differences, 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 and supplemented with amino acids (100 mg/l) as required. To repress *slp1* expression, the cells were incubated for 5 h in 29℃ in YE medium (Petrova et al., 2013). For hydroxyurea arrest and release, 12 mM hydroxyurea was added to exponentially growing cells. After 5 h, 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 ∼10-fold, as the signal associated with the oSPB reaches the cytoplasmic background.

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Image analysis and processing

Images were analysed using RodCellJ (Schmitter et al., 2013). The background was set by averaging the cytoplasmic signal in >100 mitotic cells. Only signals that were at more than a 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 two 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 a two-tailed non-parametric Mann–Whitney test.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Experiments were conceived of by P.W. and V.S., and performed by P.W. Data and statistical analysis were performed by P.W., A.C., E.C.D.R, J.R. and I.X. A.K. provided many strains prior to publication and much critical intellectual input. Image analysis software RodCell J was updated and maintained by D.Schmitter, D.Sage and M.U. The paper was written by V.S.

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Supplementary material

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