

# Ipl1-dependent phosphorylation of Dam1 is reduced by tension applied on kinetochores

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## Summary

The conserved Aurora B protein kinase (Ipl1 in *Saccharomyces cerevisiae*) is essential for ensuring that sister kinetochores become attached to microtubules from opposite spindle poles (bi-orientation) before anaphase onset. When sister chromatids become attached to microtubules from a single pole, Aurora B/Ipl1 facilitates turnover of kinetochore-microtubule attachments. This process requires phosphorylation by Aurora B/Ipl1 of kinetochore components such as Dam1 in yeast. Once bi-orientation is established and tension is applied on kinetochores, Aurora B/Ipl1 must stop promoting this turnover, otherwise correct attachment would never be stabilised. How this is achieved remains elusive: it might be due to dephosphorylation of Aurora B/Ipl1 substrates at kinetochores, or might take place independently, for example because of conformational changes in kinetochores. Here, we

show that Ipl1-dependent phosphorylation at crucial sites on Dam1 is maximal during S phase and minimal during metaphase, matching the cell cycle window when chromosome bi-orientation occurs. Intriguingly, when we reduced tension at kinetochores through failure to establish sister chromatid cohesion, Dam1 phosphorylation persisted in metaphase-arrested cells. We propose that Aurora B/Ipl1-facilitated bi-orientation is stabilised in response to tension at kinetochores by dephosphorylation of Dam1, resulting in termination of kinetochore-microtubule attachment turnover.

Supplementary material available online at  
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Key words: Chromosome bi-orientation, Microtubules, Kinetochore

## Introduction

To ensure efficient chromosome segregation during mitosis, sister chromatids must become attached to microtubules originating from opposite poles of the metaphase spindle. This state, termed bi-orientation, ensures that each daughter cell receives one copy of each chromosome when sister chromatids are separated during anaphase. Chromosomes do not automatically achieve the bi-oriented state and studies in yeast and other organisms have demonstrated that a correction mechanism ('re-orientation') is required to deal with cases where both sister chromatids have become attached to microtubules from a single spindle pole, an error termed syntelic attachment (see Tanaka, 2008). In the yeast *Saccharomyces cerevisiae*, the protein kinase Ipl1 is an essential element in this correction mechanism. In *ipl1* mutants, the majority of sister chromatid pairs fail to bi-orient (Biggins et al., 1999; He et al., 2001; Tanaka et al., 2002), instead they remain attached to microtubules originating from the old spindle pole body and both segregate to the daughter cell (Tanaka et al., 2002). The Ipl1 protein kinase is highly conserved and a similar role has been proposed for its metazoan orthologue, Aurora B (Hauf et al., 2003; Lampson et al., 2004). Ipl1 has been proposed to promote correction of syntelically attached sister chromatids to the bi-oriented state by phosphorylating proteins at the microtubule-kinetochore interface, leading to detachment of the microtubule and thereby allowing a microtubule from the opposite pole to establish a new kinetochore-microtubule interaction (Tanaka et al., 2002).

A number of kinetochore proteins have been established as *in vivo* substrates of yeast Ipl1 (Cheeseman et al., 2002), and two of these, Dam1 and Ndc80, have been implicated as targets with relevance to the remodelling of kinetochore-microtubule interactions

(for a review, see Tanaka and Desai, 2008). Dam1 is not at all well conserved outside fungi, and in metazoans, the KMN kinetochore complex containing Ndc80 has been proposed to be the major interface between the kinetochore and the microtubule, with the N-terminal domain of the conserved Ndc80 component emerging as a likely target for Aurora B in the regulation of kinetochore-microtubule interactions (Cheeseman et al., 2006; DeLuca et al., 2006). Yeast Ndc80 is also an *in vivo* target for Ipl1 (Cheeseman et al., 2002). However, since the N-terminal domain in yeast can be deleted and the Ipl1 phosphorylation sites mutated, apparently without compromising chromosome bi-orientation (Kemmler et al., 2009), the role of Ndc80 in yeast chromosome bi-orientation is currently unclear.

Dam1 forms part of a heterodecameric complex (the DASH or Dam1 complex), multiple copies of which can form rings around individual microtubules that can mediate processive movement of cargo along the microtubule (Miranda et al., 2005; Westermann et al., 2005; Westermann et al., 2006). The DASH complex might form part of the mechanism that couples a microtubule to the kinetochore, and artificially tethering the Dam1 complex to DNA is able to recapitulate many aspects of kinetochore function, including the promotion of chromosome bi-orientation (Kiermaier et al., 2009; Laceyfield et al., 2009). Four *in vivo* phosphorylation sites for Ipl1 have been mapped in Dam1. Mutation of all four sites to alanine is lethal, whereas mutation of three of these sites together with an Ipl1 phosphorylation site in Spc34 (another DASH complex component) confers temperature sensitivity. At the restrictive temperature, this double *dam1 spc34* mutant appears to recapitulate the phenotype of an *ipl1* mutant with regards to chromosome segregation (Cheeseman et al., 2002). Conversely, mutation of these

sites in Dam1 to aspartate (to mimic constitutive phosphorylation) might destabilise kinetochore-microtubule interactions, because it leads to the appearance of lagging chromosomes on the anaphase spindle (Cheeseman et al., 2002; Shang et al., 2003). Three of these sites are located in the C-terminal domain of Dam1 that is located adjacent to the microtubule lattice when the ring complex is loaded onto a microtubule (Wang et al., 2007), placing the phosphorylation sites where they could potentially influence interaction with the microtubule. However, formation of rings by the DASH complex is not necessary for dynamic attachment to microtubules, whereas mutation of a fourth Ipl1 phosphorylation site in Dam1 (Ser20) to non-phosphorylatable alanine reduces affinity of the DASH complex for microtubules in vitro (Gestaut et al., 2008). Thus, although there is some uncertainty over exactly how the DASH complex functions, there is clear evidence that it has a role in coupling kinetochores to microtubules and that it constitutes a key target of Ipl1 kinase in the re-orientation process.

