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

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Abstract

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, while the fission yeast equivalent, the Septum Initiation Network (SIN), only controls the execution of cytokinesis. The components and architecture of the SIN and MEN are highly conserved[1]. It is currently assumed that the functions of the core SIN/MEN components are restricted to their characterised roles at the end of mitosis. We now show that the NDR 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 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.

Keywords

NDR kinase; NIMA kinase; S. pombe; Wee1; Sid2; Skp1; Mitosis

S. pombe contains a single NIMA kinase, Fin1[2]. Deletion of *fin1*$^+$ (*fin1*.Δ) delays mitotic commitment[2] indicating that Fin1 emulates its *A. nidulans* counterpart in regulating the G2 to M transition[3]. We monitored Fin1 levels in cultures in which cell cycle progression had been synchronised by size selection of small, early G2 phase, cells. During mitosis, Fin1 levels paralleled the septation profile, dropping dramatically as the septation index decreased (Figure 1a, Arrow "C" for cytokinetic decline). Fin1 levels rose sharply once more at the start of the following G2 phase before rapidly declining again mid-way through this G2 phase (Fig. 1a; Arrow "G2" for G2 phase decline). Fin1 accumulation/decline is a

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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 upon 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[5] (Figure 1b). Substrate recognition by Cullin family E3 ligases is often contingent upon phosphorlyation to generate a “phospho-degron” recognition motif. Therefore, we tested 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 (Figure 1c (for characterisation of *fin1.KD* see Supplementary Figure 2a-d)) and did not oscillate as synchronised *fin1.KD* cultures transited the cell cycle (Figure 1d). Expression of a *fin1.GFP* fusion gene from a heterologous locus (wild type Fin1 kinase, 27kD 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 (Figure 1e). This ability of Fin1 activity to promote Fin1 destruction *in trans* likely arises from direct phosphorylation because Fin1 phosphorylates recombinant Fin1.KD *in vitro* (Figure 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 (Figure 1g). We therefore used the temperature and ATP analogue sensitive alleles *sid2.250* and *sid2.as4* (Supplementary Figure 2h, i-l) to assess the impact of Sid2 function upon Fin1 stability. In each case Sid2 inactivation in early G2 abolished the sharp declines in Fin1 levels that normally occurs during G2 phase and mitotic exit (Figure 1h, i). The sharp increase in Fin1 levels in early G2 phase was also absent in the *sid2.250* culture (Figure 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/sid2.as* single mutant backgrounds (Supplementary Figure 3a,b). As Sid2 function in the SIN requires association with Mob1, we assessed Fin1 behaviour in synchronised *mob1.E9* mutant cultures. Fin1 behaviour in *mob1.E9* mirrored that seen in the *sid2.250* mutant background (Figure 1j).

We studied the changes in Fin1 kinase specific activity in size-selected synchronised cultures (Figure 2a, Supplementary Figure 2b,d). Fin1 activity increased midway through G2 phase, and increased further after mitotic commitment (40-60 minutes 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 (Figure 2b).

Like its budding yeast counterpart Dbf2, Sid2 targets the consensus sequence RXXS/T[6,7]. The two RXXS/T sequences in Fin1 (S377 (**R**VT**S**), S526 (**R**KV**S**)) are phosphorylated *in vivo* (Supplementary Figure 3c-e). Antibodies that specifically recognise each site when phosphorylated (F1S377P, F1S526P) recognised 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 (Figure 2c). Their ability to recognise Fin1 precipitated from mid/late G2 phase *sid2.as4 skp1.A4* cells was dependent upon Sid2 activity (Figure 2d, Supplementary Figure 3 g, h).

Polyclonal antibodies against the non-catalytic domain of Sid2 (Supplementary Figure 2m) were used for *in vitro* Sid2 kinase assays using either casein or His tagged Fin1.KD as the *in vitro* substrate (Figure 2e, Supplementary Figure 2k, n). Both S377 and S526 of Fin1.KD (Figure 2f lanes 3-6) were phosphorylated by Sid2 immunoprecipitates; this activity was temperature dependent when Sid2 had been isolated from *sid2.250* cultures (Figure 2f lanes 1, 2). Addition of 3-MB-PP1 to the analogue sensitive kinase precipitated from *sid2.as4* cells also abolished activity towards each site (Figure 2f lanes 7, 8). The continued fluctuation of Fin1.S377AS526A levels during cell cycle progression (Supplementary Figure 3i) suggested that Sid2 might phosphorylate additional sites on Fin1. Consistently, serine 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 *skp1.A4* cells (Supplementary Figure 3f). While the context of S698 (LPG**S**) does not conform to the RXXS/T consensus[6], we note that the analagous budding yeast kinase, Dbf2, also phosphorylates sites that deviate from this *in vitro* defined consensus[8]. The recognition of Fin1 by antibodies that specifically recognise phosphorylated S698 (F1S698P) mirrored the *in vitro* and *in vivo* dependency upon Sid2 function displayed by F1S377P and F1S526P (Figure 2, c, d, f), indicating that S698 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 Figure 3j), 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 (Figure 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, while the activity did not exceed that of the Sid2.250 control early in G2 (Figure 2g; t = 180 and 200; Figure h t= 220 and t = 230), it did in the latter stages (Figure 2g, h compare *sid2.250* lanes with; g) 220-280, h) 240-280). We conclude that Sid2 activity towards Fin1 appears in G2 phase before being considerably enhanced within mitosis.

