Mob4 plays a role in spindle focusing in *Drosophila* S2 cells

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Summary

The characteristic bipolar shape of the mitotic spindle is produced by the focusing of the minus ends of microtubules at the spindle poles. The focus is maintained by the centrosome, a microtubule-nucleating organelle, as well as by proteins that are capable of focusing kinetochore fibers (K fibers) even in the absence of a centrosome. Here, we have performed a small-scale RNA interference (RNAi) screen of known or suspected pole-related proteins in *Drosophila* S2 cells. An unexpected outcome of this screen was the finding that one of the four *Drosophila* Mob proteins (a family of kinase regulators) plays a role in spindle pole organization. Time-lapse microscopy of mitotic cells depleted of *Drosophila* Mob4 by RNAi revealed that the K fibers splay apart and do not maintain their focus either in the presence or absence of functional centrosomes. The Mob4 RNAi phenotype most closely resembles that observed after depletion of the protein encoded by *abnormal spindle* (Asp), although Asp localization is not substantially affected by Mob4 RNAi. Expression of a *Drosophila* Mob4-GFP fusion protein revealed its localization to the nucleus in interphase and to spindle poles and kinetochores during mitosis. We propose that Mob4 in *Drosophila* controls a mitotic kinase that in turn regulates downstream target proteins involved in K fiber focusing at the poles.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/8/1284/DC1

Key words: RNAi, Centrosome, Microtubule, Kinetochore, γ-tubulin

Introduction

During mitosis, the microtubule network reorganizes into a bipolar spindle that ensures proper chromosome segregation by aligning the chromosomes along the metaphase plate before partitioning them equally into the two daughter cells. Because the fidelity of chromosome segregation depends on proper spindle function, the mechanisms by which microtubules self-organize into a bipolar spindle have been a topic of considerable interest (Karsenti and Vernos, 2001; Maiato et al., 2004; Nedelec et al., 2003). The centrosome is the primary microtubule organizing center (MTOC) in somatic animal cells, and it plays a fundamental role in spindle formation. The centrosome number determines both the location and number of spindle poles, as illustrated by the formation of monopolar spindles in cells with unseparated or unduplicated centrosomes (Heald et al., 1997) or of multipolar spindles in cells with multiple centrosomes (Nigg, 2002). However, functional spindles are capable of forming in the absence of bona fide centrosomes, as occurs in meiotic cells or in cells with disrupted centrosomes (Heald et al., 1996; Hinchcliffe et al., 2001; Khodjakov et al., 2000; Mahoney et al., 2006; Megraw et al., 2001).

The process of spindle formation in somatic cells begins typically with the duplication and separation of centrosomes in prophase. This separation is facilitated by bipolar kinesin motors (kinesin-5) (Lawrence et al., 2004), which establish spindle bipolarity by crossbridging and pushing apart antiparallel microtubules from the two centrosomes (Sharp et al., 2000). Following breakdown of the nuclear envelope, centrosome-nucleated microtubules invade the nuclear region, grow and shrink by dynamic instability and become selectively stabilized after connecting with kinetochores (Desai and Mitchison, 1997). Chromosomes also mediate microtubule nucleation, including polymerization at the kinetochore (Khodjakov et al., 2003; Mahoney et al., 2006; Maiato et al., 2004). Microtubules originating from both sources contribute to the formation of kinetochore fibers (K fibers), thick microtubule bundles connected directly to the kinetochore. K fibers become focused at the spindle poles adjacent to the two centrosomes through the action of microtubule crosslinking proteins [e.g. abnormal spindle protein (Asp) and NuMA] and motors (Ncd and dynein) that crossbridge the minus (distal) ends of the K fibers and transport them towards the centrosomes along astral microtubules (Goshima et al., 2005; Khodjakov et al., 2003; Maiato et al., 2004; Merdes et al., 2000; Morales-Mulia and Scholey, 2005). The centrosome, therefore, has a fundamental role not only in the nucleation of spindle microtubules but also in the collection and organization of K fibers into two focused poles before chromosome segregation in anaphase. However, some degree of pole focusing can be achieved in acentrosomal spindles through the actions of minus-end-directed motors and microtubule crosslinking proteins alone (Nedelec et al., 2003).

