Dynein and Mast/Orbit/CLASP have antagonistic roles in regulating kinetochore-microtubule plus-end dynamics

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Summary

Establishment and maintenance of the mitotic spindle requires the balanced activity of microtubule-associated proteins and motors. In this study we have addressed how the microtubule plus-end tracking protein Mast/Orbit/CLASP and cytoplasmic dynein regulate this process in *Drosophila melanogaster* embryos and S2 cells. We show that Mast accumulates at kinetochores early in mitosis, which is followed by a poleward streaming upon microtubule attachment. This leads to a reduction of Mast levels at kinetochores during metaphase and anaphase that depends largely on the microtubule minus end-directed motor cytoplasmic dynein. Surprisingly, we also found that co-depletion of Dynein rescues spindle bipolarity in Mast-depleted cells, while restoring normal microtubule poleward flux. Our results suggest that Mast and Dynein have antagonistic roles in the local regulation of microtubule plus-end dynamics at kinetochores, which are important for the maintenance of spindle bipolarity and normal spindle length.

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Introduction

Mitotic spindles are fusiform structures whose principal function is to carry out proper segregation of chromosomes during mitosis. Spindles are composed of highly dynamic microtubules that constantly exchange tubulin heterodimers with a soluble pool. This exchange results in some particular features of microtubules, such as dynamic instability, a behavior in which individual microtubule ends alternate stochastically between prolonged phases of polymerization and depolymerization (Mitchison and Kirschner, 1984) and poleward flux (Mitchison, 1989). Flux requires that microtubules polymerize at the kinetochores and depolymerize at the pole, while translocating poleward (Cassimeris, 2004). Several factors have been implicated in this complex dynamic behavior, which are required for the coordination of polymerization (Maiato et al., 2005), depolymerization (Rogers et al., 2004) and sliding of microtubules (Miyamoto et al., 2004).

Mast/Orbit/CLASP (Akhmanova et al., 2001; Inoue et al., 2000; Lemos et al., 2000) are conserved non-motor microtubule-associated proteins (MAPs) with essential roles in mitosis. They belong to a subfamily of MAPs known as plus-end tracking proteins (+TIPs) (Carvalho et al., 2003) that bind microtubules and transiently accumulate at their growing plus-ends (Akhmanova et al., 2001; Sousa et al., 2007). They also localize to kinetochores of mitotic chromosomes, centrosomes, the central spindle region and the midbody (Lemos et al., 2000; Maiato et al., 2003a; Pereira et al., 2006). Recently, it was shown that in interphase Mast has a stabilizing role on microtubules by promoting the pause state (Sousa et al., 2007). In mitosis, Mast was shown to be required for functional kinetochore-microtubule attachments, chromosome congression and maintenance of spindle bipolarity (Lemos et al., 2000; Maiato et al., 2002). Mast mutants or siRNA-treated cells form monopolar spindles because of their inability to sustain spindle bipolarity. In S2 cells, the absence of Mast results in a strong reduction in microtubule poleward flux (Buster et al., 2007; Maiato et al., 2005). Accordingly, Mast was proposed to ensure spindle bipolarity by regulating the incorporation of microtubule subunits at attached plus-ends of mature kinetochore fibers (K-fibers), counterbalancing the activity of microtubule depolymerases at the minus-ends (Maiato et al., 2005).

Interestingly, a recent study revealed that in *Schizosaccharomyces pombe* many of the phenotypes associated with interphase microtubule dynamics arising from the loss of peg1, the Mast homologue, are seen only if dynein is present (Grallert et al., 2006), suggesting a functional interplay between these two proteins. Dynein is a minus-end-directed microtubule-associated motor protein (Paschal and Vallee, 1987) that localizes to a variety of subcellular sites, including kinetochores, microtubules, cell cortex, centrosomes and microtubule plus-ends (Dujardin and Vallee, 2002; Hays et al., 1994; Pfarr et al., 1990; Sharp et al., 2000a; Siller et al., 2005; Steuer et al., 1990; Vaughan et al., 1999; Xiang et al., 2000). This widespread localization allows dynein to be involved in several distinct processes, including the organization of the minus-ends of the spindle and their tethering to the centrosomes (Compton, 1998; Maiato et al., 2005; Maiato et al., 2004). Loss of dynein causes a metaphase delay (Goshima and Vale, 2003) and slows down poleward chromosome motion in anaphase (Savoian et al., 2000; Sharp et al., 2000b), without

affecting the rate of microtubule poleward flux (Maiato et al., 2005; Yang et al., 2007). The localization of dynein at the kinetochore relies on ZW10 (Scaerou et al., 1999; Starr et al., 1998) and Spindly (Griffis et al., 2007) where it has a dual role in facilitating force generation and also in mediating the poleward transport of spindle checkpoint proteins from kinetochore, thereby promoting the inactivation of the checkpoint and mitotic exit (Howell et al., 2001; Wojcik et al., 2001). The removal of dynein from kinetochores requires at least partial microtubule-kinetochore attachment and also requires other proteins, including NudeI (Hoffman et al., 2001; King et al., 2000; Liang et al., 2007).

In this study, we investigate the role of Mast at kinetochores and explore the functional interplay with dynein in this process using *Drosophila* embryos and S2 cells. We found that Mast accumulates at kinetochores soon after NEBD where it remains at high levels until metaphase. During mid metaphase Mast starts to be removed along microtubules in a poleward streaming fashion, which is partially dependent on dynein. We then analyzed whether the presence of dynein affects spindle organization in cells where Mast is depleted. Surprisingly, we observed that after co-depletion of Mast and dynein, spindle bipolarity is maintained, suggesting that the collapse of the spindle resulting from depletion of Mast requires dynein activity. Moreover, in contrast to Mast-depleted cells where microtubule poleward flux is severely affected, we found that in mitotic cells depleted of both Mast and dynein, spindle microtubules displayed normal plus-end polymerization rates at kinetochores. Taken together, our results suggest that dynein has an important role in regulating the kinetochore levels of Mast at the metaphase-anaphase transition and that Mast and dynein have antagonistic roles in the regulation of microtubule plus-end dynamics at kinetochores.

Results

Mast shows a highly dynamic pattern of kinetochore localization during mitosis

Mast binds to centrosomes, microtubules and kinetochores during various stages of mitosis (Lemos et al., 2000). We found strong accumulation of Mast at the outer kinetochores by co-localization with the centromere marker Cid, from early prometaphase soon after NEBD, until early anaphase when it starts to accumulate also at the central spindle region (Fig. 1A). Similarly to CLASPs in HeLa cells (Maiato et al., 2003a; Pereira et al., 2006), the level of Mast at kinetochores was found to be higher at prometaphase when compared with metaphase and anaphase (Fig. 1B). Previous work with CLASP1 has determined that its kinetochore-targeting domain is localized in the C-terminus of the protein. Therefore, we used several deletion constructs of the C-terminus of Mast to determine its kinetochore-targeting motif after fusion with EGFP and transfection into S2 cells (supplementary material Fig. S1A-B). We found that the kinetochore-targeting domain of Mast is contained within the 457 C-terminal amino acids of the protein, a region that is highly conserved amongst several species (supplementary material Fig. S1C).