We addressed the functional significance of Fin1 activation by Sid2 in G2 phase by exploiting our ability to inhibit Fin1.as3 (Supplementary Figure 2e-g) and Sid2.as4 (Supplementary Figure 2h-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 (Figure 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 upon the timing of the subsequent mitosis (Figure 3b). In contrast

it delayed the appearance of mitotic spindles in both *fin1.as3* and *sid2.as4* cells (Figure 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 upon growth rate.

Neither Fin1 nor Sid2 are 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 synchronised wave of division (Figure 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 (Figure 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. Firstly, Fin1 appears to be the major 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 (Figure 4b). Secondly, the Sid2/Fin1 switch is very responsive to Sid2 activity, because phosphorylation of either 698 alone or 377 and 526 promotes mitosis (Figure 4c).

Next, we asked whether Sid2/Fin1 signaling targeted the MPF regulators Wee1 and Cdc25, by exploiting two well-characterised *cdc2* mutations; *cdc2.3w* mutants are largely insensitive to Cdc25[9] while *cdc2.1w* cells are largely insensitive to Wee1[10,11]. The peaks of mitosis arising from reactivation of either Sid2 or Fin1 were abolished by *cdc2.1w* but not *cdc2.3w* (Figure 4d), suggesting an exclusive routing via 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 Cdr1/2 upon Wee1 activity[12-15]. *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 (Figure 4e, f), indicating that the Sid2/Fin1 switch regulates Wee1 via Pom1. The means by which it does so awaits further analysis; however we note that Pom1 remained at the tips of *fin1.KD* or 3-MB-PP1 treated *fin1.as3* cells (Figure 4g).

In *S. pombe*, there is a direct correlation between cell length at septation and the timing of mitotic commitment[16]. 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 upon Pom1 control of Cdr1/2 to modulate Wee1 activity (Table 1).

In summary, we believe that activation of Sid2 midway through G2 phase phosphorylates S377, S526 and S698 of Fin1. Fin1 activation has two consequences; Fin1 destruction and the promotion of mitotic commitment via the Pom1/Cdr1/Cdr2/Wee1 cell geometry network (Figure 4h).

While Sid2/Mob1 kinase is a well characterised SIN component[1,17], 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. Firstly, 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 Figure 4a, b). Secondly, cycles of Fin1 accumulation and destruction persisted in synchronised cultures when SIN activity was abolished by the mutations *cdc11.136* and *cdc7.A20 spg1.B8* (Supplementary Figure 4c-e). In *sid4.SA1* cultures Fin1 destruction in G2 persisted, however, the mitotic accumulation of Fin1 was perturbed (Supplementary Figure 4f). Finally, induction of SIN signaling by inactivation of Cdc16[18] did not promote Fin1 destruction (Supplementary Figure 4g). 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 Figure 4h,i). This would suggest that the tight relationship proposed for Sid1 activation of Sid2 within the SIN may also couple the functions of these kinases in the control of Sid2 in G2[19]. While 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 synchronised by size selection, clearly show persistence of Fin1 destruction at the end of mitosis (Supplementary Figure 4i).

Because the diffuse distribution of Fin1 during G2 phase contrasts with the association of Sid2.GFP with the spindle pole body (SPBs)[17,20] 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 Figure 5a,b), even though Sid2 was present at this stage of the cell cycle (Supplementary Figure 5c). Thus, it is a cytoplasmic population of Sid2 that is activated mid-way through G2 to regulate a cytoplasmic population of Fin1. We assume that Fin1 activity within 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 via Pom1/Cdr1/Cdr2 is distinct from its role in regulating Plo1 recruitment to the SPB[21], as the *plo1.S402A* mutation that perturbs the latter[22] had no impact upon 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 by Sveiczer et al when an invariant "*timer*" is triggered by the completion of the variable "sizer" to commence preparations for mitotic commitment[23]. Thus, mitotic commitment in fission yeast maybe more similar to the staged antephase/mitotic commitment of mammalian somatic cells than the rapid transitions seen in *Xenopus* or Sea Urchin oocyte systems[24].