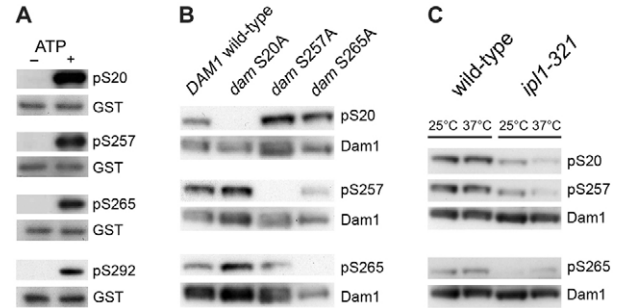
An important difference between syntelic and bi-oriented sister chromatids is that bi-oriented sister chromatids are under tension from the opposing pulling forces exerted by microtubules, whereas syntelic sister chromatids are not. Such tension has been proposed to be important for regulating kinetochore function (Nicklas and Koch, 1969; Nicklas, 1997), and more recently has been shown to drive Ipl1-dependent minichromosome bi-orientation in yeast (Dewar et al., 2004). However, once bi-orientation is established and tension is applied to sister kinetochores, turnover of kinetochore-microtubule attachment must stop so that the correct attachment is stabilised. How this occurs is unclear, but given the important role of Ipl1-dependent phosphorylation of kinetochore components for initiating this turnover, dephosphorylation of these components might prevent such turnover if it occurs specifically when tension is applied. Alternatively, kinetochore-microtubule attachments could be stabilised independently of the phosphorylation state of Ipl1 substrates at the kinetochore when tension is applied. For example, a tension-induced conformational change in kinetochores rather than a change in their biochemical properties might strengthen their grasp on the attached microtubules, similarly to fingers caught in a Chinese finger trap.

Here, we examined Ipl1-dependent in vivo phosphorylation of Dam1 using phosphospecific antibodies that recognise Ser20, Ser257 and Ser265. We show that phosphorylation is largely constrained to S phase, when chromosome bi-orientation is first established, and that levels of phosphorylated Dam1 in metaphase-arrested cells are very low. However, when sister kinetochore tension is reduced following depletion of Scc1 (also called Mcd1), phosphorylation of Dam1 persists in metaphase-arrested cells. We therefore propose that Dam1 is dephosphorylated as a result of tension applied to kinetochores and that this dephosphorylation leads to termination of the turnover of kinetochore-microtubule attachments.

## Results

### Ipl1-dependent phosphorylation of Dam1 is maximal during S phase

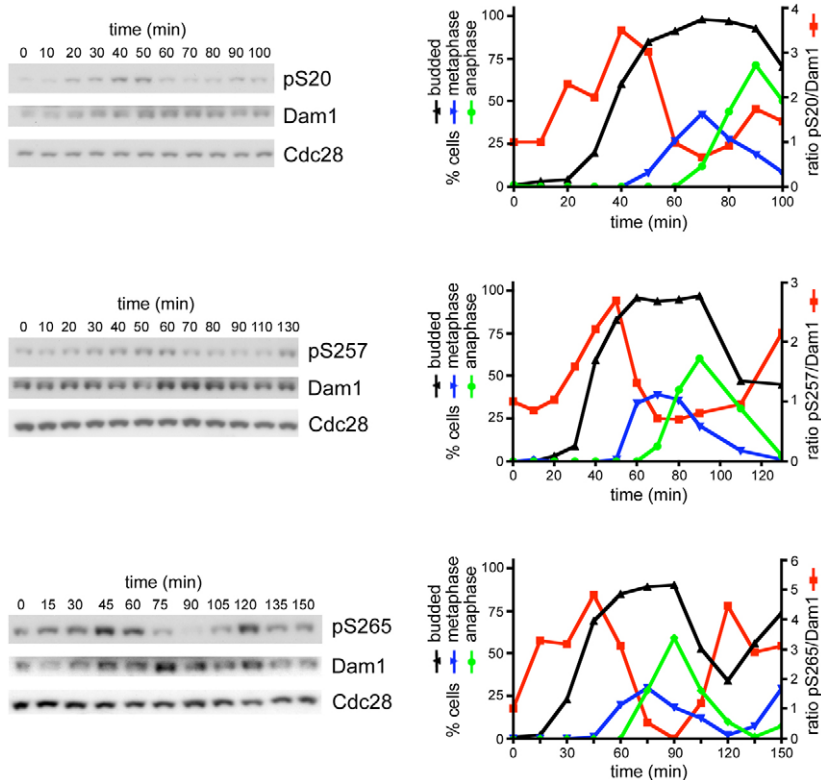
To examine Dam1 phosphorylation and its regulation in vivo, we raised phosphospecific antibodies that could recognise each of the four Ipl1 phosphorylation sites in Dam1 defined by Cheeseman and co-workers (Cheeseman et al., 2002). Western blot analysis of in-vitro-phosphorylated recombinant GST-Dam1 showed that each antibody was completely phosphospecific, because omission of ATP from the phosphorylation reaction abolished all detectable signal



**Fig. 1.** Phosphospecific antibodies demonstrating Ipl1-dependent phosphorylation of Dam1 on Ser20, Ser257, Ser265 and Ser292. (A) Western blot of recombinant GST-Dam1 following an in vitro Ipl1 kinase assay carried out with or without addition of ATP using recombinant GST-Ipl1 and GST-Sli15. Phosphospecific antibodies raised against Ser20-*P* (pS20), Ser257-*P* (pS257), Ser265-*P* (pS265) and Ser292-*P* (pS292) together with anti-GST antibody (to confirm loading) were used to probe the reactions. Only phosphorylated GST-Dam1 from reactions supplemented with ATP was recognised by the phosphospecific antibodies. (B) Protein extracts from *DAM1* wild-type (PKY140), *dam1-S20A* (PKY141), *dam1-S257A* (PKY142) and *dam1-S265A* (PKY143) strains containing the indicated *dam1* Ser to Ala mutations were prepared and analysed by western blotting with the anti-Ser20-*P*, anti-Ser257-*P* and anti-Ser265-*P* antibodies, together with a non-phosphospecific anti-Dam1 antibody to monitor overall Dam1 levels in the extracts. (C) Protein extracts from wild-type (wt; K699) and *ipl1-321* (T1654) strains were prepared and analysed as in B to examine the Ipl1 dependence of phosphorylation monitored using the phosphospecific antibodies.