We then analyzed the dynamic behavior of Mast during mitosis using transgenic *Drosophila* lines in which either the full-length EGFP-Mast protein or the C-terminal fragment EGFP-Mast-Kt2 (containing the kinetochore-targeting domain, but not the microtubule-binding domain), were expressed using the UAS-GAL4 system (Brand and Perrimon, 1993). To determine whether the full-length EGFP-Mast protein was functional, it was expressed in a $mast^4$ mutant background (Lemos et al., 2000) under the control of either a specific neuroblast promoter (MZ1061) or a ubiquitous driver Act-Gal4 (supplementary material Fig. S2). We found that full-length EGFP-Mast expressed specifically in neuroblasts could fully rescue the mitotic phenotype caused by mutations in the endogenous *mast* gene. Moreover, if expressed using the actin-Gal4 driver, most mutant cells formed normal bipolar spindles and other mitotic phenotypes associated with mutation of *mast* were substantially reduced. However, this ubiquitous driver did not rescue the lethality of *mast*, probably because of inappropriate control of EGFP-Mast expression in certain vital tissues. However, at the cellular level, EGFP-Mast could replace the function of the endogenous protein, suggesting that the presence of the EGFP-tag does not significantly affect the protein function and therefore its dynamic behavior probably reflects that of the endogenous protein. We then used laser-confocal time-lapse imaging to analyze the behavior of both EGFP-Mast and the EGFP-Mast-Kt2 deletion construct during mitotic progression in syncytial embryos (Fig. 1C-E; supplementary material Movies 1 and 2, respectively). Still images from the movies show that both the full-length protein (EGFP-Mast) and the C-terminal fragment alone (EGFP-Mast-Kt2) accumulate strongly at kinetochores from prometaphase until metaphase when the signal starts to decrease (Fig. 1C). Quantification of fluorescence intensity of EGFP-Mast and EGFP-Mast-Kt2 at kinetochores from prophase to anaphase, confirmed a highly dynamic pattern of accumulation with a decrease of the kinetochore signal starting at metaphase and continuing during anaphase (Fig. 1D,E), as seen also with kymograph analyses. These results suggest that as cells undergo metaphase-to-anaphase transition, the levels of Mast at kinetochores are significantly reduced.

Mast shows poleward streaming during metaphase and anaphase

In the previous section, we showed that the accumulation of Mast at kinetochores decreases during metaphase and anaphase. To further characterize this dynamic behavior, we first expressed either the full-length EGFP-Mast or the shorter version with the kinetochore-targeting domain EGFP-Mast-Kt2 in *Drosophila* lines simultaneously expressing the centromere marker mRFP-Cid (Fig. 2A high magnification panels; supplementary material Movie 3). The results indicate that EGFP-Mast-Kt2 localizes externally to the centromere marker from NEBD until anaphase, as expected. The full-length EGFP-Mast showed an identical localization pattern (data not shown). Moreover, analysis of the time-lapse images revealed that at metaphase and anaphase, both the complete EGFP-Mast and EGFP-Mast-Kt2 (which does not contain the microtubule-binding domain) could be seen streaming poleward along spindle microtubules (Fig. 2B; supplementary material Movies 2 and 4). Poleward streaming started at metaphase and continued during anaphase and is highly reminiscent of the dynamic behavior of Rod or ZW10, two members of the RZZ complex, as they are shed from kinetochores (Williams et al., 1992; Williams et al., 2003). To determine whether the kinetochore streaming of Mast was similar to that of the RZZ complex, we co-expressed EGPF-Mast with mRFP-Rod and followed their dynamic behavior during late syncytial divisions (Fig. 2C). Analysis of these double-expressing embryos showed that Mast and Rod follow an identical kinetochore accumulation pattern early in mitosis that is followed by streaming along microtubules during metaphase and anaphase. These observations suggest that Mast streams polewards either bound to the RZZ complex or using a similar mechanism. Quantification of poleward streaming of EGFP-Mast showed that these particles move at a velocity of 14.6±3.6 μm/minute (n=76 particles from 31 spindles of seven syncytial embryos at cycles 11 or 12), a rate very similar

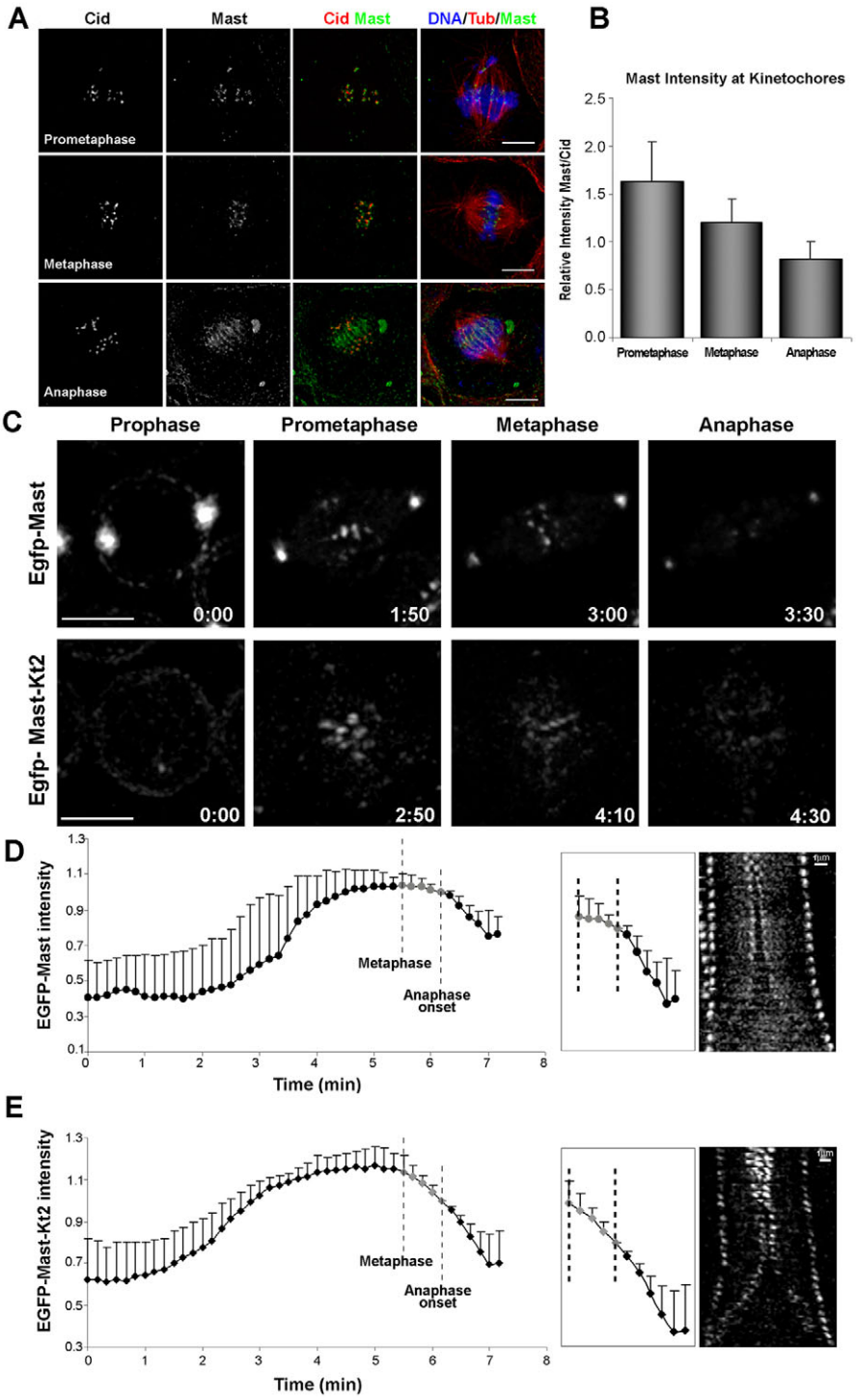

Fig. 1. Dynamic behavior of Mast during mitosis. (A) Immunostaining of Mast, Cid and α-tubulin in S2 cells during different stages of mitosis. Mast colocalizes with Cid from prometaphase until anaphase, when it is also found at the central spindle region. (B) Quantification of fluorescence intensity of kinetochore-associated Mast relative to Cid at different stages of mitosis (prometaphase, n=80; metaphase, n=79; anaphase, n=87; error bars represent s.d.; P<0.0001; n=number of kinetochores). (C) Still images from time-lapse movies of syncytial embryos expressing either EGFP-Mast or EGFP-Mast-Kt2. Time is indicated in minutes:seconds. Quantification of (D) kinetochore intensity of EGFP-Mast and (E) EGFP-Mast-Kt2 during syncytial divisions (in each case, three nuclei per embryo of ten embryos at cycle 13 were analyzed). Panels on the right show a higher magnification of EGFP-Mast and EGFP-Mast-Kt2 intensity at the metaphase-anaphase transition and the corresponding kymographs, showing the difference in kinetochore intensities as mitotic exit starts. Scale bars: 5 µm (C); 1 µm (D,E).