Methods

Cell Culture and growth

Strain used in this study are listed in Supplementary Table 1. Cells were grown and maintained according to Moreno et al. 1991[25]. 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[26]. 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 Figure 2 3-MB-PP1 at a final concentration of 20μM 3-MB-PP1 (0.04 % MeOH of total culture volume) was used to inhibit Sid2.as4 and Fin1.as3 function.

Microscopy/FACS 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 antibodies 1:25, Sid2, 1:100 and phosphohistone H3 1:100[16,27,28]. For Sid2 fluorescence, fixation for 10 minutes with 3% formaldehyde was used for optimal SPB staining, while the duration of this fixation period was extended to 30 minutes to give optimal preservation of the signal on the cytokinetic ring[29]. DNA content via FACS analysis used Cytox Green (Invitrogen) according to published procedures[30].

Genetic manipulation, Fusion protein production and antibody generation

Mutant alleles were generated via “Quick change” mutagenesis (Stratagene) and inserted into the genome with the marker switch approach using *natMX6* as a marker[31] (Supplementary Figures 2). The α-Sid2 sheep polyclonal antibody was raised by Scottish National Blood Transfusion Service against $Sid2^{1-205}$, purified from *E.coli*. Rabbit poly-clonal antibodies against Histone H3 S10 and peptides corresponding to the sequences surrounding S377, S526 and S698 in which these serines were phosphorylated were generated by Eurogentec. The generation and purification of Fin1 polyclonal antibodies has been described previously[20]. 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 recognise Cdc2[4,20]. 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 Figure 1a with Supplementary Figure 1a). Blots were quantitated using Image J. 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 normalised 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 phosphospecific antibodies were used at a dilution of 1:20. For immuno preciptations, 15µl Dynabeads A (Invitrogen, 100.02D) were loaded with 1.5 µl α-Fin1 or α-Sid2 undiluted antibody and used as per manufacturers instructions.

For the kinase assays Fin1/Sid2 was precipitated from 2×10^8 cells with polyclonal antibodies with Dynabeads A (Invitrogen) in KA buffer: 50mM Hepes, 10mM EDTA, 40mM Na-β-glycPhosh, 4mM Na_3VO_4, 50mM NaF, 0.6% NP40, 150mM NaCl, protease inhibitor cocktail (Roche), 1mM PMSF. The kinase reaction was carried out at 30°C for 30 min in KR buffer: 20 mM Hepes, 15mM KCl, 1mM EGTA, 10mM $MgCl_2$, 10mM $MnCl_2$, 0.125nM ATP, 10 µg of either recombinant Fin1.KDn6His (Figure 2a, b, c, d, Supplementary figure 2b, d, k, n) casein (Figure 1f, 2e, g), Fin1FP1[20] (Figure 2h). For assessment via ^{32}P incorporation 5µCi $^{32}P\gamma ATP$ was also added to the reaction mix. The amount of ^{32}P incorporated into the substrate was determined with a Phosphoimager (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, 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) prior to mass spectrometry.

