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The *S. pombe* cytokinesis NDR kinase Sid2 activates Fin1 NIMA kinase to control mitotic commitment through Pom1/Wee1

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Mitotic exit integrates the reversal of the phosphorylation events initiated by mitotic kinases with a controlled cytokinesis event that cleaves the cell in two. The mitotic exit network (MEN) of budding yeast regulates both processes, whereas the fission yeast equivalent, the septum initiation network (SIN), controls only the execution of cytokinesis. The components and architecture of the SIN and MEN are highly conserved[1]. At present, it is assumed that the functions of the core SIN–MEN components are restricted to their characterized roles at the end of mitosis. We now show that the NDR (nuclear Dbf2-related) kinase component of the fission yeast SIN, Sid2–Mob1, acts independently of the other known SIN components in G2 phase of the cell cycle to control the timing of mitotic commitment. Sid2–Mob1 promotes mitotic commitment by directly activating the NIMA (Never In Mitosis)-related kinase Fin1. Fin1's activation promotes its own destruction, thereby making Fin1 activation a transient feature of G2 phase. This spike of Fin1 activation modulates the activity of the Pom1/Cdr1/Cdr2 geometry network towards Wee1.

Schizosaccharomyces pombe contains a single NIMA kinase, Fin1 (ref. 2). Deletion of *fin1*$^+$ (*fin1.Δ*) delays mitotic commitment[2], indicating that Fin1 emulates its *Aspergillus nidulans* counterpart in regulating the G2 to M transition[3]. We monitored Fin1 levels in cultures in which cell cycle progression had been synchronized by size selection of small, early G2 phase, cells. During mitosis, Fin1 levels paralleled the septation profile, dropping markedly as the septation index decreased (Fig. 1a, arrow C for cytokinetic decline). Fin1 levels rose sharply once more at the start of the following G2 phase before rapidly declining again midway through this G2 phase (Fig. 1a; arrow G2 for G2 phase decline). Fin1 accumulation/decline is a G2, rather than size-dependent, event because it did not occur when cell cycle progression was arrested at START or early S phase (data not shown).

Ablation of the APC/C had no impact on Fin1 protein levels[2] (data not shown); however, Fin1 accumulated in size-selected *skp1.A4* cultures following inactivation of the ubiquitous component of all Cullin-based E3 ubiquitin ligase complexes Skp1 (ref. 4; Fig. 1b). Substrate recognition by Cullin family E3 ligases is often contingent on phosphorylation to generate a phospho-degron recognition motif. Therefore, we investigated whether any kinases associated with either G2 or septation events influenced Fin1's stability, starting with Fin1 itself. Fin1 levels were elevated in *fin1.KD* cells in which Fin1 is catalytically inactive (Fig. 1c; for characterization of *fin1.KD*, see Supplementary Fig. S2a–d) and did not oscillate as synchronized *fin1.KD* cultures transited the cell cycle (Fig. 1d). Expression of a *fin1.GFP* fusion gene from a heterologous locus (wild-type Fin1 kinase, 27 kDa larger than Fin1.KD) induced fluctuations in the stability of the Fin1.KD protein that paralleled those of the wild-type fusion protein in the same cells (Fig. 1e). This ability of Fin1 activity to promote Fin1 destruction in *trans* probably arises from direct phosphorylation because Fin1 phosphorylates recombinant Fin1.KD *in vitro* (Fig. 1f).

We combined mutations in kinases associated with either G2 or septation events with *cdc25.22*. Inactivation of Cdc25 by incubation at 36 °C arrests cell cycle progression at the G2/M boundary, after the point at which Fin1 destruction is normally triggered. Deficiency in any kinase that promotes Fin1 destruction during G2 phase would be expected to elevate Fin1 levels in these arrested cells. Fin1 levels were markedly elevated in G2-arrested *sid2.250 cdc25.22* cells (Fig. 1g). We therefore used the temperature- and ATP-analogue-sensitive alleles *sid2.250* and *sid2.as4* (Supplementary Fig. S2h,i–l) to assess the impact of Sid2 function on Fin1 stability. In each case, Sid2 inactivation in early G2 abolished the sharp declines in Fin1 levels that normally occur

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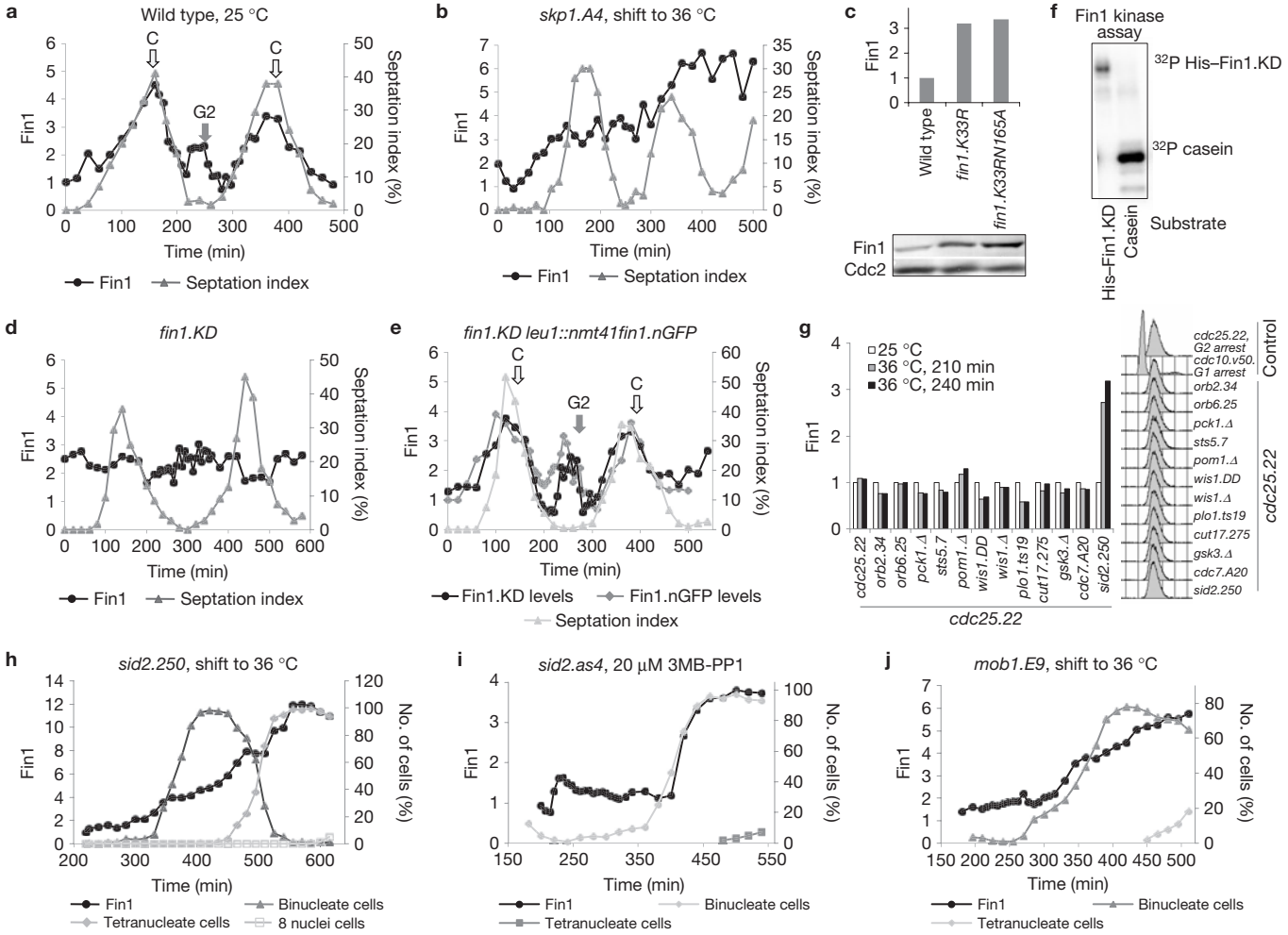

Figure 1 Fin1 kinase is destroyed twice in each cell cycle in a Cullin-, Fin1- and Sid2-dependent manner. (**a**,**b**,**d**,**e**,**h**–**j**) Fin1 levels were normalized to those of Cdc2 kinase in the same lane on the same blot and plotted against time as cells transit the cell cycle (for images of blots see Supplementary Fig. S1b). (**a**) Fin1 levels declined at two points in wild type cultures; mid-G2 (grey arrow G2) and during septation (open arrow C). Destruction was seen irrespective of whether the culture was maintained at 25 °C throughout the experiment, or shifted to 36 °C immediately after size selection (Supplementary Fig. S4c). (**b**) Oscillations in Fin1 levels were not seen after synchronized *skp1.A4* cultures were shifted to 36 °C immediately after size selection at 25 °C to inactivate Skp1. (**c**,**g**) Normalized Fin1 levels in blots of asynchronous or *cdc25.22* arrested double-mutant cultures reveal threefold increases in Fin1 levels in the *fin1.K33RN165A* kinase-dead and *sid2.250* backgrounds. (**d**) Fin1 levels did not fluctuate as *fin1.K33RN165A* cultures transited a synchronized cell cycle. (**e**) Strikingly the levels of both the inactive *fin1.K33RN165A* protein and the GFP-tagged wild-type protein oscillate as cells transit the cell cycle when a wild-type Fin1.GFP fusion protein was constitutively expressed within the same cells. (**f**) Fin1 immunoprecipitates from asynchronous cells were employed in kinase assays that used recombinant Fin1 or casein as substrates. (**g**) Left: 210 and 240 min refers to the duration of incubation at 36 °C to inactivate and arrest cell cycle progression at the G2/M boundary. Right: fluorescence-activated cell sorting profiles of DNA content demonstrate G2 arrest in all strains. (**h**–**j**) Assessing the impact of Sid2–Mob1 function on Fin1 levels in size-selected synchronized cultures. (**h**,**j**) *sid2.250* and *mob1.E9* cultures were maintained at 25 °C during transit through the first cell division before a portion of the culture was shifted to 36 °C to inactivate the kinase/regulatory subunit. (**i**) A *sid2.as4* culture was split into three after the first wave of septation was complete (Supplementary Fig. S1c) and either nothing, methanol or 3-MB-PP1 in methanol was added to a final concentration of 20 μM at time point 190. Images of blots/gels are shown in Supplementary Fig. S1.

during G2 phase and mitotic exit (Fig. 1h,i). The sharp increase in Fin1 levels in early G2 phase was also absent in the *sid2.250* culture (Fig. 1h), suggesting that Sid2 activity may also promote Fin1 production/stability at this point in the cell cycle. The levels of Fin1 in *fin1.KD skp1.A4*, and *sid2.as skp1.A4* were identical to those seen in the *fin1.KD* or *skp1.A4* or *sid2.as* single mutant backgrounds (Supplementary Fig. S3a,b). As Sid2 function in the SIN requires association with Mob1, we assessed Fin1 behaviour in synchronized *mob1.E9* mutant cultures. Fin1 behaviour in *mob1.E9* mirrored that seen in the *sid2.250* mutant background (Fig. 1j).