(Fig. 1A). When used to probe extract from asynchronous yeast cells, the antibody recognising phosphorylated serine 292 (Ser292-*P*) proved of insufficient quality to give a useable signal (not shown) and so was not used further, but the antibodies recognising Ser20-*P*, Ser257-*P* and Ser265-*P* each gave a good signal that was abolished when cell extract was prepared from mutant strains in which the respective site in Dam1 had been mutated to alanine to prevent phosphorylation (Fig. 1B). Phosphorylation of the three sites detected by these antibodies was largely dependent on Ipl1 kinase, because the signal obtained with each antibody was greatly reduced in extracts prepared from the temperature-sensitive *ipl1-321* mutant following growth at 37°C (Fig. 1C). The *ipl1-321* mutant is defective for chromosome bi-orientation at 37°C (Biggins et al., 1999; Tanaka et al., 2002) and the mutant protein has very little kinase activity when assayed in vitro (Kotwaliwale et al., 2007; Makrantonis and Stark, 2009). In fact even when extracts were made from cells grown at 25°C, the phosphospecific signal obtained using all three antibodies was reduced in *ipl1-321* extracts compared with wild-type controls (Fig. 1C).

We next examined whether Dam1 phosphorylation showed cell cycle dependency by synchronising yeast cells in G1 using  $\alpha$ -factor and then releasing them into a new cell cycle (Fig. 2A-C). Progress through the cell cycle was monitored by quantifying bud emergence (which is a robust marker for the initiation of S phase) (Schwob and Nasmyth, 1993), short metaphase spindles and long anaphase spindles. These latter two parameters were determined by using strains expressing yellow fluorescent protein (YFP)-tagged tubulin. Although there was some inter-experiment variability in the basal level of phosphorylation observed in G1 cells, phosphorylation of Ser20, Ser257 and Ser265 each increased markedly at the point of bud emergence. Phosphorylation reached a peak after 40-50 minutes, by which time most cells had budded, and then phosphorylation levels fell as cells established metaphase spindles. Phosphorylation



**Fig. 2.** Dam1 shows cell-cycle-stage-specific phosphorylation on Ser20, Ser257 and Ser265. Yeast cells expressing YFP-Tub1 (T2749) were synchronised in G1 and released at 25°C. Samples were taken for western blotting, spindle morphology determination and budding index determination. Dam1 phosphorylation on Ser20 (pS20), Ser257 (pS257) and Ser265 (pS265) was examined by western blotting with the three phosphospecific antibodies and the overall level of Dam1 (Dam1) was examined using anti-Dam1 antibody. Cdc28 was also visualised as a loading control. Relative phosphorylation of Dam1 at each site was estimated by plotting the ratio of each phosphospecific signal to the Dam1 signal at each time point, normalising the ratios to an arbitrary value of 1.0 at the zero time point in each case (red squares). The proportion of budded cells (black triangles), proportion of cells containing a metaphase spindle (blue triangles) and proportion of cells containing an elongated anaphase spindle (green diamonds) are also plotted.

remained low during anaphase spindle elongation, but then increased again as cells entered a new cell cycle. No obvious differences were noted between the three sites in terms of their kinetics of phosphorylation. Thus Dam1 phosphorylation at each site shows cell-cycle-stage dependence, with maximal phosphorylation occurring in the window defined by bud emergence and the establishment of a metaphase spindle.

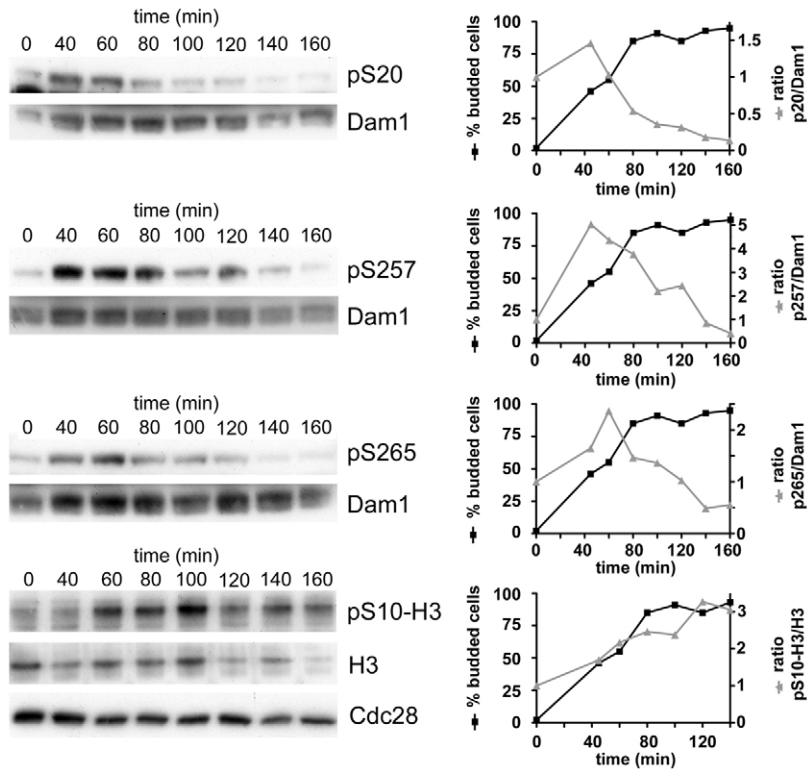
In cycling cells released from  $\alpha$ -factor arrest, cells quickly enter a second cell cycle following completion of anaphase. To confirm that the low stoichiometry of phosphorylation in post-S-phase cells was a feature of the metaphase state, we generated synchronous yeast cultures using a strain where cells could be arrested in metaphase because *CDC20*, which encodes an activator of APC/C required for progression into anaphase, had been placed under control of the methionine-repressible *MET3* promoter. Such cells were released from  $\alpha$ -factor arrest into methionine-containing medium such that Cdc20 was not expressed, leading to a prolonged arrest in metaphase. Fig. 3 shows that phosphorylation of Dam1 on all three sites peaked during S phase, as expected, but then remained low for at least 60 minutes in metaphase-arrested cells. Since virtually all chromosomes have become bi-oriented by the time a yeast cell reaches metaphase (Tanaka et al., 2002), these data confirm that Dam1 phosphorylation on all three sites is minimal during metaphase and are consistent with the notion that Dam1 phosphorylation is reduced once chromosomes have become bi-oriented. In parallel to monitoring Dam1 phosphorylation, we also examined the phosphorylation state of histone H3 on Ser10. Phosphorylation of this site is also Ipl1-dependent (Hsu et al., 2000) but has been reported to peak in metaphase. Fig. 3 shows that histone H3 phosphorylation appeared later than Dam1 phosphorylation, and the relative level of phosphorylation continued to increase as cells arrested in metaphase. This is in contrast to Dam1 phosphorylation, indicating that phosphorylation of the two substrates by Ipl1 is

