

Mars promotes dTACC dephosphorylation on mitotic spindles to ensure spindle stability

Shengjiang Tan,^{1,2} Ekaterina Lyulcheva,^{1,2} Jon Dean,¹ and Daimark Bennett^{1,2}

¹Department of Zoology, Oxford University, Oxford OX1 3PS, England, UK

²School of Biological Sciences, Biosciences Building, University of Liverpool, Liverpool L69 7ZB, England, UK

Microtubule-associated proteins (MAPs) ensure the fidelity of chromosome segregation by controlling microtubule (MT) dynamics and mitotic spindle stability. However, many aspects of MAP function and regulation are poorly understood in a developmental context. We show that *mars*, which encodes a *Drosophila melanogaster* member of the hepatoma up-regulated protein family of MAPs, is essential for MT stabilization during early embryogenesis. As well as associating with spindle MTs in vivo, Mars binds directly to protein phos-

phatase 1 (PP1) and coimmunoprecipitates from embryo extracts with minispindles and *Drosophila* transforming acidic coiled-coil (dTACC), two MAPs that function as spindle assembly factors. Disruption of binding to PP1 or loss of *mars* function results in elevated levels of phosphorylated dTACC on spindles. A nonphosphorylatable form of dTACC is capable of rescuing the lethality of *mars* mutants. We propose that Mars mediates spatially controlled dephosphorylation of dTACC, which is critical for spindle stabilization.

Introduction

Microtubule-associated proteins (MAPs) ensure the fidelity of chromosome segregation during cell division by controlling the formation and stability of spindle microtubules (MTs). Because disruption of spindle formation can promote genomic instability, an understanding of MAP function and regulation is central to dissecting basic mechanisms of tumorigenesis and would be invaluable in designing new therapies for the treatment of cancer. Although much progress has been made in understanding the functions of spindle-associated MAPs in the last few years, many aspects of their role or regulation remain to be fully elucidated. Human hepatoma up-regulated protein (HURP) has been described as a highly charged MAP that can bind directly to MTs in vitro and enhance their polymerization (Santarella et al., 2007). In vivo, HURP is part of a Ran-dependent complex that stabilizes mitotic MTs and is required for the formation and function of bipolar mitotic spindles (Koffa et al., 2006; Sillje et al., 2006; Wong and Fang, 2006). However, it is not known how HURP-associated proteins functionally interact with one another in a developmental context to support normal cellular function.

Mars, a *D. melanogaster* sequence homologue of HURP, was previously identified as a protein phosphatase 1 (PP1) binding protein, implicating reversible phosphorylation in the control of Mars or Mars-associated proteins (Bennett and Alpey, 2004; Yang et al., 2005). In this paper, we report the essential role of *mars* during early embryogenesis, its interactions with other MAPs, and its key role in promoting protein dephosphorylation on mitotic spindles to ensure spindle stability.

Results and discussion

HURP is a component of the mitotic spindle apparatus. To determine the cell cycle distribution of Mars, we generated a Mars-specific antibody and used it to stain syncytial embryos undergoing nuclear division. In prophase, Mars antibody staining was predominantly around the centrosome. In metaphase and anaphase, Mars was localized on spindle MTs in a gradient along the pole-to-pole axis with more intense staining at the centrosome-proximal regions (Fig. 1 A). This is distinct from the distribution of HURP, which has been shown to be predominantly at chromatin-proximal regions until telophase, when levels sharply decline (Koffa et al., 2006; Sillje et al., 2006; Wong and Fang, 2006). In telophase, we observed a low level of discrete Mars staining at the midbody (not depicted), but the majority of Mars protein appeared to be spread over the nuclear envelope where it persisted during interphase (Fig. 1 A and Fig. S1, available at

Correspondence to Daimark Bennett: daimark.bennett@liverpool.ac.uk

Abbreviations used in this paper: dTACC, *Drosophila* transforming acidic coiled coil; FM, FLAG-Myc; HURP, hepatoma up-regulated protein; MAP, microtubule-associated protein; Msps, minispindles; MT, microtubule; p-dTACC, phosphorylated dTACC; PP1, protein phosphatase 1.

The online version of this paper contains supplemental material.