We studied the changes in Fin1 kinase specific activity in size-selected synchronized cultures (Fig. 2a and Supplementary Fig. S2b,d). Fin1 activity increased midway through G2 phase, and increased further after mitotic commitment (40–60 min before septation). Addition of the ATP analogue 3-MB-PP1 to size-selected *sid2.as4 skp1.A4* cultures abolished both the G2 and mitotic enhancement of Fin1 activity, supporting the view that Sid2 activity in G2 phase of the cell cycle promotes Fin1 activation (Fig. 2b).

Similarly to its budding yeast counterpart Dbf2, Sid2 targets the consensus sequence RXXS/T (refs 5,6). The two RXXS/T sequences in

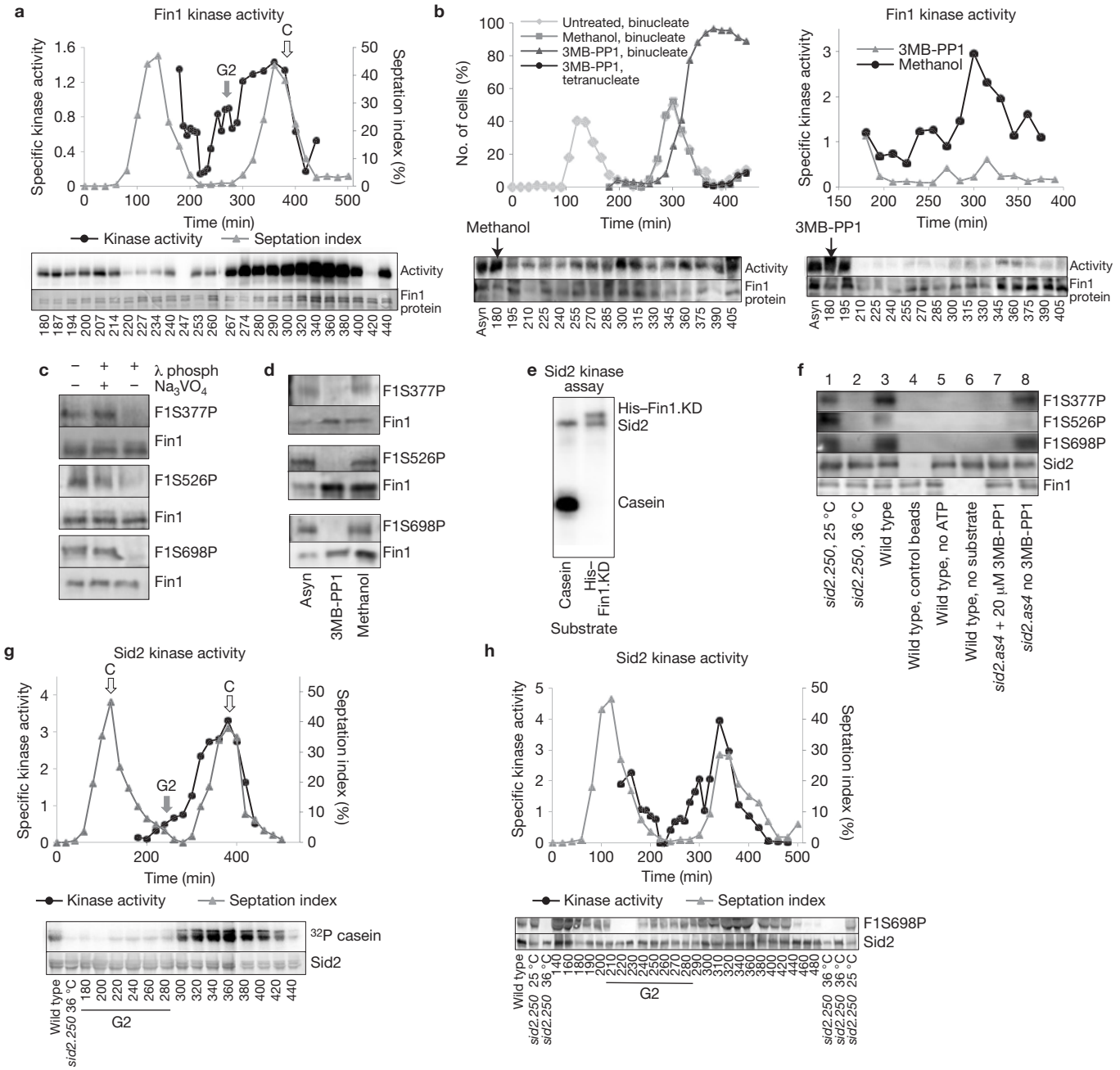

Figure 2 Sid2 phosphorylation of Fin1 on Ser 377, 526 and 698 promotes Fin1 activity in G2 phase before a peak of each kinase activity accompanies mitotic progression. (**a**,**b**) Fin1 kinase assays from size-selected cultures in which His-tagged Fin1.KD was labelled with [$\gamma-^{32}$P]ATP to quantify activity that is plotted alongside the septation profile. Wild-type (**a**) and small G2 *sid2.as4 skp1.A4* (**b**) cells were isolated from a culture grown at 25 °C and immediately shifted to 36 °C to inactivate Skp1 (and so preserve activated Fin1) at $t = 0$. The culture was split in two and 20 μM 3-MB-PP1 (left kinase assay) or solvent alone (right kinase assay) was added after the first division at $t = 180$. (**c**,**d**) Fin1 immunoprecipitates from asynchronous *skp1.A4* (**c**) or cell-size-selected *sid2.as4 skp1.A4* (**d**) cultures were split in two and probed with antibodies that recognize the indicated phosphorylation sites or polyclonal antibodies that recognize the non-catalytic domain of Fin1. See Supplementary Fig. S3g for details of the scheme used for each of the three identical cultures used to generate the samples and Supplementary Fig. 3h for the phenotypic characterization of one of the three cultures. Samples from asynchronous cultures are run in the left lane in each case to provide a reference standard. (**e**) Sid2 immunoprecipitates were isolated from asynchronous cultures and employed in *in vitro* kinase assays using [$\gamma-^{32}$P]ATP and either recombinant Fin1.KD or casein as indicated. (**f**) Sid2 was isolated from the indicated strains and combined with recombinant Fin1.KDnHis before blotting with the indicated antibodies. (**g**) The incorporation of ^{32}P into casein from [$\gamma-^{32}$P]ATP was used to monitor Sid2 activity in size-selected wild-type cultures. (**h**) Sid2 immunoprecipitates were processed as for **g** with the exception that the shorter Fin1.FP1 (non-catalytic carboxy-terminal domain[20]) was used as a substrate and the F1S698P antibody was used to develop the assay with the secondary reagent BCIP. The loading of the Sid2.250 36 °C sample in the second to last lane was four times that in other lanes to ensure that the basal level dictated by the reduced level of Sid2.250 protein in the 36 °C sample was representative of the reference point for normalization. (**g**,**h**) Plots show the activity per unit protein (that is, specific activity).

