Plo1 phosphorylates Dam1 to promote chromosome bi-orientation in fission yeast

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Accepted 28 November 2011
Journal of Cell Science 125, 1645–1651

doi: 10.1242/jcs.096826

Summary

The fungal-specific heterodecameric outer kinetochore DASH complex facilitates the interaction of kinetochores with spindle microtubules. In budding yeast, where kinetochores bind a single microtubule, the DASH complex is essential, and phosphorylation of Dam1 by the Aurora kinase homologue, Ipl1, causes detachment of kinetochores from spindle microtubules. We demonstrate that in the distantly related fission yeast, where the DASH complex is not essential for viability and kinetochores bind multiple microtubules, Dam1 is instead phosphorylated on serine 143 by the Polo kinase homologue, Plo1, during prometaphase and metaphase. This phosphorylation site is conserved in most fungal Dam1 proteins, including budding yeast Dam1. We show that Dam1 phosphorylation by Plo1 is dispensable for DASH assembly and chromosome retrieval but instead aids tension-dependent chromosome bi-orientation.

Key words: Dam1, Kinetochore, Microtubule, Polo kinase

Introduction

Accurate segregation of chromosomes to daughter cells requires that chromosomes are correctly bi-oriented before anaphase onset. Bi-orientation is ensured by dynamic changes in protein phosphorylation at the spindle–kinetochore interface, mediated by several kinases including the Polo-like kinase Plk1, chromosomal passenger protein Aurora B (Ark1 in fission yeast) and Mps1. The role of Aurora B in promoting chromosome bi-orientation has been studied most extensively. Chromosome bi-orientation generates tension across kinetochores, separating centromerically associated Aurora B from the outer kinetochore where it phosphorylates components of the microtubule-binding KMN complex, including Ndc80 and Knl1 (also known as Spc105) (Liu et al., 2009). Phosphorylation of these proteins weakens the affinity of KMN for microtubules, facilitating correction of microtubule–kinetochore interactions (Welburn et al., 2010).

Substrates of Plk1 and Mps1 that are involved in promoting chromosome bi-orientation have been more difficult to elucidate. It is known that Plk1 promotes bi-orientation by phosphorylating kinetochores of unaligned or unattached chromosomes to generate a phosphoepitope recognised by the 3F3/2 antibody (Ahonen et al., 2005). Several kinetochore Plk1 targets have been identified, but the precise mechanism by which Plk1 promotes bi-orientation and the identity of the 3F3/2 epitope in many systems remains unclear (Elowe et al., 2007; Wong and Fang, 2007). Two complications are that human Mps1 (which is also known as TTK) and Plk1 kinases share a similar substrate consensus motif and that loading of the 3F3/2 substrate to kinetochores requires prior assembly and activity of Mps1 (Dou et al., 2011; Wong and Fang, 2006).

In *Saccharomyces cerevisiae* the heterodecameric outer kinetochore DASH complex facilitates microtubule binding of Ndc80 in vitro. Purified *S. cerevisiae* kinetochores lacking DASH are defective in forming stable microtubule attachments when a force is applied comparable to that imposed by the spindle (Akiyoshi et al., 2010; Lampert et al., 2010; Tien et al., 2010). The DASH complex is conserved in other fungi but not in metazoa. The requirement for DASH is related to the number of microtubule-binding sites per kinetochore (Burrack et al., 2011; Thakur and Sanyal, 2011). Although the DASH complex is essential for viability in budding yeast, where kinetochores bind to a single microtubule, it is not required for viability in fission yeast, where kinetochores bind to between two and four microtubules (Liu et al., 2005; Sanchez-Perez et al., 2005). Despite this, *S. pombe* cells lacking DASH display defects in chromosome segregation and are profoundly impaired in kinetochore retrieval, possibly because this occurs on a single microtubule (Franco et al., 2007; Gachet et al., 2008; Liu et al., 2005; Sanchez-Perez et al., 2005).

The *S. cerevisiae* Aurora homologue, Ipl1, phosphorylates Dam1 at multiple sites, which causes microtubule detachment from the kinetochore (Cheeseman et al., 2002). However the Ipl1 phosphorylation sites are not conserved in *S. pombe* Dam1 or in Dam1 proteins in most other fungi (Buttrick and Millar, 2011). This persuaded us to investigate how the DASH complex is regulated in fission yeast.