to the dynein-mediated transport observed for either Rod or Spindly (Basto et al., 2004; Griffis et al., 2007). These results show that during the metaphase-anaphase transition Mast and Rod have very similar dynamic behavior and therefore their poleward streaming could be due to a similar molecular mechanism.

Dynein is required to remove Mast from kinetochores

Rod is part of the RZZ complex (Rod, ZW10 and Zwilch), which is known to leave the kinetochore in a dynein-dependent manner (Wojcik et al., 2001). Since Mast shows a similar poleward streaming pattern, we sought to determine whether Mast also depends on dynein for its removal from kinetochores. To test this hypothesis, we depleted either ZW10 or dynein from S2 cells using RNAi and then analyzed the localization of endogenous Mast by immunostaining using a specific anti-Mast antibody (Fig. 3). Both ZW10 and dynein were efficiently depleted (92% and 88%, respectively) from S2 cells after 120 hours of treatment (Fig. 3A). Then we determined the level of Mast at kinetochores in metaphase of control, dynein- or ZW10-depleted cells treated with the proteasome inhibitor MG132 to prevent control cells from exiting mitosis (Fig. 3B). The level of

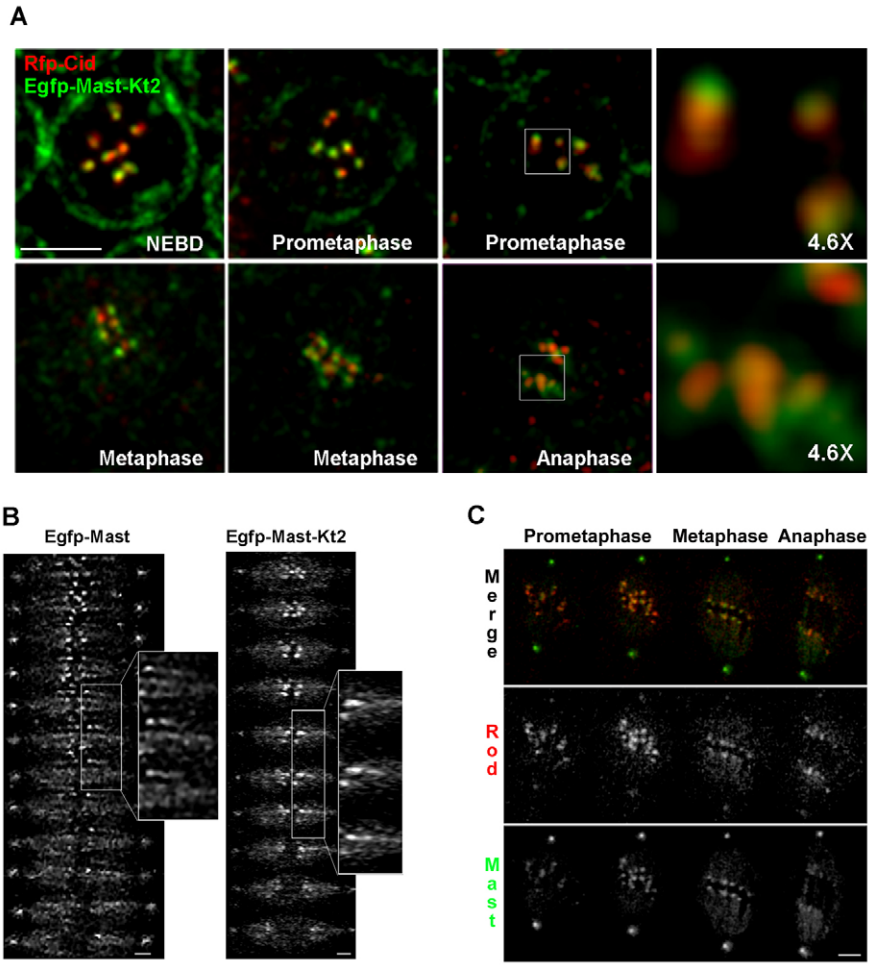

Fig. 2. Poleward streaming of Mast from kinetochores during metaphase and anaphase. (A) High magnification image of a nucleus from a syncytial embryo expressing EGFP-Mast-Kt2 and mRFP-Cid to label centromeres. These two proteins partially colocalize from NEBD to early anaphase. Mast localizes outside the centromere marker Cid. High magnification images are shown on the right. Scale bar: 5 µm. (B) Kymographs from time-lapse movies of embryos expressing either EGFP-Mast showing or EGFP-Mast-Kt2. High magnification images show details of the poleward streaming of EGFP-tagged particles during metaphase and anaphase. Scale bars: 2 µm. (C) High magnification images of nuclei from a syncytial embryo expressing EGFP-Mast and mRFP-Rod showing that these two proteins mostly colocalize and have the same poleward streaming pattern along microtubules during metaphase and anaphase. Scale bar: 2 µm.

Mast at kinetochores was quantified by measuring the fluorescence intensity ratio of Mast relative to the constitutive centromere marker Cid (Fig. 3C). The results indicate that in the absence of either dynein or ZW10, kinetochores show significant differences on Mast intensity ($P<0.001$) exhibiting higher peak levels when compared with control cells, suggesting that removal of Mast from kinetochores depends largely on dynein activity. In agreement, a recent study in mammalian cells showed significant kinetochore retention of CLASP1 after expression of the dynein tail that displaces the motor-containing dynein heavy chain from kinetochores (Varma et al., 2008). Given that dynein requires microtubules for the removal of kinetochore-associated proteins (Holzbaur and Vallee, 1994; Howell et al., 2001), the level of Mast at kinetochores in cells lacking microtubules should be independent of dynein. To test this, control cells and cells depleted of either ZW10 or dynein were incubated with colchicine to remove microtubules and the fluorescent intensity ratio between Mast and Cid was determined (Fig. 3D). The results show that indeed there are no significant differences in the peak intensities between Mast levels at kinetochores of cells lacking either ZW10 or dynein and control cells, indicating that in the absence of microtubules, accumulation of Mast at kinetochores is independent of these two proteins. Thus, as for other known dynein cargo proteins, Mast removal from kinetochores appears to require microtubules. Taken together, these results indicate that removal of Mast from kinetochores depends upon microtubule attachment and dynein motor activity.