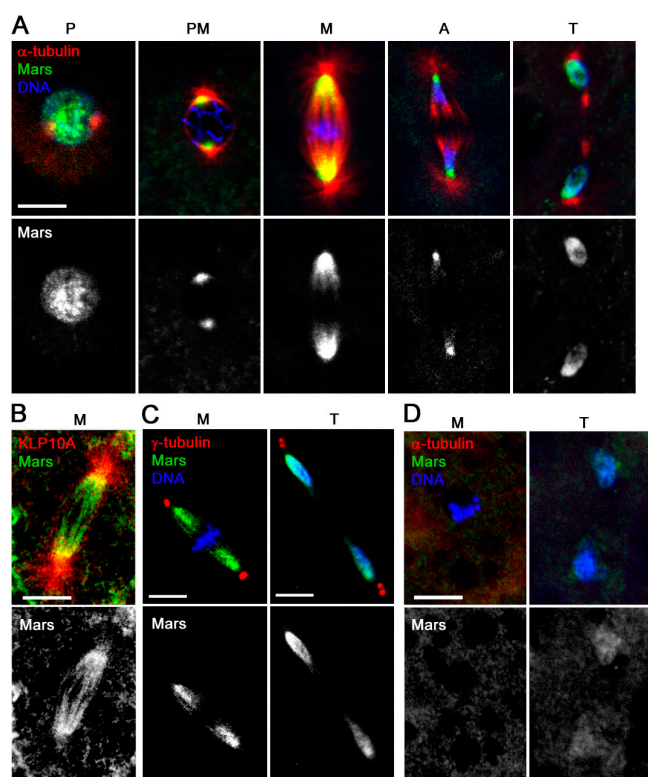


Figure 1. Mars localizes to spindle MTs. (A) Fixed wild-type embryos stained to reveal the distribution of Mars (green), α -tubulin (red), and DNA (blue) during mitosis. (B and C) Mars is concentrated at MT minus ends. Fixed wild-type embryos stained to reveal distribution of Mars (green) and either KLP10A (red; B) or γ -tubulin (red) and DNA (blue; C). (D) An embryo treated with 500 μ g/ml colchicine before fixation to depolymerize MTs. Under these conditions, Mars staining (green) disappears from the spindle during metaphase but can be seen on the nuclear envelope at telophase. P, prophase; PM, prometaphase; M, metaphase; A, anaphase; T, telophase. Bars, 10 μ m.

<http://www.jcb.org/cgi/content/full/jcb.200712080/DC1>). Double staining for Mars and either Klp10A, which is primarily localized at focused minus ends where it promotes depolymerization and poleward flux (Rogers et al., 2004), or γ -tubulin, which marks the face of the centrosome and nucleates MT polymerization (Jeng and Stearns, 1999), confirmed that Mars is localized at MT minus ends but not at the centrosome (Fig. 1, B and C). Mars' localization during mitosis was completely disrupted upon treatment with colchicine to depolymerize MTs, indicating that Mars associates with spindle MTs (Fig. 1 D).

To determine the *in vivo* role of *mars*, we generated a null allele of *mars*, *mars*^l, by imprecise excision of a *P* element transposon (referred to as *mars*^P hereafter), which we found inserted in the *mars* 5' untranslated region (Fig. 2, A and C). *mars*^P flies express full-length Mars protein at a much lower level than wild type (Fig. 2 B). *mars*^l flies fail to produce Mars protein, which is consistent with molecular analysis revealing that ~0.84 kb of the coding region, including the translation start site, is deleted in this mutant (Fig. 2, B and C). *mars* mutant flies are viable but female sterile. Notably, eggs laid by *mars*^l, *mars*^P, and *mars*^{l/P} flies show a greatly reduced ability to hatch (Fig. 3 A). As we were able to visualize sperm tails in early-arrested embryos laid by *mars* mutant females (unpublished data), we conclude that the sterility

observed in *mars* mutants is caused by arrest in embryogenesis after fertilization. The viability of embryos laid by *mars* mutant mothers was restored by moderate overexpression of *mars*^{WT} (wild-type *mars*) in the female germline (Fig. 3 A), indicating that the failure of *mars* mutant embryos to develop is caused by disruption of the *mars* transcription unit.

To determine the cause of the lethality of *mars* mutant embryos, we fixed embryos from wild-type and *mars* mutant females and examined the distribution of nuclei and MTs. Embryos lacking maternal *mars* arrested during early embryogenesis after no more than five nuclear divisions. 81.9% ($n = 144$) of 15–45-min embryos laid by *mars*^l mothers exhibited at least two discrete DNA-containing regions (Fig. 3, B and C). The first of these was localized to the embryonic cortex and resembled a polar body, most likely containing the unused products of meiosis II (Wilson and Borisy, 1998). One or more additional DNA-containing regions, each surrounded by a bipolar spindle, were also observed more centrally, indicating that the vast majority of mutant embryos pass through meiosis to form one or more mitotic figure. Notably, the spindle structures in *mars*^l mutant embryos were very small and weak, albeit still bipolar (Fig. 3 C). Most spindles had at least one detached centrosome, possibly because of weakened spindle–centrosome interactions (Fig. 3, C–E). We also observed unaligned chromosomes in the *mars*^l mutant, which is indicative of insufficient MT attachment or tension (Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200712080/DC1>). Embryos laid by *mars*^{l/P} mothers resembled those from *mars*^l mothers, except the phenotypes were somewhat less severe (Fig. 3, C–E). Quantitation of α -tubulin staining revealed no significant difference between astral MTs in embryos laid by wild-type and *mars*^{l/P} mothers ($P = 0.416$), whereas cold-resistant kinetochore MTs were destabilized in embryos laid by *mars*^{l/P} mothers (Fig. S2 B). Approximately 51% of embryos laid by *mars*^P homozygous mothers ($n = 264$) failed to develop beyond embryogenesis. Many showed terminal phenotypes at or shortly after gastrulation, presumably as a consequence of primary defects during the early cleavage divisions. Collectively, analysis of loss-of-function mutants suggest that *mars* has a role in spindle MT stabilization. HURP has bundling activity *in vitro* and *in vivo* (Koffa et al., 2006; Sillje et al., 2006; Wong and Fang, 2006). Correspondingly, strong overexpression of *mars*^{WT} in syncytial embryos resulted in enlarged spindles with ectopic MT fibers (Fig. 3 F), suggesting that *mars* is limiting for MT bundling.

mars was originally isolated from a two-hybrid screen for putative PP1 binding proteins and contains a canonical PP1 binding motif (K/R,x,V,x,F; Bennett and Alpey, 2004; Bennett et al., 2006). To verify interactions with PP1, we performed pulldown experiments between Mars and PP1 α 87B, the major *D. melanogaster* PP1 isoform (Dombrádi et al., 1990; Baksa et al., 1993; Kirchner et al., 2007). We found that GST-tagged full-length Mars^{WT} interacted efficiently with HA-tagged PP1 α 87B from crude *D. melanogaster* embryo extracts (Fig. 4 A), whereas cells expressing GST alone did not bind (not depicted). We also observed efficient binding between endogenous PP1 and FLAG-Myc (FM)-tagged Mars^{WT} by coimmunoprecipitation from *D. melanogaster* embryo extracts (Fig. 4 B). To assess the