Results and Discussion

Fission yeast Dam1 is phosphorylated during early mitosis

To determine whether Dam1 is phosphorylated during mitosis, extracts were prepared from log-phase and mitotically arrested *dam1-13myc nda3-KM311* cells. In arrested cells, some Dam1 migrated with slower mobility in SDS-PAGE gels containing 50 μM Phos-Tag acrylamide (Fig. 1A). The slower band was abolished by λ phosphatase treatment and was not detected in the absence of Phos-Tag acrylamide (Fig. 1B), indicating this band represents phosphorylated Dam1. Dam1 phosphorylation was not observed in mitotically arrested cells lacking the DASH

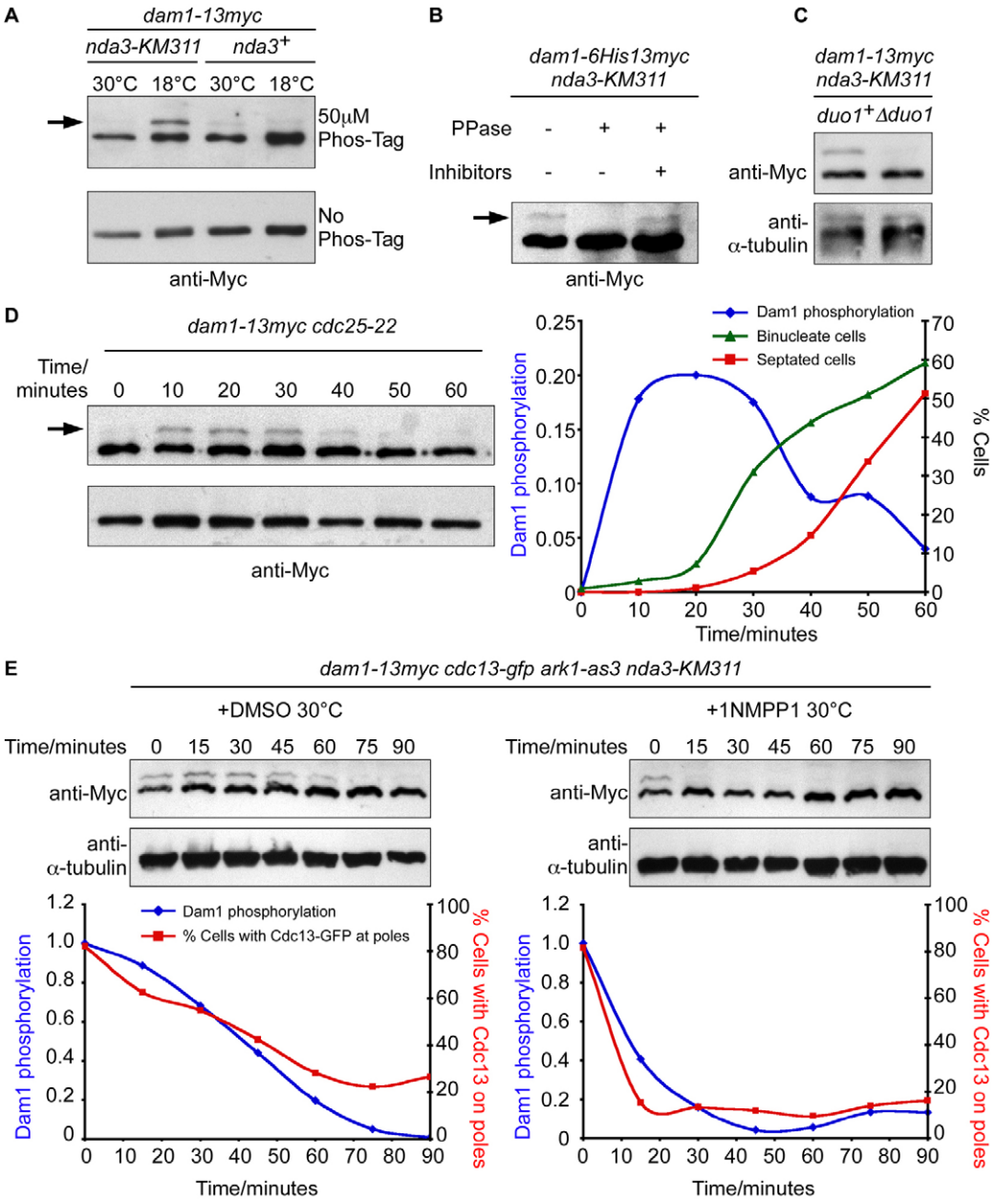

Fig. 1. Dam1 is phosphorylated in mitosis. (**A**) Extracts from log-phase *nda3*$^{+}$ *dam1-13myc* and *nda3-KM311 dam1-13myc* cells grown at 30℃, or from the same cells following incubation at 18℃ for 6 hours, were separated by SDS-PAGE in the presence or absence of 50 μM Phos-Tag acrylamide and probed by western blot using anti-Myc antibodies. The arrow indicates the shifted band corresponding to phosphorylated Dam1. (**B**) Extracts were prepared from mitotically arrested *nda3-KM311 dam1-6His13myc* cells. Dam1 was enriched on Ni^{2+}-resin and incubated in the presence or absence of λ-phosphatase and phosphatase inhibitors. Dam1 phosphorylation was monitored as above. (**C**) Extracts were prepared from mitotically arrested *nda3-KM311 dam1-13myc Δduo1* cells. Phosphorylation of Dam1 was monitored as in A except that blots were re-probed with anti-α-tubulin antibodies as a loading control. (**D**) G2-arrested *cdc25-22 dam1-13myc* cells were released to 25℃ and samples taken at intervals. The graph shows Dam1 phosphorylation as the ratio of phosphorylated:unphosphorylated Dam1. Cells were fixed at each time point to assess mitotic progression (binucleates and septa). (**E**) *nda3-KM311 ark1-as3 dam1-13myc cdc13-gfp* cells were synchronised by incubation at 18℃ and shifted to 30℃ in the presence of DMSO (left panels) or 5 μM 1NMPP1 (right panels). At the indicated times extracts were prepared and Dam1 phosphorylation assessed (top panels) or cells were fixed and the percentage of cells with SPB-associated Cdc13 assessed (red squares, bottom panels) and plotted against relative Dam1 phosphorylation (normalised to 1.0 at time 0; blue diamonds, bottom panels).

component Duo1 (Fig. 1C), indicating that DASH complex formation is essential for Dam1 phosphorylation.

To establish whether Dam1 is phosphorylated during an unperturbed mitosis, arrested *cdc25-22* cells were released into mitosis. Dam1 was not phosphorylated in G2, but its phosphorylation increased rapidly on mitotic entry. The timing of phosphorylation was coincident with an increase in binucleate cells and prior to an increase in septation (Fig. 1D), suggesting that Dam1 is dephosphorylated during anaphase. To assess this more directly we monitored Dam1 phosphorylation and Cdc13