Organization of the metaphase spindle in the absence of Mast and dynein

The collapse of the spindle after depletion of Mast is due to the inability to incorporate subunits at the microtubule plus-ends and the constant removal at the minus-end by kinesin 13 depolymerases (Rogers et al., 2004; Maiato et al., 2005; Buster et al., 2007). In the previous section, we showed that dynein is required to regulate Mast levels at kinetochores during the metaphase-anaphase transition. These observations raise the possibility that dynein might affect Mast activity at kinetochores. To test this, we performed depletion of Mast alone or together with dynein or ZW10 (supplementary material Fig. S3) and analyzed the effect upon spindle organization (Fig. 4). Analysis of spindle morphology in the different RNAi treatments revealed three major phenotypes, which we classified as monopolar spindles (with microtubules emanating from a single MTOC), bipolar spindles (with a typical fusiform shape) or multipolar spindles (with more than two MTOCs) (Fig. 4A). Quantification of these phenotypes showed that the most frequent spindle shape in control and in dynein-depleted cells was bipolar (93±4.5% and 95.7±1.1%, respectively) whereas depletion of Mast alone caused mostly monopolar spindles (62.3±24.5%) (Fig. 4B). However, after simultaneous depletion of Mast with either dynein or ZW10, we observed a significant rescue of spindle bipolarity from 31.3±22.6% in Mast alone to 77.2±8.8% and 75.1±11% in cells depleted of Mast and dynein or Mast and ZW10, respectively (Fig. 4B). More significantly, the frequency of

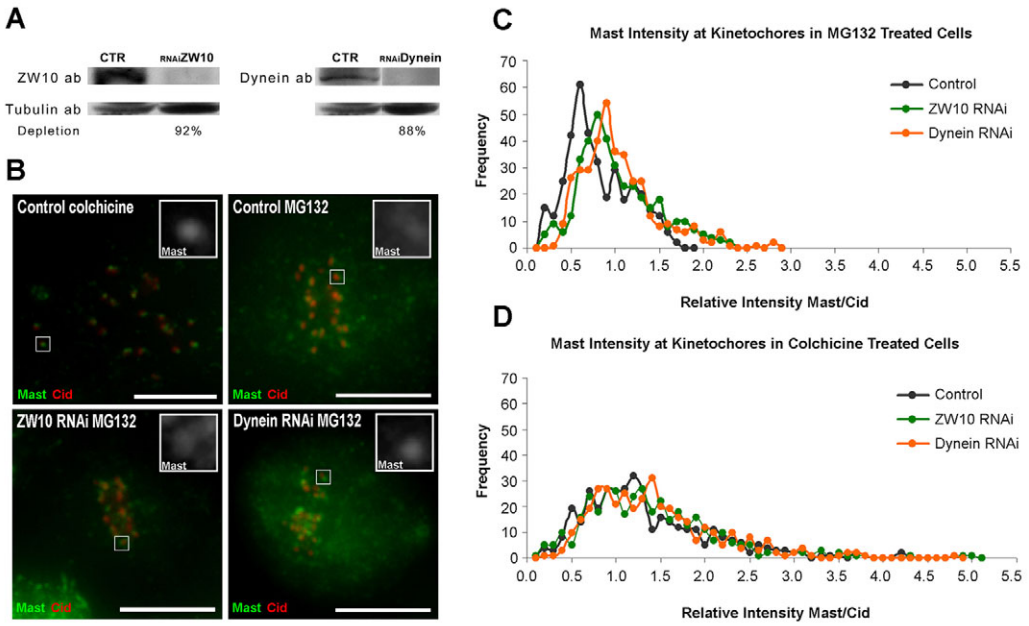

Fig. 3. Role of dynein in removal of Mast from kinetochores. (A) Western blot of total protein extracts from control and from cells treated with ZW10 or dynein siRNAs. After 120 hours of treatment, the depletion levels were 92% and 88%, upon knockdown of ZW10 and dynein, respectively. Tubulin was used as a loading control. (B) Immunofluorescence of S2 cells labelled for Mast (green) and for the centromere marker Cid (red) showing higher levels of Mast accumulation at kinetochores in cells treated with ZW10 and dynein siRNAs and also stronger accumulation in control cells treated with colchicine to remove microtubules (see insets for higher magnification images). (C) Quantification of Mast intensity at kinetochores of S2 cells arrested in metaphase with MG132 (D) or in prometaphase with colchicine. Relative Mast:Cid intensity was measure in kinetochores of control cells (black) and in ZW10 (green) or dynein (orange) siRNA-treated cells (n=375 or n=388 kinetochores in each case, for cells treated with MG132 or colchicine, respectively).

monopolar spindles, the most common phenotype observed after depletion of Mast, was significantly reduced (62.3±24.5%) when compared with cells in which dynein or ZW10 are also depleted (19.3±8.8% or 9.4±7.8%) (Fig. 4B). Given that in the absence of ZW10 or dynein there is abnormal accumulation of Mast at kinetochores (see above), we then analyzed the bipolar spindles of cells depleted of Mast and dynein in more detail, by comparing the length of the metaphase spindle with that of control cells and cells depleted of either dynein or ZW10 alone (Fig. 4C). Since centrosomes are usually detached from the spindle in dynein-depleted cells (Robinson et al., 1999), the length of all the spindles was determined by measuring the distance between the opposite kinetochore microtubule minus-ends. The results indicated that after depletion of ZW10 or dynein, cells consistently exhibited metaphase spindles (10 μm or 11 μm, respectively) that were significantly longer than in control cells (8 μm, P<0.001) or in cells depleted of both Mast and dynein, which exhibited significantly shorter spindles (6 μm, P<0.001).

Spindle bipolarity can be rescued either by causing the reformation of the bipolar spindle after it has collapsed, or it can be a true rescue in which the spindle never collapses and is therefore capable of maintaining bipolarity. To determine how spindle bipolarity is rescued in double-depleted cells, we depleted both Mast and dynein from cells expressing GFP-tubulin and analyzed the organization of the spindles over long periods of time (Fig. 4D). The results indicate that these spindles first reached a normal spindle length (8 μm), which then over time reduced to almost 6 μm. After the spindle had reduced in size, it remained at that constant length for a long period and some of these cells were able to exit mitosis into anaphase where the spindle elongated and only rarely collapsed (Fig. 4D and data not shown). Taken together, these observations indicate that in the absence of dynein, Mast-depleted cells can form bipolar spindles, and although they have a reduced length, they are able to maintain bipolarity over long periods and do not collapse. Moreover, these results suggest first, that maintenance of spindle bipolarity relies on antagonistic activities between Mast and dynein, and second, that inappropriate accumulation of Mast at kinetochores might be responsible for the elongated metaphase spindles observed when ZW10 or dynein are absent.

Depletion of dynein or Mast and dynein simultaneously does not affect functional microtubule-kinetochore attachment

Mast is involved in microtubule plus-end polymerization and therefore, in its absence, the spindle is thought to collapse because of continued depolymerization at the minus-end while the kinetochore remains firmly attached to microtubule bundles. However, the absence of dynein is thought to compromise microtubule-kinetochore interactions, resulting in a decrease in the inter-kinetochore distance and a consequent delay in exiting mitosis because of loss of tension across sister kinetochores (Varma et al., 2008; Yang et al., 2007). Thus, we analyzed the stability of microtubule-kinetochore attachment and its effects upon interkinetochore distances in the absence of dynein or dynein and Mast (Fig. 5). Control or siRNA-treated cells were then subjected to cold treatment and the interaction between microtubule bundles and kinetochores was quantified (Fig. 5A,B). The results indicated that there is no significant difference between control and siRNA-treated cells and therefore, loss of dynein or dynein and Mast appeared to have no significant effect upon microtubule-kinetochore attachment. To analyze the impact of these two proteins in generating tension across sister kinetochores, we then measured the inter-kinetochore distance of metaphase cells lacking dynein, or both Mast and dynein, and compared these with both untreated control