Mass spectrometry

Samples were run on 4-12% NuPAGE bis-Tris gel (Invitrogen), Fin1 bands were excised and digested with either 20ng sequencing-grade trypsin (Sigma-Aldrich), 400 ng LysN (Associates of Cape Cod) or 350 ng Elastase (Calbiochem) in 100µl 40mM ammonium bicarbonate, 9% (v/v) acetonitrile at 37°C for 18hrs. The peptides were separated utilising a Nano-Acquity UPLC system (Waters) using a Waters NanoAcquity BEH C18 column (75µm ID, 1.7µm, 25cm) with a gradient of 1 to 25% (v/v) of acetonitrile, 0.1% formic acid over 30 minutes at a flow rate of 400nl/min. The LTQ-Orbitrap XL mass spectrometer was operated in parallel data dependent mode where the MS survey scan was performed at a nominal resolution of 60, 000 (at m/z 400) resolution in the Orbitrap analyser between m/z range of 400-2000. The top 6 precursors were selected for CID in the LTQ at normalised collision energy of 35% utilising multi-stage activation at m/z 98.0, 49.0 … 32.7 Da.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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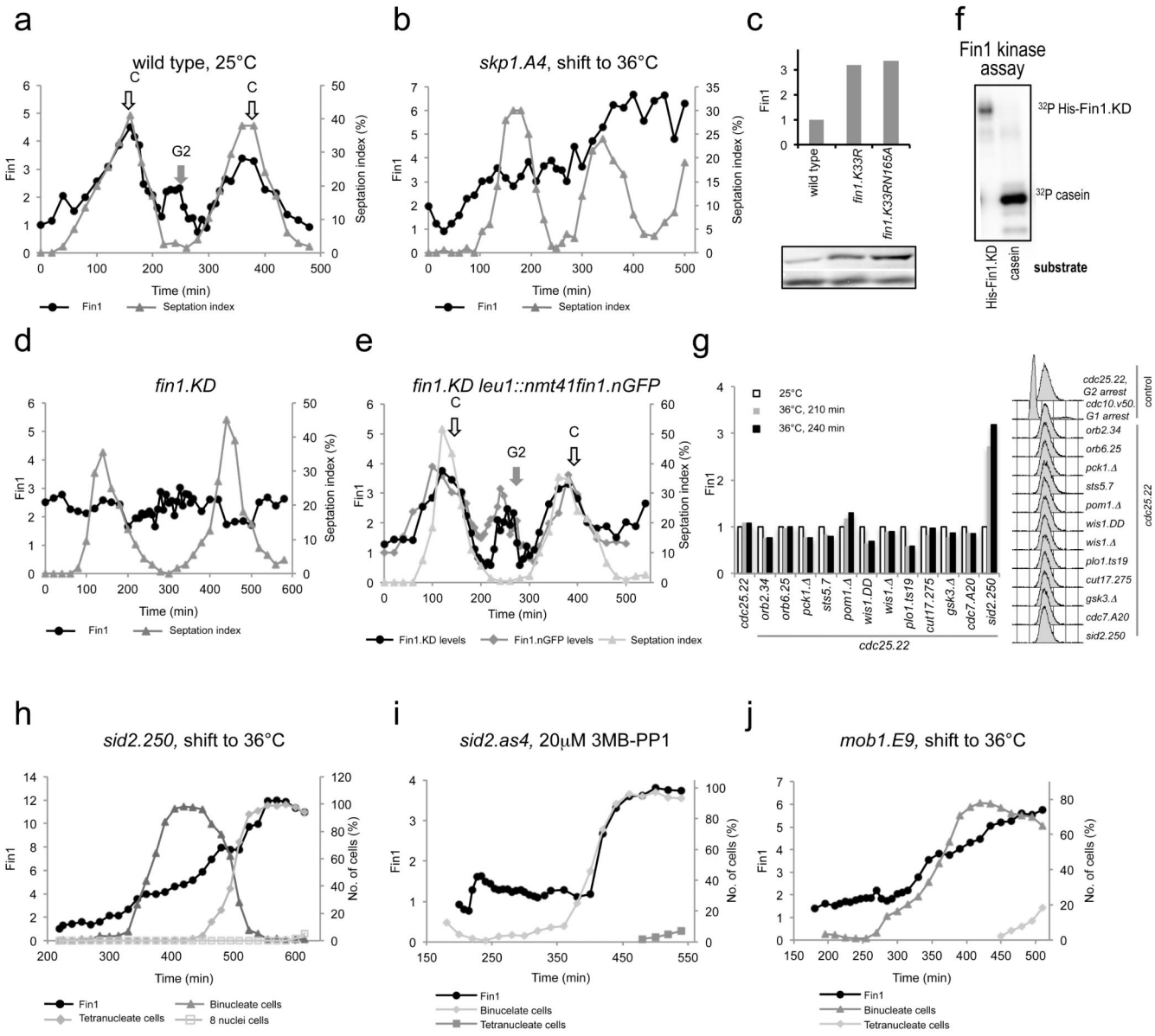

Figure 1. Fin1 kinase is destroyed twice each cell cycle in a Cullin, Fin1 and Sid2 dependent manner

(a, b, d, e, h-j) Fin1 levels were normalised 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 Figure 1b). (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 Figure 4c). (b) Oscillations in Fin1 levels were not seen after synchronised *skp1.A4* cultures were shifted to 36°C immediately after size selection at 25°C to inactivate Skp1. (c, g) Normalised Fin1 levels in blots of asynchronous or *cdc25.22* arrested double mutant cultures reveal three fold increases in Fin1 levels in the *fin1.K33RN165A* "kinase dead" and *sid2.250* backgrounds. (d)

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Fin1 levels did not fluctuate as *fin1.K33RN165A* cultures transited a synchronised 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 or casein as substrates. (g) Left: 210 and 240 mins refers to the duration of incubation at 36°C to inactivate and arrest cell cycle progression at the G2/M boundary. Right: FACS profiles of DNA content demonstrate G2 arrest in all strains. (h-j) Assessing the impact of Sid2/Mob1 function upon Fin1 levels in size selected synchronised 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 Figure 1c) and either nothing, methanol or 3-MB-PP1 in methanol were added to a final concentration of 20μM at time point 190.