Here, through the use of RNA interference (RNAi), we have depleted 24 proteins that have been implicated in spindle pole organization in *Drosophila* S2 cells, a well-characterized cell line for RNAi and mitosis (Goshima et al., 2007; Bettencourt-Dias et al., 2005; Goshima and Vale, 2003; Morales-Mulia and Scholey, 2005; Rogers et al., 2002). One of the ‘hits’ from this mini-RNAi screen was *Drosophila* Mob4, an uncharacterized member of the highly conserved Mob protein family (Lai et al., 2005). Our experiments reveal that *Drosophila* Mob4 is required for both centrosome separation and focusing of K fibers. These findings represent the first demonstration of a Mob family protein serving a role in the formation of the mitotic spindle.
Results
Screen of centrosome-related proteins for mitotic defects in S2 cells
We examined RNAi phenotypes for 24 Drosophila proteins that have been implicated in spindle pole organization in Drosophila or other organisms. While this list probably represents only a subset of the proteins involved in Drosophila spindle pole formation, our intention was to uncover new functions for known or suspected pole proteins. We examined the mitotic index, mitotic spindles and γ-tubulin localization to the spindle poles in Drosophila S2 cells after a 4-day RNAi treatment of candidate spindle pole proteins (the screen was repeated with 7-day RNAi and produced identical results). The efficacy of RNAi was examined for seven proteins for which we could obtain antibodies (supplementary material Fig. S1). In these cases, substantial (>80%) protein depletion was observed. We suspect that protein depletion occurred for all of the RNAi treatments, as these results are consistent with our prior (Goshima and Vale, 2003; Rogers et al., 2003) and our unpublished observations, where comparable RNAi-induced depletion was observed for approximately 40 proteins tested by immunoblot analysis.

The results from the screen are shown in Table 1 and more details are described in supplementary material Table S2 and supplementary material Figs S1 and S2. While this screen was being performed, similar work described phenotypes for γ-tubulin and γ-tubulin-associated subunits (Goshima et al., 2007; Vérollet et al., 2006), which largely agreed with the results from this screen. Several RNAi treatments did not alter the mitotic index or spindle morphology.