(cyclin B) destruction in mitotically arrested *nda3-KM311 ark1-as3 dam1-13myc cdc13-GFP* cells released to the permissive temperature in the presence or absence of 1NMPP1 [a cell-permeable ATP homologue which specifically inhibits Ark1-as3 (Hauf et al., 2007)]. In both situations dephosphorylation of Dam1 occurred co-incidentally with Cdc13 destruction, although more rapidly when Ark1 was inhibited (Fig. 1E), presumably because inactivation of Ark1 ectopically silences the spindle assembly checkpoint (SAC) (Vanoosthuyse and Hardwick, 2009).

Dephosphorylation of Dam1 could be a direct consequence of Ark1 inactivation, de-repression of a phosphatase under the control of Ark1, or as a consequence of anaphase-promoting complex/cyclosome (APC/C) activation. To distinguish these possibilities we repeated the above experiment at 18℃ to prevent spindle formation. Under these conditions addition of 1NMPP1 triggered destruction of Cdc13 after 10 minutes. However, little Dam1 dephosphorylation had occurred by 30 minutes, and even after 1 hour, a considerable portion of Dam1 remained phosphorylated (supplementary material Fig. S1A). This suggests that dephosphorylation of Dam1 does not occur solely as a consequence of APC/C activation. Secondly, we overexpressed the SAC component Mad2 in *ark1-as3 dam1-13myc cdc13-gfp* cells to arrest them in mitosis with short spindles. Addition of 1NMPP1 did not cause appreciable Dam1 dephosphorylation, suggesting that efficient Dam1 dephosphorylation requires both spindle microtubules and passage through anaphase, but is only indirectly under the control of Ark1 (supplementary material Fig. S1B).

Plo1 phosphorylates Dam1 on S143

Because Dam1 lacks conserved phosphorylation sites for Ark1, we examined whether other mitotically active kinases are responsible for Dam1 phosphorylation. We found no effect on Dam1 phosphorylation in metaphase-arrested cells lacking Mph1, Bub1 or Fin1 (Fig. 2A). Strikingly, we were unable to observe phosphorylation of Dam1 in metaphase-arrested *plo1-ts35* cells, even at the permissive temperature for the *plo1-ts35* mutant (Fig. 2B). This defect was rescued by expression of wild-type *plo1* but not by expression of a *plo1* allele [*plo1(YQL508AAA)*] that disrupts the interaction of the polo-box domain (PBD) with phosphorylated target proteins and, hence, is unable to rescue temperature sensitivity of the *plo1-ts35* allele (Fig. 2C; supplementary material Fig. S2). These results strongly suggest that Plo1 is the Dam1 kinase in *S. pombe*.