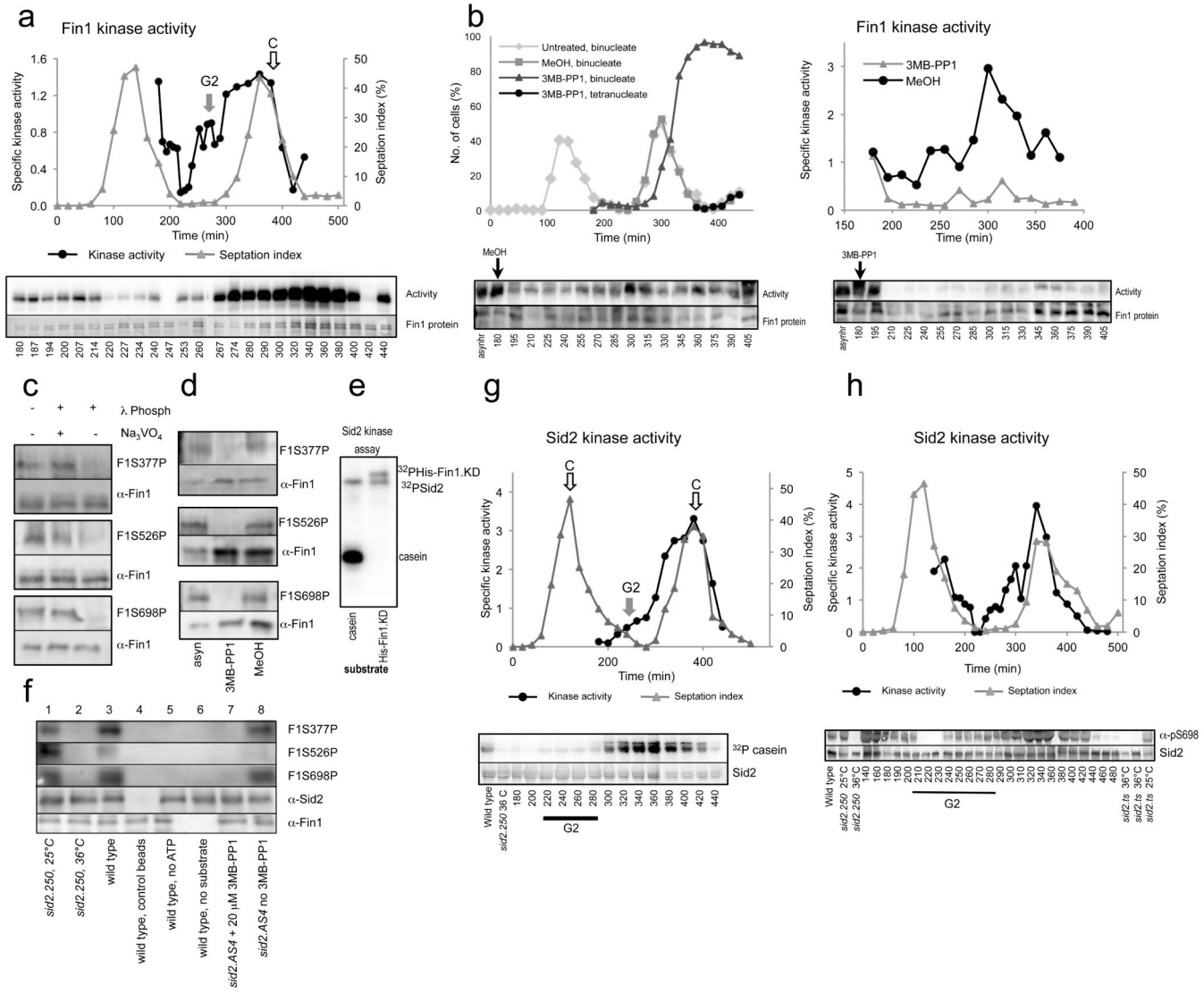

Figure 2. Sid2 phosphorylation of Fin1 on serines 377, 526 and 698 promotes Fin1 activity in G2 phase before a peak of each kinase activity accompanies mitotic progression