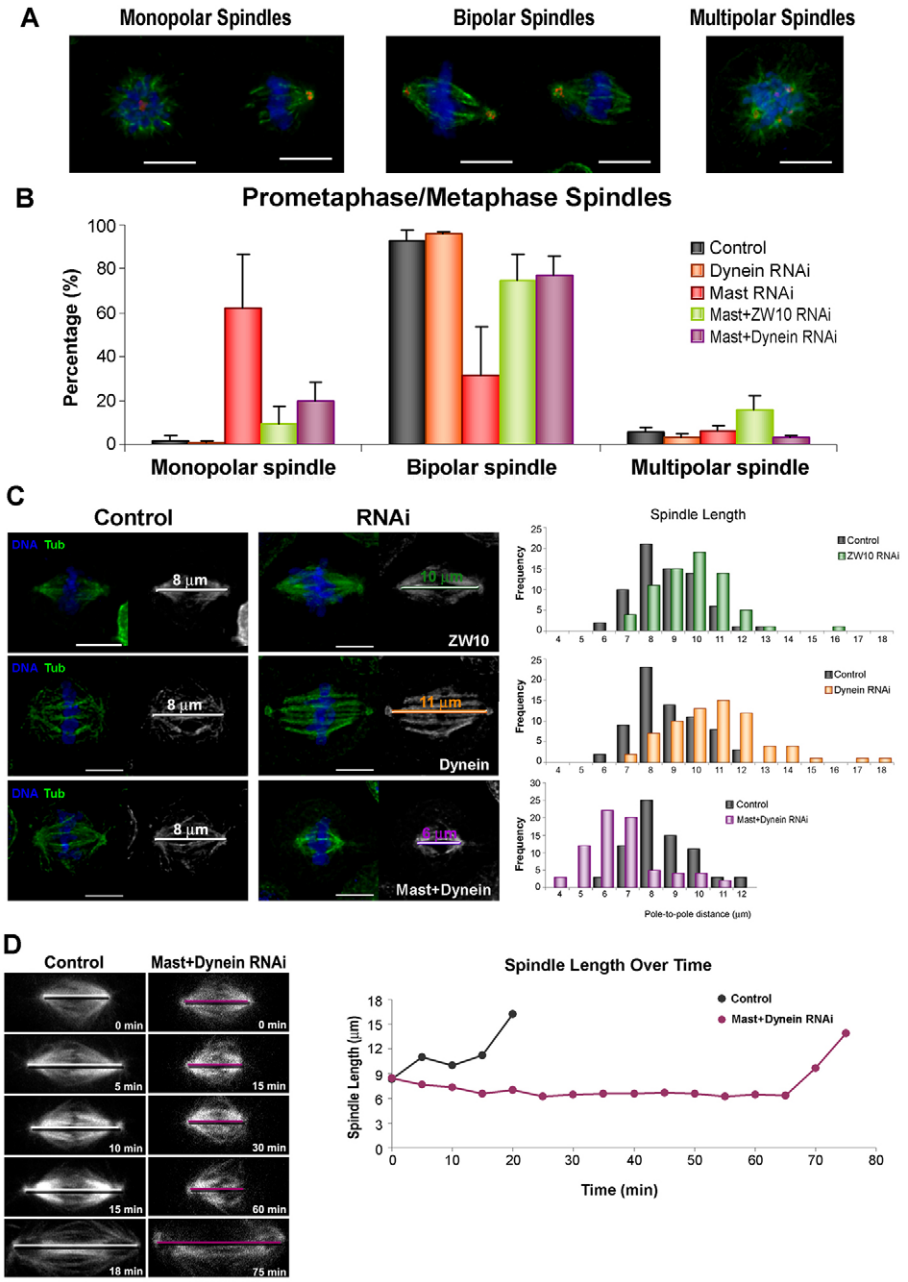

Fig. 4. Spindle organization in cells after depletion of dynein and Mast. (A) S2 cells labelled for γ-tubulin (red), α-tubulin (green) and DNA (blue) showing the three major spindle phenotypes found in cells depleted for Mast alone or in combination either with dynein or ZW10. The spindles were morphologically classified as monopolar, bipolar or multipolar. (B) Frequency of the three spindle phenotypes found in control cells (black), and in cells depleted of dynein (orange), Mast (red) and double-depleted Mast+ZW10 (green) or Mast+dynein (violet). At least 400 mitotic cells were quantified for each treatment. (C) Metaphase spindles of control or siRNA-treated S2 cells from asynchronous cultures labelled for tubulin (green) and DNA (blue). The lines represent pole-to-pole distances. Quantification of the frequency distribution of spindle length for each siRNA treatment is shown on the right (n=72 cells in each case). Note that cells depleted of both Mast and dynein have smaller spindles than those seen in control cells. (D) Still images from representative movies of control and dynein+Mast-depleted S2 cells expressing GFP-tubulin. Images were acquired every 30 seconds. Time zero was defined as the time the bipolar spindle is formed. Note that after simultaneous depletion of both Mast and dynein, the spindle remains short for long periods of time. Quantification of spindle length over time is shown on the right. Scale bars: 5 µm (A,C).

cells and control cells treated with colchicine (lack of tension) (Fig. 5C,D). The results revealed that in all cases, the inter-kinetochore distance was similar to untreated control cells and only statistically different from colchicines-treated cells when microtubules are not present (P<0.001) (Fig. 5D). Indeed, previous studies also show that loss of dynein at kinetochores after depletion of Spindly, have no effect upon the inter-kinetochore distance in S2 cells (Griffis et al., 2007). These results suggest that the absence of these two proteins, alone or simultaneously, has no significant impact either on microtubule-kinetochore attachment or tension generation in *Drosophila* cells.

Rates of microtubule flux are normal in cells depleted of Mast and dynein

Previous results have shown that Mast has an essential role in the incorporation of tubulin heterodimers into the plus-ends of mature K-fibers (Maiato et al., 2005) and that the collapse of the spindle in Mast-depleted cells requires the activity of KLP10A, a microtubule minus-end depolymerase (Buster et al., 2007; Laycock et al., 2006). In *Xenopus* extracts, kinesin-13 depolymerases are no longer targeted to spindle poles in the absence of dynein (Gaetz and Kapoor, 2004) and it is therefore possible that the rescue of spindle bipolarity in S2 cells depleted of Mast and dynein is also due to KLP10A mislocalization. However, after simultaneous depletion of Mast and dynein, we found that KLP10A localization to spindle poles was unaltered (supplementary material Fig. S4). In agreement, Kif2A also remained concentrated at the minus-ends of K-fibers after dynein-dynactin inhibition in mammalian PtK1 cells (Cameron et al., 2006). Since microtubule minus-end depolymerases are normally localized, an alternative possibility is that spindles are bipolar in cells lacking Mast and dynein, because plus-end microtubule polymerization was restored. To test this