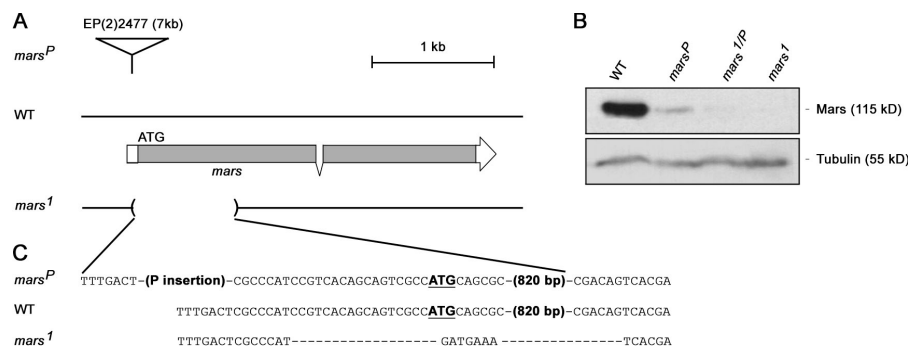


Figure 2. Molecular defects in *mars* mutants.

(A) *mars* genomic region in *mars^P*, wild type, and *mars¹*. The coding region of *mars* is represented by shading. *mars^P* contains a P element insertion, EP(2)2477, in the *mars* untranslated region. *mars¹* has a 0.84-kb deletion that removes the *mars* initiation codon and part of the coding region. (B) Mars protein produced in embryos from wild-type, *mars^P*, *mars^{1/P}*, and *mars¹* flies. Total protein was analyzed by immunoblotting using Mars antibody. *mars^P* produced full-length Mars protein in a much lower amount, whereas *mars¹* failed to produce any detectable protein. Blotting with α -tubulin antibodies controlled for loading. (C) Genomic sequence of wild-type and *mars* mutant strains. The initiation codon (ATG) of *mars* is bold and underlined.

importance of the putative PP1 binding motif for interaction with PP1, we tested a mutant form of Mars in which phenylalanine 839 was replaced with alanine (Mars^{F839A}) in our pulldown assay. The ability of Mars^{F839A} to bind PP1 was greatly reduced compared with wild-type Mars (Fig. 4 A), indicating that Phe839 is crucial for interaction with PP1. Immunoprecipitation with an HURP antibody followed by immunoblotting with PP1 antibody showed that endogenous human PP1 and HURP

also interacted efficiently with each other in HeLa cell extracts (Fig. 4 C), suggesting that binding to PP1 is an evolutionarily conserved property of HURP proteins.

Binding to PP1 prompted us to test functional interactions between *mars* and *PP1* in vivo. The ability of embryos laid by *mars* or *PP1a87B* heterozygotes to hatch resembled that of the wild type (Fig. 4 D). Embryos laid by flies transheterozygous for *mars* and *PP1a87B* showed a significantly reduced hatch