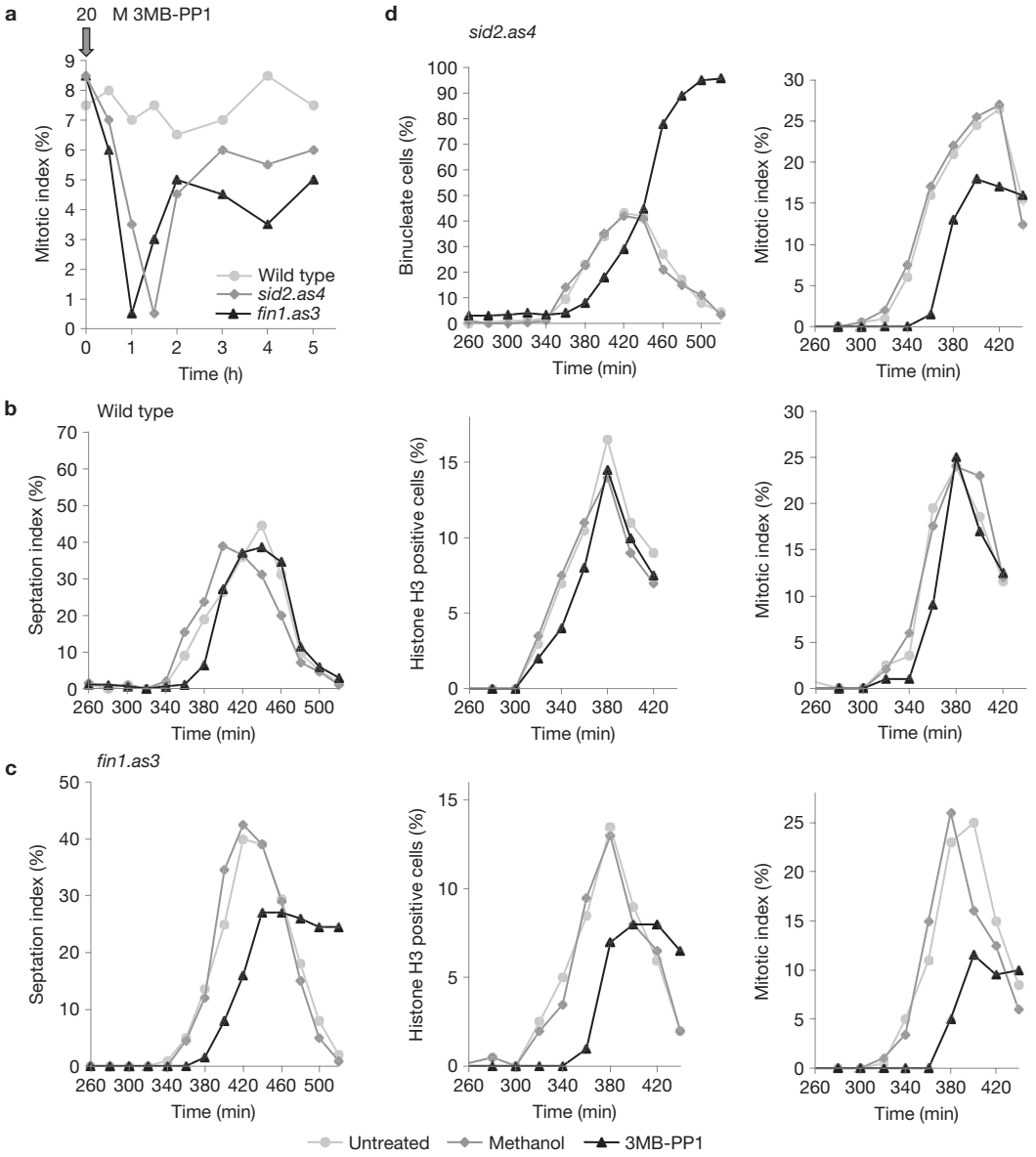

Figure 3 Inhibition of Sid2 or Fin1 delays mitotic commitment. (**a**) 3-MB-PP1 was added to asynchronous cultures of wild-type, *fin1.as3* and *sid2.as4* cells, and the mitotic index monitored by anti-α-tubulin immunofluorescence at the indicated times. The analogue transiently inhibited mitotic commitment of *fin1.as3* and *sid2.as4* but not wild-type cells. (**b**–**d**) Wild-type, *fin1.as3* and *sid2.as4* cultures were synchronized with respect to cell cycle progression by size selection and split into three equal cultures after the first round of septation. Methanol or 3-MB-PP1 (to a final concentration of 20 μM) in methanol was added to two of these sub-cultures at 160 min. Commitment to mitosis was monitored by the spindle index or phospho-histone H3 reactivity, as indicated. Addition of solvent alone had no impact on cell cycle progression whereas addition of analogue in solvent delayed mitotic commitment in *fin1.as3* and *sid2.as4* cells, but had no impact on wild-type cells.

Fin1 (Ser 377 (**R**VT**S**) and Ser 526 (**R**KV**S**)) are phosphorylated *in vivo* (Supplementary Fig. S3c–e). Antibodies that specifically recognize each site when phosphorylated (F1S377P, F1S526P) recognized Fin1 that had been immunoprecipitated from *skp1.A4* cells grown at 36 °C, but failed to do so when the precipitate had been treated with λ phosphatase (Fig. 2c). Their ability to recognize Fin1 precipitated from mid/late G2 phase *sid2.as4 skp1.A4* cells was dependent on Sid2 activity (Fig. 2d and Supplementary Fig. S3g,h).

Polyclonal antibodies against the non-catalytic domain of Sid2 (Supplementary Fig. S2m) were used for *in vitro* Sid2 kinase assays using either casein or His-tagged Fin1.KD as the *in vitro* substrate (Fig. 2e and Supplementary Fig. S2k,n). Both Ser 377 and Ser 526 of Fin1.KD (Fig. 2f, lanes 3–6) were phosphorylated by Sid2 immunoprecipitates; this activity was temperature dependent when Sid2 had been isolated from *sid2.250* cultures (Fig. 2f, lanes 1 and 2). Addition of 3-MB-PP1 to the analogue-sensitive kinase precipitated from *sid2.as4* cells also abolished activity towards each site (Fig. 2f, lanes 7 and 8). The continued fluctuation of Fin1.S377AS526A levels during cell cycle progression (Supplementary Fig. S3i) suggested that Sid2 might phosphorylate additional sites on Fin1. Consistently, Ser 698 was phosphorylated in mass spectra of recombinant Fin1.KD that had been incubated with Sid2 *in vitro* and in spectra of Fin1 precipitated from

Table 1 Cell length measurements support the model depicted in Fig. 4h.

Cell length at division	Cell length in micrometres
Anaphase cells	
Wild type, 36 °C, 2 h	13.0 ± 1.7
fin1.Δ, 36 °C, 2 h	14.0 ± 2.1
sid2.250, 36 °C, 2 h	15.6 ± 2.2
Septating cells	
Wild type	12.5 ± 1.8
fin1.Δ	13.3 ± 2.0
fin1.K33R	15.0 ± 2.2
fin1.KD	15.9 ± 1.6
fin1.S698A	13.9 ± 1.3
fin1.S698E	12.5 ± 1.3
fin1.2A	12.9 ± 1.4
fin1.2E	12.8 ± 1.2
fin1.3A	14.9 ± 1.9
fin1.3E	11.7 ± 1.7
cdc2.1w	7.9 ± 0.9
fin1.Δ cdc2.1w	8.1 ± 1.5
fin1.KD cdc2.1w	8.1 ± 0.7
cdc2.3w	8.0 ± 1.4
fin1.Δ cdc2.3w	10.7 ± 2.8
fin1.KD cdc2.3w	11.3 ± 4.0
pom1.Δ	11.1 ± 1.0
pom1.Δfin1.KD	11.4 ± 1.9
pom1.Δfin1.3A	11.0 ± 1.4
pom1.Δfin1.3E	10.9 ± 1.8
cdr1.Δ	18.4 ± 1.3
cdr1.Δfin1.KD	18.0 ± 1.3
cdr2.Δ	19.1 ± 1.5
cdr2.Δfin1.KD	19.3 ± 1.3
fin1.3A cdr2.Δ	18.9 ± 1.9
fin1.3E cdr2.Δ	18.4 ± 2.3

Cell length at division following treatment with ATP analogues		Cell length in micrometres
Septating cells		
Wild type		
	Untreated	12.5 ± 1.8
	3-MB-PP1, 2 h	12.4 ± 1.3
	3-MB-PP1, 4 h	12.8 ± 1.3
	3-MB-PP1, 8 h	12.3 ± 1.0
fin1.as3		
	Untreated	13.8 ± 1.4
	3-MB-PP1, 2 h	13.2 ± 1.0
	3-MB-PP1, 4 h	15.2 ± 1.8
	3-MB-PP1, 8 h	15.7 ± 1.0
fin1.as3 cdc2.1w		
	Untreated	7.7 ± 0.9
	3-MB-PP1, 2 h	7.8 ± 0.8
	3-MB-PP1, 4 h	8.2 ± 0.9
	3-MB-PP1, 8 h	8.0 ± 0.9
fin1.as3 cdc2.3w		
	Untreated	10.8 ± 1.1
	3-MB-PP1, 2 h	11.0 ± 1.2
	3-MB-PP1, 4 h	11.9 ± 1.4
	3-MB-PP1, 8 h	12.0 ± 1.5
fin1.as3 pom1.Δ		
	Untreated	11.5 ± 1.4
	3-MB-PP1, 2 h	11.6 ± 1.6
	3-MB-PP1, 4 h	12.0 ± 1.6
	3-MB-PP1, 8 h	12.0 ± 1.6
fin1.as3 cdr1.Δ		
	Untreated	17.3 ± 1.1
	3-MB-PP1, 2 h	17.4 ± 1.6
	3-MB-PP1, 4 h	18.3 ± 1.5
	3-MB-PP1, 8 h	18.2 ± 1.6
fin1.as3 cdr2.Δ		
	Untreated	19.4 ± 1.4
	3-MB-PP1, 2 h	19.9 ± 1.2
	3-MB-PP1, 4 h	21.1 ± 1.6
	3-MB-PP1, 8 h	20.2 ± 1.6
fin1.as3 syncnhronous		
	420 min	16.1 ± 1.1
	460 min	17.5 ± 1.1
	580 min	18.8 ± 1.4
	620 min	18.9 ± 1.2

For cell length at division, cells were grown in EMM2 medium to an attenuance of 0.1 at 595 nm at 25 °C unless otherwise indicated and stained with either DAPI/calcofluor to identify anaphase cells, or calcofluor alone to identify septating cells, as indicated. For cell length at division following treatment with 20 μM 3MB-PP1, cells were grown in EMM2 medium to an attenuance of 0.1 at 595 nm at 25 °C and stained with calcofluor to identify septating cells.

skp1.A4 cells (Supplementary Fig. S3f). Although the context of Ser 698 (LPG**S**) does not conform to the RXXS/T consensus[5], we note that the analogous budding yeast kinase, Dbf2, also phosphorylates sites that deviate from this *in vitro*-defined consensus[7]. The recognition of Fin1 by antibodies that specifically recognize phosphorylated Ser 698 (F1S698P) mirrored the *in vitro* and *in vivo* dependency on Sid2 function exhibited by F1S377P and F1S526P (Fig. 2c,d,f), indicating that Ser 698 is directly phosphorylated by Sid2 in G2 phase. Integration of the triply mutated *fin1.S377AS526AS698A* (*fin1.3A*) allele at the *fin1* locus generated a protein whose levels remained constant as cells transited the cell cycle (Supplementary Fig. S3j), supporting the view that direct phosphorylation of all three sites by Sid2 regulates Fin1 activity *in vivo*.