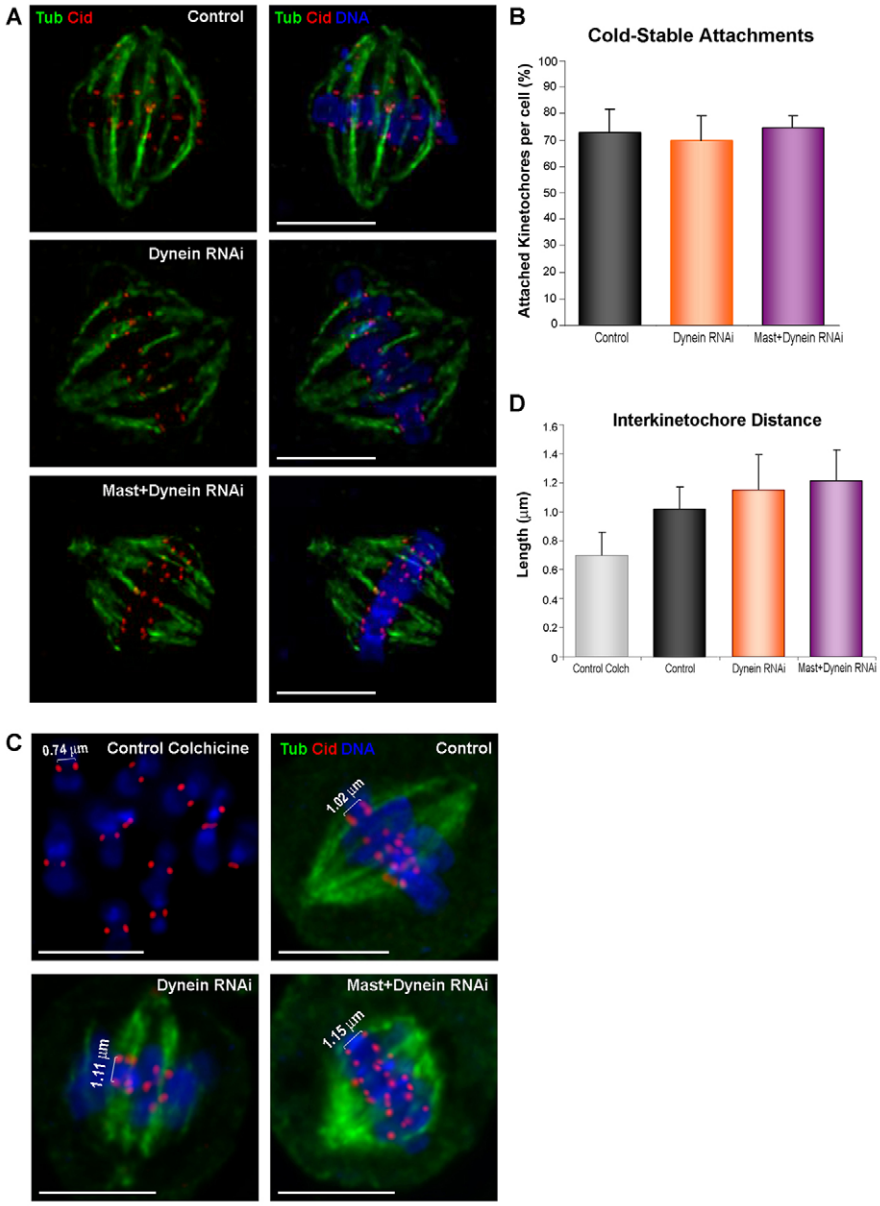

Fig. 5. Role of dynein and Mast on the stability of kinetochore-microtubule attachments and generation of tension across sister kinetochores. To determine the microtubule-kinetochore stability, control or siRNA-treated cells were subjected to cold treatment, fixed and stained to reveal cid (red), α-tubulin (green) and DNA (blue). (B) Quantification of microtubule-kinetochore stability shown as a percentage of attached kinetochores per cell. All the kinetochores in ten cells were analyzed for each condition. Results are means ± s.d. (C) Control and siRNA-treated cells were fixed and stained to revealed cid (red), α-tubulin (green) and DNA (blue), Kinetochore pairs were identified by following Cid dots through the stacks and the interkinetochore distance quantified. As a control for loss of tension across sister kinetochores, control cells were treated with colchicine to depolymerize microtubule before fixation. (D) Quantification of inter-kinetochore distance in control cells treated with colchicine (0.70±0.15 µm, *n*=142) (grey), in untreated cells (1.02±0.16 µm, *n*=137) (black), and in cells treated with dynein RNAi (1.15±0.24 µm, *n*=93) (orange) and Mast+dyenin RNAi (1.21±0.21 µm, *n*=126) (violet). Results are means ± s.d. Scale bars: 5 µm.

possibility, we analyzed the microtubule plus-end polymerization rate on K-fibers after depletion of either Mast alone or in combination with dynein by fluorescence speckle microscopy in S2 cells stably expressing low levels of GFP-tubulin and mCherry-Cid (Fig. 6). The kymographs were aligned using the mCherry-Cid signal at kinetochores as a reference, allowing us to determine the rate of plus-end microtubule polymerization (Fig. 6A,B). The results confirm that after depletion of Mast alone, microtubule plus-end polymerization was indeed significantly reduced (0.50±0.41 µm/minute, *n*=10 cells) when compared with either control or dynein-depleted cells (1.49±0.10 µm/minute, *n*=35 cells or 1.53±0.10 µm/minute, *n*=26 cells, respectively), as previously shown (Buster et al., 2007; Maiato et al., 2005). However, after co-depleting Mast and dynein, we found that microtubule plus-end polymerization fully recovered, with values that were not significantly different from that of control cells (1.35±0.10 µm/minute, *n*=32 cells). These data further support the hypothesis that the rescue of spindle bipolarity is not due to the absence of KLP10A at the spindle poles, because it was shown that cells lacking KLP10A or Mast and KLP10A have significantly reduced flux rates (Buster et al., 2007; Ganem et al., 2005; Rogers et al., 2004). These results suggest that during metaphase Mast has an important role in facilitating microtubule plus-end polymerization when dynein is present.

Discussion

Mast localizes at kinetochores in a microtubule-independent manner and its removal requires dynein activity

Our data show that Mast localizes to the external region of the kinetochore and that this targeting is microtubule independent and requires the conserved C-terminal region of the protein, similarly to the human CLASP1 protein (Maiato et al., 2003a). We also show that, like CLASP1 (Maiato et al., 2003a), Mast has a dynamic pattern of localization at kinetochores during mitosis and accumulates at high levels during prometaphase but then starts to rapidly decrease during metaphase and anaphase. From the analysis of transgenic

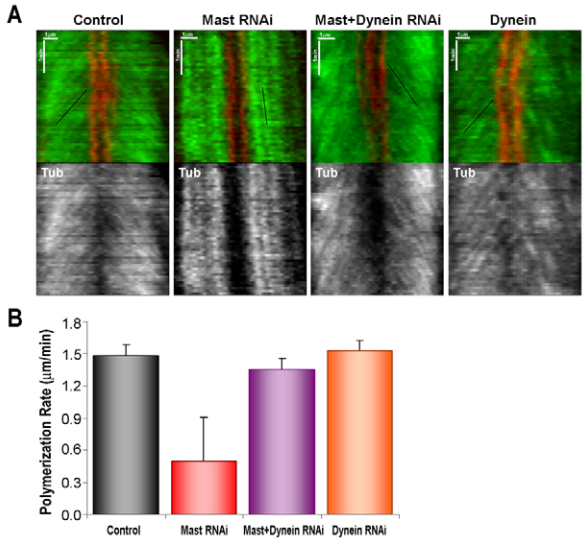

Fig. 6. Effect of Mast and dynein on microtubule plus-end polymerization rate. (A) Kymographs of control cells and cells depleted for Mast and dynein, individually or simultaneously. These kymographs were obtained from movies of cells expressing low levels of GFP-tubulin and coexpressing mCherry-Cid used to align the kymographs. Black lines represent movement of tubulin speckles relative to kinetochores. Scale bars: 1 μm (horizontal); 1 minute (vertical). (B) Quantification of polymerization rates (mean ± s.d.) in control cells (1.49±0.10 μm/minute, *n*=35) (black), and in cells treated with Mast RNAi (0.50±0.42 μm/minute, *n*=10) (red), Mast+dynein RNAi (1.35±0.10 μm/minute, *n*=32) (violet) and dynein RNAi (1.53±0.10 μm/minute, *n*=26) (orange).

flies expressing EGFP-Mast, we show that like many other proteins that localize transiently to the kinetochore, including Rod (Basto et al., 2004), Zw10 (Williams et al., 1996), dynein (King et al., 2000) and CLIP-190 (Dzhindzhev et al., 2005), a significant fraction of Mast leaves the kinetochores by poleward streaming along microtubules.