differentially regulated. To provide extra confirmation that the phosphorylation we were detecting in these synchronous cultures was Ipl1 dependent, we used all three phosphospecific antibodies to probe extracts from synchronous cultures of *IPL1* wild-type and *ipl1-321* mutant cells released from  $\alpha$ -factor arrest at 37°C to a metaphase arrest as above. Fig. 4A confirms that the S-phase peak of Dam1 phosphorylation at each of the three sites could only be seen in the wild-type cells.

Next, we addressed whether the phosphorylated Dam1 that we could detect by western blotting was likely to be microtubule and/or kinetochore associated. We attempted unsuccessfully to perform indirect immunofluorescence localisation of phosphorylated Dam1 in samples of fixed cells. However, since loading of the DASH complex to the kinetochore-microtubule interface is dependent on microtubules (Li et al., 2002; Tanaka et al., 2007), we treated  $\alpha$ -factor-synchronised cultures with the microtubule-depolymerising agent nocodazole. Fig. 4B shows that appearance of phosphorylated Dam1 in S-phase cells was largely dependent on the presence of microtubules and was reduced to near-background levels in nocodazole-treated cells. It is therefore the microtubule and/or kinetochore-associated pool of Dam1 that appears to be the *in vivo* target for Ipl1-dependent phosphorylation, which is consistent with the localisation of Ipl1 at, or in the vicinity of, kinetochores from G1 until anaphase onset (Tanaka et al., 2002; Buvelot et al., 2003; Shimogawa et al., 2009).

#### Dam1 phosphorylation remains high when sister kinetochore tension is reduced

The reduction of Dam1 phosphorylation around the time when bi-orientation is established raises the possibility that this reduction is actually caused by the tension applied on sister kinetochores. To test this, we generated cells in which sister chromatid tension was reduced by allowing cells to replicate their DNA without



**Fig. 3.** Phosphorylation of Dam1 is reduced in metaphase. Yeast cells dependent on *pMET3-CDC20* (T2748) were synchronised in G1 and released at 25°C in the presence of methionine so that they arrested before anaphase. Dam1 phosphorylation on Ser20 (pS20), Ser257 (pS257) and Ser265 (pS265) was examined by western blotting with the three phosphospecific antibodies and the overall level of Dam1 (Dam1) examined using anti-Dam1 antibody. Relative phosphorylation of Dam1 at each site was estimated by plotting the ratio of each phosphospecific signal to the Dam1 signal at each time point, normalising the ratios to an arbitrary value of 1.0 at the zero time point in each case (grey triangles). Histone H3 phosphorylation on Ser10 (pS10-H3) and total histone H3 (H3) were also examined by western blotting and relative Ser10 phosphorylation estimated in the same manner. The proportion of budded cells is also plotted (black squares). The level of Cdc28 was examined as a loading control.