Table 1. Spindle phenotype and γ-tubulin localization analysis after RNAi

<table>
<thead>
<tr>
<th>Targeted gene product</th>
<th>Relative mitotic index</th>
<th>Spindle phenotype</th>
<th>% abnormal spindles (in %), (n)</th>
<th>Spindles with localized γ-tubulin (in %), (n)</th>
<th>Knockdown confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>Monopolar/anastral</td>
<td>24±9 (496)*</td>
<td>100±0 (114)</td>
<td></td>
</tr>
<tr>
<td>γ-tubulin (23C)</td>
<td>3.3±0.7*</td>
<td>Monopolar/anastral</td>
<td>98±2 (279)</td>
<td>3±2 (118)</td>
<td></td>
</tr>
<tr>
<td>Dgrip223</td>
<td>2.8±0.9*</td>
<td>Monopolar/anastral</td>
<td>98±3 (303)</td>
<td>14±5 (146)</td>
<td></td>
</tr>
<tr>
<td>Dgrip17</td>
<td>2.9±0.6*</td>
<td>Monopolar/anastral</td>
<td>95±3 (371)</td>
<td>11±5 (133)</td>
<td></td>
</tr>
<tr>
<td>Dgrip128</td>
<td>2.6±0.3*</td>
<td>Monopolar/anastral</td>
<td>84±1 (285)</td>
<td>94±3 (125)</td>
<td></td>
</tr>
<tr>
<td>Dgrip163</td>
<td>2.0±0.7*</td>
<td>Monopolar/anastral</td>
<td>76±5 (301)</td>
<td>89±7 (166)</td>
<td></td>
</tr>
<tr>
<td>Dgrip71</td>
<td>2.5±0.3*</td>
<td>Monopolar/anastral</td>
<td>85±7 (385)</td>
<td>96±1 (82)</td>
<td></td>
</tr>
<tr>
<td>Dgrip75</td>
<td>2.3±0.3*</td>
<td>Monopolar/anastral</td>
<td>82±1 (331)</td>
<td>91±5 (129)</td>
<td></td>
</tr>
<tr>
<td>Msp5</td>
<td>3.2±0.4*</td>
<td>Multiple defects</td>
<td>97±2 (292)</td>
<td>99±1 (135)</td>
<td></td>
</tr>
<tr>
<td>Msp5 and Asp</td>
<td>2.0±0.6*</td>
<td>Bipolar/anastral</td>
<td>88±5 (134)</td>
<td>95±4 (111)</td>
<td></td>
</tr>
<tr>
<td>Centrosomin</td>
<td>1.0±0.2</td>
<td>Normal</td>
<td>93±5 (246)</td>
<td>10±1 (108)</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>1.2±0.7</td>
<td>Detached/unfocused spindles</td>
<td>91±6 (222)</td>
<td>98±3 (109)</td>
<td></td>
</tr>
<tr>
<td>Dmob4</td>
<td>1.1±0.4</td>
<td>Monopolar/monoastral bipolar</td>
<td>73±7 (342)</td>
<td>96±6 (83)</td>
<td></td>
</tr>
<tr>
<td>Mps1</td>
<td>0.5±0.2*</td>
<td>Monopolar/bipolar/other</td>
<td>51±2 (122)</td>
<td>99±2 (63)</td>
<td></td>
</tr>
<tr>
<td>γ-tubulin (37C)</td>
<td>0.9±0.5</td>
<td>Normal</td>
<td>33±1 (235)</td>
<td>99±2 (85)</td>
<td></td>
</tr>
<tr>
<td>Dgrip232</td>
<td>1.2±0.1</td>
<td>Normal</td>
<td>26±8 (152)</td>
<td>100±1 (79)</td>
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</tr>
<tr>
<td>Dgrip79</td>
<td>0.7±0.6</td>
<td>Normal</td>
<td>29±1 (176)</td>
<td>100±0± (84)</td>
<td></td>
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<tr>
<td>D-TACC</td>
<td>1.2±0.7</td>
<td>Normal</td>
<td>25±7 (241)</td>
<td>100±0± (101)</td>
<td></td>
</tr>
<tr>
<td>Centrin 1</td>
<td>0.6±0.1</td>
<td>Normal</td>
<td>15±2 (190)</td>
<td>99±2 (105)</td>
<td></td>
</tr>
<tr>
<td>Centrin 2</td>
<td>0.6±0.1</td>
<td>Normal</td>
<td>26±1 (174)</td>
<td>100±0± (77)</td>
<td></td>
</tr>
<tr>
<td>Niki/Nek2</td>
<td>1.1±0.1</td>
<td>Normal</td>
<td>30±5 (168)</td>
<td>100±0± (89)</td>
<td></td>
</tr>
<tr>
<td>CP60</td>
<td>0.9±0.3</td>
<td>Normal</td>
<td>36±2 (260)</td>
<td>94±2 (116)</td>
<td></td>
</tr>
<tr>
<td>CP190</td>
<td>0.9±0.2</td>
<td>Normal</td>
<td>24±6 (241)</td>
<td>99±2 (77)</td>
<td></td>
</tr>
<tr>
<td>Dmob1</td>
<td>0.8±2.4</td>
<td>Normal</td>
<td>25±2 (247)</td>
<td>100±0± (93)</td>
<td></td>
</tr>
<tr>
<td>Dmob2</td>
<td>1.0±0.2</td>
<td>Normal</td>
<td>24±10 (286)</td>
<td>100±0± (19)</td>
<td></td>
</tr>
<tr>
<td>Dmob3</td>
<td>1.3±0.4</td>
<td>Normal</td>
<td>24±3 (484)</td>
<td>95±1 (148)</td>
<td></td>
</tr>
<tr>
<td>Pericentrin</td>
<td>0.8±0.3</td>
<td>Normal</td>
<td>24±12 (206)</td>
<td>100±0± (99)</td>
<td></td>
</tr>
</tbody>
</table>

For complete gene information, including CG-identification numbers and RNAi primer sequences, see supplementary material Table S1. Cells were analyzed on day 4 or 5 of RNAi treatment. Values of mean ± s.d. are from three individual experiments unless noted otherwise, (n) number of spindles observed.

Dmob1, Dmob2, Dmob3, Dmob4; Drosophila Mob1 (CG13852), Mob2 (CG11711), Mob3 (CG4946), Mob4 (CG3403), respectively. They have been identified as Drosophila Mob proteins based on homology to the Saccharomyces cerevisiae Mob1p (He et al., 2005).