The C-terminus of *S. cerevisiae* Dam1 participates in inter-complex interactions, and is highly phosphorylated by Ipl1 (Cheeseman et al., 2002; Ramey et al., 2011). Alleles of *S. pombe dam1* where the C-terminus of the protein is truncated show a striking resistance to the microtubule poison thiabendazole (TBZ) suggesting this is a key regulatory domain of Dam1 (Sanchez-Perez et al., 2005). Importantly, whereas DASH formation was unperturbed in *dam1(1-127)* cells, we detected no change in the mobility of Dam1(1-127)–13myc in mitotically arrested cells, suggesting that the Plo1 phosphorylation site is located in the C-terminal 28 amino acids of Dam1 (supplementary material Fig. S3). This region contains three highly conserved serine or threonine residues (Fig. 2D,E). To determine whether any of these residues is phosphorylated in mitosis, each was individually mutated to alanine residues, and migration of the mutant proteins was analysed in *nda3-KM311* cells. This revealed that S143 is the major Dam1 phosphorylation site during mitosis (Fig. 2D). Importantly, the region surrounding S143 precisely matches the consensus sequence for Polo-like kinases (Nakajima et al., 2003), suggesting Plo1 directly phosphorylates Dam1 (Fig. 2E). To establish whether Dam1 S143 was indeed a bona fide Plo1 target, antibodies were generated to specifically recognise Dam1 phosphorylated on S143 (supplementary material Fig. S4A). These antibodies readily recognised Dam1 protein purified from *E. coli* and incubated with Plo1 in vitro, indicating that Dam1 S143 is phosphorylated by Plo1 (Fig. 2F; supplementary material Fig. S4B). Unfortunately, these antibodies were not of sufficient titre to recognise phosphorylated Dam1 S143 in total cell extracts (supplementary material Fig. S4A; data not shown).

Polo-like kinases are often targeted to their substrates by association to a neighbouring phosphoepitope through the C-terminal PBD (Elia et al., 2003a). As the Plo1 PBD is required for Dam1 phosphorylation (Fig. 2C), we speculated as to the identity of the Polo-box-binding domain. Polo-box binding epitopes are often created by Cdk1 phosphorylation (Elia et al., 2003b), and the DASH subunit Ask1 is a Cdk1 target in *S. cerevisiae* (Li and Elledge, 2003). Interestingly, *S. pombe* Ask1 is phosphorylated on T136, which not only matches the Cdk1 consensus, but also strongly corresponds to the optimum polo-box binding motif (Elia et al., 2003b; Wilson-Grady et al., 2008) (supplementary material Fig. S4C). We found that DASH complex formation or localisation was unaffected in *ask1-T136A* mutants (supplementary material Fig. S4D). However, Dam1 S143 phosphorylation was not observed in mitotically arrested *ask1-T136A* cells, suggesting that phosphorylation of Ask1 T136 generates the polo-box-binding site required for phosphorylation of Dam1 S143 by the kinase Plo1 (Fig. 2G).

Mitotic phosphorylation is reversed during anaphase by several phosphatases, including PP1, PP2A and Cdc14 (Wurzenberger and Gerlich, 2011). In *S. cerevisiae*, phosphorylation of Dam1 by Ipl1 is reversed by Glc7 (the PP1 phosphatase) when tension is applied to kinetochores by spindle microtubules (Keating et al., 2009; Pinsky et al., 2006). We speculate that Dis2, one of two PP1 catalytic subunits in *S. pombe*, could dephosphorylate Dam1 S143 given that Dis2 localises to the kinetochore and is required for both correct chromosome segregation and silencing the SAC (Alvarez-Tabares et al., 2007; Meadows et al., 2011; Ohkura et al., 1989). However, we were unable to assess this for technical reasons (supplementary material Table S1). Nevertheless dephosphorylation of Dam1 occurred normally in *clp1(C286S)* cells, which express a catalytically inactive allele of the Clp1 (Cdc14-like) phosphatase, and *Δppa2* cells, which lack the major PP2A catalytic subunit in *S. pombe* (Kinoshita et al., 1993) (supplementary material Fig. S5). These phosphatases are, therefore, not required for Dam1 dephosphorylation.

Dam1 S143 phosphorylation aids tension-dependent chromosome bi-orientation

DASH function requires assembly of the complex from ten subunits (Liu et al., 2005; Sanchez-Perez et al., 2005). To examine whether Plo1 phosphorylation of Dam1 is required for DASH assembly, DASH was purified using tandem affinity purification. We found that Dam1–13myc and Dam1(S143A)–13myc show a similar affinity to Ask1–TAP, indicating that Dam1 phosphorylation is not required for DASH complex