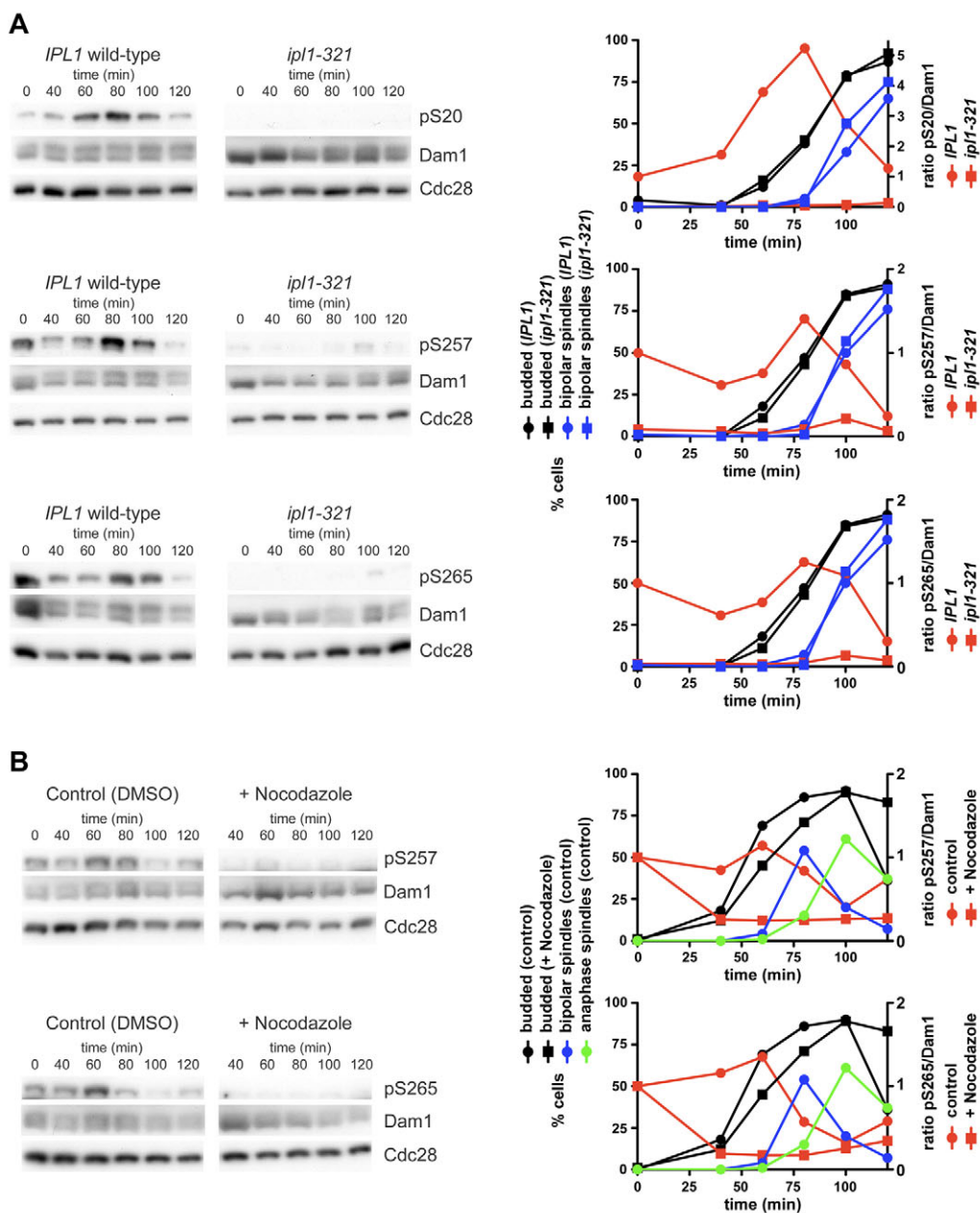
establishing proper sister chromatid cohesion. This was achieved by placing expression of Scc1, a critical component of the cohesin complex that is loaded onto DNA during S phase (Uhlmann and Nasmyth, 1998), under control of the galactose-inducible *GAL* promoter in strains also dependent on *pMET3-CDC20*. Cells were synchronised in G1 using  $\alpha$ -factor in methionine-free, galactose-containing medium and then released to a metaphase arrest in medium containing glucose (to prevent Scc1 expression) and methionine (to block Cdc20 expression). Control cultures were treated similarly but released using galactose-containing medium, such that Scc1 was expressed to enable normal establishment of cohesion as cells passed through S phase. Depletion of Scc1 in this manner does not prevent the establishment of kinetochore-microtubule interactions (Tanaka et al., 2000). Control cells expressing Scc1 showed the normal S-phase peak of Dam1 phosphorylation on Ser20, Ser257 and Ser265 (Fig. 5A). However, in Scc1-depleted cells, phosphorylation on all three sites persisted during cell cycle arrest in metaphase (because of Cdc20 depletion). Lack of sister chromatid cohesion (and hence reduced tension) under these conditions was confirmed by showing that the arms of chromosome XV were no longer associated (Fig. 5B). Thus, the loss of Dam1 phosphorylation that we observe as cells enter metaphase is dependent on establishment of proper sister chromatid cohesion during the preceding S phase, and is consistent with a requirement for sister chromatid tension to keep Ipl1-dependent Dam1 phosphorylation low once sister chromatids have bi-oriented.

## Discussion

Phosphorylation of Dam1 by Ipl1 has been proposed to be a key element in the re-orientation mechanism that resolves syntelic sister chromatids so they achieve the correct, bi-oriented state, and a number of *in vivo* phosphorylation sites have been identified (Cheeseman et al., 2002; Zhang et al., 2005). Here, we used

phosphospecific antibodies that recognise three of these sites to confirm that they are indeed phosphorylated, and have demonstrated that this phosphorylation is cell-cycle-stage dependent. Specifically, using synchronous cell cultures, we found that phosphorylation of these three sites in Dam1 was maximal in the window between initiation of S phase and establishment of a metaphase spindle. The centromeric regions of yeast chromosomes are replicated early during S phase (McCarroll and Fangman, 1988), at which time chromosomes transiently detach and then quickly reattach to microtubules (Kitamura et al., 2007). Soon afterwards, Ipl1 promotes turnover of kinetochore-microtubule attachments, until bi-orientation is established shortly after formation of a bipolar spindle at the end of S phase (Tanaka et al., 2002). Dam1 phosphorylation is therefore maximal at a time coincident with the stage when syntelic chromosomes must be re-oriented. Since Dam1 phosphorylation is prevented in the absence of microtubules, we infer that phosphorylation is occurring on Dam1 that is associated with microtubules and/or kinetochores, as expected if phosphorylation is regulating the kinetochore-microtubule interface. The persistence of very low levels of phosphorylated Dam1 during an extended metaphase arrest emphasises the conclusion that Dam1 phosphorylation is much reduced by the time all chromosomes are bi-oriented.

