Dialogue between centrosomal entrance and exit scaffold pathways regulates mitotic commitment

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Summary

The fission yeast scaffold molecule Sid4 anchors the septum initiation network, to the spindle pole body (SPB – centrosome equivalent) to control mitotic exit events. A second SPB associated scaffold, Cut12, promotes SPB associated Cdk1-Cyclin B to drive mitotic commitment. Signals emanating from each scaffold have been assumed to operate independently to promote two distinct outcomes. We now find that signals from Sid4 contribute to the Cut12 mitotic commitment switch. Specifically, phosphorylation of Sid4 by NIMA^{Fin1} reduces Sid4 affinity for its SPB anchor, Ppc89, while enhancing Sid4’s affinity for Casein Kinase 1\(\delta\) (CK1\(\delta\)). The resulting phosphorylation of Sid4 by the newly docked CK1\(\delta\) recruits Chk2^{Cds1} to Sid4. Chk2^{Cds1} then expels the Cdk1-Cyclin B antagonistic phosphatase Flp1/Clp1 from the SPB. Flp1/Clp1 departure can then support mitotic commitment when Cdk1-Cyclin B activation at the SPB is compromised, by reduction of Cut12 function. Such integration of signals emanating from neighbouring scaffolds shows how centrosomes/SPBs can integrate inputs from multiple pathways to control cell fate.

Introduction

Cdk1-Cyclin B activity is restrained through Wee1 kinase phosphorylation of Cdk1 until Cdc25 phosphatase removes this phosphate to promote mitosis. Cdk1-Cyclin B activation then promotes polo kinase activity to further boost Cdc25 and inhibit Wee1 activity (Hagan and Grallert, 2013). In some systems engagement of Polo feedback control influences the rate of mitotic commitment, while in others Polo activity sets its timing (Lane and Nigg, 1996; Gavet and Pines, 2010; Karaiskou et al., 1999; 2004; Kumagai and Dunphy, 1996; Aspinall
In fission yeast, the recruitment of protein phosphatase 1 (PP1) to the SPB component Cut12 sets the level of Polo\(^{Plo1}\) activity, both locally on the SPB, and globally throughout the cell (Mulvihill et al., 1999; MacIver et al., 2003; Grallert et al., 2013b; a). Blocking PP1 recruitment to Cut12 removes the requirement for Cdc25 (Grallert et al., 2013b). When Cut12 function is compromised by shifting the temperature sensitive cut12.1 mutant to the restrictive temperature of 36\(^\circ\)C, Polo\(^{Plo1}\) activity falls and the new SPB, that has been generated by conservative duplication, fails to nucleate microtubules, leading to cell cycle arrest with a monopolar spindle (Bridge et al., 1998; Tallada et al., 2009a; MacIver et al., 2003) (Fig. 1A). The activation of this non-functional SPB by enhancement of Cdc25 levels, shows that the inactivity of the new SPB arises from a failure to locally activate Cdk1-Cyclin B on this SPB (Tallada et al., 2007; 2009b). Thus, the cut12.1 monopolar phenotype is a measure of the local role played by Cut12 in Cdk1 activation on the SPB (Fig. 1B).

In addition to its role in driving the cell into mitosis, Polo\(^{Plo1}\) kinase is recruited to the SPB component Sid4 to control the timing of cytokinesis and septation by activating the septum initiation network (SIN) (Ohkura et al., 1995; Tanaka et al., 2001; Morrell et al., 2004) (Fig. 1B). The SIN is a G protein regulated network whose components and architecture are analogous to the core of metazoan Hippo Signalling and the budding yeast Mitotic Exit Network (MEN) (Simanis, 2015). Cnm67 and Sid4 are homologous MEN and SIN components that act in an identical fashion to anchor the budding and fission yeast networks to the SPB. The amino-termini of Sid4/Cnm67 dock the orthologous SIN/MEN scaffolds Cdc11/Nud1, while the carboxyl termini anchor the complexes to their
respective SPBs via the Pfam motif 11778 (Tomlin et al., 2002; Rosenberg et al., 2006; Morrell et al., 2004; Krapp et al., 2001; Klenchin et al., 2011; Schäerer et al., 2001; Elliott et al., 1999; Adams and Kilmartin, 1999). The anchor for Sid4 in the fission yeast SPB is Ppc89 (Rosenberg et al., 2006) (Fig. 1B). Recruitment of the RING finger Ubiquitin ligase Dma1 to Sid4 delays septation when spindle function is perturbed (Murone and Simanis, 1996; Guertin et al., 2002; Johnson and Gould, 2010; Johnson et al., 2013).

To date, the mitotic entrance signalling events emanating from Cut12 have been assumed to operate independently of the signalling events on Sid4 (Fig. 1B, upper). However, we now find that a signalling relay on Sid4, promotes mitotic commitment. Thus, the decision to commit to mitosis integrates inputs from both Cut12 and Sid4 (Fig. 1B, lower). We discuss how the incorporation of the DNA replication checkpoint kinase Chk2\textsuperscript{Cds1} offers further potential for signals emanating from Sid4 to integrate inputs from replication/repair pathways into cell cycle control, as reported for DNA checkpoint control by human centrosome components (Griffith et al., 2008; Barr et al., 2010).

**Results**

*sid4 mutations overcome the mitotic commitment defect of cut12.1*

Because Sid4 recruits Polo\textsuperscript{Plo1} (Morrell et al., 2004), we combined the temperature sensitive Sid4 inactivating *sid4.SA1* mutation (Balasubramanian et al., 1998) with the temperature sensitive *cut12.1* mutation in an attempt to study the impact of compromising Cut12 function upon Plo1.GFP recruitment to the SPB in isolation of any influence of Sid4 upon Plo1.GFP recruitment.
Unexpectedly, the cut12.1 sid4.SA1 cells formed bipolar spindles to complete successive rounds of mitosis (Fig. 1C). The inclusion of the sid4.SA1 mutation had enabled the new SPBs to activate Cdk1-Cyclin B. We therefore asked whether this cut12.1 suppression was a specific feature of a Sid4 defect or whether deficiencies in other SIN components would similarly suppress the SPB activation defect of cut12.1, however none did (Fig. 1D). This independence from SIN function prompted us to isolate 4 sid4.soc (suppressor of cut12.1) mutations that suppressed cut12.1 yet retained full SIN function (Fig. 1D and Fig. S1A). All soc mutations resided in the C-terminal SID domain (Pfam 11778) that anchors Sid4 to the core SPB component Ppc89 (Morrell et al., 2004; Tomlin et al., 2002; Rosenberg et al., 2006) (Fig. 1E, F). cut12.1 suppression by sid4.13myc and sid4.TdTom alleles, in which the “tag” had been fused to the C terminus of Sid4 (Krapp et al., 2001; Grallert et al., 2006) further highlighted the ability of changes in the carboxyl terminal region of the mitotic exit anchor Sid4 to compensate for local Cdk1-Cyclin B activation deficiency in mitotic commitment of cut12.1 (Fig. 1D). sid4.GFP did not suppress cut12.1.

**Sid4.T584E compromises SIN function and suppresses cut12.1**

Mapping sites of phosphorylation on Sid4 revealed phosphate on T584 (data not shown). The proximity of T584 to the sid4.soc1 and sid4.soc4 mutations (Fig. 1F) prompted us to test the hypothesis that the soc mutations suppress cut12.1 because they constitutively emulate a change in conformation/function that is normally transiently invoked by T584. If true, mutations that constitutively alter T584 phosphorylation should also suppress cut12.1. A repeated inability to generate the phospho-mimetic sid4.T584E mutation at the native locus suggested
that this mutation maybe lethal. We therefore modified the \( \text{sid}^4 \) locus to \( \text{sid}^4.T584E \) in a host strain in which wild type \( \text{sid}^4 \) was expressed from the \( \text{hph.171k} \) locus under the control of the thiamine repressible \( \text{nm}t81 \) promoter (Fig. 2A) (Basi et al., 1993; Fennessy et al., 2014). This strain was viable at all temperatures when \( \text{sid}^4 \) was expressed, yet dead above 20°C when thiamine had been added to repress \( \text{sid}^4 \) expression. Outcrossing the \( \text{nm}t81: \text{sid}^4 \) confirmed the presumed lethality of \( \text{sid}^4.T584E \) as colony formation failed at all temperatures (data not shown). The recessive temperature sensitive phenotype of \( \text{sid}^4.T584E \) \( \text{nm}t81: \text{sid}^4 \) cells in the presence of transcription repressing thiamine suggests either that the levels of wild type Sid4 required to support \( \text{sid}^4.T584E \) viability are extremely low, or, we suspect, that \( \text{sid}^4.T584E \) alone is unable to support the challenges of either spore viability or germination. All experiments with \( \text{sid}^4.T584E \) therefore use \( \text{sid}^4.T584E \) \( \text{nm}t81: \text{sid}^4 \) in the presence of thiamine.