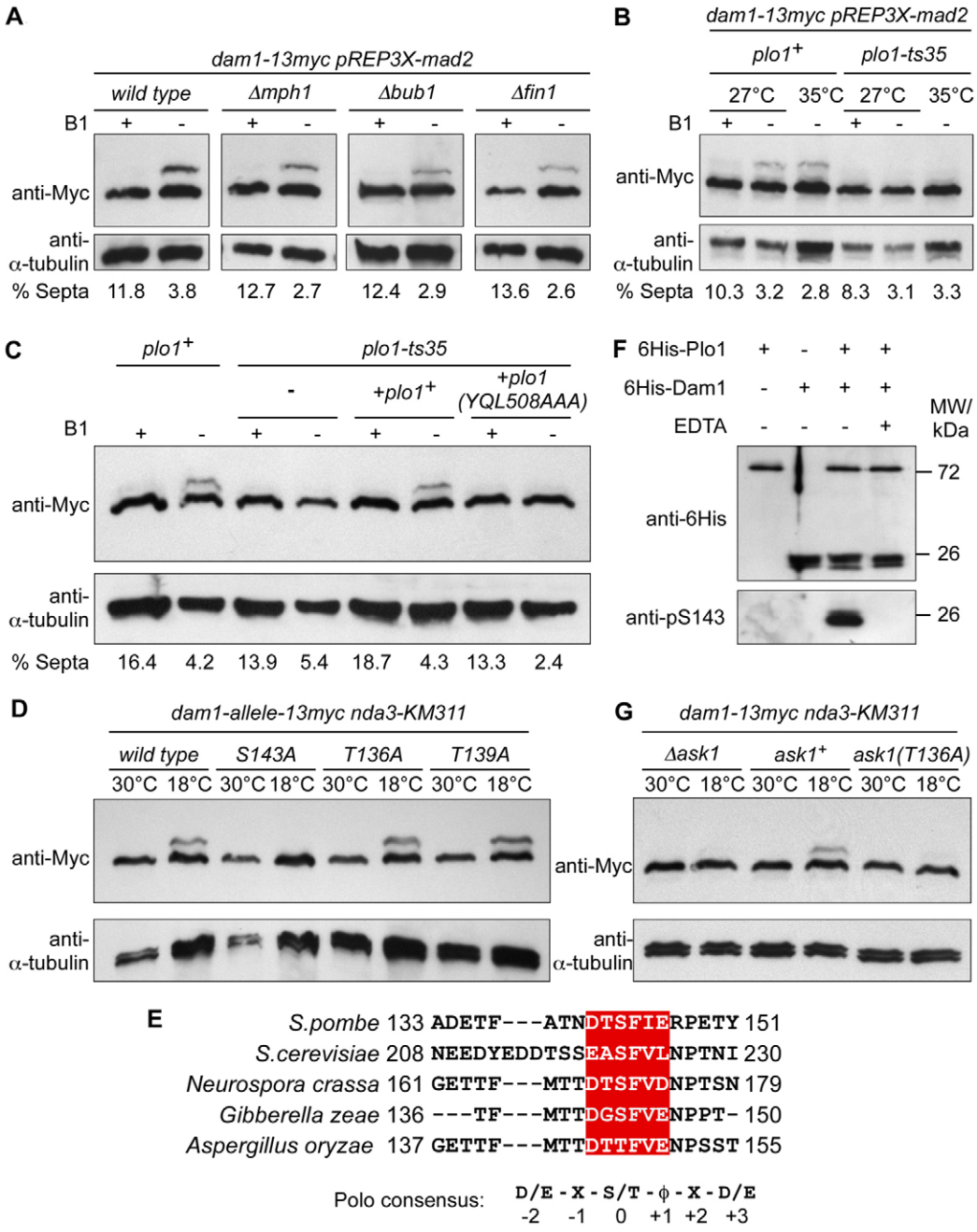

Fig. 2. Plo1 phosphorylates Dam1 on S143. (**A**) *dam1-13myc pREP3X-mad2* cells with indicated genes deleted were grown to mid-log phase either in the presence (+ B1) or absence (– B1) of thiamine. The percentage of cells with septa was measured by fluorescence microscopy. (**B**) *dam1-13myc pREP3X-mad2* and *plo1-ts35 dam1-13myc pREP3X-mad2* cells were grown to mid-log phase at 27℃, and then incubated either in the presence (+ B1) or absence of thamine (– B1) at either the permissive (27℃) or restrictive (35℃) temperature. (**C**) *dam1-13myc pREP3X-mad2* or *plo1-ts35 dam1-13myc pREP3X-mad2* cells expressing either nothing, *plo1*$^{+}$ or *plo1(YQL508AAA)* were incubated at 27℃ in the presence (+ B1) or absence (– B1) of thiamine to induce Mad2 and Plo1 expression. (**D**) Extracts were prepared from log-phase (30℃) or mitotically arrested (18℃) *nda3-KM311 dam1-allele-13myc* cells bearing alanine residue replacements within the C-terminal tail of Dam1. In A–D, Dam1 phosphorylation was monitored as Fig. 1. (**E**) The phosphorylation site for Plo1 in *S. pombe* Dam1 is conserved across several subphyla of Ascomycota and matches the consensus site for Plk1 (Nakajima et al., 2003). (**F**) Purified His_6-tagged (6His) Plo1 and Dam1 were incubated in the presence of ATP for 30 minutes at 30℃. Proteins were detected by western blot using anti-His_6 antibodies. Dam1 S143 phosphorylation was detected using phosphorylation-specific antibodies. (**G**) Extracts were prepared from log phase (30℃) or mitotically arrested (18℃) *nda3-KM311 dam1-13myc* cells with the indicated *ask1* allele. Dam1 phosphorylation was monitored as in Fig. 1.