We quantified the cell-cycle-dependent variation in Sid2 activity by using either ^{32}P incorporation into casein or F1698P reactivity of Fin1.KD (Fig. 2g,h). The signal from Sid2 precipitated from *sid2.250* cells that had been incubated at the restrictive temperature of 36 °C defined the baseline level of activity in each assay. Sid2 specific activity in size-selected synchronous cultures increased significantly following mitotic commitment. Importantly, whereas the activity did not exceed that of the Sid2.250 control early in G2 (Fig. 2g, $t = 180$ and 200; Fig. 2h, $t = 220$ and $t = 230$), it did in the later stages (Fig. 2g,h; compare *sid2.250* lanes with: 220–280 (Fig. 2g) and 240–280 (Fig. 2h)). We conclude that Sid2 activity towards Fin1 appears in G2 phase before being considerably enhanced during mitosis.

We addressed the functional significance of Fin1 activation by Sid2 in G2 phase by exploiting our ability to inhibit Fin1.as3 (Supplementary Fig. S2e–g) and Sid2.as4 (Supplementary Fig. S2h–l) function with the ATP analogue 3-MB-PP1. Analogue addition to asynchronous *sid2.as4* or *fin1.as3* cultures prompted an immediate, but transient, decline in mitotic index (Fig. 3a). Cell size at division increased during the recovery from this transient G2 arrest (Table 1), indicating that inhibition of Sid2 or Fin1 delayed the timing of mitotic commitment until a new size threshold for division was met. Addition of analogue to size-selected wild-type cultures immediately after completion of the first synchronous division had little impact on the timing of the

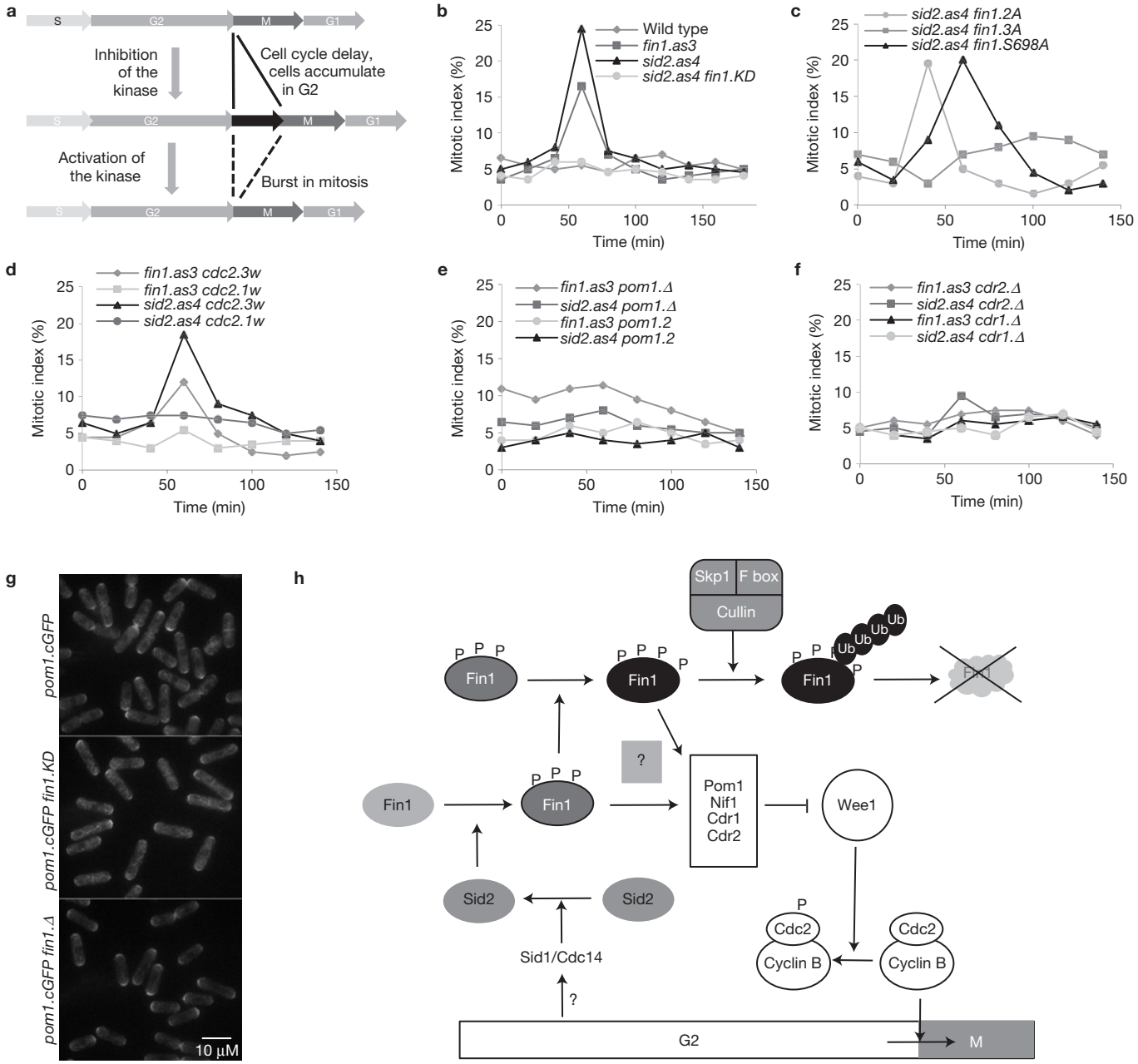

Figure 4 Sid2 and Fin1 target Cdr1/Cdr2/Pom1 to control mitotic commitment through Wee1. (**a**) Schematic detailing the analogue wash-out approach. (**b**–**f**) 5 h after the addition of 3-MB-PP1 to early log-phase cultures of the indicated strains cells were filtered from the culture and re-suspended at the same density in growth medium that contained no inhibitor. Restoration of Sid2 and Fin1 function induced a burst of mitosis. Importantly, restoration of Sid2 function failed to induce mitotic commitment when Fin1 kinase was inactivated by the *fin1.KD* mutations (**b**). (**c**) Restoring Sid2 activity in strains harbouring mutation of the candidate Sid2 phosphorylation sites in Fin1 suggests that Sid2 can activate Fin1 by phosphorylating the serine at either 377, 526 or 698. Both Sid2 and Fin1 were able to induce mitosis when the *cdc2.3w* mutation compromised sensitivity to Cdc25, but not when the *cdc2.1w* mutation compromised Wee1 inhibition of Cdc2 (**d**), or the functions of Pom1, Cdr1 or Cdr2 are ablated (**e**,**f**). (**g**) Pom1.GFP signals in the indicated strain backgrounds. (**h**) A schematic depicting the model for G2/M control by Sid2/Fin1. P represents phosphorylation, and Ub represents ubiquitin conjugation. Fin1 is activated in G2 by phosphorylation by Sid2. This promotes mitotic commitment through modulation of the geometry network; however, the exact mechanism remains to be determined. Activated Fin1 promotes its own destruction, thereby limiting its activity temporally. Our present lack of understanding of the means by which Fin1 regulates the Pom1/Cdr1/Cdr2/Wee1 cell geometry network is represented by incorporating all members of this pathway that are required for Fin1 to regulate mitotic commitment within a single rectangle. The question mark to the left of this rectangle reflects our lack of knowledge as to whether it is the Sid2 or the auto-phosphorylated form of Fin1 that is responsible for the control of the cell geometry network. The question mark beneath Sid1–Cdc14 reflects our ignorance as to the nature of the cue in G2 phase that triggers this pathway. See main text for further details.

subsequent mitosis (Fig. 3b). In contrast it delayed the appearance of mitotic spindles in both *fin1.as3* and *sid2.as4* cells (Fig. 3c,d). Importantly, the length of septating *fin1.as3* cells increased following analogue addition (Table 1), confirming that the delayed appearance of mitotic cells represented a true delay in mitotic commitment rather than an impact on growth rate.

Neither Fin1 nor Sid2 is essential for mitotic progression at 25 °C. If Sid2 activation of Fin1 promotes mitotic commitment, inhibition of either kinase would create a population of late G2 cells that had grown beyond the point in G2 at which the kinase normally promotes mitotic commitment. Restoration of kinase activity to those cells that occupy this newly created 'permissive' window within the extended G2 would be expected to promote immediate entry of this cohort into mitosis, resulting in a synchronized wave of division (Fig. 4a). We therefore transiently inhibited each kinase by analogue addition before restoring activity by filtration and resuspension into fresh medium devoid of analogue one generation time later. In each case restoration of kinase activity was followed by a peak of mitotic cells (Fig. 4b) confirming that each kinase promotes mitotic commitment.

Further analogue wash-out experiments enabled us to draw two important conclusions about the relationship between Sid2 and Fin1. First, Fin1 seems to be the main target for Sid2 kinase in mitotic commitment control because inactivation of Fin1 abolished the burst of mitosis in a *sid2.as4 fin1.KD* analogue wash-out experiment (Fig. 4b). Second, the Sid2/Fin1 switch is very responsive to Sid2 activity, because phosphorylation of either 698 alone or 377 and 526 promotes mitosis (Fig. 4c).

Next, we examined whether Sid2/Fin1 signalling targeted the maturation-promoting factor regulators Wee1 and Cdc25, by exploiting two well-characterized *cdc2* mutations; *cdc2.3w* mutants are largely insensitive to Cdc25 (ref. 8) whereas *cdc2.1w* cells are largely insensitive to Wee1 (refs 9,10). The peaks of mitosis arising from reactivation of either Sid2 or Fin1 were abolished by *cdc2.1w* but not *cdc2.3w* (Fig. 4d), suggesting an exclusive routing through Wee1. Consistently, *wee1.Δ* also blocked the mitotic induction in analogue wash-out experiments (data not shown).

Pom1 kinase controls the timing of mitotic commitment by regulating the inhibitory impact of Cdr1/Cdr2 on Wee1 activity[11–14]. *pom1.Δ*, *pom1.2*, *cdr1.Δ* or *cdr2.Δ* (but not *blt1.Δ*, data not shown) mutations mimicked *cdc2.1w* in blocking the wave of mitosis (Fig. 4e,f), indicating that the Sid2/Fin1 switch regulates Wee1 through Pom1. The means by which it does so awaits further analysis; however, we note that Pom1 remained at the tips of *fin1.KD* or *fin1.Δ* cells (Fig. 4g).