\( \text{sid}^4.T584E \) temperature sensitive lethality is associated with the appearance of multi-nucleated cdc- (cell division cycle) sin- (septum initiation network) cells at temperatures above 20°C (Fig. 2B, arrows). This sin- deficiency appeared to account for the lethality of \( \text{sid}^4.T584E \) because enhancement of SIN signalling through inactivation of the SIN inhibitory component Cdc16 (Minet et al., 1979; Fankhauser et al., 1993) raised the \( \text{sid}^4.T584E \) restrictive temperature from 25°C to 32°C (Fig. 2C).

Having developed a strategy to study the \( \text{sid}^4.T584E \) phenotypes we found that \( \text{sid}^4.T584E \) did indeed emulate the \( \text{sid}^4.\text{soc} \) mutants in suppressing the SPB.
activation defect of cut12.1 (Fig. 2D). By contrast, cut12.1 monopolar spindle counts were insensitive to incorporation of the sid4.T584V mutation to block phosphorylation (Fig. 2D, Fig. S1B).

**The NIMA kinase Fin1 phosphorylates T584**

Antibodies that only recognise Sid4 when phosphorylated on T584 were generated to identify the kinase responsible for T584 phosphorylation (Fig. 2E). Johnson et al. have shown how the recruitment of the ubiquitin ligase Dma1 to Sid4 generates a complex ladder of Sid4-Ubiquitin conjugates on Western blots that collapse to simple smear of phosphorylated isoforms over the unphosphorylated protein upon deletion of dma1+ (Johnson and Gould, 2010). We therefore repeated their approach of incorporating a dma1.Δ deletion allele in all strains in which Sid4 phosphorylation was monitored by western blotting in this study. Synchronisation of mitotic progression through transient inhibition of Cdc25 revealed a peak in T584 phosphorylation just before septation (data not shown). We therefore used samples from this mitotic stage to monitor the impact of candidate kinases upon T584 phosphorylation. T584 phosphorylation was abolished by the addition of the ATP analogue 3BrPP1 to cells harbouring an analogue sensitive version of the NIMA related kinase Fin1 (Grallert et al., 2012) (Fig. 2E). As the NDR kinase Sid2 activates Fin1 to control mitotic commitment in Pom1 dependent manner (Grallert et al., 2012), either one of these three kinases could be the kinase that directly phosphorylates T584 of Sid4. We therefore used recombinant Sid4 in *in vitro* kinase assays of molecules isolated from yeast cells to show that Fin1 alone was able to directly
phosphorylate T584 (Fig. 2F). This ability of Fin1 to phosphorylate T584 relied upon prior activation by Sid2 kinase, in vivo, before the kinase assay (Fig. 2G).

**T584 phosphorylation reduces Sid4 affinity for the SPB anchor Ppc89 to invoke the SIN phenotype**

We next wanted to address the means by which Fin1 phosphorylation of T584 could alter Sid4 function. As the phosphorylation site sits within the carboxyl terminal domain, that binds to the core SPB component Ppc89 (Rosenberg et al., 2006), we asked whether SPB association of Sid4 was influenced by T584 phosphorylation? We exploited an assay developed for the budding yeast Sid4 homologue Cnm67 in which fusion to the C terminal domain directs GFP to the SPB (Fig. 3A) (Klenchin et al., 2011). Induction of analogous fusion proteins in which GFP was fused to residues 466-660 of Sid4 gave an SPB signal (Fig. 3B). The signal persisted when 584 was mutated to valine, but was abolished by mutation to either glutamic or aspartic acid to mimic phosphorylation (Fig. 3C). Precipitation of these fusion proteins with GFP-trap® (Rothbauer et al., 2008) confirmed that Ppc89 association with the wild type Sid4 fragment was disrupted by phosho-mimetic T584D and T584E (Fig. 3D). This disruptive impact of the phosho-mimetic mutations was reiterated in the established ability of C-terminal fragment of sid4 (466-660 a.a) to interact with ppc89+ in yeast two hybrid assay (Fig. 3E, Fig. S1C) (Rosenberg et al., 2006). Significantly, while mutation of T584 to valine had no impact upon the strength of the two-hybrid interaction, the association of T584A was compromised to suggest that valine more accurately represents the non-phosphorylated state of T584 than
alanine. We conclude that T584 phosphorylation regulates Sid4 affinity for Ppc89.

Fusion of sequences encoding a GFP tag to the 3' end of the *sid4.T584E* ORF generated a Sid4.T584EGFP fusion protein whose SPB association was notably diminished at 19°C and completely absent at 36°C (Fig. 4A), even though *sid4.T584E* protein levels were unaffected by the shift from 19°C and 36°C (Fig. 4B). As the amino terminus of Sid4 anchors the SIN to the SPB (Morrell et al., 2004), it seemed likely that the *sid4.T584E* sin− phenotype arose from the inability to recruit the SIN to the SPB (Fig. 2B,C). Certainly, the recruitment of the SIN component Cdc11.GFP mirrored the behaviour of Sid4.T584EGFP in failing to bind the SPB at 36°C and associating weakly at 19°C (Fig. 4C). We therefore asked whether we could restore SIN function to Sid4.T584E protein by artificially anchoring Sid4.T584E to the SPB by combining *sid4.T584EGFP* with a *ppc89.GBP* allele in which the sequences encoding the single chain llama antibody GFP binding protein (GBP) (Rothbauer et al., 2008; 2006) were fused to the C terminus of Ppc89 (Fig. 4D). Strikingly, these *sid4.T584EGFP ppc89.GBP* cells, were viable at 36°C (Fig. 4E). We conclude that *sid4.T584E* loss of viability does indeed arise from sin− deficiency generated by the inability to recruit Sid4 to the SPB. This rescue experiment provides the first concrete, non-correlative, evidence that anchorage to the SPB is essential for SIN function (Simanis, 2015).

*Recruitment of CK1δ to Sid4, not departure of Sid4 from the SPB, suppresses cut12.1*
We next asked whether cut12.1 suppression by sid4.T584E was also a consequence of Sid4 departure from the SPB. Combining sid4.T584EGFP ppc89.GBP with cut12.1 showed that suppression was maintained when Sid4.T584EGFP was anchored to the SPB (Fig. 5A). In a second approach we asked whether complete removal of Sid4 from the cell would suppress cut12.1? To bypass the essential requirement for Sid4 we directly anchored the SIN scaffold Cdc11.GFP to SPB associated Ppc89.GBP (Fig. S2A) before deleting sid4+ from this background (sid4.Δ). Introduction of cut12.1 to this cdc11.GFP ppc89.GBP sid4.Δ background confirmed that cut12.1 suppression did not arise from the failure of Sid4.T584E to bind the SPB and so the suppression of cut12.1 must be the consequence of a distinct property conferred upon Sid4 by this phospho-mimetic mutation (Fig. 5A).

Johnson et al, proposed that events at the C-terminus of Sid4 recruit CK1δHHp1 and CK1δHHp2 (Johnson et al., 2013). As mutation of T584 had a profound impact upon cell fate, we tested the association between the sequences encoding the C-terminal fragment of Sid4 (466-660 a.a) and those encoding Hhp1 and Hhp2 in the yeast two-hybrid assay. A robust interaction between Sid4 and both hhp1+ and hhp2+ was detected when T584 was mutated to the phospho-mimetic residues E/D (Fig. 3E, Fig. S1C), whereas wild type or T584V sequences failed to promote viability. Sid4.T584A exhibited a strong interaction with hhp1+ and a weak interaction with hhp2+ to consolidate the view that valine rather than alanine is an appropriate mutation to mimic the non-phosphorylated state of T584.
If the phospho-mimetic Sid4.T584E protein supresses cut12.1 through the inappropriate recruitment of CK1δHhp1/CK1δHhp2, then removal of these Casein Kinases should abrogate this suppression. Given the robust functional redundancy between CK1δHhp1 and CK1δHhp2 (Ishiguro et al., 2010), it was not surprising that individual deletion of either hhp1+ or hhp2+ had no impact upon cut12.1 suppression by sid4.T584E (Fig. 5B). We therefore inhibited an analogue sensitive Hhp2.AS kinase in a hhp1.Δ deletion background (Gregan et al., 2007). Strikingly, suppression of the monopolar spindle defect of cut12.1 by sid4.T584E was fully reversed by ablation of CK1δ activity (Fig. 5B).