To date no studies have shown that Mast and dynein interact directly; however, several studies have described interactions between CLIP-190 or CLIP-170 (mammalian orthologue) and dynein (Dujardin et al., 1998; Dzhindzhev et al., 2005; Lansbergen et al., 2004; Tanenbaum et al., 2006). Indeed, it was observed that, in addition to its role in localizing CLIP-190 to kinetochores, dynein also seems to be responsible for its removal from kinetochores (Dzhindzhev et al., 2005). The same kind of dynein dependence was described for mammalian CLIP-170 (Dujardin et al., 1998; Tanenbaum et al., 2006). It was observed that in addition to dynein, CLIP-190 also binds Mast (Mathe et al., 2003). Since CLASPs are CLIP-associated-proteins that bind to CLIP-170 through its conserved C-terminal domain (the same domain that targets Mast to kinetochores) (Akhmanova et al., 2001), it was proposed that CLIP-170 might be a linker for dynein cargo (Dujardin et al., 1998). Therefore, it is very likely that Mast and CLASP indirectly leave the kinetochore in a dynein-dependent manner via CLIP190.

Mast and dynein regulate spindle organization

MAPs have been involved in the regulation of spindle length given their capacity to change the balance of dynamic behavior and forces of spindle microtubules. Previously, it was demonstrated in S2 cells that the absence of microtubule stabilizers results in shorter spindles, and the absence of microtubule depolymerases results in elongated spindles (Goshima et al., 2005). In particular, regulators present at kinetochores appear to affect spindle length more severely than those located at the poles (Goshima et al., 2005). As Mast is required for microtubule plus-end incorporation in mature K-fibers, we analyzed its role in the overall organization of the spindle after depletion of either ZW10 or dynein that results in abnormally high levels of Mast at kinetochores. We hypothesized that higher levels of Mast at kinetochores might allow more incorporation of subunits at K-fibers, which would result in an abnormal spindle length. Indeed, we observed that in the absence of either dynein or ZW10, the spindle length increases significantly. However, the polymerization rate at kinetochores does not increase significantly in the absence of dynein, suggesting that higher levels of Mast at kinetochores during metaphase do not affect spindle length directly. Interestingly, recent studies have identified Spindly, a new regulator of dynein recruitment to kinetochores that is essential for congression, timely mitotic exit and maintenance of correct spindle length (Griffis et al., 2007; Gassmann et al., 2008). However, although the molecular mechanism involved has not yet been identified, we can speculate that the absence of kinetochore dynein might be responsible for this effect.

The observation that co-depletion of Mast and dynein results in cells that are able to form bipolar spindles is surprising and suggest that these two proteins have opposite effects upon spindle organization. Moreover, the rescue of spindle bipolarity in these double-depleted cells is probably due to the absence of dynein specifically at kinetochores, because in cells in which Mast and ZW10 are depleted, where only the kinetochore dynein is displaced, the results are identical. Interestingly, analysis of mutants in the Mast orthologue of *Arabidopsis*, an organism that lacks dynein (Ambrose et al., 2005), show mostly normal cell division in which chromosomes align and segregate with no apparent defects; however, the spindles are significantly shorter (Ambrose et al., 2007). Similarly, our results show that in the absence of Mast and dynein the spindles although bipolar are shorter than in control cells and are maintained for long periods. These short spindles resemble those found in EB1-depleted cells, a protein known to be essential for the interaction of astral microtubules with the cell cortex (Goshima et al., 2005; Rogers et al., 2002). Therefore, it is possible that the spindles from cells lacking Mast and dynein are shorter because of the absence of any interaction between astral microtubules and the cell cortex.

Mast facilitates kinetochore-fiber subunit incorporation in the presence of dynein

Studies in S2 cells have demonstrated that after depletion of Mast, tubulin poleward flux of K-fibers is strongly reduced because the incorporation of tubulin subunits at the microtubule plus-ends of mature K-fibers is shut down (Buster et al., 2007; Maiato et al., 2005). Although collapse of the spindle is prevented by co-depleting Mast and the minus-end microtubule-destabilizing kinesin KLP10A, this does not restore K-fiber flux (Buster et al., 2007; Laycock et al., 2006), suggesting that antagonizing activities regulate bipolarity independently of flux and that Mast is indeed an essential protein for K-fiber flux. However, our results demonstrate that Mast is not involved in the direct incorporation of tubulin at the plus-ends of kinetochore microtubules, but rather regulates this process. Indeed, recent analysis of microtubule flux fully supports these results. Depletion of Mast and centromere-associated microtubule depolymerases KLP67A or KLP59C, also results in restoration of microtubule flux (Buster et al., 2007).

However, although the microtubule-depolymerizing nature of either KLP67A or KLP59C could in principle explain the rescue of flux when they are co-depleted with Mast, dynein has never been shown to promote microtubule depolymerization at kinetochores. Therefore, our results suggest that dynein affects flux indirectly, for instance by affecting the kinetochore function and/or targeting of a depolymerizing enzyme. Indeed, theoretical work supports the idea that dynein located at the kinetochore assists microtubule depolymerases in suppressing rescue events by inserting the plus-ends of kinetochore microtubules into the kinetochore structure (Civelekoglu-Scholey et al., 2006; Gadde and Heald, 2004).

Dynein is also known to be required to target CLIP-190/CLIP-170 to the kinetochore (Dujardin et al., 1998; Dzhindzhev et al., 2005). Although the absence of CLIP-170/CLIP-190 does not affect microtubule dynamics, CLIP-170 appears to facilitate the initial formation of kinetochore-microtubule attachments, possibly through direct capture of microtubules at the kinetochore (Tanenbaum et al., 2006). Accordingly, in mammalian cells the absence of dynein or CLIP-170 causes cells to exhibit spindles with a substantially reduced number of kinetochore microtubules when subjected to cold treatment (Li et al., 2007; Yang et al., 2007), and cells lacking CLIP-170 also display high levels of kinetochore Mad1 (Tanenbaum et al., 2006). However, contrary to mammalian cells, our results suggest that S2 cells lacking dynein have relatively stable K-fibers because they were able to resist cold treatment and to generate normal inter-kinetochore tension. We therefore propose that the main function of dynein and Mast is to regulate kinetochore-microtubule dynamics and not attachment. Accordingly, our results suggest that dynein is essential, not for kinetochore-microtubule attachment per se, but to generate the proper interactions between the plus-end of microtubules and the kinetochore proteins, namely depolymerases. Recent studies in mammalian cells and *C. elegans* suggest that this motor has an important role in pushing the plus-ends of microtubules into the kinetochore structure allowing the maturation of the attachments (Civril and Musacchio, 2008; Gassmann et al., 2008; Varma et al., 2008). Since Mast is required for tubulin incorporation only in mature K-fibers (Maiato et al., 2005), microtubule plus-end incorporation in the absence of both proteins (Mast and dynein) might be restored because microtubules are no longer pushed into the kinetochore structure and expose their plus-ends to the action of depolymerases.