formation (supplementary material Fig. S6A). Consistent with this, Ask1–GFP localisation was unperturbed in *dam1(S143A)* cells, demonstrating that Dam1 phosphorylation is not required for DASH localisation (supplementary material Fig. S6B). However, *dam1(S143A)* cells showed similar levels of TBZ resistance to *dam1(1-127)* cells, consistent with phosphorylation

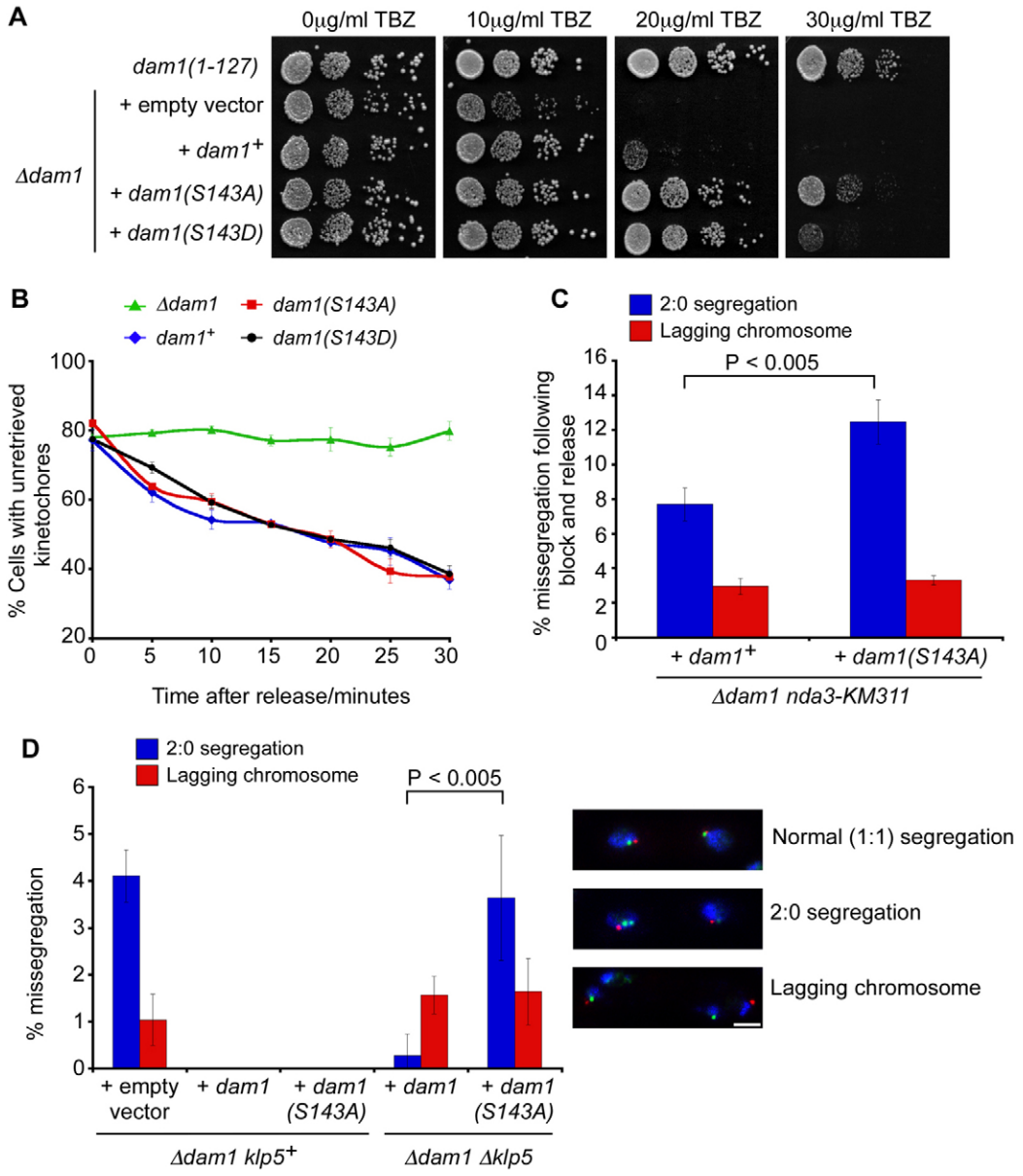

Fig. 3. Dam1 phosphorylation promotes chromosome bi-orientation. (**A**) Serial dilutions of log-phase cells ($\sim 10^4$, 10^3, 10^2, 10^1 cells from right to left) were spotted onto YES medium plates containing the indicated concentrations of TBZ and grown for 2 days at 30°C. (**B**) Kinetochore retrieval was assessed in *nda3-KM311 dad1-gfp sid4-tdtomato* as described previously (Grishchuk and McIntosh, 2006). Results are means ± s.e.m. ($n=4$). (**C**) Mitotically arrested *nda3-KM311 Δdam1 cen2-gfp sid4-tdtomato* cells containing no addition (*Δdam1*), or *dam1*$^+$ or *dam1(S143A)* alleles were released to permissive temperature. Cells were fixed and scored for either chromosome non-disjunction (2:0 segregation) or lagging sister chromatids. Results are means ± s.d. ($n=4$). (**D**) Cells with the genotypes detailed were synchronised in G2 and released into anaphase. Chromosome segregation was monitored by GFP bound to the *lys1* locus and Cdc11–CFP and scored as in C. Results are means ± s.d. ($n=4$).

at S143 having a major regulatory function at the Dam1 C-terminus (Fig. 3A).

We have previously shown that DASH is essential for retrieval of unclustered kinetochores in fission yeast (Franco et al., 2007). To determine whether Dam1 phosphorylation is required for this process, we monitored the retrieval of kinetochores from mitotically arrested *nda3-KM311* cells following release to permissive temperature. In agreement with prior studies, we find that most unclustered kinetochores are retrieved by 30 minutes and that Dam1 is essential for this process. However, we found that kinetochore retrieval in *dam1(S143A)* cells was indistinguishable from that in wild type (Fig. 3B).

Recaptured chromosomes are initially attached to one SPB and must be re-oriented on the spindle following retrieval to the SPB. To determine whether kinetochore bi-orientation requires Plo1-mediated phosphorylation of Dam1, the centromere of chromosome 2 was marked with GFP (cen2–GFP) to monitor re-orientation. Following release of mitotically arrested *nda3-KM311* cells to the permissive temperature, 8% of wild-type cells segregated both cen2–GFP markers to the same SPB,

presumably because microtubule function is somewhat perturbed (Fig. 3C). Importantly, under the same conditions, 13% of *dam1(S143A)* cells segregated both cen2–GFP markers to the same SPB (Fig. 3C). This is comparable to the defect observed in *Δsgo2* cells following *nda3-KM311* block and release (Vanoosthuyse et al., 2007).