To confirm that it is indeed the recruitment of CK1δ by Sid4.T584E that confers cut12.1 suppression, we recruited CK1δHhp2.GFP to an otherwise wild type Sid4.GBP anchor on the SPB. Not only did this surrogate recruitment of CK1δHhp2.GFP to Sid4.GBP confer robust suppression of cut12.1 (Fig. 5D, Fig. S2B), but it was dependent on kinase activity, because suppression was analogue sensitive when the version of CK1δHhp2 that was recruited incorporated the analogue sensitizing mutation (Fig. 5D and Fig. S2B). We conclude that sid4.T584E suppression of cut12.1 arises from the inappropriate recruitment of CK1δ activity to Sid4.

**CK1δ directed phosphorylation of Sid4 on both T275 and S278 supresses cut12.1**

Johnson et al. showed how Sid4 phosphorylation at positions T275 and S278 by CK1δHhp1/CK1δHhp2 recruits the ubiquitin ligase Dma1 to block septation when spindle function is perturbed (Johnson et al., 2013). To determine whether T275
and S278 phosphorylation was the means by which CK1δ\(^{\text{Hhp1}}\)/CK1δ\(^{\text{Hhp2}}\) recruitment to Sid4 suppressed *cut12.1*, we tested all permutations of phospho-mimetic mutations of the endogenous locus T275 and S278 for *cut12.1* suppression. *Sid4.T275ES278D* suppressed the monopolar spindle defect and lethality of *cut12.1* (Fig. 5E, Fig. S2C, D). Importantly, these phospho-mimetic mutations by-passed the requirement for T584 phosphorylation as *sid4.T275ES278DT584V* at the endogenous locus suppressed *cut12.1* (Fig. 5E).

Antibodies that only recognise Sid4 when phosphorylated on both T275 and S278 (Fig. 6A) revealed a T275S278 phosphorylation signal that began in G2 to persist until the peak of septation (Fig. 6B). T275S278 dual phosphorylation was dependent on T584 phosphorylation because it is absent from *sid4.T584V* cells (Fig. 6A), yet constitutively present throughout *sid4.T584EGFP ppc89.GBP* cell cycles (Fig. 6C, D). We conclude that *sid4.T584E* promotes inappropriate phosphorylation of Sid4 on both T275 and S278 and that it is this phosphorylation that supresses the mitotic commitment defect of *cut12.1*.

**Recruitment of Chk2\(^{\text{Cds1}}\) to T275S278 phosphorylated Sid4 suppresses cut12.1**

Because T275S278 phosphorylation generates a docking site for the FHA domain of Dma1 we asked whether *sid4.T584E* and *sid4.T275ES278D* suppressed *cut12.1* because they inappropriately recruited Dma1 to Sid4 (Johnson et al., 2013)？ Unexpectedly, *dma1\(^{+}\)* deletion had no impact upon *cut12.1* suppression (Fig. 7A, Fig. S3A). Testing the other FHA domain proteins, Csc1 (Singh et al., 2011), Chk2\(^{\text{Cds1}}\) (Murakami and Okayama, 1995; Lindsay et al., 1998; Boddy et al., 1998)
for an impact upon cut12.1 suppression revealed a reliance upon chk2cds1 (Fig. 7A, Fig. S3A). A positive reaction between the FHA domain of Chk2\textsuperscript{Cds1} and Sid4 in the yeast two hybrid assay was abolished by mutation of either chk2cds1 FHA domain (H81A) (Tanaka and Russell, 2001) or sid4.T275AS278A (Fig. 7B, Fig. S3B). We therefore used the bimolecular fluorescence complementation (BiFc) to monitor the association between Sid4 and Chk2\textsuperscript{Cds1} \textit{in vivo}. In this assay two halves of YFP are fused to candidate proteins. If these partners reside within a common complex, the two halves can dock to generate a functional fluorescent protein (Hu et al., 2002). BiFc analysis with Chk2\textsuperscript{Cds1}:nYFP and Sid4.cYFP gave a dim SPB signal whose intensity increased markedly when dma1.\Delta removed competition for T275S278 docking from Dma1 (Fig. 7C). Importantly, no signal was detected when T275S278 could not be phosphorylated to create an FHA anchor in sid4.T275AS278AcYFP chk2cds1.nYFP dma1.\Delta cells (Fig. 7D, Fig. S3C). Co-immunoprecipitation confirmed the association between Chk2\textsuperscript{Cds1} and Sid4 as Chk2\textsuperscript{Cds1}.GFP precipitated with wild type Sid4 and Sid4.T275ES278D but not with Sid4.T275AS278A (Fig. 7E).

We next asked whether it was the recruitment of Chk2\textsuperscript{Cds1} to phosphorylated Sid4 that supressed the mitotic SPB activation defect of cut12.1. As permanent anchorage of Chk2\textsuperscript{Cds1}.GFP to Sid4.GBP compromised fitness (data not shown), we used the thiamine repressible nmt81 promoter to induce different levels of chk2cds1.GFP expression in a sid4.GBP cut12.1 background. The failure to activate the new SPB was supressed in a dose dependent manner (Fig. 7F).

\textit{Chk2\textsuperscript{Cds1} directed eviction of Flp1 from the SPB supresses cut12.1}
Hydroxyurea (HU) inhibits ribonucleotide reductase to deplete the nucleotides required for DNA replication. HU treatment prompts Chk2\textsubscript{Cds1} to evict the Cdc14 family phosphatase Flp1/Clp1 from the SPB (Díaz-Cuervo and Bueno, 2008; Broadus and Gould, 2012). Cdc14 family phosphatases dephosphorylate the SP/TP motifs that are targeted by Cdk1-Cyclin B (Mocciaro and Schiebel, 2010). Furthermore, \textit{S. pombe} Flp1/Clp1 inactivates Cdc25 (Wolfe and Gould, 2004; Esteban et al., 2004). Thus, Chk2\textsubscript{Cds1} eviction of Flp1 from the pole is an attractive mode for suppression of \textit{cut12.1} by Sid4 because it would act at two levels to reduce the local threshold for Cdk1-Cyclin B activation at the SPB. We therefore monitored the distribution of Flp1.GFP in strains that bore simultaneous mutation of T275 and S278 following HU addition, to determine whether the kinase relay on Sid4 we have defined underpins Flp1 eviction. Strikingly, the phospho-blocking \textit{sid4.T275AS278A} mutation emulated the impact of Chk2\textsubscript{Cds1} removal in abolishing the HU invoked eviction of Flp1.GFP from the spindle poles (Fig. 8, Fig. S4). Importantly, the \textit{flp1.9A} allele that blocks Chk2\textsubscript{Cds1} mediated eviction from the SPB (Díaz-Cuervo and Bueno, 2008) compromised the ability of \textit{sid4.T275ES278D} to suppress the Cdk1-Cyclin B activation defect of \textit{cut12.1} (Fig. 8D, Fig. S4). We conclude that the phosphorylation of Sid4 at position T584 recruits CK1\textsubscript{Hhp1}/CK1\textsubscript{Hhp2} to promote an association of Chk2\textsubscript{Cds1} with Sid4 that ejects Flp1 from the SPB.

**Discussion**

We show how a phosphorylation cascade along Sid4 is able to compensate for Cdk1-Cyclin B activation defects arising from the \textit{cut12.1} mutation. The chain of events is initiated by Fin1 phosphorylation of T584 to promote CK1\textsubscript{δ}}
recruitment (Fig. 9). CK1δ phosphorylation generates a docking site for the FHA domain of Chk2\(^{Cds1}\), from which Chk2\(^{Cds1}\) is able to execute its characterised role of evicting the Cdc14 phosphatase from the SPB (Díaz-Cuervo and Bueno, 2008; Broadus and Gould, 2012). De-phosphorylation of Cdc25 by Flp1 reduces Cdc25 activity (Wolfe and Gould, 2004; Esteban et al., 2004) and Cdc14 family phosphatases dephosphorylate the SP/TP motifs that are targeted by Cdk1-Cyclin B (Mocciaro and Schiebel, 2010). Thus, Flp1 eviction will reduce the local threshold for Cdk1-Cyclin B on the new SPB to support activation of the defective new SPB of cut12.1 cells (Fig. 9, Fig. 1B).

This dialogue between two independent scaffolds reinforces the robustness of the mitotic switch. Activation of Fin1 impacts at two points in this core switch: the eviction of PP1 from Cut12 (Grallert et al., 2013b) and the recruitment of Chk2\(^{Cds1}\) to Sid4. Fin1 also promotes mitotic commitment via a third, less defined, route through the Pom1 cell geometry network (Grallert et al., 2012). As protein kinases, CK1δ\(^{Hhp1}\), CK1δ\(^{Hhp2}\) and Chk2\(^{Cds1}\) each have the potential to emulate Fin1 in hitting further targets in the switch to enhance the robustness and bi-stable nature of the mitotic commitment switch.