(a, b) Fin1 Kinase assays from size selected cultures in which His tagged Fin1.KD was labeled with ^{32}PγATP to quantitate activity that is plotted alongside the septation profile. (a) wild type (b) Small G2 *sid2.as4 skp1.A4* 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 assay) or solvent alone (right assay) 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 recognise the indicated phosphorylation sites or polyclonal antibodies that recognise the non-catalytic domain of Fin1. See Supplementary Figure 3g for details of the scheme used for each of the three identical cultures used to generate the samples and Supplmentary Figure 3h for the phenotypic characterisation 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

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and employed in *in vitro* kinase assays utilising ^{32}PγATP and either recombinant Fin1.KD or casein as indicated. Plots show activity per unit protein (i.e. specific activity) f) Blots with the indicated antibodies of *in vitro* kinase assays in which the indicated forms of Sid2 were isolated from the respective strains and combined with recombinant Fin1.KDnHis. (g) The incorporation of ^{32}P into casein from ^{32}PγATP was used to monitor Sid2 activity in size selected wild type cultures. (h) Sid2 immunoprecipitates were processed as for panel g with the exception that the shorter Fin1.FP1 (non-catalytic C terminal domain[21]) 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 normalisation.

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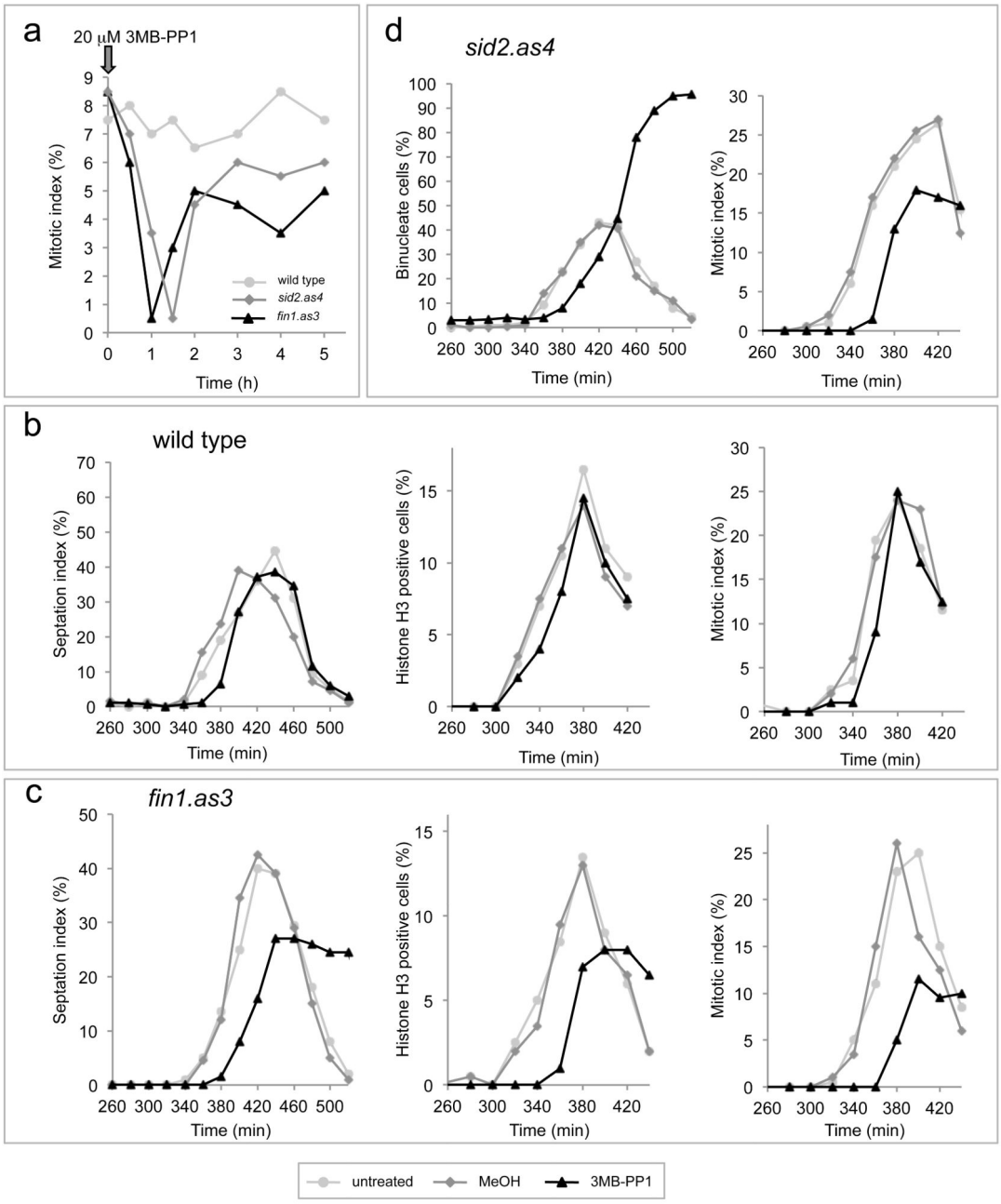