From our data, we propose that at metaphase, dynein controls the level or function of Mast and other microtubule regulators, potentially microtubule depolymerases, to allow tubulin incorporation and flux on K-fibers. This dynein-mediated regulation might be important for the switch from polymerizing to depolymerizing state of kinetochore microtubules that occurs during anaphase.

Materials and Methods

Cell culture and western blotting

Drosophila S2 cells were cultured at 25°C in Schneider's insect medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD). For western blot total extracts from 1×10^6 cells were used. After washing with PBS, cells were resuspended in SDS-PAGE sample buffer. The samples were boiled for 10 minutes at 95°C and proteins separated by SDS-PAGE and blotted to a nitrocellulose membrane. Membranes were incubated with primary antibodies diluted to the following concentrations: anti-α-tubulin mouse (DM1A, Sigma), 1:8000; anti-dynein mouse (P1H4) (McGrail and Hays, 1997), 1:10,000; anti-Mast rabbit (IP726α) (Lemos et al., 2000), 1:100; anti-ZW10 rabbit (Williams et al., 1992), 1:1500. The HRP anti-mouse and anti rabbit secondary antibodies (Amersham) were visualized using the ECL system.

RNA interference

RNAi depletion of Mast, dynein and ZW10 was done as previously described (Maiato et al., 2003b), except that the amount of dsRNA per well was 30 μg. The siRNA fragment of Mast and dynein was the same as previously described (Maiato et al., 2004; Maiato et al., 2002). The primers used to amplifyZW10 were: 5′-TAA TAC GAC TCA CTA TAG GGT GGC ACC TAC GTT CGA TT-3′ and 5′-TAA TAC GAC TCA CTA TAG GGA TCA TGC AGC GTG GGA AG-3′. Five days after the addition of the siRNA, cells were collected and processed for western blot, immunofluorescence analysis or in vivo recording.

Immunofluorescence of *Drosophila* S2 cells and data analysis

For immunofluorescence, 2×10^5 cells were centrifuged onto slides at 123 ***g*** for 5 minutes, fixed in 3.7% formaldehyde (Sigma) in PHEM (60 mM PIPES, 25 mM HEPES pH 7.0, 10 mM EGTA, 4 mM $MgSO_4$) for 12 minutes and detergent-extracted with PBST 0.5% (0.5% Triton X-100 in PBS) three times for 5 minutes. In some experiments, cells were fixed with cold methanol for 10 minutes at –20°C and then rehydrated with 0.1% PBST. Blocking was performed in PBST 0.1% with 10% FBS for 30 minutes at room temperature. Cells were incubated for at least 1 hour at room temperature or overnight at 4°C in primary antibodies that were diluted in blocking solution to the following concentrations: anti-α-tubulin mouse (B512, Sigma), 1:5000; anti-γ-tubulin mouse (GTU88, Sigma), 1:500; anti-Cid rat (C.E.S. and Sore Steffensen, IBMC, University of Porto, Portugal, unpublished), 1:2000; anti-Mast rabbit IP726 (Lemos et al., 2000), 1:10; anti-KLP10A (Rogers et al., 2004), 1:150. After washing three times with 0.1% PBST, cells were incubated with secondary antibodies used according to the manufacturer's instructions. DNA was stained with Vectashield medium containing DAPI (Vector). For microtubule depolymerization, cells were incubated for 4 hours with 30 μM colchicine (Sigma) and for proteasome inhibition, for 2 hours with 20 μM MG132 (Calbiochem). Cold treatment was performed by incubating cells on ice for 10 minutes. Fluorescence images were acquired on an AxioImager Z1 microscope (Carl Zeiss, Germany) using an Axiocam MR ver.3.0 (Carl Zeiss, Germany), deconvolved with Huygens Essential version 3.0.2p1 (Scientific Volume Imaging, Hilversum, The Netherlands), projected using ImageJ 1.3v software (http://rsb.info.nih.gov/ij/) and processed with PhotoShop CS (Adobe Microsystems, CA). Mast intensity levels at kinetochores of S2 cells treated with either MG132 or colchicine were measured using ImageJ 1.3v software. The intensity of Mast and Cid was measured in individual kinetochores for each cell. After subtracting the respective backgrounds, it was expressed as a ratio of Mast:Cid intensity for each kinetochore. Spindle length quantification was obtained by measuring the distance between spindle poles using the software Axiovision 4.5 (Carl Zeiss, Germany).

Transfection of S2 cells

pMT-EGPF-Kt1 to pMT-EGPF-Kt6 were derived from the full-length pMT-EGPF-Mast described in Lemos et al. (Lemos et al., 2000). S2 cells were transfected using the calcium phosphate method (Sousa et al., 2007), incubated with colchicine and analyzed by immunofluorescence.

Fly stocks and time-lapse analysis

Mast^4 was previously described (Lemos et al., 2000). UASp-EGPF-Mast and UASp-EGPF-Kt2 transgenic flies were generated by standard P-element-mediated germline transformation. To drive transcription in embryos, the maternal-α-tub VP16 GAL4 driver was used (Bloomington Stock Centre). Embryos at 0-2 hours were collected, manually dechorionated and mounted in oil holocarbon 700 (Sigma). Live images were acquired every 10 seconds for EGFP-Mast or EGFP-Mast-Kt2 kinetochore intensity measurements using a laser-scanning confocal microscope Leica SP2 AOBS SE and processed using Software LCS 2.61 (Leica Microsystems, Wetzlar, Germany) or every 2 seconds for determination of EGFP-Mast poleward velocity using a spinning disk confocal system Andor Revolution XD (ANDOR Technology). Syncytial embryos expressing UASp-EGPF-Mast or UASp-EGPF-Kt2 undergoing cycle 13 were used for kinetochore fluorescence quantification and the different movies were aligned by the anaphase onset. Quantitative analysis was performed using ImageJ 1.3v software. To test the ability of the EGFP-Mast transgene to complement mutations in the endogenous *mast* gene, the transgene was expressed in a mutant background (*mast*^{4}/*mast*^{4}) under the control of the Act5C-Gal4 driver (Bloomington) (w; UAS-EGPF-Mast/Act-Gal4;*mast*^{4}/*mast4*) or the neuroblast-specific driver MZ1061 (a gift from Joachim Urban) (MZ1061; UAS-EGFP-Mast; *mast*^{4}/*mast4*). Flies from the same cross lacking the driver (w; UAS-EGPF-Mast/Cyo;*mast*^{4}/*mast4*) were used as controls. Third instar brains were dissected in 0.7% NaCl, fixed in acetic acid (45% and 60%), squashed and stained with DAPI for quantification.

Time-lapse fluorescence imaging of S2 cells

S2 cells stably expressing GFP-tubulin (kindly provided by Ron Vale) were incubated with dsRNA and plated after 120 hours for at least 30 minutes on glass coverslips previously coated with 0.5 μg/μl concanavalin A (Sigma). When required, coverslips were placed in special incubations chambers (Rose et al., 1958) and images were collected every 30 seconds using a spinning disk confocal system Andor Revolution XD (ANDOR Technology).

Quantification of microtubule flux

The flux rate was measured by fluorescent speckle microscopy (FSM) (Waterman-Storer et al., 1998) in S2 cells stably expressing low levels of GFP-tubulin and mCherry-Cid from a leaky metallothionein promoter without induction. By collecting images every 5 seconds using a spinning disk confocal system (ANDOR Technology) we could follow the movement of the fluorescent speckles within the spindle. This movement was represented on a kymograph, keeping the labelled kinetochores as the reference point. The slope of the lines obtained from individual speckles was used to calculate flux rates on kinetochore microtubules. All kymographs were analyzed using a program from a custom routine written in Matlab (Natick, MA) (A.J.P. and H.M., unpublished results).

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