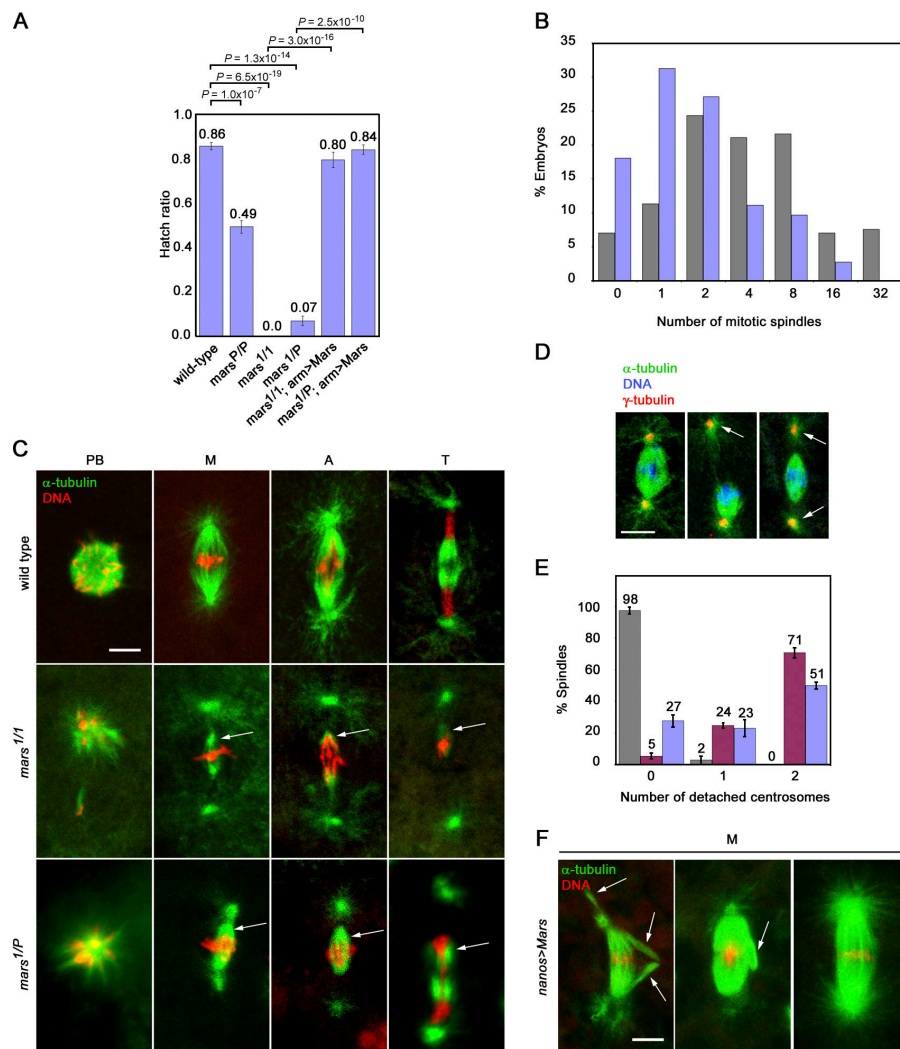
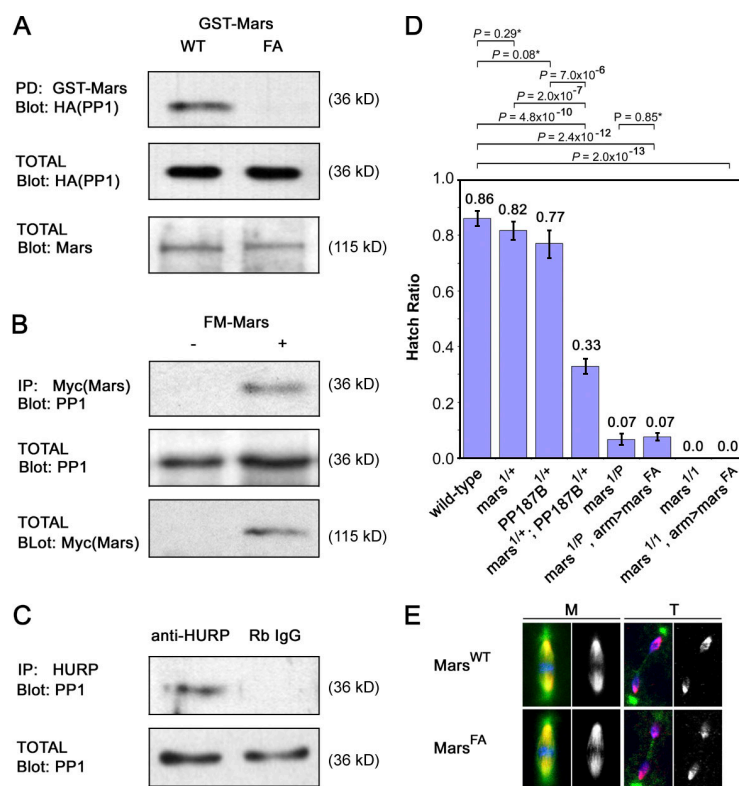


Figure 3. *mars* is essential for spindle stability.

(A) Hatch ratios, plotted as the mean \pm standard error of five experiments, showing greatly reduced ability of embryos laid by *mars* mutant females to hatch compared with wild type. Ectopic expression of *UASP-FM-mars^{WT}* under control of the *arm-GAL4* driver (*arm>Mars*) rescues this phenotype. P-values determined from student's *t* tests are shown above the graph. (B) Graph showing significant reduction in mean number of mitotic spindles in 15–45-min embryos laid by *mars¹* mutant mothers (blue; *n* = 144 embryos) compared with wild-type mothers (gray; *n* = 185 embryos; *P* = 2.9×10^{-10}). 81.9% of *mars¹* mutant embryos progress through meiosis to form one or more mitotic figure. (C) Images of mitotic spindles from embryos laid by wild-type, *mars¹*, and *mars^{1/P}* mothers showing small spindles in *mars* mutants (arrows) and loss of centrosome attachment to the spindle body. Loss of chromosome attachment to monopolar MTs is apparent in polar bodies. PB, polar body; M, metaphase; A, anaphase; T, telophase. (D) Images of spindles from mutant embryos, showing detached centrosomes (arrows). (E) Bar graph showing percentage of spindles with detached centrosomes from wild type (gray), *mars¹* (red), and *mars^{1/P}* (blue), plotted as the mean \pm standard error of three experiments. (F) Representative images showing effects of strong ectopic expression of *mars^{WT}* with *nanos-GAL4^{VP16}*. Compared with the wild type (C, top), these embryos display abnormally robust spindles, which often have aberrantly aligned MT fibers at metaphase (arrow). Bars, 10 μ m.

Figure 4. Binding to PP1 is essential for Mars function. (A) Mars binds to PP1 α 87B in a GST pull-down (PD) assay. Binding requires Phe839 of Mars. *arm-GAL4 UAS-HA-PP1 α 87B* fly extracts were incubated with lysates from *Escherichia coli* expressing GST-Mars^{WT} or Mars^{F839A}. Mars complexes were purified on glutathione-Sepharose and immunoblotted with HA to test for coprecipitation of PP1 α 87B. Blots of total extracts confirmed equal levels of HA-PP1 and GST-Mars in these experiments. (B) Mars coprecipitates PP1 from *D. melanogaster* embryonic nuclear extracts. *arm-GAL4* (–) and *arm-GAL4 UAS-FM-mars* (+) embryo extracts were subjected to immunoprecipitation (IP) with Myc antibodies followed by immunoblotting with PP1 antibodies. Blots of total extracts confirmed levels of PP1 and FM-tagged Mars. (C) HURP coprecipitates PP1 from HeLa cell nuclear extracts. Immunoprecipitation of extracts with rabbit HURP antibody or with rabbit random IgG (Rb IgG) was followed by immunoblotting with PP1 antibodies to test for binding. (D) Hatch ratios, plotted as the mean \pm standard error of seven experiments. Loss of one copy of *PP1 α 87B* enhances *mars*^{1/+} to semilethality. Ectopic *mars*^{FA} fails to rescue lethality of embryos laid by *mars* mutant mothers. P-values indicated by an asterisk are not statistically significant. (E) Ectopic Mars^{WT} and Mars^{F839A} have identical distributions on mitotic spindles. Fixed embryos from *arm>mars*^{WT} or *arm>mars*^{F839A} mothers were stained to reveal the distribution of α -tubulin (green), FLAG-tagged Mars (red and greyscale), and DNA (blue) during mitosis. M, metaphase; T, telophase.