In *S. pombe*, there is a direct correlation between cell length at septation and the timing of mitotic commitment[15]. Blocking Fin1 function by mutational or chemical ablation of kinase activity, or gene deletion, increased cell length at septation, indicating a delay in mitotic commitment (Table 1). Blocking Sid2 function with the *sid2.250* mutation similarly delayed mitotic commitment at 36 °C (Table 1). Mutations that either blocked, or mimicked, phosphorylation of Fin1 by Sid2, increased or reduced cell length at division, respectively (Table 1). Importantly, loss of function of any of the proteins predicted by the analogue wash-out experiments to act after Fin1 either abolished the delay to mitotic commitment arising from deletion of *fin1*$^+$ (*pom1.Δ*, *cdc2.1w*) or were not additive with it (*cdr1.Δ*, *cdr2.Δ*), confirming that Fin1 relies on Pom1 control of Cdr1/Cdr2 to modulate Wee1 activity (Table 1).

In summary, we believe that activation of Sid2 midway through G2 phase phosphorylates Ser 377, Ser 526 and Ser 698 of Fin1. Fin1 activation has two consequences: Fin1 destruction and the promotion of mitotic commitment through the Pom1/Cdr1/Cdr2/Wee1 cell geometry network (Fig. 4h).

Although Sid2–Mob1 kinase is a well-characterized SIN component[1,16], three arguments suggest that the population of Sid2/Mob1 that drives the G2 control of Fin1 is not operating within the context of the SIN. First, restoration of Sid2 activity in analogue wash-out experiments promoted a burst of mitosis when SIN function was ablated in *sid4.SA1* and *cdc11.136* cells at 36 °C (Supplementary Fig. S4a,b). Second, cycles of Fin1 accumulation and destruction persisted in synchronized cultures when SIN activity was abolished by the mutations *cdc11.136* and *cdc7.A20 spg1.B8* (Supplementary Fig. S4c–e). In *sid4.SA1* cultures, Fin1 destruction in G2 persisted; however, the mitotic accumulation of Fin1 was perturbed (Supplementary Fig. S4f). Finally, induction of SIN signalling by inactivation of Cdc16 (ref. 17) did not promote Fin1 destruction (Supplementary Fig. S4g). The impact of conditional mutations in Sid1–Cdc14 kinase were more complex as it is required for the accumulation and degradation of Fin1 in early G2 phase (Supplementary Fig. S4h,i). This would suggest that the tight relationship proposed for Sid1 activation of Sid2 in the SIN may also couple the functions of these kinases in the control of Sid2 in G2 (ref. 18). Whereas the Fin1 profile of *sid1.c14* cells shows only a marginal decline in Fin levels, the data for the *cdc14.332* strain, which is more easily synchronized by size selection, clearly show persistence of Fin1 destruction at the end of mitosis (Supplementary Fig. S4i).

As the diffuse distribution of Fin1 during G2 phase contrasts with the association of Sid2.GFP with the spindle pole body[16,19] (SPB) at this time, we used the polyclonal Sid2 antibodies to re-examine the distribution of Sid2. Unlike the Sid2.GFP fusion protein, wild-type, untagged, Sid2 did not associate with interphase SPBs (Supplementary Fig. S5a,b), even though Sid2 was present at this stage of the cell cycle (Supplementary Fig. S5c). Thus, it is a cytoplasmic population of Sid2 that is activated midway through G2 to regulate a cytoplasmic population of Fin1. We assume that Fin1 activity in an individual cell in G2 is transient because Fin1 activation promotes its own destruction. When this control is missing, G2 phase is extended by 50%. This role for Fin1 in promoting mitotic commitment through Pom1/Cdr1/Cdr2 is distinct from its role in regulating Plo1 recruitment to the SPB (ref. 20), as the *plo1.S402A* mutation that perturbs the latter[21] had no impact on the ability of Fin1 or Sid2 to promote mitotic commitment in analogue wash-out experiments (data not shown). The timing of Sid2/Fin1 activation is strikingly reminiscent of the point in G2 phase proposed when an invariant timer is triggered by the completion of the variable sizer to commence preparations for mitotic commitment[22]. Thus, mitotic commitment in fission yeast may be more similar to the staged antephase/mitotic commitment of mammalian somatic cells than the rapid transitions seen in *Xenopus* or sea urchin oocyte systems[23]. □

METHODS

Methods and any associated references are available in the online version of the paper at www.nature.com/naturecellbiology

Note: Supplementary Information is available on the Nature Cell Biology website

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AUTHOR CONTRIBUTIONS

A.G. and I.M.H. conceived the study. A.G. did all of the experimentation with the exception of the mass spectrometry. Y.C. and D.L.S. did the mass spectrometry. V.S. provided essential advice and strains for the analysis of SIN function. A.G. and I.M.H. wrote the manuscript with comments and advice from V.S.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Cell culture and growth. The strains used in this study are listed in Supplementary Table S1. Cells were grown and maintained according to ref.[24]. Appropriately supplemented EMM2 synthetic medium was used for all experiments, except cell length measurements, which were conducted with prototrophic strains in unsupplemented EMM2. Centrifugal elutriation was used to isolate small G2 cells[25]. ATP analogues (Toronto Research Chemicals) were dissolved in methanol to generate 50 mM stock solutions that were subsequently added to cultures. For all data presented outwith Supplementary Fig. S2, 3-MB-PP1 at a final concentration of 20 μM (0.04 % methanol of total culture volume) was used to inhibit Sid2.as4 and Fin1.as3 function.

Microscopy and fluorescence-activated cell sorting analysis. Anti-tubulin/Sad1, anti-Sid2 and anti-histone H3 phospho-serine 10 immunofluorescence and calcofluor staining were conducted using established procedures after antibody dilution: TAT1 1:80, AP9.2 Sad1 1:25, Sid2 1:100 and phospho-histone H3 1:100 (refs 15,26,27). For Sid2 fluorescence, fixation for 10 min with 3% formaldehyde was used for optimal SPB staining, and the duration of this fixation period was extended to 30 min to give optimal preservation of the signal on the cytokinetic ring[28]. DNA content through fluorescence-activated cell sorting analysis used Cytox green (Invitrogen) according to published procedures[29].

Genetic manipulation, fusion protein production and antibody generation. Mutant alleles were generated by QuikChange mutagenesis (Stratagene) and inserted into the genome with the marker switch approach using *natMX6* as a marker[30] (Supplementary Fig. S2). The Sid2 sheep polyclonal antibody was raised by the Scottish National Blood Transfusion Service against recombinant $Sid2^{1-205}$ that had been purified from *Escherichia coli*. Rabbit polyclonal antibodies against histone H3 Ser 10 and peptides corresponding to the sequences surrounding Ser 377, Ser 526 and Ser 698 in which these serines were phosphorylated were generated by Eurogentec. The generation and purification of Fin1 polyclonal antibodies has been described previously[19]. Recombinant Fin1.KDn6His was purified from *E. coli*.

Immunoblotting and kinase assays. To monitor Fin1 levels, all blots were cut in half, one half developed with anti-Fin1 antibodies and the other with PN24 antibodies that recognize Cdc2[19,31]. Blots were developed with BCIP and scanned or developed with ECF (Amersham) and imaged with a Pharos FX Molecular Imager (BioRad). Identical profiles were obtained when fluorescent antibodies were used rather than BCIP as a substrate for the secondary antibody (compare Fig. 1a with Supplementary Fig. S1a). Blots were quantified using ImageJ. A mask was drawn around each band on the blot. The total counts were recorded when the mask was both over the band and again immediately below it, in the blank region of the lane. Subtraction of the second value from the first gave the protein level. In all cases, Fin1 levels were normalized to those of Cdc2 in the same lane on the same blot. The level of Fin1 observed immediately after elution of wild-type cells was set at 1 and all other levels were quantified relative to this basal level. For western blotting, Fin1, Sid2 and Cdc2 antibodies were used at a dilution of 1:500; the three phospho-specific antibodies were used at a dilution of 1:20. For immunoprecipitations, 15 μl Dynabeads A (Invitrogen, 100.02D) was loaded with 1.5 μl Fin1 or Sid2 undiluted antibody and used as per the manufacturer's instructions.

For the kinase assays, Fin1/Sid2 was precipitated from 2×10^8 cells with polyclonal antibodies with Dynabeads A (Invitrogen) in KA buffer: 50 mM HEPES, 10 mM EDTA, 40 mM Na-β-glycPhosh, 4 mM Na_3VO_4, 50 mM NaF, 0.6% NP40, 150 mM NaCl, protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulphonyl fluoride. 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 $MgCl_2$, 10 mM $MnCl_2$, 0.125 nM ATP and 10 μg of either recombinant Fin1.KDn6His (Fig. 2a–d and Supplementary Fig. S2b,d,k,n) casein (Figs 1f and 2e,g) or Fin1FP1 (ref. 19) (Fig. 2h). For assessment through ^{32}P incorporation, 5 μCi[$\gamma-^{32}$P]ATP was also added to the reaction mix. The amount of ^{32}P incorporated into the substrate was determined with a Phosphorimager (BioRad).

To identify *in vivo* phosphorylation sites on Fin1, the protein was precipitated from 2×10^{11} *skp1.A4* cells with polyclonal α-Fin1 antibodies. To identify *in vitro* Sid2-phosphorylation sites on Fin1, a Sid2 kinase assay was carried out as described in the previous paragraph using 50 μg recombinant Fin1.KDn6His as a substrate having isolated Sid2 from 6×10^8 cells. The Fin1.KD protein was isolated from the reaction mix using Ni-magnetic beads (Qiagen) before mass spectrometry.

Mass spectrometry. Samples were run on 4–12% NuPAGE bis-Tris gel (Invitrogen); Fin1 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 NanoAcquity 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.