The concept of dialogue between scaffolding proteins on the SPB addresses the long-standing question as to “why should mitotic entry and exit be regulated from the spindle pole?” Centrosomal scaffold dialogue can integrate inputs from diverse signalling networks with a limited number of neighbouring molecules in order to generate a single coherent output that can then be amplified throughout the cell. Alongside centrosomal signalling in cell cycle and DNA checkpoint
controls, the overriding impact of the spindle assembly checkpoint signal emanating from a single kinetochore provides another example of the dramatic impact that co-ordinated singling from a single centre can have (Lara-Gonzalez et al., 2012).

Sid4 is a classic example of a centrosomal scaffold that sits at the intersection of multiple signalling pathways. Sid4’s role in recruiting Cdc11 to anchor the SIN is well established (Tomlin et al., 2002; Krapp et al., 2001). The activation of Sid4 anchored SIN signalling on the newer of the two SPBs, late in anaphase B, triggers cytokinesis and mitotic exit (Sohrmann et al., 1998; Grallert et al., 2004). In addition to driving the recruitment of Chk2Cds1 in the pathway that sets the level of Cdk1-Cyclin B activity at the SPB that we define here, dual phosphorylation at T275 and S278 independently recruits Dma1, in the distinct control that maintains mitotic arrest in response to spindle microtubule perturbation (Tomlin et al., 2002; Johnson et al., 2013; Johnson and Gould, 2010). Our demonstration that Sid4 anchors Chk2Cds1 to expel Flp1 from the SPB when DNA replication is incomplete, now puts Sid4 into DNA checkpoint signalling in an echo of the role played by pericentrin, CDK5RAP2 and AKAP450 in checkpoint signalling in human cells (Griffith et al., 2008; Barr et al., 2010).

We believe that further studies will reveal similar networks in mitotic controls in other systems as both CK1δ and Chk2 are recruited to human centrosomes (Sillibourne et al., 2002; Tsvetkov et al., 2003). Although it is clear that CK1δ activity regulates the timing of mitotic commitment of human cells by targeting Wee1 for destruction (Penas et al., 2014), before itself being degraded by the
APC/C\textsuperscript{Cdh1} (Penas et al., 2015), it remains to be established whether it is this centrosomal pool of CK1δ that mediates these controls. Chk2 is activated in mitosis independently of any stimulation from its role in DNA damage responses (Stolz et al., 2010). As for fission yeast Chk2\textsuperscript{Cds1}, Chk2 association with \textit{Drosophila} centrosomes is reliant upon the function of its FHA domain (Takada et al., 2015). Critically, phosphorylation of human BRCA1 by Chk2 recruits PP6-SAP3 to counteract the activation of Aurora A at centrosomes (Stolz et al., 2010; Ertych et al., 2014; 2016). The abnormal enhancement of centrosomal Aurora A that arises from Chk2 depletion elevates microtubule dynamics to promote chromosome loss in the classic CIN (Chromosome INstability) phenotype associated with tumours (Ertych et al., 2016; 2014). Strikingly Aurora kinase also plays a key role in triggering the Cut12 mediated activation of polo at the fission yeast SPB at the core of the mitotic commitment controls addressed in our study (Hálová and Petersen, 2011; Petersen and Hagan, 2005; Petersen and Nurse, 2007).

Such extensive similarities between fission yeast and human centrosomal CK1δ/Chk2/AuroraA/Polo signalling define some simple dependency relationships that can be probed in higher systems to unravel the complex usage of the spindle pole as a hub at which to co-ordinate signalling events that determine cell fate.

**Methods:**

**Yeast culture and growth**

Strains used are listed in Table S1. Cell culture and maintenance (Petersen and Russell, 2016) was carried out as follows. Unless specified, yeast strains were
streaked on YES (yeast extract supplemented with the amino acids, adenine, histidine, leucine, uracil and lysine) plates from frozen glycerol stocks at -80°C and grown at permissive temperature (25°C) until single colonies are formed. One single colony was then used to start overnight starter liquid cultures in EMM2 (Edinburgh Minimal Media 2; EMM2) and grown at 25°C. Main cultures were inoculated from overnight starter liquid cultures and allowed to grow for at least 17 hours (~3 generations) at 25°C. *sid4.T584E* mutants were streaked on YES plates and grown at 20°C and for starter and liquid culture experiments cells were grown in EMM2 with 40 µg ml⁻¹ thiamine at 19°C.

Centrifugal elutriation was used to isolate small G2 cells (Hagan et al., 2016). Briefly, a 1 litre starter culture of each prototrophic strain was diluted to generate 7 litres of culture at a density of 3.8 x 10⁶ 19 hours later. These 7 litres were loaded into the 40 ml chamber of a Beckman Elutriator JE-5.0 rotor in a modified J6 centrifuge over 27 minutes. 200 ml of media was collected from the exhaust tube 23 minutes after loading began. Incremental reduction of the centrifuge speed eluted small G2 cells from the asynchronous population in the chamber. During elutriation the 200 ml of exhaust media was added to the media flowing through the chamber. The freshly eluted G2 cells were immediately diluted into pre-warmed EMM2 to generate a 2.5 litre culture at a cell density of 1 x 10⁶ cells ml⁻¹. The synchrony of cell division in the culture was monitored by scoring the septation index through staining with the fluorescent cell wall stain calcofluor white (Hagan, 2016a).

For protein induction using the thiamine repressible *nmt1* derived promoters (Maundrell, 1990), cells were grown at early log phase (1 x 10⁶ – 2 x 10⁶ cells ml⁻¹) at 25°C in EMM2 with 20 µM thiamine before being harvested via
centrifugation at 1300 g for 2 min. The cell pellets were subsequently washed with 10 ml of EMM2 without thiamine. After two further washes cells were inoculated into EMM2 without thiamine. Cells were subsequently grown at 25°C for 24 hours before analysis.

For the experiments with attenuated \textit{cds1.GFPn} expression levels, 5 \(\mu\)M, 2 \(\mu\)M, 1 \(\mu\)M, 0.5\(\mu\)M or no thiamine was added back to the culture after the thiamine washes and cultures were grown for a further 24 hours at 25 \(^\circ\)C before being shifted to 36\(^\circ\)C.

For the inhibition of analogue sensitive kinases, ATP analogues 1NM-PP1 and 3BrB-PP1 (Toronto Research Chemicals, Dalton Pharma Services) were dissolved in methanol to generate 10 mM and 50 mM stock solutions respectively before addition to media at the concentrations specified in the figure legends.

\textbf{Live cell imaging}

All strains used for live cell imaging were prototrophs. The starter and main culture for live imaging of cells were both grown in filter sterilised EMM2.

Unless specified, the cultures were grown at 25\(^\circ\)C to a cell density of \(1 \times 10^6\) cells ml\(^{-1}\) before being harvested via centrifugation at 1800 g for 2 minutes at room temperature and re-suspended in 500 \(\mu\)l of EMM2. 10 \(\mu\)l of cells were mixed with plant lectin (Sigma, cat. L1395-5mg) to a final concentration of 0.1 mg ml\(^{-1}\) and attached onto glass coverslips for 3 minutes before the coverslips were washed three times with 1 ml of EMM2 to remove any unattached cells. The coverslips were subsequently mounted in a FCS2 chamber (Bionetics). All live imaging was conducted on a DeltaVision Core system (Applied Precision) fitted with a
Zeiss 100x, 1.45 NA objective, a Cascade II:1024 EMCCD Camera (Photometrics, Tucson, AZ, USA), and the 89000 ET-Sedat Quad filter set (Chroma, Vermont, U.S.A.) and controlled by Softworx software (Applied Precision). Images shown are maximal projection of sections composed of 30 slices (0.3 µm apart) that were taken and processed with Imaris (Bitplane) software. For the bimolecular fluorescence complementation (BiFc) images that monitored the interaction between Cds1.nYFP and Sid4.cYFP, individual slices of image stacks were digitally de-convolved using Huygens Remote Manager (Ponti et al., 2007) before maximal projection of the de-convolved image stacks were generated with Imaris (Bitplane) software.

For TRITC lectin labelling of live cells, 100 µl of cells were labelled with 0.01 mg ml⁻¹ TRITC conjugated lectin (Sigma, cat. L5264-2MG) in filter sterilised EMM2 at room temperature for 5 minutes. The cells were subsequently centrifuged at 850 g for 2 min and washed three times with filter sterilised EMM2. Labelled and unlabelled cells were mixed with a ratio of 1 to 1 and 10 µl of this cell mixture was attached onto glass coverslips as described above.