ratio (Fig. 4 D). PP1 is a pleiotropic enzyme. To test the specific role of Mars-bound PP1, we introduced FM-tagged Mars^{F839A} into flies to create Mars complexes lacking PP1. Ectopic expression of Mars^{F839A} at comparable levels to those of Mars^{WT} failed to rescue the embryonic lethality of embryos laid by *mars*¹ or *mars*^{1/P} mothers (Fig. 4 D). Collectively, these data suggest that binding to PP1 is critical for Mars function. Identical distributions of Mars^{F839A} and Mars^{WT}, as determined by FLAG antibody staining, indicate that PP1 binding is not necessary for normal Mars localization on the mitotic spindle (Fig. 4 E).

HURP has been shown to interact with other MAPs, including TPX2 and XMAP215 in *Xenopus laevis* (Koffa et al., 2006), suggesting that its role in the stabilization of spindle MTs may be partly mediated via interactions with other spindle assembly factors. In immunoprecipitation assays, we found that Mars associates with two proteins that are known to cooperate with each other to stabilize MTs during cell division (Lappin et al., 2002): the *D. melanogaster* XMAP215 homologue encoded by *minispindles* (*msps*) and *Drosophila* transforming acidic coiled-coil (dTACC) protein (Fig. 5 A). In syncytial embryos, dTACC is concentrated at centrosomes during mitosis but is also found on spindle MTs (Gergely et al., 2000) where it colocalizes with Mars (unpublished data). Mars staining was not affected in embryos that produce no detectable dTACC protein (Fig. S3 A, available at <http://www.jcb.org/cgi/content/full/jcb.200712080/DC1>; Lee et al., 2001). Conversely, the ability of dTACC to associate with MTs was largely unaffected in *mars*^{1/P} mutant embryos (Fig. 5 B). Therefore, although Mars and dTACC associate with one another, they do not appear to be dependent on each other for their localization.

Phosphorylation of dTACC on Ser863 is critical for stabilization of the minus ends of centrosome-associated MTs dur-

ing mitosis (Barros et al., 2005). Although dTACC is found on both the centrosome and mitotic spindle, phosphorylated dTACC (p-dTACC) is tightly localized to the centrosomes (Barros et al., 2005), suggesting that once phosphorylated, p-dTACC is either unable to exchange with the soluble pool of dTACC or is rapidly dephosphorylated when it leaves the centrosome. The role of dephosphorylated TACC is not known, but it may function to stabilize MTs through lateral interactions with MTs or interactions with MT plus ends. The localization of Mars toward the minus ends of spindle MTs and association with both dTACC and PP1 prompted us to examine the involvement of Mars in maintaining low levels of p-dTACC on the spindle.

To examine the effect of Mars on dTACC phosphorylation, we stained *mars* mutant embryos with an antibody that specifically recognizes dTACC phosphorylated at Ser 863 (p-dTACC). *mars* mutant embryos showed increased levels of p-dTACC on the mitotic spindles compared with the wild type (Fig. 5, B–D). On careful examination of these mutants, we noticed some spindles that looked normal but possessed elevated levels of p-dTACC (Fig. S3 B), indicating that increased p-dTACC was not simply a secondary consequence of aberrant spindle structure. Levels and distribution of total dTACC appeared normal in *mars* mutants (Fig. 5 B), although we cannot rule out that global ratios of dTACC/p-dTACC are affected. We used *mars*^{F839A} to examine whether Mars promotes the dephosphorylation of dTACC by binding to PP1. Embryos with moderate levels of ectopic Mars^{WT} in embryos laid by either *mars*¹ or *mars*^{1/P} mothers were essentially wild type in appearance and had little or no p-dTACC staining on mitotic spindles. In contrast, *mars* mutant embryos ectopically expressing Mars^{F839A} at comparable levels to those of ectopic Mars^{WT} retained elevated p-dTACC staining on spindles (Fig. 5 B and not depicted).