Msps, minispindles protein; Asp, abnormal spindle protein.

γ-tubulin (23C) and γ-tubulin (37C): two isoforms of the γ-tubulin gene are present in Drosophila. The 23C isoform is expressed ubiquitously, whereas the 37C isoform is primarily expressed in the ovaries and in embryos.

Relative mitotic index: Percentage of nuclei that stained positive for phosphorylated histone H3 (a marker of mitotic cells), primarily scored using an automated microscope (see Materials and Methods). Numbers give the average fold increase in mitotic index for at least three separate RNAi experiments (mean ± s.d.) relative to the average mitotic index of untreated control cells (1.41±0.47, derived from untreated cells in three independent experiments). The average mitotic index of untreated cells from each experiment was determined from measurements of 90 separate wells. *P<0.005 (t-test), fold increase in mitotic index is statistically different from untreated cells.

As reported previously (Goshima and Vale, 2003), S2 cells often show aberrant mitotic spindle phenotypes. Some S2 cells form monopolar spindles that subsequently can be converted to a bipolar spindle by a rescue process in which microtubules become organized to form a second, acentrosomal pole (~10%). These monastral bipolar spindles are capable of entering anaphase and segregating chromosomes.

Spindles with localized γ-tubulin: Values are the mean ± s.d. of three experiments, except that for Dgrip223, which was obtained from a single experiment. n, number of mitotic cells.

+, protein reduction after RNAi confirmed using specific antibodies; –, not tested because antibodies were unavailable; ○, expression of these genes was at background levels in the S2 cell line by Affimatrix DNA microarray analysis (J. Hollien and J. Weissman, personal communication), suggesting that they are not expressed in this cell line at significant levels.
In some cases (e.g. Dgrip79, Dgrip223, centrin2, Niki and germ-line specific γ-tubulin37C), the gene expression was low or at background levels, as determined by DNA microarray analysis (Table 1). Recent work described a phenotype of centrosomal antigen dispersion for Nek2 depletion in S2 cells (Prigent et al., 2005); however, the reported penetrance of this phenotype was low (~ 30% of cells, which was only slightly above wild-type background levels) and most likely was not sufficiently robust to be scored by our criteria (see Materials and Methods). For other genes, we used double RNAi treatment to explore whether the lack of an RNAi phenotype might be due to redundancy with another gene product. For example, the fly genome contains multiple splice variants of pericentrin (Zimmerman et al., 2004), centrin (Beisson and Wright, 2003) and two NEK kinase-like proteins, Nek2 and Niki (Prigent et al., 2005). We performed double RNAi of these related proteins and still failed to observe any significant effects on mitotic index or spindle morphology (data not shown). Thus, these proteins either might not have a significant function in S2 cell mitosis or they might not be sufficiently depleted by RNAi.

An interesting and unexpected outcome of this screen was obtained with RNAi targeting Drosophila Mob4, which has not been low or at background levels, as determined by DNA microarray analysis (Table 1). Recent work described a phenotype of centrosomal antigen dispersion for Nek2 depletion in S2 cells (Prigent et al., 2005); however, the reported penetrance of this phenotype was low (~ 30% of cells, which was only slightly above wild-type background levels) and most likely was not sufficiently robust to be scored by our criteria (see Materials and Methods). For other genes, we used double RNAi treatment to explore whether the lack of an RNAi phenotype might be due to redundancy with another gene product. For example, the fly genome contains multiple splice variants of pericentrin (Zimmerman et al., 2004), centrin (Beisson and Wright, 2003) and two NEK kinase-like proteins, Nek2 and Niki (Prigent et al., 2005). We performed double RNAi of these related proteins and still failed to observe any significant effects on mitotic index or spindle morphology (data not shown). Thus, these proteins either might not have a significant function in S2 cell mitosis or they might not be sufficiently depleted by RNAi.