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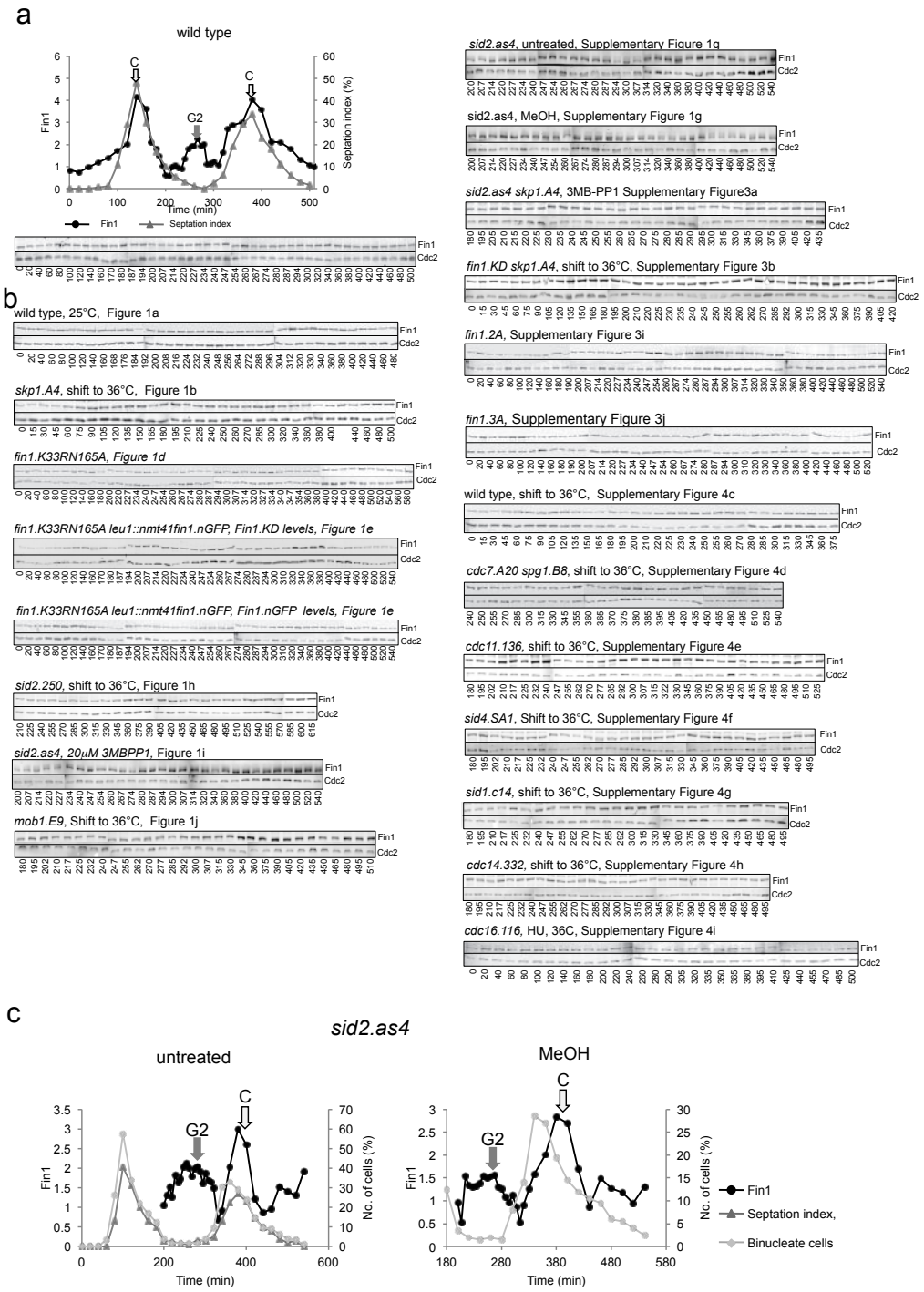

Figure S1 *Fin1 levels; protein blot data.* a) Fin1 levels were normalised to the level of Cdc2 kinase in the same lane on the same blot and plotted against time as cells transit the cell cycle. Fin1 was destroyed twice as wild type cells transited the cell cycle; mid-G2 (Grey arrow: G) and during septation (open arrow: C). The blot used to generate the plot is shown below the graph. This blot was developed with the fluorescent substrate ECF, while those for all other synchronised cultures in this manuscript were performed with the chromogenic substrate BCIP. The profile in a) is indistinguishable from the plot in Figure 1a in which BCIP was used to develop the blot. b) The blots that were quantified to generate the data shown in the indicated figures. (c) Fin1 levels and septation profiles of the portions of the culture shown in in Figure 1i that received no treatment (left) or to which an equivalent volume of methanol had been added to that bearing 3-MB-PP1 in Figure 1i.