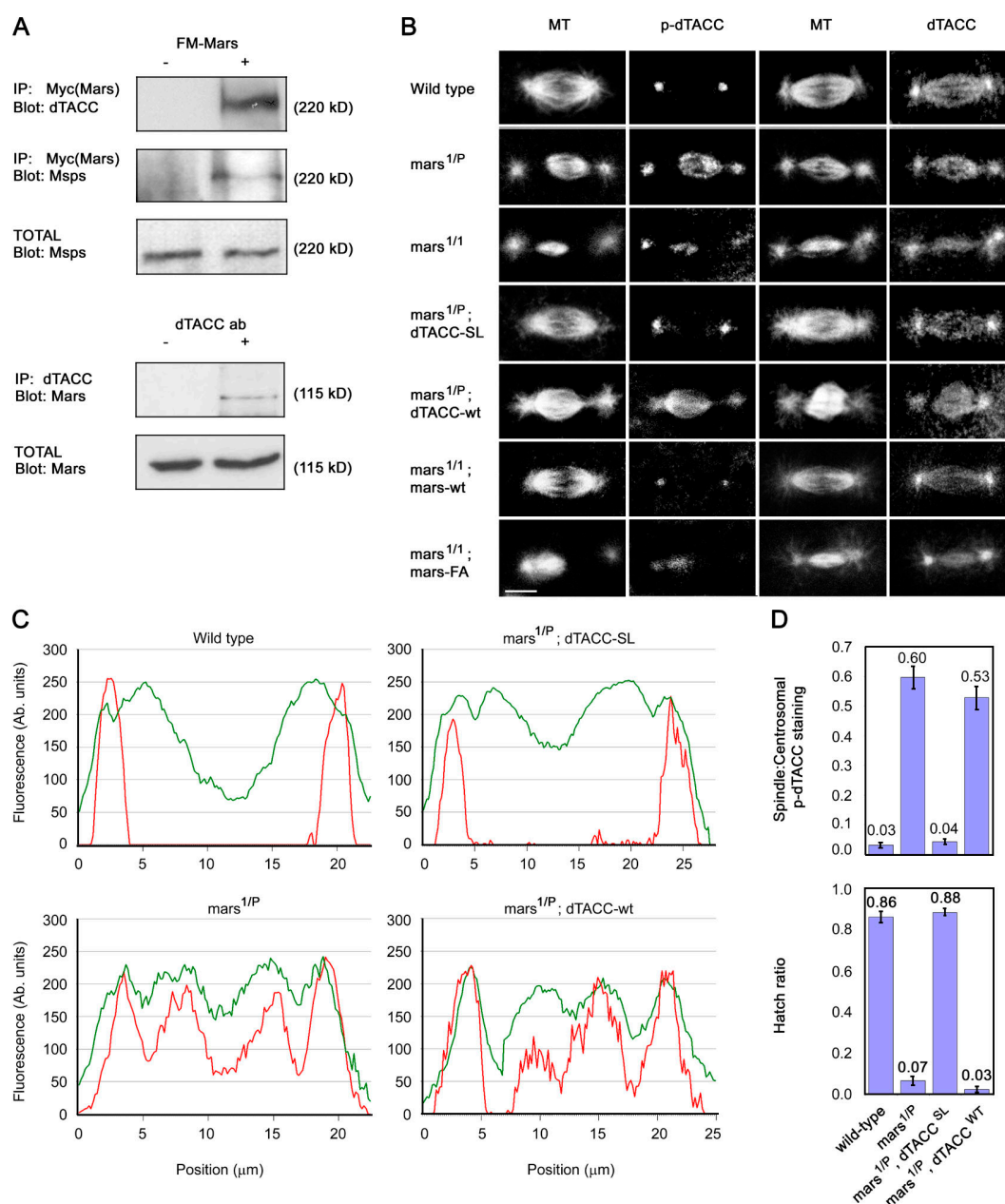


Figure 5. An essential role of *mars* is to promote dTACC dephosphorylation. (A) Mars coprecipitates dTACC and Msps from *D. melanogaster* embryonic nuclear extracts. Immunoprecipitation of *arm-GAL4* (–) and *arm>mars*^{WT} (+) embryo extracts with Myc antibody, followed by immunoblotting with dTACC and Msps antibodies, showed binding to FM-tagged Mars. Immunoprecipitation with dTACC antibody followed by immunoblotting with Mars antibody confirmed dTACC binding. (B) Distribution of dTACC and p-dTACC on mitotic spindles in embryos from mothers of different genotypes, as indicated. In wild type, p-dTACC is only found at the centrosome but in *mars* mutants, p-dTACC abnormally accumulates on mitotic spindles. Total dTACC staining is largely unaffected in *mars* mutants. A nonphosphorylatable mutant form of dTACC (*dTACC*^{SL}), but not wild-type dTACC (*dTACC*^{WT}), restores spindle structure and normal distribution of p-dTACC in a *mars*^{1/P} mutant background. Similarly, *arm>mars*^{WT}, but not *arm>mars*^{FA}, restores normal spindle structure and p-dTACC staining in a *mars*¹ background. Bar, 10 μm. (C) Linescans of fluorescence intensity (arbitrary units) across spindles from embryos of different genotypes, as indicated. The distribution of p-dTACC (red trace) is shown relative to α-tubulin (green trace). (D) Top graph shows quantification of ratio of spindle/centrosomal p-dTACC staining. *dTACC*^{SL}, but not *dTACC*^{WT}, restores a normal p-dTACC ratio in embryos from *mars*^{1/P} mothers. Bottom graph shows that *dTACC*^{SL}, but not *dTACC*^{WT}, rescues lethality of embryos laid by *mars*^{1/P} mothers. Hatch ratios are plotted as the mean ± standard error from *n* = 5 experiments.