Figure 3. Inhibition of Sid2 or Fin1 delays mitotic commitment

(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 synchronised with respect to cell cycle progression by size selection and split into three equal cultures after the first round of septation. Methanol (MeOH), or 3-MB-PP1 (to a final concentration of 20 μM) in MeOH were added to two of these sub-cultures at 160 minutes. Commitment to mitosis was

monitored by the spindle index or phospho-histone H3 reactivity, as indicated. Addition of solvent alone had no impact upon cell cycle progression while addition of analogue in solvent delayed mitotic commitment in *fin1.as3* and *sid2.as4* but had no impact upon wild type cells.

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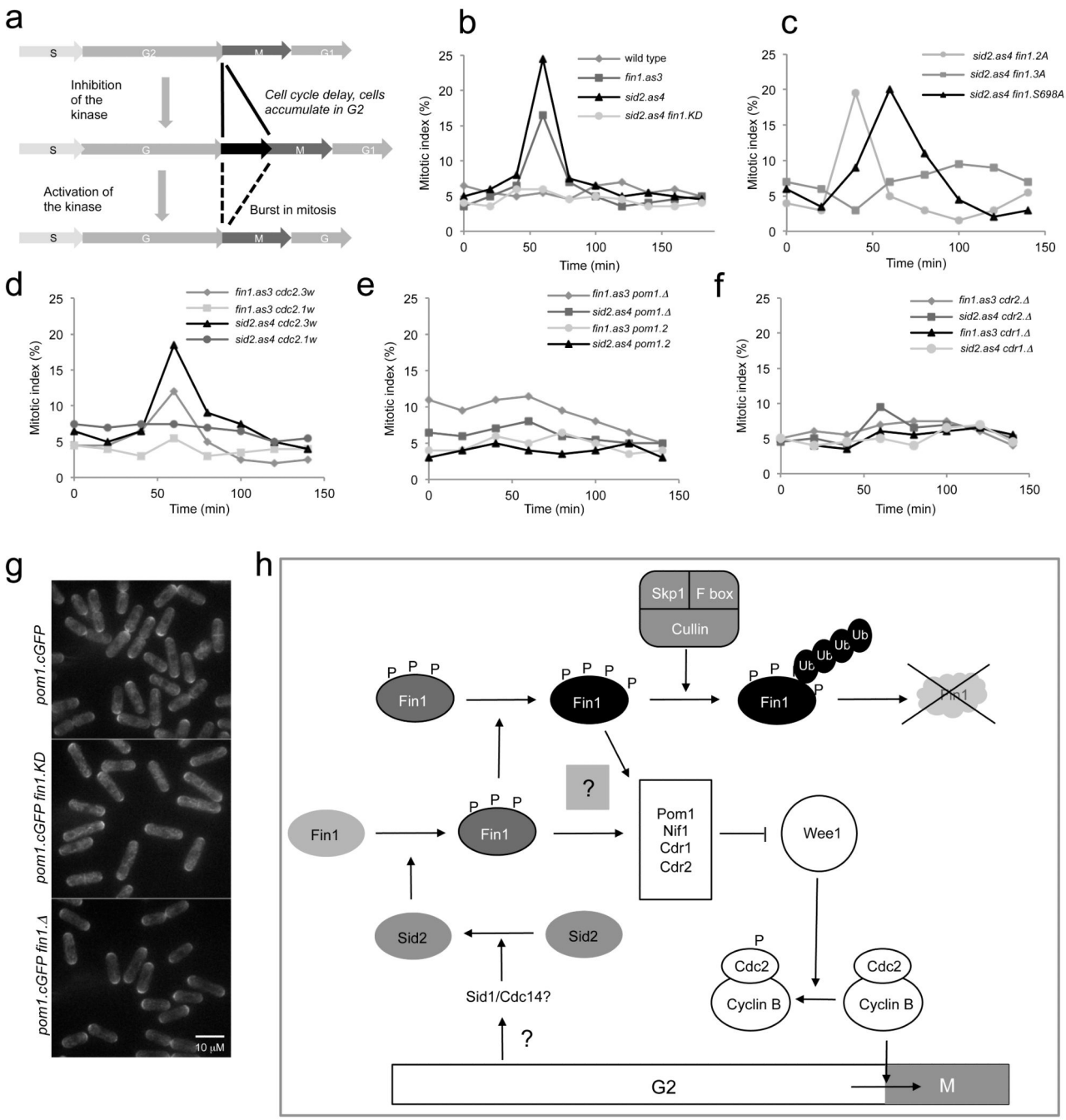