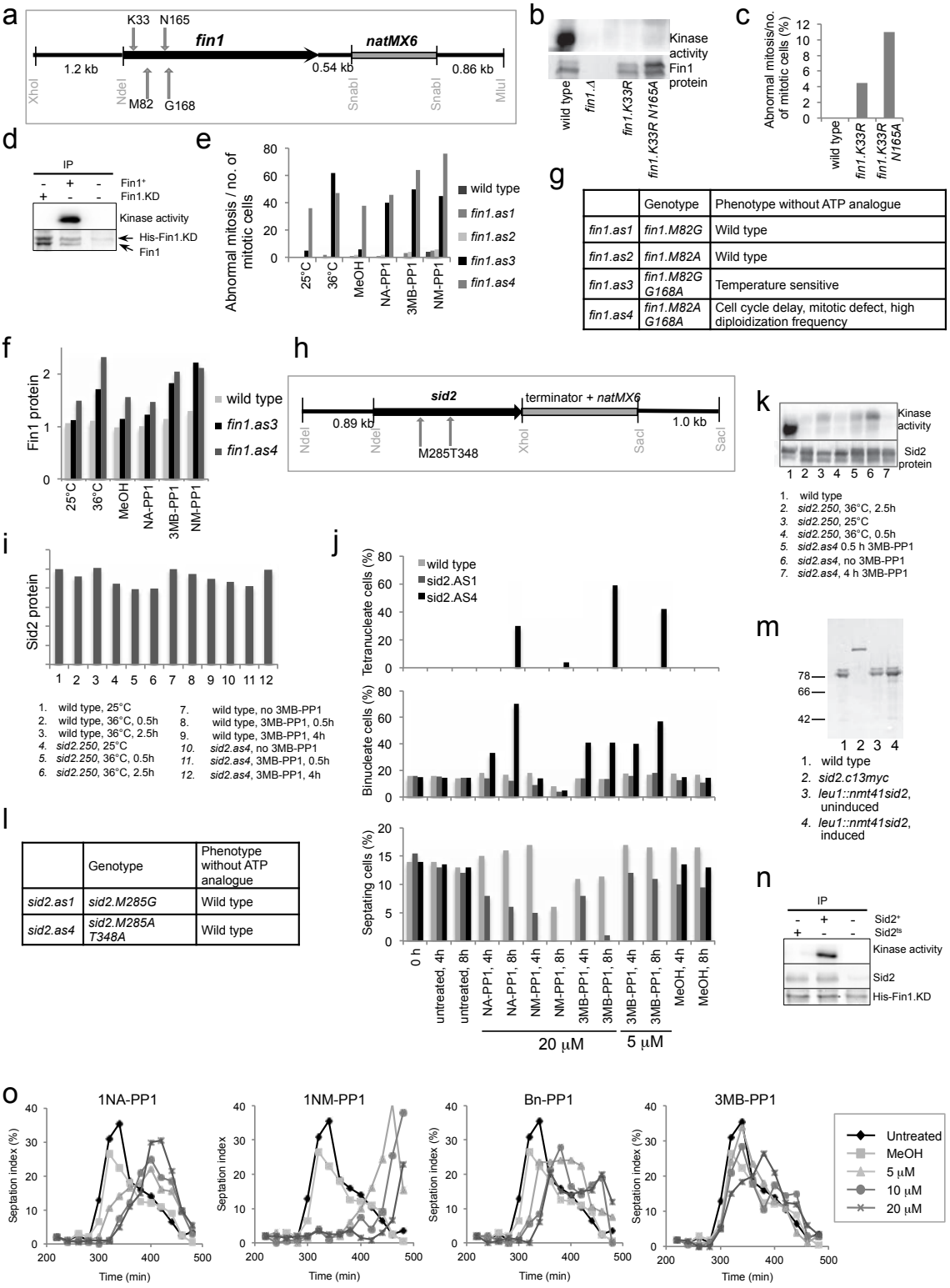

Figure S2 *Fin1and Sid2 reagents and the impact of ATP analogues upon cell cycle progression.* a-g) Fin1 reagents. a-d) *fin1.K33RN165A* encodes a catalytically inactive protein. a) A cartoon of the construct used to integrate *fin1* mutant alleles at the native locus indicating the location of the residues within the catalytic domain that were mutated to abolish kinase activity (K33, N165) or generate analogue sensitive alleles (M82, G168). The selection of the two sites for kinase inactivation was based upon the previous work in many labs that has established that point mutation of either the conserved lysine in domain II to arginine or the asparagine of domain VIB to alanine severely compromises kinase activity[1]. b) Fin1 kinase assays in which the activity of wild type Fin1 and Fin1.K33R or Fin1.K33RN165A mutant proteins against casein is assessed. c) The spindle formation defect after 3 hours at 37°C of the double *fin1.K33RN165A* mutant was greater than that of the single *fin1.K33R.* Similarly Fin1 protein accumulated to a greater extent in *fin1.K33RN165A* than in *fin1.K33R* cells (Figure 1c). Finally, cell length measurements indicate a greater defect in the double *fin1.K33RN165A* than single *fin1.K33R* mutant (Table 1). We conclude that the *fin1.K33RN165A* allele encodes a catalytically inactive molecule and is therefore referred to as *fin1.KD* (for kinase dead) throughout the manuscript. d) Fin1 kinase assays in which the catalytically inactivated Fin1 substrate is mixed with immunoprecipitates from the indicated strains; the substrate on it's own possesses no intrinsic kinase activity. e) A graph scoring the frequency of abnormal mitoses 3h following addition of analogue to the *fin1.asx* alleles indicated. f) Fin1 levels following normalisation to control Cdc2 protein levels in the indicated strains 3 hours after the addition of the indicated ATP analogues to early log phase cultures at 25°C and 36°C. g) A table showing the mutation sites and phenotypes of the different *fin1.as* alleles. (h-n) *Sid2 reagents* (h) A cartoon of the construct used to integrate *sid2* alleles at the native locus indicating the location of the residues within the catalytic domain (M285, T348) that were selected for mutation to generate analogue sensitive alleles according to the published data from the Shokat lab[2,3]. (i) Sid2 protein levels (normalised to those of Cdc2 in the same lane on the same blots) in the indicated strains after the indicated manipulations. (j) Plots of the indicated phenotypes at the times shown after the addition of the indicated ATP analogues or solvent alone to the indicated *sid2.as* alleles. (k) *in vitro* Sid2 kinase assays in which Sid2 immunoprecipitates from the indicated strains were used to phosphorylate casein with ^{32}PγATP demonstrate that this assay specifically detects Sid2 kinase and no other activity. (l) A table showing the mutation sites and phenotypes of the different *sid2.as* alleles. (m) Characterisation of Sid2 antibodies. The slower migration of the band recognised by affinity purified Sid2 antibodies in wild type cells in a *sid2.13myc* background and the enhancement of the band upon induction of the expression of an integrated version of the *sid2*$^+$ gene at the *leu1* locus (*leu1::nmt41sid2*) indicates that these antibodies recognise the Sid2 protein. n) Sid2 kinase assays in which the catalytically inactivated Fin1 substrate is mixed with immunoprecipitates from the indicated strains. The right hand lane indicates that the substrate on it's own possesses no intrinsic kinase activity under the reaction conditions used to monitor Sid2 activity. o) Impact of ATP analogue addition upon the septation profile of wild type cells. A wild type culture was synchronised with respect to cell cycle progression by size selection and split into equal portions to which the indicated analogues were added at the indicated concentrations. Septation was used as a read out of cell cycle progression. While the timing of septation was perturbed by the addition of the indicated concentrations of 1-NA-PP1, 1-NM-PP1 and Bn-PP1 in this assay, 3-MB-PP1 had the least impact. The modest impact of 20 μM MB upon the septation profile was not reproducible (see Figure 3) and probably arose because of the batch analysis approach in this extensive experiment. We therefore believe that in the assays used in this study had no impact upon the timing of mitotic commitment or the rate of mitotic progression. These specific conditions for which we believe this assertion holds true were the addition of 20 μM MB to asynchronous or size selected cultures grown to early log phase in the minimal medium EMM2 to which the supplements Leucine, Uracil, Adenine and Histidine had all been added to a final concentration of 200 mg ml^{-1}. It is likely that in other conditions such as live cell imaging where there are considerable levels of UV induced stress and conditions in many cases are anaerobic, different levels of sensitivity may apply.

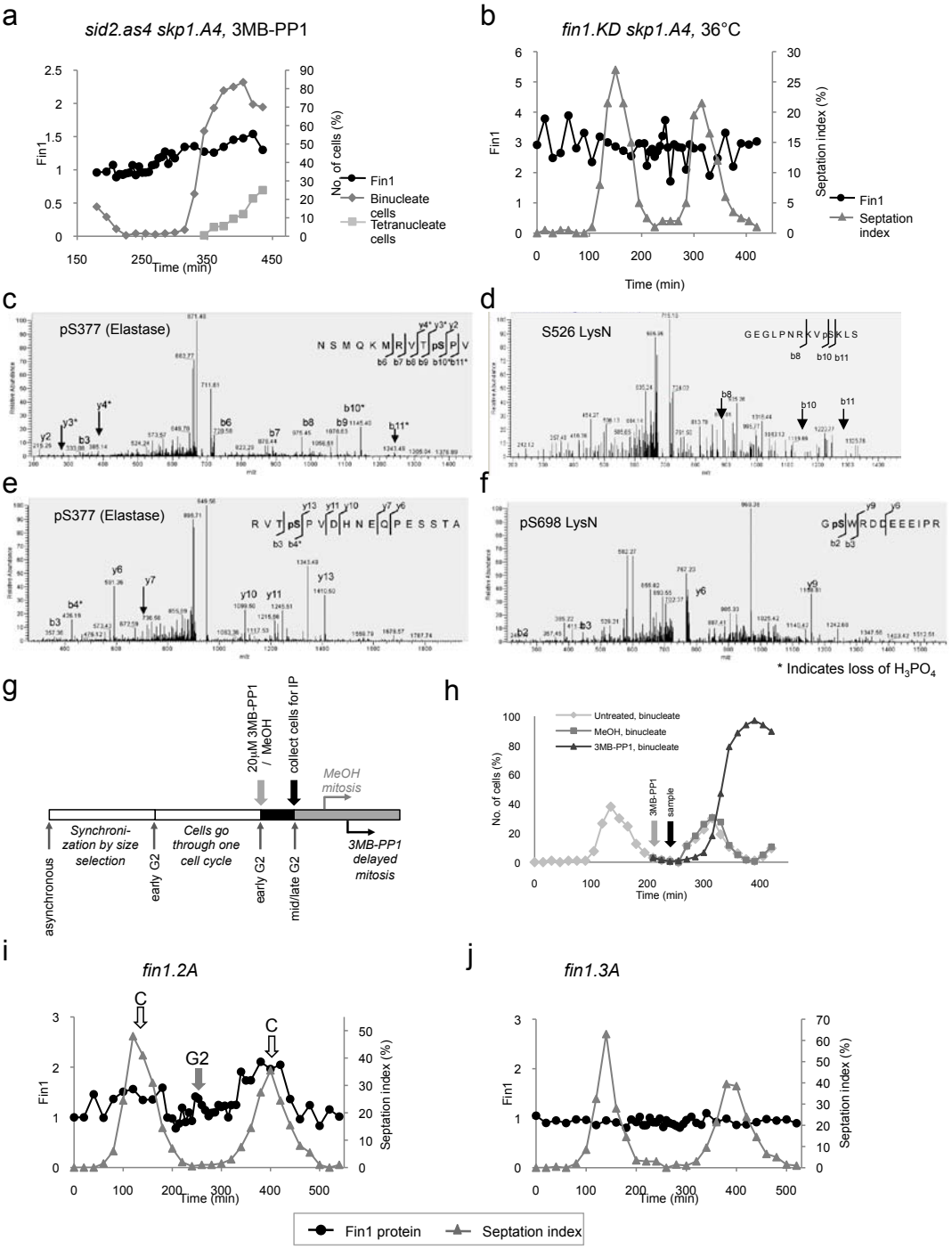

Figure S3 *Phosphorylation of S377, S526 and S698 of Fin1 by Sid2.* The indicated strains were synchronised by size selection at 25°C. Each culture was split in two with one half shifted to 36°C while the other was maintained at 25°C. (a) For the *sid2.as4 skp1.A4* mutant in panel a the shift was performed 180 minutes after size selection and the ATP analogue 3MB-PP1 was added to a final concentration of 20 μM to each culture at this time. For the *fin1.KD skp1.A4* culture the shift was performed immediately after splitting the culture following size selection. (c-f) Mass spectrometric analysis of Fin1 phosphorylation sites. (c, d) Product ion spectra from two independent elastase generated peptides (sequences denoted). Product ions of particular analytical note are labelled with those fragments showing H_3PO_4 loss further denoted with *. Both spectrum (c) and (d) confirm the respective sequences and location of the site of phosphorylation unambiguously as S377. (e, f) Product ion spectra from two LysN generated peptides. Product ions of particular analytical note are labelled. The spectrum in panel (e) confirms the denoted sequence and the location of the site of phosphorylation unambiguously as S526. The spectrum in (f) confirms the denoted sequence and the location of the site of phosphorylation unambiguously as S698. (g) A cartoon illustrating the procedure for sampling for the blots in Figure 2d. Small G2 cells were isolated by centrifugal elutriation. After one round of division the culture was split into two and the ATP analogue 3-MB-PP1 in MeOH or the solvent alone were added. 30 minutes later Fin1 was isolated by immunoprecipitation and probed with the phospho-specific and generic Fin1 antibodies to give the blots shown in Figure 2d. (h). The phenotypic characterisation of one of the three cultures used to create the samples for panel d of Figure 2d. (i-j) Fin1 protein levels as *fin1.2A* (i) or *fin1.3A* (j) cultures for which small G2 cells were isolated from asynchronous cultures at time 0. While modest changes in Fin1.2A protein levels were observed, no changes were seen in Fin1.3A levels.