**Immunofluorescence**

Tubulin and Sad1 staining were conducted using established procedures (Hagan, 2016b; Hagan and Hyams, 1988). Cells were fixed by the addition of freshly prepared, pre-warmed, 30% formaldehyde solution in PEM (100 mM piperazine-N,N-bis(2-ethanesulfonic acid) (PIPES, sodium salt) 1 mM EGTA, 1 mM MgSO₄ pH 6.9) to the culture to a final concentration of 3.7%. 30 seconds later glutaraldehyde was added to a final concentration of 0.2%. After fixation for 30 – 120 minutes, and three washes in PEM, cells were washed once in PEMS (PEM
+ 1.2M sorbitol), before digestion of the cell wall with Zymolyase 100-T in PEMS. After permeabilisation in PEM 1% Triton X-100, a further 3 washes in PEM, cells were re-suspended in PEM 1 mg ml$^{-1}$ NaBH$_3$ for 5 minutes after which a further three washes in PEM were followed by re-suspension in PEMBAL (PEM + 1% BSA (globulin free), 1% NaN$_3$ and 100 mM lysine HCl) for 30 minutes. Cells were pelleted once more before re-suspension in PEMBAL with affinity purified polyclonal Sad1 antibodies (Hagan and Yanagida, 1995) at a concentration of 1 in 100 alongside tissue culture supernatant containing the TAT1 monoclonal antibody (Woods et al., 1989) (gift from K. Gull, University of Oxford) at a dilution of 1 in 80. Incubation overnight at room temperature with agitation was followed by three washes in PEMBAL and re-suspension in PEMBAL containing appropriate secondary antibodies (fluorescein isothiocyanate (FITC) conjugated goat anti-mouse specific IgG antibodies to detect TAT1 and Cy3 conjugated goat anti-rabbit specific IgG antibodies to recognise Sad1). After a further 5 hours at room temperature with agitation, a final three PEMBAL washes were followed by a wash in PBS 1% NaN$_3$ and re-suspension in PBS 1% NaN$_3$ 0.2 μg ml$^{-1}$ DAPI (4',6-Diamidino-2-phenylindole). Imaging was conducted on the DeltaVision core system (Applied Precision) described above. The representative images shown in the manuscript are maximal projection of sections composed of 30 slices (0.3 μm apart) were taken and processed with Imaris (Bitplane) software.

**Genetic Manipulation**

Mutant *sid4* alleles were generated via mutagenesis using the Phusion system (New England Biolabs) and inserted into the genome using the *kanMX6* at the 3’
UTR as a marker immediately after the stop codon. For the split BiFC assay, sid4+ was tagged with the C-terminal fragment of Venus YFP while cds1+ was tagged with the N-terminal fragment of Venus YFP (Grallert et al., 2013b). Both sid4+ and cds1+ loci was tagged at the C-terminus using PCR tagging (Bähler et al., 1998; Grallert et al., 2013b).

For the generation of sid4.T584E and sid4.T275ES278D phospho-mutants, the TADH natMX6 or TADH kanMX6 markers from the pFA6 series of tagging vectors were inserted at the STOP codon of mutated genomic sid4 sequences. A fragment containing each mutant sid4 ORF, the TADH and marker within 400 bp of flanking sequences were transformed into a strain containing an additional copy of sid4+ integrated into the hph.171k locus under the control of the thiamine repressible promoter nmt81 (Fennessy et al., 2014). All strains bearing sid4 phospho-mutant alleles were backcrossed at least 3 times and prototrophs were isolated before use in experiments.

For the generation of stably integrated inducible ectopic genes, the gene of interest was cloned into the pINTH81 vector series via Nde1/BamH1 restriction sites and integrated into the hph.171k locus (Fennessy et al., 2014).

Generation of unmarked flp1.9A and hhp2.as alleles with native UTRs: The mutation conferring analogue sensitivity upon Hhp2 (M85A) was introduced via site directed mutagenesis into sequences encoding the Hhp2 ORF amplified from genomic DNA alongside 250 bp of flanking DNA and Not1 sites. The flp1.9A construct was made by generating a synthetic gene (Life Technologies, ThermoFisher Scientific) in which all Chk2Cds1 consensus phosphorylation sites, reported by Díaz-Cuervo and Bueno in the Flp1 sequence were mutated to alanine within 205 bp of flanking genomic regions straddled by Not1 sites (Díaz-
Cuervo and Bueno, 2008). Each mutant gene including the flanking 250 bp UTR was excised on a Not1 fragment and transformed into an \textit{rpl42.SP56Q} \textit{S. pombe} host in which the ORF had been disrupted with \textit{rpl42+} and the integration event was selected for by plating on plates containing 100 µg ml\(^{-1}\) cyclohexamide (Fennessy et al., 2014). The loci of cyclohexamide resistant mutants were amplified via PCR and sequenced for confirmation of gene sequences. Each mutant allele was backcrossed 3 times and a prototroph, without the \textit{rpl42.SP56Q} mutation, was isolated for use in experiments. The \textit{flp1.9AGFP} allele was generated via PCR tagging \textit{flp1.9A} allele with GFP at the C-terminus (Bähler et al., 1998).

\textbf{Bacterial expression of full length Sid4 and purification}

The \textit{sid4+} ORF was cloned into pET41 vector and transformed into BL21 (NEB, cat. C2527I) competent \textit{E. coli} cells together with the pLysS vector (Studier et al., 1990). \textit{E. coli} cells resistant to chloramphenicol and ampicillin were selected and grown in LB (Luria-Bertani) liquid medium at 37\(^{\circ}\)C. When the OD at 600 nm reached 0.4, 1 mM IPTG (Isopropyl \(\beta\)-D-1-thiogalactopyranoside) was added to induce \textit{sid4+} expression. Cells were harvested after 1 hour of induction at 37 \(^{\circ}\)C Sid4 was purified from inclusion bodies by separating the proteins of inclusion bodies in a 5mm thick 10% acrylamide SDS PAGE gel and excising the protein band corresponding to Sid4 before electro-elution into elution buffer (16.8 mM Na\(_2\)HPO\(_4\).12H\(_2\)O, 11.4 mM NaH\(_2\)PO\(_4\).2H\(_2\)O, 0.0288 % SDS, pH 7.4).

\textbf{Antibody generation}
Sheep polyclonal antibodies against Sid4 and GFP were raised by Diagnostics Scotland, using full-length recombinant proteins purified from *E. coli* as the antigen. Affinity purification of sheep polyclonal antibodies were performed on beaded agarose gel columns containing covalently linked purified full length Sid4+ or GFP peptide. The columns were generated using the AminoLink Plus Immobilization Kit (ThermoFisher Scientific, cat. 44894) and purification was performed according to manufacturers instructions with the following modifications: Purified antibodies were eluted in 900 µl fractions in elution buffer (0.5 M NaCl, 0.05 M glycine, 100 µg ml⁻¹ BSA, pH 2.3) and 100 µl of neutralisation buffer (0.5 M Na₂HPO₄) was subsequently added to each fraction. A total of 10 fractions were collected. Fractions containing purified antibodies were identified via loading of 10 µl samples from each fraction on a 10% acrylamide SDS PAGE gel and stained with Coomassie Blue. Fractions containing high concentrations of antibodies were pooled and glycerol was added to a final concentration of 50% before being snap frozen using liquid N₂ and stored at –80°C.

Rabbit polyclonal antibodies that specifically recognised peptides corresponding to Sid4 peptides when phosphorylated on T584 or simultaneously on both T275 and S278 were generated and affinity purified by Eurogentec.

**Yeast two-hybrid**

Vectors, strains and media used in the yeast two-hybrid assay were obtained from TaKaRa (Matchmaker Gold Yeast Two-Hybrid System, cat. 630489). Full length or fragments of *sid4*+, *cds1*+, *hhp1*+, *hhp2*+ and *ppc89*+ were cloned into either into pGADT7 AD Vector or pGBK7 DNA-BD Vector via the Nde1 and
BamH1 restriction sites. The pGADT7 AD Vector based constructs were transformed into Y187 Yeast Strain according to manufacturer’s instructions and selected in SD-Leu agar plates. The pGBK7 AD Vector based constructs were transformed into Y2HGold Yeast Strain according to the manufacturer’s instructions and selected in SD-Trp agar plates. Single colonies were isolated from each transformation and crosses were made between Y187 and Y2HGold on YPD agar plates and grown overnight at 30°C to generate diploids. Diploids were selected using SD-Trp-Leu agar plates. To perform yeast two-hybrid, single colonies of diploid strains containing both pGBK7 and pGADT7 constructs were grown for at least 4 hours at 30°C in SD-Trp-Leu media before OD\textsubscript{600} was used to calculate the plating of indicated cell numbers indicated on SD-Trp-Leu and SD-Trp-Leu-His and grown at 30°C for 2 to 3 days.