An interesting and unexpected outcome of this screen was obtained with RNAi targeting Drosophila Mob4, which has not been studied previously in Drosophila. This RNAi phenotype consisted of a high percentage of monopolar (32%; Fig. 1B) and poorly formed monastral bipolar (41%) and bipolar spindles (Fig. 1C), yet the mitotic index of the population was normal (Table 1). Mob4 proteins are implicated as regulators of kinases (Frenz et al., 2000). In Saccharomyces cerevisiae, Mob1p has been implicated in spindle pole body (SPB) duplication and the mitotic checkpoint through regulation of the Mps1p kinase (Fisk et al., 2004; Jones et al., 2005; Luca and Winey, 1998; Stucke et al., 2002). However, a role for a Mob protein in mitosis in higher eukaryotes has not been described. In Drosophila, there are four Mob-like proteins (25-40% similarity to S. cerevisiae Mob1p), including CG11711, the protein previously named in FlyBase as Mob1. During the course of our study, He et al. (He et al., 2005) reported similar findings, referring in their paper to CG13852 (mats or Dmob1), CG11711 (Dmob2), CG4946 (Dmob3) and CG3403 (Dmob4). Here, the Drosophila Mob proteins are referred to as Mob1, Mob2, Mob3 and Mob4. In our screen, only Drosophila Mob4 had an effect on spindle morphology, which is somewhat surprising as it is the family member most distantly related to yeast Mob1p.

A particular feature of the Mob4-depleted cells was that their spindles had disorganized poles with spliced K fibers. A similar phenotype is also observed after depletion of abnormal spindle protein (Morales-Mulia and Scholey, 2005; do Carmo Avides and Glover, 1999; Saunders et al., 1997) (Fig. 1D), although the spaying of K fibers is more dramatic after RNAi of Asp. We reproduced this phenotype using dsRNA sequences corresponding to different parts of the mRNA sequence encoding Mob4 (supplementary material Fig. S3), indicating that the phenotype is specific and is not caused by an off-target RNAi effect. We thus focused our effort on characterizing the Mob4 RNAi phenotype.

Live-cell imaging of spindle formation after Mob4 RNAi reveals defects in maintenance of pole focusing

We next examined the dynamics of mitotic spindles after RNAi depletion of Mob4 using GFP-tubulin-expressing cells and time-lapse microscopy. In control cells (Fig. 1E and supplementary
Depletion of Mob4 increased the proportion of single-centrosome cells relative to untreated cells but did not generate a significant population of acentrosomal cells, as did RNAi of SAK. (B) RNAi of SAK led to loss of centrosomes and astral microtubules from spindle poles, and co-depletion of Mob4 with SAK noticeably exacerbated spindle fiber unfocusing at acentrosomal poles (right). (α-tubulin, red; Dgrip84, green; DNA, blue; bar, 5 μm.) (C) Histogram showing the distribution of spindle pole focal breadth (yellow line; the distance between the minus ends of the outermost K fibers) at acentrosomal poles in SAK-depleted cells (blue) and in cells depleted of both Mob4 and SAK by double RNAi (red). The mean focal breadth in Mob4-SAK co-depleted cells was 5.0±2.4 μm (n=246), whereas that in cells depleted of SAK alone was 1.9±1.4 μm (n=261; P<0.0001). (Error bars indicate s.d. of mean value of two independent experiments.)

Mob4 has a role in focusing of K fibers independent of centrosomes

Live-cell imaging of mitotic spindle formation in cells depleted of Mob4 revealed a frequent loss of K fiber bundling at spindle poles with centrosomes or at acentrosomal poles of monastral bipolar spindles. We therefore reasoned that the primary role of Mob4 in pole focusing might be in K fiber self-assembly rather than in centrosome-mediated organization. To test this hypothesis, we analyzed K fiber unbundling in Mob4-RNAi-treated cells depleted of functional centrosomes by RNAi of SAK/PLK4, a polo-like kinase crucial for duplication of centrioles (Bettencourt-Dias et al., 2005). RNAi of SAK increased the frequency of cells with zero or 1 centrosome (Fig. 2A), as reported by Bettencourt-Dias and colleagues. In fixed-cell assays, SAK-depleted cells often had either a small, unorganized array of microtubules surrounding the condensed chromosomes, or bipolar spindles with aligned chromosomes but completely lacking astral microtubules (Fig. 2B). These spindle types were both present in cells co-depleted of Mob4 and SAK. However, the poles of the bipolar spindles were more disorganized and splayed apart than were poles after SAK RNAi treatment alone. We analyzed pole focusing quantitatively by