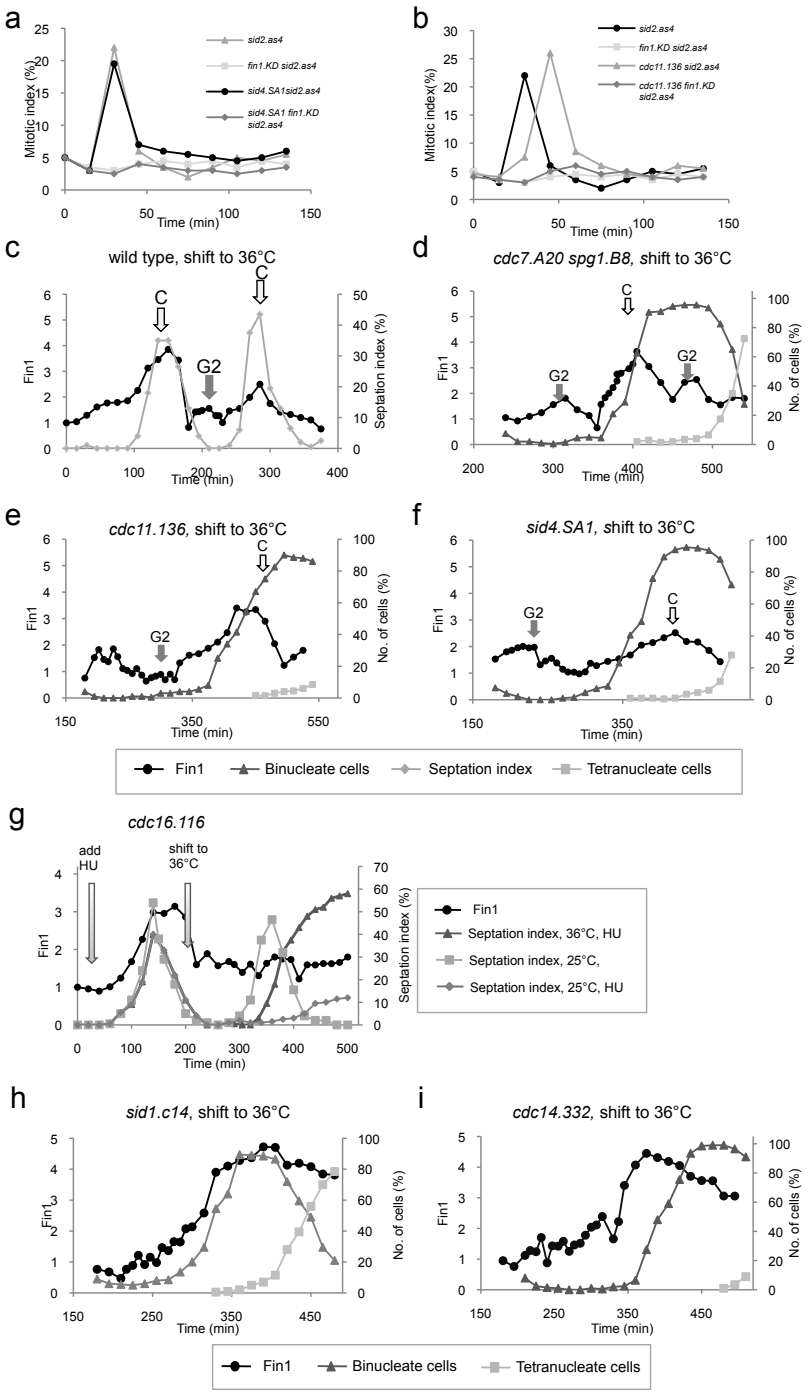

Figure S4 *SIN function is not required to generate the cycles of Fin1 accumulation/destruction.* (a, b) "Analogue washout" experiments as executed for Figure 4b-f with the indicated strains. Restoration of Sid2 kinase activity was able to induce mitotic commitment in strains in which the function of the SIN anchor SPB protein Sid4 or the scaffold molecule Cdc11 had been compromised by conditional mutation. The lack of induction in these backgrounds when Fin1 is catalytically inactive indicates that loss of either protein does not lead to a re-routing of the controls from Sid2 to another primary target. (c-f, h,i) Plots of Fin1 levels in size selected cultures of the indicated strains. Culture were split into two after the first round of septation and one half was shifted to 36°C while the other was maintained at 25°C for the duration of the experiment (data not shown). Both phases of destruction were maintained in size selected wild type cultures (c). Perturbation of SIN function by mutation of the G protein/Pak kinase cassette Spg1/Cdc7 (d), the scaffold protein Cdc11 (e) or the SPB anchor protein Sid4 (f), had no impact upon the cycles in Fin1 levels. (g) SIN induction according to an approach detailed previously[17]. Hydroxyurea was added to small G2 wild type or *cdc16.116* cells 60 minutes after they had been isolated from an asynchronous population by centrifugal elutriation. 230 minutes later each culture was split into two and one half shifted to 36°C. This temperature shift inactivated the SIN inhibitor Cdc16 driving SIN activation and consequently induced septation (grey triangles) in the S phase arrested *cdc16.116* culture, but did not impact upon SIN function in the wild type control. Fin1 levels were not altered by this form of ectopic induction of SIN activity. Sid1 (h) and Cdc14 (i) are required for the G2 phase accumulation and destruction of Fin1.

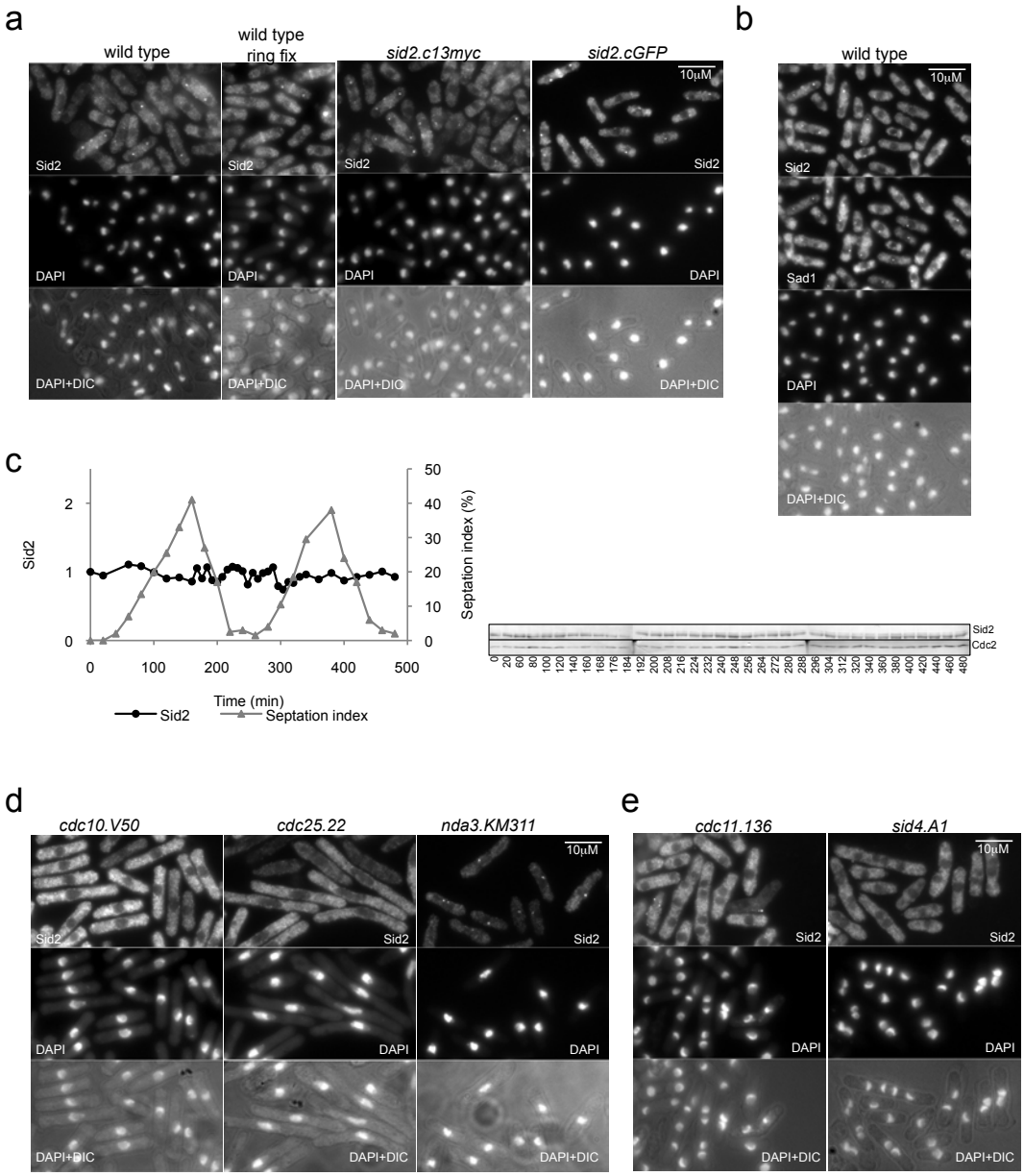

Figure S5 *Sid2 recruitment to mitotic but not interphase SPBs.* a, b, d, e) Sid2 immunofluorescence/DAPI/combined DAPI and differential interference contrast (DIC) images of the indicated strains. For all panels in the figure the "SPB fix" (10 mins 3% formaldehyde) was used with the exception of the indicated panel in (a) where "ring fix" (30 mins 3% formaldehyde) was employed. For (b) the immunofluorescence imaging combined Sid2 and Sad1 imaging as indicated to show that the Sid2 signal was absent from short, uninucleate, G2 cells in which a clear Sad1 signal could be detected. (a) In addition to staining of the cytokinetic ring, the Sid2 signal was seen on the mitotic SPBs of *sid2*$^+$ and *sid2.c13myc* cells. In contrast, the ring staining was accompanied by association with the SPBs irrespective of cell cycle status in the *sid2.GFP* background. We conclude that the finding that Sid2 is recruited to SPBs throughout the cell cycle[16] is misleading as it is based upon a behavioural shift in the Sid2.GFP fusion protein that is generated by the addition of the GFP sequences. Rather we suggest that Sid2 binds to SPBs of mitotic cells from just after the separation of the two SPBs following mitotic commitment until the degradation of the septum (staining persists on the SPBs while the background fluorescence in cells indicates that the primary septum is full formed). (c) Sid2 levels in western blots of whole cell extracts (right panel) remain constant as cells transit the cell division cycle (left). (d) Sid2 immunofluorescence of *cdc10.v50, cdc25.22* or *nda3.KM311* cells after, 5, 4.5 or 9 hours at their restrictive temperatures, respectively, consolidates the view that Sid2 only associates with the SPBs during mitosis (*nda3.KM311*), but neither G1 (*cdc10.v50*) nor G2 (*cdc25.22*) phase of the cell cycle. We conclude that Sid2 associates with mitotic but not interphase SPBs and that this association with the SPB is regulated at the level of recruitment and not synthesis/stability. (e) Given that the fusion of GFP to Sid2 kinase influences it's recruitment to the SPB we assessed the dependency of the wild type (Sid2$^+$) protein upon the function of the SPB anchor and SIN scaffold molecules Sid4 and Cdc11. Like Sid2.GFP, wild type *Sid2*$^+$ relies upon these molecules to dock to the SPB.

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