**Western blotting**

Total protein extracts were prepared via trichloroacetic acid (TCA) precipitation and dissolved in 1× SDS buffer (50 mM Tris-Cl pH 8, 2% (w/v) sodium dodecyl sulphate, 0.1% (w/v) bromphenol blue, 10% (v/v) glycerol, 100 mM β-mercaptoethanol) (Grallert and Hagan, 2017a). The proteins were separated on either 10% acrylamide or NuPAGE 4-12% Bis-tris (Life technologies, cat. NP0322) gels. Working concentrations of affinity purified anti-Sid4 were around 1 in 1000. Tissue culture supernatant TAT1 was used at a dilution of 1 in 500, cdc2 antibody (Abcam, cat. Ab5467) was 1 in 1000, while in house antibodies raised to and affinity purified with recombinant GFP were used at 1 in 1000. Alkaline-phosphatase-coupled secondary antibodies (Sigma) were used for all
blots followed by direct detection with NBT/BCIP substrates on nitrocellulose membranes.

Detection with Sid4 antibodies that recognise Sid4 when phosphorylated on T584 and simultaneously on both T275 and S278 were performed on immunopurified Sid4 from TCA precipitated whole cell extracts. 2 × 10^8 cells were used for each immunoprecipitation (Grallert and Hagan, 2017b). The cells were lysed in the presence of 200 μl of 20% TCA and 0.5 ml ice cold acid washed glass beads using a Yasui Kikai Multi-bead Shocker at 4°C (2500 rpm for 30 sec in a pre-cooled at 4 deg). The precipitated proteins were pelleted by centrifugation at 14,000 g at 4 °C and washed 2 x 1 ml with 0.1% ice cold TCA. The pelleted protein was subsequently dissolved in 100 μl of IP buffer (50 mM Tris-Cl pH 8, 50 mM NaCl, 1mM EDTA, 20 mM Na-β-glycerophosphate, 0.1 mM Na₃VO₄, 1 mM DTT, 0.2 % Triton-X100, protease inhibitor cocktail (Roche, cat. 11836153001), PhosSTOP (Roche, cat. 4906845001), 1 mM PMSF) containing 2% SDS and heated to 80 °C for 10 minutes. The denatured protein suspension was diluted further with 1 ml of IP buffer +1% Triton X-100 and centrifuged at 14,000 g at 4 °C to clear the denatured protein cell lysate. Denatured Sid4 was immunopurified using Sid4 antibodies and Pierce Protein A/G Magnetic Beads (ThermoFisher, cat. 88803). The primary antibody was diluted 1 in 20 in blocking buffer 1 (1% BSA, 100 mM lysine HCl, 10 nM NaH₂PO₄, 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween-20) and the alkaline-phosphatase-coupled secondary rabbit antibody (Sigma, cat. A3687) was diluted 1 in 10,000 and probed in blocking buffer 2 (1% BSA, 100 mM lysine HCl, 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween-20). Blots were detected with NBT/BCIP substrates on PVDF membranes.
**Co-immunoprecipitation of recombinant GFPn.Sid4(466-660) fragments and Ppc89.3Pk.**

Expression of N-terminally GFP tagged sid4 fragments (amino acids 466-660) (Fig. 2a) was induced by culture in thiamine free medium at mid-log phase for 24 hours at 25°C to a final cell density of $4 \times 10^6$ cells ml$^{-1}$. Cells were subsequently harvested via centrifugation at 5500 g for 1 minute and washed once with ice cold STOP buffer (10 mM EDTA, 50 mM NaF, 150 mM NaCl, 1 mM NaN$_3$) (Simanis and Nurse, 1986), centrifuged at 11,000 g for 1 minute at room temperature to remove the supernatant and snap frozen in liquid N$_2$ for storage at $\sim$ 80°C. For $4 \times 10^8$ cells, 200 µl of IP1 buffer (50 mM Tris-Cl pH 8, 50 mM NaCl, 1mM EDTA, 20 mM Na-β-glycerophosphate, 0.1 mM Na$_3$VO$_4$, 1 mM DTT, 100 mM lysine HCl, 0.5% Triton-X100, protease inhibitor cocktail (Roche, cat. 11836153001), 1 mM PMSF) was used (Grallert and Hagan, 2017b). The GFP tagged Sid4 fragments were precipitated using GFP-trap-M® (Chromotek, cat. Gtm-20) and co-precipitation of Ppc89.3Pk was assayed by Western blotting with monoclonal antibodies against the SV5 Pk epitope (Bio-Rad, cat. MCA1360) at a 1 in 1000 dilution from stock.

**Co-immunoprecipitation of Sid4 and Chk2$^{Cds1,GFP}$**

The approach of LaCava et al. was used to identify conditions for co-precipitation (LaCava et al., 2016). Cells were grown in EMM2 at 25°C to a cell density of $4 \times 10^6$ cells ml$^{-1}$ and subsequently harvested via centrifugation at 5500 g for 1 minute and washed once with ice cold STOP buffer (10 mM EDTA, 50 mM NaF, 150 mM NaCl, 1 mM NaN$_3$, 1 mM PMSF). After further centrifugation at 5500 g
for 1 minute the supernatant was removed and the cell paste passed through a 10 ml syringe into liquid N\textsubscript{2} to form frozen yeast cell “noodles”. The frozen cells were subsequently ground in a 6870 Freezer/Mill (SPEX SamplePrep) in 10 × 2 min grinding cycles set at level 10 with 2 min breaks at each interval. The ground cell powder was weighed and aliquoted into screw capped tubes, pre-cooled by immersion in liquid N\textsubscript{2}. 4 × 100 mg of ground cells were re-suspended in 400 µl of IP2 buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 50 mM NaF, 60 mM Na-β-glycerophosphate, 0.2 mM Na\textsubscript{3}VO\textsubscript{4}, 10% glycerol, 1 mM DTT, 2 mM EDTA, 0.2 % Triton X-100, protease inhibitor cocktail (Roche, cat. 11836153001), 1 mM PMSF). The re-suspended cell lysate was centrifuged at 14,000 g at 4 °C for 5 minutes and the cleared lysate was pooled into a single tube. Chk2\textsuperscript{cds1}.GFP was precipitated using GFP-trap-MA \textsuperscript{®} (Chromotek, cat. Gtma-20) and co-precipitation of Sid4 was assayed by Western blotting.

**Protein kinase assays**

Assessment of the activities of Fin1, Sid2 and Pom1 towards recombinant Sid4 were conducted as reported previously with the exception that recombinant Sid4 (see above) was substituted for the previously described substrates (Grallert et al., 2012; Bähler and Nurse, 2001). Fin1/Sid2 was precipitated from 2×10\textsuperscript{8} cells with polyclonal antibodies with Dynabeads A (Invitrogen) in KA buffer (50 mM HEPES, 10 mM EDTA, 40 mM Na-β-glycerophosphate, 4 mM Na\textsubscript{3}VO\textsubscript{4}, 50 mM NaF, 0.6% NP40, 150 mM NaCl, protease inhibitor cocktail (Roche, cat. 11836153001) and 1mM PMSF). The kinase reaction was carried out at 30°C for 30 min in KR buffer (20 mM HEPES, 15 mM KCl, 1 mM EGTA, 10 mM MgCl\textsubscript{2},
10 mM MnCl₂, 0.125 nM ATP and 10 μg of recombinant Sid4). For Pom1 assays 1.5 x 10⁸ cells were harvested by filtration, washed in STOP buffer and once in POM buffer (2.5 mM HEPES pH 7.4, 1% Triton X 100, 10% glycerol, 50 mM KCH₃CO₂, 50 mM NaF, 60 mM Na-β-glycerolphosphate, 2 mM EDTA, 1 mM di-thiothreitol, 0.1 mM Na₃VO₄, 15 mM p-nitrophenylphosphate, protease inhibitor cocktail (Roche, cat. 11836153001), 1 mM PMSF, 2 mM benzamidine) before re-suspension in POM buffer. After disruption by agitation with glass beads in a Yasui Kikai Multi-bead Shocker (2500 rpm for 30 sec in a pre-cooled at 4°C), and clearing of the cell supernatant, 9-E10 monoclonal antibodies that recognize the myc epitope were added along with Dynabeads A (Invitrogen) before incubation for 2 h with agitation at 4°C. After two washes in POM buffer and one in AB buffer (25 mM MOPS pH 7.0, 60 mM Na-β-glycerolphosphate, 7 mM MgCl₂, 7 mM MnCl₂, 0.1 mM Na₃VO₄, protease inhibitor cocktail (Roche cat. 11836153001), 2 mM benzamidine), the beads were re-suspended in AB buffer plus 10 μg of recombinant Sid4, 10 mM ATP and incubated at 30°C for 20 mins. Control experiments with the validated substrates ensured that the conditions to assay activity had been established (data not shown).