To test whether promoting the dephosphorylation of dTACC is a critical function of *mars*, we examined whether the sterility of *mars* mutants could be rescued by a nonphosphorylatable form of dTACC (*dTACC*^{S863L}) expressed under control of the *dTACC* promoter (Barros et al., 2005). *dTACC*^{S863L}, but not *dTACC*^{WT} (wild-type dTACC), restored a normal distribution of p-dTACC on mitotic spindles (Fig. 5, B–C). Quantification of p-dTACC

staining confirmed that *dTACC*^{S863L} restored a normal ratio of spindle/centrosomal p-dTACC staining in *mars* mutant embryos (Fig. 5 D). When we examined hatching of these embryos, we found that the lethality of embryos laid by *mars*^{1/P} mothers was rescued by *dTACC*^{S863L} but not *dTACC*^{WT} (Fig. 5 D). Collectively, these data indicate that dephosphorylation of dTACC on the spindle is an essential function of *mars*. Homozygous *mars*¹ mutants

were not rescued by *dTACC*^{S863L} (unpublished data), suggesting that residual Mars protein in *mars*^{1P} embryos may play a dTACC-independent role, such as MT bundling, or that the level of ectopic *dTACC*^{S863L} was insufficient to compensate for elevated p-dTACC in a *mars*¹ background.

In summary, we have shown that *mars*, which encodes a *D. melanogaster* sequence homologue of HURP, is critical for mitotic spindle structure and chromosome segregation during early embryogenesis. The primary defect in *mars* mutants appears to be loss of spindle MT stability, whereas overexpression of *mars* leads to the production of enlarged spindles with ectopic MTs. These data are consistent with a role for *mars* in MT bundling/stabilization similar to that described for its human homologue HURP. However, our identification of Mars as an interacting subunit of PP1 suggests a novel mechanism by which this family of proteins can maintain normal spindle structure in vivo. Binding of PP1 to Mars implicates PP1 in dephosphorylation of Mars or a Mars-associated protein. Although dTACC may be a substrate of PP1, it is also possible that Mars-bound PP1 may indirectly stimulate dephosphorylation of dTACC on the spindle by activating another protein phosphatase or inactivating a dTACC kinase such as Aurora-A, a known target of PP1 during mitosis (Katayama et al., 2001).

Our genetic experiments indicate that promoting dephosphorylation of dTACC on mitotic spindles is an essential role of Mars. Why is it important to maintain dephosphorylated TACC on the spindle? One possibility is that *mars* functions to ensure that MT stabilization mediated by p-dTACC only occurs at the centrosome, allowing a more dynamic spindle. This is hard to test however, because the mechanism by which p-dTACC stabilizes MTs is unclear. The effect of dTACC on MT assembly appears to be mediated by effector proteins, such as Msps, as TACC has not been described to possess MT stabilizing activity on its own (Kinoshita et al., 2005). Phosphorylation of dTACC might help activate effectors because dTACC mutated at Ser863 is able to recruit Msps to the centrosome but not promote MT assembly (Barros et al., 2005). However, it is not known which aspect of Msps activity is affected by dTACC phosphorylation or to what extent the effect of dTACC phosphorylation is context dependent. It is conceivable that dTACC stabilizes spindle MTs by establishing lateral interactions with MTs or interactions with plus ends and that these functions of dTACC are impaired when phosphorylated at Ser863.

Is *mars* a functional homologue of HURP? We have confirmed that various aspects of *mars* and HURP function are conserved, including spindle stabilization and binding of Mars to Msps and PP1. However, Mars and HURP display apparently distinct spindle localizations, suggesting that there may be differences in how these proteins are used during cell division. This may reflect a wider difference in the organization of MAPs that control MT stability and the formation of bipolar spindles in flies and humans.

Spindle defects caused by lack of TACC phosphorylation or by alterations in TACC or HURP protein levels may lead to genetic instability and are implicated in cancer progression (Raff, 2002; Barros et al., 2005; Brittle and Ohkura, 2005). Our data indicate that spatially controlled dephosphorylation also plays a positive role in TACC function, suggesting that deregulation

of either phosphorylation or dephosphorylation of TACC may also be involved in the molecular pathology of cancer by compromising the fidelity of chromosome segregation.

Materials and methods

Fly strains

EP(2)2477, referred to here as *mars*^P, is a homozygous viable *P* element insertion in the 5' untranslated region of *mars*. *GFP-dTACC*^{WT} and *GFP-dTACC*^{S863L} (gift from J. Raff, The Gurdon Institute, Cambridge, England, UK) have been previously described (Barros et al., 2005). Other fly stains are described in FlyBase (<http://www.flybase.org>).

Isolation and characterization of a *mars*-null allele

Isolation of a null allele of *mars* by *P* element excision from *mars*^P was performed as follows. Jumpstarter *y w/Y*; isogenic *mars*^P/CyO, *P(Delta2-3)* males were crossed with *y w*; *Tf/CyO* females. From each cross, only one *w* revertant male, *y w*; *mars*^{*}/CyO, in which the *P* element was excised, was individually crossed back to *y w*; *Tf/CyO* females. To determine the molecular lesion in the *mars*¹ mutant, genomic DNA surrounding the original *mars*^P insertion site was amplified from *mars*¹ homozygotes by PCR using flanking primers and sequenced.

Statistical analysis

We used unpaired two-tailed *t* tests to compare mean hatch ratios of eggs from different strains and unpaired one-tailed *t* tests to compare mean number of mitotic spindles in wild-type and *mars*¹ mutant embryos.

Site-directed mutagenesis and ectopic expression

mars^{F839A} was constructed by PCR-based site-directed mutagenesis. For ectopic expression in flies, full-length *mars*^{WT} and *mars*^{F839A} were subcloned into pPFMW (*Drosophila* Genomics Resource Center, Indiana University, USA), a modified version of pUASP (Rorth, 1998) that contains an N-terminal 3xFLAG 6xMyc (FM) tag. *UASP-FM-mars* flies were made by *P* element-mediated germline transformation into a *w*¹¹¹⁸ strain by Genetic Services, Inc. Embryos were provided with moderate levels of tagged *Mars*^{WT} and *Mars*^{F839A} by ectopic expression of *UASP-FM-mars* transgenes in the germline using *arm-GAL4* (Sanson et al., 1996) or *nanos-GAL4*^{VP16} (Van Doren et al., 1998).