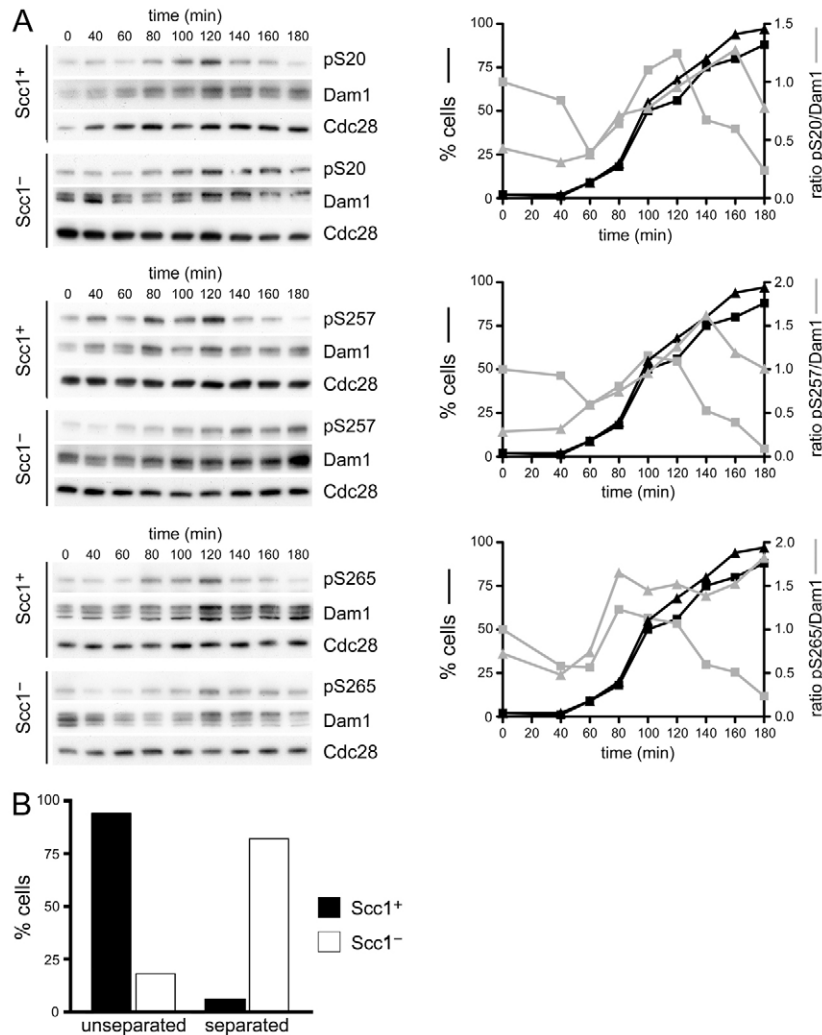
Tension exerted on sister kinetochores by the opposing pulling forces from microtubules when sister chromatids are bi-oriented has been proposed as a major factor in the stabilisation of kinetochore-microtubule interactions. Tension might both regulate the correction mechanism that converts syntelic chromosomes to the bi-oriented state and be a signal to the spindle assembly checkpoint to delay anaphase until all chromosomes are correctly attached to the spindle (see Pinsky and Biggins, 2005; Kelly and Funabiki, 2009). We have shown that by reducing such tension in cells depleted of cohesin, phosphorylation of Dam1 remains high



**Fig. 4.** Dam1 phosphorylation depends on Ipl1 and microtubules. (A) Ipl1 dependence of Dam1 phosphorylation. Wild-type (T2748; circles) and *ipl1-321* (T2863; squares) yeast cells were synchronised in G1 and released at 37°C to a metaphase arrest. Samples were taken for western blot analysis using anti-Ser20-P (pS20), anti-Ser257-P (pS257), anti-Ser265-P (pS265) and anti-Dam1 (Dam1) antibodies. Cdc28 was also visualised as a loading control. Relative Dam1 phosphorylation at each site was estimated as described in the legend to Fig. 2 except that for each dataset, the samples from both strains were processed concurrently on the same western blot. The ratios of Dam1-P to Dam1 for both strains (red) were normalised using a value of 1.0 for the zero time point of the wild-type strain to enable direct comparison of the two datasets. Values were plotted together with the proportion of budded cells (black) and of cells with a bipolar spindle (blue). (B) Phosphorylation of Dam1 depends on microtubules. Yeast cells expressing YFP-Tub1 (T2749) were synchronised in G1 and released at 25°C in the presence of 15 µg/ml nocodazole (squares), adding an equivalent volume of drug vehicle (DMSO, 1% v/v final concentration) to the control culture (circles). Samples were taken for western blotting, spindle morphology determination and budding index determination. Dam1 phosphorylation on Ser257 (pS257) and Ser265 (pS265) was examined by western blotting with anti-S257-P and anti-S265-P antibodies and the overall level of Dam1 examined using anti-Dam1 (Dam1). Cdc28 was also visualised as a loading control. Dam1 phosphorylation was estimated as described in A, normalising values from both cultures using the zero time point of the control culture (set arbitrarily to 1.0) to enable direct comparison of the two datasets (red). Black, proportion of budded cells; blue, proportion of cells with a metaphase spindle; green, proportion of cells with an elongated anaphase spindle. No spindles were evident in nocodazole-treated cells owing to microtubule depolymerisation. Therefore, 0% data points for spindle morphologies in nocodazole-treated have been omitted.

during metaphase, supporting the idea that Ipl1-dependent chromosome reorientation is regulated by sister chromatid tension. However, if tension-dependent stabilisation of kinetochore-microtubule interactions resulted from structural changes in the

kinetochore, there would be no requirement for tension-dependent regulation of Dam1 dephosphorylation: it would be sufficient to induce Dam1 phosphorylation to initiate re-orientation but there would be no need to invoke dephosphorylation as part of the



**Fig. 5.** Phosphorylation of Dam1 persists in the absence of sister chromatid cohesion. (A) Cells dependent on *pGAL-SCC1* and *pMET3-CDC20* (T6984) were grown in galactose-containing medium, synchronised in G1 and then released to a metaphase arrest in the presence of methionine and either galactose (*SCC1* expressed, *Scc1*<sup>+</sup>, squares) or glucose (*SCC1* repressed, *Scc1*<sup>-</sup>, triangles). Dam1 phosphorylation on Ser20 (pS20), Ser257 (pS257) and Ser265 (pS265) was examined by western blotting with the three phosphospecific antibodies and the overall level of Dam1 examined using anti-Dam1 antibody. Cdc28 was also visualised as a loading control. Relative phosphorylation of Dam1 at each site (grey) was quantified as described in the legend to Fig. 2. For each site, blots from the *Scc1*<sup>+</sup> and *Scc1*<sup>-</sup> cultures were processed concurrently and the ratio of phosphorylated Dam1 to total Dam1 normalised to a value of 1.0 at time zero in the *Scc1*<sup>-</sup> culture to enable a direct comparison of *Scc1*<sup>+</sup> and *Scc1*<sup>-</sup> cells. The proportion of budded cells (black) is also indicated. (B) Quantification of metaphase-arrested cells (160-minute time point) for presence of associated (unseparated) or separated chromosome XV sister chromatids (GFP-labelled *HIS3* locus on the right arm of chromosome XV) in *Scc1*<sup>+</sup> and *Scc1*<sup>-</sup> cultures. The high proportion of pre-anaphase sister chromatid separation confirms loss of cohesion in the *Scc1*<sup>-</sup> regime.