We next examined the effect of the *dam1(S143A)* allele in other genetic backgrounds in which spindle formation or chromosome bi-orientation is impaired. Dam1 is essential for viability in the absence of Klp5 and Klp6, components of the *S. pombe* kinesin-8 heterodimer (Sanchez-Perez et al., 2005). *Δklp5* cells exhibit hyper-stable microtubules and SAC-mediated delay in mitosis with abnormally long metaphase spindles (Garcia et al., 2002; West et al., 2001). Notably, alleles disrupting the C-terminus of Dam1 cause slow growth in the absence of Klp5/6 (Griffiths et al., 2008). This phenotype was also observed in *dam1(S143A) Δklp5* double mutants (Table 1). To characterise this further we assayed segregation of a GFP-tagged chromosome 1 in these cells. Whereas *dam1(S143A)* cells show no chromosome segregation defects in the presence of Klp5, *dam1(S143A) Δklp5* cells showed appreciable defects, with both chromosome 1 markers segregating to the same pole in 3.6% of cells (Fig. 3D). More dramatically, we found that both Dam1 phosphorylation and Plo1 activity were essential for viability in the absence of Dis2 (Table 1). The lethality of *Δdam1 Δdis2* and *dam1(S143A) Δdis2* double mutants was not rescued by deleting the SAC component *mad3*. Lethality of these strains is therefore not due to persistent SAC arrest. Instead we conclude that Dam1 phosphorylation on S143 by Plo1 aids chromosome bi-orientation in situations where establishment of amphitelic microtubule–kinetochore connections is compromised.

In an attempt to mimic constitutive phosphorylation of Dam1, we also made S143 to aspartic acid replacements. Dam1(S143D) did not adversely effect kinetochore retrieval and did not cause lethality in *Δdis2* cells (Fig. 3B; Table 1). However, we also note that *dam1(S143D)* cells displayed TBZ resistance, albeit to a lesser extent than *dam1(S143A)* or *dam1(1-127)* cells, and impaired growth in a *Δklp5* background (Fig. 3A; Table 1). We therefore propose that Dam1(S143D) is not a true phosphorylation mimic, and *dam1(S143D)* presents an intermediate phenotype between *dam1(S143A)* and wild type. Similar results were seen in *dam1(S143E)* cells (data not shown).

Table 1. Genetic interactions with *dam1* mutants

Strain	Wild type	*Δdis2*	*Δklp5*
Wild type	++++	+++	++++
Δdam1	+++	−	−
dam1(1-127)	++++	−	++
dam1(T136A)	++++	+++	ND
dam1(T139A)	++++	+++	ND
dam1(S143A)	++++	−	++
dam1(S143D)	++++	+++	++
Δmad3 Δdam1	+++	−	ND
Δmad3 dam1(1-127)	++++	−	ND
Δmad3 dam1(S143A)	++++	−	ND
plo1-ts35 (23℃)	+++	−	ND

Viability of double mutants assessed as: ++++, wild type growth; +++, mild growth defect; ++, severe growth defect; +, forms inviable micro-colonies; − cells die in first cell cycle; ND, not determined.