Immunoprecipitation and GST-pulldown experiments

We subjected lysates from *arm-GAL4* or *arm-GAL4 UASP-FM-mars* flies to immunoprecipitation with dTACC (gift from J. Raff; Gergely et al., 2000) or Myc antibody (A14 rabbit polyclonal; Santa Cruz Biotechnology, Inc.). After adsorption on protein G bound to GammaBind Plus Sepharose (GE Healthcare), we analyzed immunoprecipitates and total cell extracts by immunoblotting with Myc (9E10 mouse monoclonal; Santa Cruz Biotechnology, Inc.), α -tubulin (DM1a; Sigma-Aldrich), Msps (gift from H. Ohkura, University of Edinburgh, Edinburgh, Scotland, UK; Cullen et al., 1999), PP1 α 87B (gift from P.T. Cohen, University of Dundee, Dundee, Scotland, UK; Helps et al., 2001), or dTACC (Gergely et al., 2000) antibodies. For GST-pulldown experiments, full-length *mars* ORF was subcloned into pDEST-15 (Invitrogen) for expression in *E. coli* in frame with an N-terminal GST tag. *mars*^{F839A} was made by PCR-based site-directed mutagenesis and subcloned into pDEST-15 in the same way. Constructs were sequenced to confirm that they contained no sequence errors. To test binding to PP1, bacterial cell lysates expressing GST-tagged *Mars*^{WT} or *Mars*^{F839A} were incubated with *arm-GAL4 UAS-HA-PP1* α 87B (Vereshchagina et al., 2004) *D. melanogaster* embryo extracts, and GST-labeled protein was precipitated with GST Bind Resin (EMD). Precipitates were examined by immunoblotting with HA antibody (12CA5; Roche). To test binding between HURP and PP1, HeLa cell nuclear extracts were subjected to immunoprecipitation with HURP antibody (gift from I.W. Mattaj, European Molecular Biology Laboratory, Heidelberg, Germany; Koffa et al., 2006). Precipitates were analyzed by immunoblotting with PP1 antibody (Helps et al., 2001).

Mars antibodies and immunofluorescence

Anti-peptide antibodies against Mars were raised by Eurogentec in rabbits by simultaneous immunization with two peptides: LVPEGTKTPPPRESN (residues 512–526) and TLRNRRVNLRPSEFM (residues 906–921). Embryos were fixed with either methanol or formaldehyde and were processed for immunofluorescence as described previously (Huang and Raff, 1999). Colchicine treatment of embryos before fixation was as previously described (Gergely et al., 2000). Antibodies used for indirect immunofluorescence

were as follows: FLAG (rabbit polyclonal; Sigma-Aldrich), KLP10A (gift from D. Sharp, Albert Einstein College of Medicine, Bronx, NY; Rogers et al., 2004), dTACC (Gergely et al., 2000), p-dTACC (gift from J. Raff; Barros et al., 2005), α -tubulin (DM1a; Sigma-Aldrich) and γ -tubulin (rabbit polyclonal or GTU88 monoclonal; Sigma-Aldrich). Secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen), Cy3, or Cy5 (Jackson ImmunoResearch Laboratories) were used at 1:500–1,000 dilutions. DNA was counterstained with 1 μ g/ml propidium iodide (Sigma-Aldrich).

Image acquisition and processing

Fixed embryos, mounted in 85% glycerol and 2.5% *n*-propylgallate in PBS, were examined using a microscope (Eclipse E800; Nikon) with either a 40 \times 1.3 NA Plan Fluor or a 60 \times 1.4 NA Plan Apo objective and a scanning confocal system (Radiance Plus; Bio-Rad Laboratories) equipped with LaserSharp 2000 software (Bio-Rad Laboratories). Images were imported to Photoshop (Adobe) and adjusted for brightness and contrast uniformly across entire fields. Quantitation of astral MTs was performed by making maximum intensity projections of 8–12 image stacks that were taken at 0.5- μ m intervals from 15–90-min methanol-fixed embryos stained with α -tubulin, γ -tubulin, and DNA. The projections were imported into AQM Advance 6 software (Kinetic Imaging Ltd.), and mean α -tubulin intensities were measured within a circle around each centrosome from spindles in metaphase. The mean pixel intensity of astral MTs from at least 25 embryos of each genotype was calculated. All measurements were corrected for variation in staining conditions by subtracting the background intensity. A similar approach was taken to quantify p-dTACC staining, except that measurements were also taken at four points on the spindle, both proximal and distal to the centrosome. Linescans were generated using LSM510 software (Carl Zeiss, Inc.). The mean intensity through the center of the unprocessed spindle images, parallel to the long axis of the structure, was calculated for each channel of fluorescence by the software.

Online supplemental material

Fig. S1 shows individual channel images of Mars distribution during mitosis. Fig. S2 shows both misaligned chromosomes on mitotic spindles in embryos laid by *mars* mutant mothers and reduction of cold-resistant kinetochore MTs in embryos laid by *mars*^{1/P} mothers. Fig. S3 shows both the spindle localization of Mars in dTACC mutant embryos and elevated p-dTACC spindle staining on a normal looking spindle from an embryo laid by *mars*^{1/P}. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200712080/DC1>.

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