**Mass spectrometry**

Sid4 protein was isolated from *cdc2.3w wee1.50* cells (cells undergoing mitotic catastrophe (Russell and Nurse, 1987)) by denaturing immunoprecipitation according to (Grallert and Hagan, 2017c). 4 x 10¹⁰ cells were isolated by centrifugation from mid-log phase cultures that had been grown overnight at 25°C before incubation at 36°C for 90 minutes. After washing in STOP buffer,
cells were re-suspended in 2 mL ice cold 20% Trichloro-acetic acid before snap freezing in liquid nitrogen to produce yeast “noodles”. After re-suspension of the powder generated by cell disruption in a SamplePrep 6870 freezer mill (SPEX) in 40 mL ice cold 5% TCA, and centrifugation at 20 000g for 10 mins at 4°C, the supernatant was discarded before a further wash with 0.1% TCA. The pellet was re-suspended in 10 mL IP buffer (100 mM Tris, pH8, 50 mM NaCl, 1 mM EDTA, 20 mM Na-β-glycerolphosphate, 0.1 mM Na3VO4, 50 mM NaF, 0.2% SDS, 2 mM PMSF, protease inhibitor cocktail (Roche, cat. 11836153001)) containing 4% SDS. After 3 min at 70°C 90 mL of ice-cold IP buffer containing 1% Triton X-100 was added before centrifugation at 20 000g for 10 mins at 4°C. Denatured Sid4 was immunopurified from this supernatant using Sid4 antibodies chemically conjugated to Pierce Protein A/G Magnetic Beads (ThermoFisher, cat. 88803). Isolated Sid4 was subsequently analysed via mass spectrometric analysis according to (Unwin et al., 2005). Samples were run on 4–12% NuPAGE bis-Tris gel (Invitrogen); Sid4 bands were excised and digested with either 20 ng sequencing-grade trypsin (Sigma-Aldrich), 400 ng LysN (Associates of Cape Cod) or 350 ng elastase (Calbiochem) in 100 μL 40 mM ammonium bicarbonate with 9% (v/v) acetonitrile at 37 °C for 18 h. The peptides were separated using a Nano-Acquity UPLC system (Waters) using a Waters Nano Acquity BEH C18 column (75 μm inner diameter, 1.7 μm, 25 cm) with a gradient of 1–25% (v/v) of acetonitrile with 0.1% formic acid over 30 min at a flow rate of 400 nL min⁻¹. The LTQ-Orbitrap XL mass spectrometer was operated in parallel data-dependent mode where the mass spectrometry survey scan was performed at a nominal resolution of 60,000 (at m/z 400) in the Orbitrap analyser over an m/z range of 400–2,000. The top 6 precursors were selected for collision-induced
dissociation in the LTQ at a normalized collision energy of 35% using multi-stage activation at m/z 98.0, 49.0 and 32.7 Da.

**Summary of Supplemental Materials**

Supplementary Figures 1-5 support the data presented in Figures 1-8 while Supplementary Table 1 lists the strains used in the study.

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Cell Cycle control by spindle pole scaffolds

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Figure Legends

Figure 1: C terminal mutation of sid4 suppresses the cut12.1 SPB activation defect

A) Representative images of immuno-fluorescence to reveal tubulin, the spindle pole marker Sad1 and chromatin 3 hours after the temperature of an early log-phase culture was shifted from 25°C to 36°C in EMM2, n = 5. Arrows: the 2 SPBs.

B) A cartoon summarising the SPB molecules upon which this study focuses. The representation is highly stylised because the mode of anchoring to the SPB core remains unclear for Ppc89 and Cut12, although. Cut12 is known to promote mitotic commitment (red arrows), while the anchorage of Sid4 to the SPB by Ppc89 enables Sid4 to anchor Cdc11 to the SPB. As Cdc11 recruits the Septum Initiation Network (SIN) to the new SPB in anaphase, the recruitment of Cdc11 to Sid4 supports the events of mitotic exit such as septation and the formation of the equatorial microtubule organising centre (Simanis, 2015; Heitz et al., 2001).

C, D) Representative graphs indicating the frequency of spindle mono-polarity in samples of the indicated strains 3 hours after early log phase cultures were
shifted from 25°C to 36°C. For each strain 100 cells with spindle staining were scored as being either bi-polar or monopolar n = 3. Note that the SPB activation delay of cut12.1 means that mono-polarity gives an underestimate of the incidence of SPB activation defects (Tallada et al., 2009b). See also Fig. S1A. E) A schematic of the characterised associations of the indicated SPB components. The core SPB and SIN are indicated in grey. It is not clear whether only one or both of the components of the Sid4 dimer binds to Cdc11 or Ppc89. The coiled coil regions in Ppc89 have prompted us to show Ppc89 as a dimer, however, we note that homo-dimerization is yet to be demonstrated. F) The position of key mutations within an alignment of the sequences of the C termini of Sid4 from *Schizosaccharomyces* species and *Pneumocystis murina*.

**Figure 2: Mutagenic mimicry of phosphorylation at T584 imparts a sin− phenotype**

A) A scheme showing the configuration of sid4.T584E strains. B) Representative images of tubulin immunofluorescence as in Fig. 1A of sid4.T584E cells 3 hours after an early-log phase culture was shifted from 19°C to 36°C. The bi-nucleate interphase cells, indicated by arrows, arise from cytokinesis/septation failure in the previous cell cycle due to abolition of SIN function, n = 3. See also Fig. S2B. C) Spot tests of the indicated strains after growth on minimal (EMM2 – no thiamine sid4+ expressed), or rich (YES) medium (contains thiamine to repress sid4+) at the indicated temperatures. D) Representative monopolar counts as in Fig. 1C,D. See also Fig. S1B. n = 3 E-H) Western blots exploiting poly-clonal antibodies that recognise; Sid4 when phosphorylated on either T584, or simultaneously on both T275 and S278, or all species of either Sid4, or Fin1, or the myc epitope tags on
Pom1 as indicated. E) Blots of Sid4 immunoprecipitates of de-natured samples from cdc25.22 dma1.Δ cultures returned to 25°C, 4.25 hours after a shift to 36°C. As T584 phosphorylation peaks 40 minutes after release (data not shown), the impact of compromising kinase activities upon T584 phosphorylation was monitored at this time point in the figure. F) Fin1 and Sid2 were isolated with poly-clonal antibodies, while Pom1 with antibodies against the myc epitope, for kinase assays to identify which kinase could directly phosphorylate recombinant, full length Sid4 purified from E. coli. G) Polyclonal antibodies precipitated Fin1 from asynchronous cultures of the indicated strains for in vitro kinase assays with recombinant Sid4. T584 phosphorylation was detected with the antibodies used in E.

**Figure 3: T584 phosphorylation switches Sid4 affinity for Ppc89 to affinity for CK1δHhp1/CK1δHhp2**

A) A scheme showing the configuration of strains expressing fusions between the carboxyl-terminus of Sid4 and GFP to monitor affinity for the SPB in (B). B) Maximum projections of Z stacks of GFP fluorescence that span the diameter of the cell. C) Qualitative assessment of the impact of the indicated mutations upon the ability of the Sid4-GFP fusion protein to be recruited to the SPB. D) GFP-Trap® precipitation of the Sid4-GFP fusions used in A-C) reveal association of wild type and phospho-blocking T584V fusions with Ppc89.3Pk (detected with 336 antibodies against the Pk epitope). E) Yeast two-hybrid assays using the indicated baits and prey suggest that phosphorylation switches the affinity of unphosphorylated Sid4 for Ppc89 to an affinity for CK1δHhp1/CK1δHhp2, n = 3. For full dilution series see Fig. S1C.
**Figure 4: sid4.T584E compromises anchorage of Sid4 and Cdc11 to the SPB**

A) Representative fluorescence images showing how the SPB affinity of the T584 phospho-mimetic Sid4.T584EGFP fusion protein is reduced at 36°C. The cell walls of wild type *sid4.GFP* cells were stained by re-suspension in red lectin before being mixed with unstained *sid4.T584EGFP* cells and mounting the mixture for capture of a series of slices in the Z axis that were merged to give the maximum projection shown. n = 3. B) Western blots of mid-log phase cultures of the indicated strains in which the transcription of an ectopic copy of *sid4+* at the *hph.171* locus was either repressed (Sid4-) by the inclusion of 20 μM thiamine or de-repressed by the removal of thiamine 24 hours before sampling (Sid4+). n = 3. C) Representative fluorescence images of Cdc11.GFP in the indicated strains at the temperatures shown. Both fields show a mixture of *sid4+ cdc11.GFP* and *sid4.T584E cdc11.GFP* cells. The *sid4+ cdc11.GFP* cells were stained by transient re-suspension in red fluorescent lectin to identify them in the mixed field of view to highlight the reduction in fluorescence intensity arising from the *sid4.T584E* mutation, n = 3. D) The scheme for the “anchorage” of Sid4.T584EGFP to the SPB for the spot tests in E that show that *sid4.T584E* sin- lethality arises from Sid4 departure from the SPB. E) Spot tests in which serial dilution of the indicated strains were placed onto agar plates and incubated at 36°C.