The equivalent residue in *S. cerevisiae* Dam1 (S221) is phosphorylated by Mps1 in vitro (Buttrick and Millar, 2011; Shimogawa et al., 2006), although this has yet to be confirmed in vivo. Because Plk1 and Mps1 share a similar consensus motif in vitro (Dou et al., 2011), and Mph1 does not apparently phosphorylate Dam1 in *S. pombe* (Fig. 2A), it is conceivable that Dam1(S221) is phosphorylated by Cdc5 (Polo) in budding yeast. Dam1(S221) phosphorylation in *S. cerevisiae* is proposed to promote kinetochore association with the microtubule plus end (Shimogawa et al., 2006); however, we found no evidence of Dam1(S143) phosphorylation being required for kinetochore positioning in *S. pombe* (supplementary material Fig. S6). Interestingly, bacterially expressed *S. cerevisiae* DASH preferentially associates with GTP-tubulin in the absence of regulation by phosphorylation (Westermann et al., 2005).

The mode of Dam1 regulation could reflect the number of microtubule–kinetochore binding sites in each organism. *S. cerevisiae* only has a single microtubule-binding site per kinetochore. Incorrect microtubule attachments are corrected by releasing microubules from the kinetochore through Ipl1 phosphorylation of kinetochore components, including Dam1 (Cheeseman et al., 2002). *S. pombe* kinetochores, like those of higher eukaryotes, attach multiple (between two and four) microtubules (Ding et al., 1993). Individual kinetochores might therefore simultaneously make correct and incorrect microtubule attachments. We propose that Dam1 phosphorylation by Plo1 during prometaphase and metaphase creates a permissive state that allows the correction of individual microtubule mal-attachments without creating monotelically attached kinetochores. We suggest that dephosphorylation of Dam1 during anaphase enhances co-operative binding of the DASH and Ndc80 complexes to microtubules to disfavour turnover of microtubule–kinetochore attachments.

Materials and Methods

Cell culture

Media, growth and maintenance of strains were as described previously (Moreno et al., 1991). Strains used are listed in supplementary material Table S2. Experiments were performed at 30℃ unless otherwise stated. G2-synchronised *his7:gfp-lacI lys1:lacO* cells were isolated from a 10–40% lactose gradient and anaphase cells wes enriched by incubating in YES medium for 90 minutes.

Strain construction

dam1 and *ask1* alleles were generated by overlap extension PCR (Ho et al., 1989) as described previously (supplementary material Fig. S7). C-terminal tagging of *dam1* was performed by PCR-based gene targeting (Bahler et al., 1998). Oligonucleotides used are given in supplementary material Table S3.

Western blotting

Proteins from cell lysates were precipitated in 20% trichloroacetic acid and solubilised in SDS sample buffer. For phosphatase experiments, cells were lysed in 6 M guanidine hydrochloride, pH 8. His_6-tagged proteins were isolated on Probond resin (Invitrogen) and washed with λ phosphatase buffer before incubation with λ phosphatase (NEB) in the presence or absence of 50 mM sodium fluoride and 10 mM sodium orthovanadate. His_6–Dam1 was purified from *E. coli* BL21-CodonPlus(DE3)-RIL cells transformed with pTRCHisA-dam1 under hybrid conditions using Probond resin according to the manufacturer's instructions. His_6–Plo1 was purified as previously described (Papadopoulou et al., 2008). Concentration of purified proteins was estimated by using the Bradford assay. Proteins were detected on western blots using anti-Myc (9E10-Cambridge Biosciences), anti-His_6 (4D11-Abcam), anti-TAP (Thermo Scientific) and anti-Tat1 (a gift from Keith Gull) antibodies. Antibodies against Dam1 phosphorylated at S143 were raised and purified by Eurogentec (see supplementary material Fig. S4). Purification of DASH using Ask1–TAP was performed essentially as described by (Gould et al., 2004). DASH was freed from washed calmodulin beads by boiling in SDS sample buffer.

Fluorescence microscopy

Cells were analysed by fluorescence microscopy as described previously (Buttrick et al., 2011).

Acknowledgements

We thank K. Gull, I. Hagan, K. Harwick, S. Hauf, X. He, C. McInerny, H. Ohkura, S. Sazer, Y. Watanabe and M. Yanagida for sharing strains and reagents. This work was supported by a UK Medical Research Council programme grant [grant number G0601118 to J.B.A.M.]. Deposited in PMC for release after 6 months.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.096826/-/DC1

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