mechanism. Since we have found that Dam1 is rapidly dephosphorylated as cells enter metaphase but that this dephosphorylation is tension responsive (i.e. reduced tension maintains Dam1 in its phosphorylated state), it is more likely that changes in the phosphorylation state of Dam1 not only promote kinetochore detachment from microtubules, but also contribute to the stabilisation of kinetochore-microtubule attachments when tension is applied.

Tension-dependent regulation of kinetochore phosphoepitopes was first proposed following studies with the 3F3/2 antibody, which reacts strongly with metazoan kinetochores that lack tension (Nicklas et al., 1995). Recently, the *Xenopus* 3F3/2 epitope has been shown to be generated by polo-like kinase phosphorylation of BubR1 (Wong and Fang, 2007) and might correspond to Ser676 in human BubR1, whose polo-dependent phosphorylation regulates the stability of kinetochore-microtubule interactions (Elowe et al., 2007). Neither this residue nor the CDK phosphorylation site required to prime its phosphorylation appear to be conserved in the yeast homologue of BubR1 (Mad3). However, our finding that the three Ipl1 phosphorylation sites in Dam1 show tension-dependent regulation of their phosphorylation state suggests that although the details might differ, tension-dependent regulation of kinetochore phosphoepitopes is nonetheless a conserved theme in the regulation of kinetochore-microtubule interactions.

The reduction in Dam1 phosphorylation as cells enter metaphase with bi-oriented chromosomes could in principle result from regulation of Dam1 phosphorylation, dephosphorylation, or both. Type 1 protein phosphatase (Glc7) has been proposed to be important for opposing Ipl1-dependent phosphorylation in yeast (Hsu et al., 2000; Pinsky et al., 2006), and the temperature-sensitive *glc7-10* allele (Sassoon et al., 1999) has been shown to affect Dam1 phosphorylation, at least as assessed by changes in the pattern of isoforms observed by SDS-PAGE in asynchronous cultures (Pinsky et al., 2006). However, by combining either *glc7-10* or a different temperature-sensitive *glc7* allele (*glc7-127*) (Baker et al., 1997) with *pMET3-CDC20*, in experiments similar to that shown in Fig. 3, we failed to detect any persistence of Dam1 phosphorylation in metaphase-arrested cells at restrictive temperatures. However, as previously described (Hsu et al., 2000), phosphorylation of Ser10 on histone H3 was enhanced in such mutants (data not shown). Thus, Glc7 might not have a major role in the tension-dependent dephosphorylation of Dam1 at the specific sites that we have examined. However, there are a number of ways by which tension could regulate Ipl1-dependent phosphorylation of Dam1 (see Kelly and Funabiki, 2009). On the one hand, tension might directly regulate Ipl1 activity by promoting conformational changes in Ipl1 itself or in its activators such as Sli15 and Bir1 (Sandall et al., 2006). Conversely, tension might lead to physical separation of Ipl1 from

key substrates at the kinetochore-microtubule interface, such as Dam1 (Tanaka et al., 2002; Shimogawa et al., 2009), and recent data from human cells supports the latter model. Thus in U2OS cells, phosphorylation of an Aurora B substrate could be manipulated by repositioning it relative to Aurora B (localised in the inner centromere region), whereas repositioning Aurora B closer to the kinetochore-microtubule interface in live cells destabilised microtubule-kinetochore interactions (Liu et al., 2009). Both 'tension-sensor' and 'kinase-substrate delocalisation' mechanisms work only if the proposed alterations, caused by tension, lead to a change in the phosphorylation state of Aurora-B/Ipl1 substrates. Whether such changes in phosphorylation state actually occur was uncertain, but in this study we show that the phosphorylation state of Dam1 is indeed responsive to changes in tension.

## Materials and Methods

### Strain construction and culture conditions

All yeast strains used in this study are listed in supplementary material Table S1. To generate PKY140, the *TRP1* marker was amplified from pFA6a-TRP1 (Longtine et al., 1998) using primers Dam1F1Mark and Dam1R1Mark (supplementary material Table S2) and inserted downstream of *DAMI1*. PKY141, PKY142 and PKY143 were generated from PKY140 genomic DNA using the method of Toulmay and Schneiter (Toulmay and Schneiter, 2006) and primers Dam1S20F, Dam1S257F and Dam1S265F, respectively (supplementary material Table S2) in combination with Dam1R1Mark for the PCR step. Media used for growth of yeast strains were made according to standard protocols (Amberg and Strathern, 2005). Cells were synchronised in G1 by addition of 1.25 µg/ml  $\alpha$ -factor (CRUK peptide synthesis service), checking microscopically that at least 95% of cells were arrested (usually 2 hours) before collecting on a filter (Whatman ME28, 1.2 µm), washing three times with milliQ water with the aid of a vacuum pump and then quickly transferring them to fresh growth medium. In experiments where *Sec1* was depleted, cells expressing *SCC1* from the *GALI10* promoter were arrested with  $\alpha$ -factor for 1 hour in YPA medium supplemented with 2% (w/v) raffinose and 2% (w/v) galactose (*SCC1* expressed), then cells were recovered by filtration, washed and then transferred to YPAD medium containing  $\alpha$ -factor for a further 2 hours. The cells were then washed free of  $\alpha$ -factor and released in to YPAD medium. Cells dependent on p*MET3-CDC20* were grown and arrested in metaphase in the presence of 2 mM methionine as described (Uhlmann et al., 2000). For quantification of the fraction of budded cells and the proportion with metaphase and anaphase spindles, samples of at least 100 cells were examined from each sample. Spindle morphology was determined using strains expressing YFP-*TUB1* (Yeast Resource Centre, Seattle, WA). When a spindle was clearly present it was defined as a metaphase spindle if less than ~1.5 µm in length and as an anaphase spindle if it had clearly elongated well beyond this size.