**Figure 5: Recruitment of CK1δHhp1/CK1δHhp2 to T584 phosphorylated Sid4 supresses cut12.1 mono-polarity.**

A, B, D, E) Representative monopolar spindle counts of the indicated strains as for Fig. 1C in each case n = 3. See also Fig. S2. C) The scheme for the “anchorage”
of CK1δ^{His} and CK1δ^{His}_2.AS to the SPB to suppress the spindle activation defect of cut12.1.

**Figure 6: Phosphorylation on T584 promotes cell cycle dependent phosphorylation of both T275 and S278**

A) Antibodies raised against a peptide corresponding to Sid4 simultaneously phosphorylated on T275 and S278 used to blot Sid4 preparations immunoprecipitated from denatured extracts of the indicated strains. Immunoprecipitates were incubated for 30 minutes at 30 °C with λ phosphatase, its inhibitor or buffer alone as indicated. B-D) Small G2 dma1.Δ (B), sid4.GFP ppc89.GBP dma1.Δ (C) or sid4.T584EGFP ppc89.GBP dma1.Δ (D) cells were isolated from mid-log phase cultures by centrifugal elutriation at t=0 and aliquots taken at the indicated intervals to monitor septation and NETO with calcofluor white or phosphorylation on both T275 and S278. Control samples 40 minutes after cdc25.22 dma1.Δ control cultures were released from 4.25 h arrest at 36°C as indicated.

**Figure 7: Recruitment of Chk2^{Cds1} to T275S278 phosphorylated Sid4 compensates for the SPB activation defect of cut12.1**

A, F) Representative plots of monopolar spindle counts as for Fig. 1C. See also Fig. S3A. In each case n = 3 B) Yeast 2-hybrid comparisons of the indicated constructs. For full dilution series see Fig. S3B. Mrc1 is a validated Chk2^{Cds1} partner (Tanaka and Russell, 2001). C, D) BiFc assays of fluorescence generated between Chk2^{Cds1}.nYFP and Sid4.cYFP in the indicated strains. Each field shows cells from cultures of different strains as indicated. Cell walls of the strain
named in red having been stained by transient suspension in red fluorescent lectin to identify it in the mixed field. The images are maximum projections of de-convoluted Z stacks that span the diameter of the cell. See also Fig. S3C. n = 3
E) GFP Trap® precipitates from the indicated Chk2\textsuperscript{Cds1}.GFP \textit{dma1Δ} strains probed with Sid4 antibodies to detect co-precipitating Sid4. Co-precipitation was abolished by simultaneous phospho-blocking mutation at 275 and 278. n = 3
F) A switch from 20 μM thiamine to the indicated concentrations 24 hours before the temperature shift to 36°C de-repressed transcription of the Chk2\textsuperscript{Cds1}.GFPn in a dose dependent manner, n = 3

**Figure 8: Blocking phosphorylation on T275 and S278 of Sid4 to block**

\textit{Chk2\textsuperscript{Cds1}} recruitment abolishes \textit{Chk2\textsuperscript{Cds1}}'s ability of to evict Flp1.GFP from the SPB

A, B) Representative fluorescence and brightfield (BF) images of \textit{flp1.GFP ppc89.tdTom} cells that reveal how the eviction of Flp1.GFP from the SPB is abolished in the \textit{sid4.T275AS278A} background that prevents Chk2\textsuperscript{Cds1} recruitment to the SPB. C) Quantification of the data in A, B. n = 3 For controls see Fig. S4. D) Frequency of mono-polarity in the indicated strains as for Fig. 1B. Error bars in C, D: SD for 3 independent experiments.

**Figure 9: Model: Flp1 eviction by a Sid4 mediated NIMA\textsuperscript{Fin1}, \textit{CK1δHhp1/CK1δHhp2}, \textit{Chk2\textsuperscript{Cds1}} relay to boost the impact of Cdk1-Cyclin B activation at the SPB**

Phosphorylation of T584 by Fin1 reduces Sid4 affinity for Ppc89 and support binding to \textit{CK1δHhp1/CK1δHhp2}. The CK1δ kinases then phosphorylate T275 and
S278 to promote the recruitment of Chk2<sup>Cds1</sup>. As we see a BiFc signal between Chk2<sup>Cds1</sup> and Sid4, that is sensitive to competition from Dma1 on the SPB (Fig. 7C,D, Fig. S3C), we assume that the loss of affinity for Ppc89 on the SPB (A) is rapidly followed by a de-phosphorylation event after CK1δ kinases have phosphophorylated T275 and S278 to create the docking site for Chk2<sup>Cds1</sup>, or that Sid4 anchorage to the SPB is retained while CK1δ kinases phosphorylate T275 and S278, for example by the phosphorylation of only one Sid4 molecule within a Sid4 dimer (B) or, through more complex higher order associations of Sid4 molecules that await characterisation. The T584 phosphatase that would be an essential component in panel A could equally well operate in the scheme shown in B to remove phosphate from T584 while Chk2<sup>Cds1</sup> is anchored to Sid4 phosphorylated on T275 and S278. Sid4 anchored Chk2<sup>Cds1</sup> phosphorylates Flp1 phosphatase to reduce its affinity for the SPB thereby lowering antagonism towards Cdk1-Cyclin B phosphorylation events on the SPB. The consequence of this cascade is a reduction in the threshold to convert the SPB into a mitotic state. The SPB matures from an early G2 state with no potential to invoke mitosis (upper panel; red SPB), to a state with all three kinases in cycles of activation on the SPB (middle panel; amber), to the mitotic commitment state of Flp1 expulsion from the SPB (lower panel; green). We assume that the tipping of the balance between mid-G2 and commitment arises from alterations in the phosphatase activities that dephosphorylate T584, T275 and S278.
**Chan et al. Figure 2**

**A**

Diagram of genetic loci: 
- thiamine repressible
- hph.171k
- nmt81
- sid4\(^{+}\)
- natR
- native locus
- sid4.T584E
- kanR

**B**

Images showing:
- sid4.T584E
- DNA
- Tubulin

**C**

EMM2

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**D**

Graph showing percentage of monopolar spindles:
- sid4\(^{+}\)
- -
- +

Cut12.1: Sid4.T584\(^{+}\)
- sid4.T584E
- sid4.584V
- sid4.T584E

**E**

Western blots showing:
- T584P
- Sid4

**F**

**in vitro** kinase assays:
- Fin1
- Sid2
- Pom1
- T584P

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* 20µM 3MB-PP1 added to assay

**G**

Western blots showing:
- T584P
- Fin1

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* 3MB-PP1 added for 3 hours

Samples 30 minutes after cdc25.22 release

**legend:**
- Filled circle
- Empty circle
- Arrow

**Note:**
- 20 µM 3BrB-PP1 added 1h before release
- 20 µM 3BrB-PP1 added at release
Chan et al. Figure 4

A

B

C

D

E

Sid4.GFP

Red = wt
Unlabeled = T584E
BF

4 μm

19 °C

36 °C

kDa

sid4.GFP

sid4.T584E

19 °C

36 °C

2h

+sid4*

Sid4

α-tubulin

Cdc2

190

135

100

80

58

46

32

25

+sid4*

-sid4*

sid4.T584EGFP

sid4.T584EGFP ppc89GBP

Cdc11.GFP

Red wt Unlabeled T584E

Bright Field
**Figure 6**

A.

- **cdc25.22 dma1.Δ**
- Samples 30 minutes after cdc25.22 release
- ****20 μM 3BrB-PP1 added 1h before release
- *20 μM 3BrB-PP1 added at release

B.

- Frequency (%)
- Time (min)
- T275S278P
- Sid4

C.

- Frequency (%)
- cdc25.22 dma1.Δ
- T275S278P
- Sid4

D.

- Frequency (%)
- cdc25.22 dma1.Δ
**Figure 8**

**A**
- **Wild type**
  - 12 mM HU
  - Flp1.GFP
  - Ppc89.tdTomato
  - BF

**B**
- **sid4.T275AS278A**
  - 12 mM HU
  - Flp1.GFP
  - Ppc89.tdTomato
  - BF

**C**
- Graph showing Fip1.GFP SPB foci (%)
  - Before treatment
  - 4h 12 mM HU

**D**
- Graph showing monopolar spindle (%)
  - cut12.1
Early G2

Mid G2

Committed to Mitosis