Figure 4. Sid2 and Fin1 target Cdr1/Cdr2/Pom1 to control mitotic commitment via Wee1

(a) Cartoon detailing the analogue washout approach. (b-f) 5 hours 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 cartoon depicting the model for G2/M control by Sid2/Fin1. P represents phosphorylation, while Ub represents Ubiquitin conjugation. Fin1 is activated in G2 by phosphorylation by Sid2. This promotes mitotic commitment via 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 current 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 box. The question mark to the left of this box 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 text for further details.

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Table 1

Cell length measurements support the model depicted in the cartoon in figure 4 h

Cell length at division			*Cell length at division following treatment with ATP analogues*			
		Cell length in µm				Cell length in µm
Ana-phase cells	wild type, 36°C, 2h	13.0±1.7	Septating cells	wild type	untreated	12.5±1.8
	finl.Δ, 36°C, 2h	14.0±2.1			3-MB-PP1, 2h	12.4±1.3
	sid2.250, 36°C, 2h	15.6±2.2			3-MB-PP1, 4h	12.8±1.3
Septating cells	wild type	12.5±1.8			3-MB-PP1, 8h	12.3±1.0
	finl.Δ	13.3±2.0		*fin1.as3*	untreated	13.8±1.4
	fin1.K33R	15.0±2.2			3-MB-PP1, 2h	13.2±1.0
	finl.KD	15.9±1.6			3-MB-PP1, 4h	15.2±1.8
	fin1.S698A	13.9±1.3			3-MB-PP1, 8h	15.7±1.0
	fin1.S698E	12.5±1.3		*fin1.as3 cdc2.1w*	untreated	7.7±0.9
	fin1.2A	12.9±1.4			3-MB-PP1, 2h	7.8±0.8
	fin1.2E	12.8±1.2			3-MB-PP1, 4h	8.2±0.9
	fin1.3A	14.9±1.9			3-MB-PP1, 8h	8.0±0.9
	fin1.3E	11.7±1.7		*fin1.as3 cdc2.3w*	untreated	10.8±1.1
	cdc2.1w	7.9±0.9			3-MB-PP1, 2h	11.0±1.2
	finl.Δ *cdc2.1w*	8.1±1.5			3-MB-PP1, 4h	11.9±1.4
	finl.KD cdc2.1w	8.1±0.7			3-MB-PP1, 8h	12.0±1.5
	cdc2.3w	8.0±1.4		*fin1.as3 pom1*.Δ	untreated	11.5±1.4
	finl.Δ *cdc2.3w*	10.7±2.8			3-MB-PP1, 2h	11.6±1.6
	finl.KD cdc2.3w	11.3±4.0			3-MB-PP1, 4h	12.0±1.6
	poml.Δ	11.1±1.0			3-MB-PP1, 8h	12.0±1.6
	poml.Δ *finl.KD*	11.4±1.9		*fin1.as3 cdr1*.Δ	untreated	17.3±1.1
	poml.Δ *fin1.3A*	11.0±1.4			3-MB-PP1, 2h	17.4±1.6
	poml.Δ *fin1.3E*	10.9±1.8			3-MB-PP1, 4h	18.3±1.5
	cdr1::ura4	18.4±1.3			3-MB-PP1, 8h	18.2±1.6
	cdr1::ura4 finl.KD	18.0±1.3		*fin1.as3 cdr2*.Δ	untreated	19.4±1.4
	cdr2.Δ	19.1±1.5			3-MB-PP1, 2h	19.9±1.2
	cdr2.Δ *finl.KD*	19.3±1.3			3-MB-PP1, 4h	21.1±1.6
	fin1.3A cdr2.Δ	18.9±1.9			3-MB-PP1, 8h	20.2±1.6
	fin1.3E cdr2.Δ	18.4±2.3		*fin1.as3* syncnh-ronous	420 min	16.1±1.1
					460 min	17.5±1.1
					580 min	18.8±1.4
					620 min	18.9±1.2

Cell length at division: Cells were grown in EMM2 medium to optical density (OD^{595}) of 0.1 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.

Cell length at division following treatment with ATP analogues: Cells were grown in EMM2 medium to optical density (OD^{595}) of 0.1 at 25°C and stained with calcofluor to identify septating cells. The three columns on the right show lengths at the indicated times after the addition of the ATP analogue 3-MB-PP1.

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