### In vitro phosphorylation of GST-Dam1

Full-length *DAMI1* was amplified by PCR from yeast genomic DNA using high fidelity DNA polymerase and primers GCGTGGATCCAGCGAAGATAAAGCTAAATTAG and GTACCCCGGGTCATCTGAAGGGGGGCTT, then cloned into pGEM-T-easy (Promega). After verification by DNA sequencing, it was excised using *Bam*HI and *Sma*I (sites underlined in the primer sequences) and inserted into pGEX-2T (Smith and Johnson, 1988), yielding pGEX-2T-Dam1. Recombinant GST-Ipl1 and GST-Sli15 were prepared as described (King et al., 2007) and recombinant GST-Dam1 was prepared using pGEX-2T-Dam1 and a similar procedure. Kinase assays were performed by incubating 0.2 µg purified GST-Ipl1 and 0.04 µg purified GST-Sli15 with 2 µg of GST-Dam1 in buffer containing 50 mM Tris-HCl (pH 7.5), 0.1% (v/v)  $\beta$ -mercaptoethanol, 0.1 mM EGTA, 10 mM MgCl<sub>2</sub> and 100 µM ATP for 15 minutes at 30°C. The reaction was stopped by the addition of 2× sample buffer and analysed by western blotting, using an anti-GST antibody (Sigma G-1160) to confirm equivalent loading of GST-Dam1.

### Anti-Dam1 antibodies

Polyclonal anti-Dam1 antibodies were generated in rabbits by Diagnostics Scotland (Edinburgh, UK) using GST-Dam1 expressed and purified as above. Affinity purification of antibodies for use in western blotting was carried out by binding to and elution from GST-Dam1 immobilised on nitrocellulose as described (Pringle et al., 1991), and were used at a dilution of 1:10,000. The rabbit anti-Dam1 antibody prepared by Cheeseman and colleagues (Cheeseman et al., 2002) was used in some experiments as an alternative.

### Phosphospecific anti-Dam1 antibodies

Synthetic peptides were synthesised with N-terminal cysteine residues corresponding to the regions of Dam1 surrounding Ser20 (CRSATEYRLSIGSAPT), Ser257 (CNTNSKLRKRSILHTIR), Ser265 (CHTIRNSIASGADLP) or Ser292 (CHPNN-

RISLGSQAAR) either with or without the presence of a phosphate group on the relevant serine residue (underlined). The phosphopeptides were coupled to keyhole limpet hemocyanin following treatment of the latter with iodoacetic acid N-hydroxysuccinimide ester (IAA-NHS) as described (Field et al., 1998). Polyclonal antibodies were then raised against each conjugated phosphopeptide in sheep by Diagnostics Scotland. Phosphospecific antibodies were affinity purified from the sheep sera on matrices prepared by conjugating the phosphopeptides to Affigel-10 following derivatisation with IAA-NHS as described previously, and the antibodies were typically eluted at a concentration of 100–400 µg/ml. To ensure phosphospecificity, eluted antibodies were treated for 60 minutes with 2% (w/v) dried yeast cell protein, which was prepared according to Harlow and Lane (Harlow and Lane, 1988) by acetone precipitation from a strain in which the corresponding phosphorylation site was mutated to alanine: T4444 for anti-Ser20-P and BP635 for anti-Ser257-P and anti-Ser265-P (supplementary material Table S1). All antibodies were used for western blotting at a concentration of 0.5 µg/ml in TBST (Tris-buffered saline plus 0.1% Tween 20) containing 5% (w/v) dried skimmed milk. Before use, these solutions of the anti-Ser20 and anti-Ser257 antibodies were also pre-treated for 30 minutes with 5.0 µg/ml of the corresponding non-phosphorylated peptide.

### Western blot analysis

Yeast cells were lysed and prepared for SDS-PAGE by sequential treatment with alkali and trichloroacetic acid, as described previously (Mekhail et al., 2008). Following sample analysis by SDS-PAGE, proteins were transferred to PVDF membrane using CAPS buffer [10 mM CAPS-NaOH and 10% (v/v) methanol, pH 11]. After transfer, the membrane was washed in TBST and then blocked for 30 minutes in TBST with 5% (w/v) milk. The membrane was incubated with primary antibody solution (prepared as above) either for 1 hour at room temperature or overnight at 4°C. Following three 10 minute washes with TBST, the membrane was incubated for 1 hour in secondary antibody coupled to HRP. The membrane was washed three times for 10 minutes in TBS-T, and bands were visualised using X-ray film following incubation in enhanced chemiluminescence (ECL, Millipore) according to the manufacturer's instructions. Equivalent loading of samples was assessed using anti-Cdc28 antibodies (sc-28550, Santa Cruz Biotechnology) as a control. Ser10 phosphorylated histone H3 was detected using a rabbit phosphospecific antibody (Millipore, #06-570) whereas total histone H3 was detected using a ChIP-grade rabbit antibody from Abcam (ab1791), both used at 1:1000 final concentration. ImageJ (Rasband, 1997-2009) was used to quantify the intensity of bands following scanning using an Epson V750 Pro scanner in transmission mode. To estimate the relative level of Dam1 phosphorylation, the phosphospecific signal obtained at each time point was normalised using the signal obtained using rabbit-anti-Dam1 antibody and then these ratios expressed relative to the ratio at time zero (cells at the point of  $\alpha$ -factor release), which was arbitrarily set to a value of 1.0. Each lane on each western blot contains proteins extracted from the cells in ~1.25 ml of culture.

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