Fig. 2. Drosophila Mob4 (Dmob4) has a minor role in the maintenance of centrosome number and functions in spindle pole organization in the absence of centrosomes. (A) Bars indicate the percentage of mitotic cells with 0, 1, 2, 3, 4 or ≥5 centrosomes (Dgrip84-stained foci that nucleate astral microtubules) in untreated cells (tan; n=401) or cells treated with RNAi to Mob4 (green; n=408) or SAK polo-like kinase (blue; n=400). (Error bars indicate s.d. of mean value of two independent experiments.)
measuring the width of the spindle fiber minus ends at these centrosome-free poles and found that SAK/Mob4 co-depletion increased the focal width by 2.5-fold compared with poles in cells depleted of SAK alone (histograms shown in Fig. 2C). Thus, Mob4 plays a role in K fiber focusing even in the absence of functional centrosomes.

Mob4 and Asp exhibit similar RNAi phenotypes, but Mob4 is not required for Asp localization

In addition to our present work on Mob4, three other proteins have been found to be required for spindle pole focusing in Drosophila cells: dynein, the kinesin-related protein Ncd (both minus-end-directed motor proteins) and Asp. To determine whether Mob4 function might be related to that of dynein, Ncd or Asp, we compared the RNAi spindle pole unfocusing phenotypes of each. As described in previous studies (Goshima et al., 2005; Maiato et al., 2004; Morales-Mulia and Scholey, 2005), RNAi of dynein heavy chain (Dhc64C) primarily disrupted centrosome attachment to K fiber minus ends, with less of an effect on K fiber focusing, whereas RNAi of Ncd generated the opposite phenotype; and depletion of Asp affected both parameters significantly (Fig. 3). The Mob4 RNAi phenotype showed an increase in both centrosome detachment and K fiber unfocusing (Fig. 3B,C). Thus, the spindle unfocusing RNAi phenotype of Mob4 more closely resembled that of Asp than of Ncd or dynein, showing both defective K fiber focusing and loss of centrosome attachment at the poles, although the magnitudes of these defects were greater for Asp than for Mob4. Double RNAi of Asp and Mob4 produced the same phenotype as depletion of Asp alone (data not shown).

The quantitative measurements described above as well as time-lapse imaging suggests that the RNAi phenotype of Mob4 is more similar to Asp than to dynein or Ncd. (A) Representative mitotic spindle morphology after RNAi of the genes indicated. (α-tubulin, red; Dgrip84, green; DNA, blue; bar, 5 µm.) (B) Quantitation of K fiber unfocusing in metaphase spindles. The relative mean width of K fiber minus ends – the distance between the minus ends of the outermost K fibers at each pole (blue line in inset) relative to control cells [average 2.08±0.03 µm (mean±s.d.)] – is shown after RNAi of the gene(s) indicated. RNAi of Mob4, Asp or Ncd induced significant increases in the focal width of K fibers relative to that of control cells, whereas Dhc64C had a minimal effect. Co-depletion of Dhc64C and Ncd had a strong synergistic effect, whereas the synergism in cells co-depleted of Dhc64C and either Asp or Mob4 was minimal. [Error bars indicate the s.d. of the average K fiber unfocusing distance measured in three independent experiments (n>45 spindles for each trial).] (C) Quantitation of centrosome detachment in the same spindles measured in (B). The distance of the gap between the centrosome (Dgrip84-staining foci that nucleate astral microtubules) and the minus end of the K fiber lying closest to the centrosome (green line in inset), relative to control cells [average 1.92±0.07 µm (mean±s.d)] is shown. RNAi of Mob4, Asp or Dhc64C, but not Ncd, caused an increase in centrosome detachment (statistically significant difference for Mob4 RNAi and control cells; P<0.0001). Co-depletion of Dhc64C and either Asp or Mob4 produced a synergistic increase in centrosome detachment, whereas no synergism was observed for co-depletion of Dhc64C and Ncd.
was examined. In this case, Ncd and Dhc64C RNAi synergize to produce significantly greater K fiber unfocusing than is seen for RNAi of either protein alone, while the combined effect of either Asp or Mob4 co-depletion with Dhc64C is only modestly increased relative to the unfocusing defect seen after RNAi of any of the three individually (Fig. 3B). Thus, the synergistic outcomes of double RNAi experiments with Dhc64C reveal similar trends for Asp and Mob4 compared with Ncd. Thus, the phenotypes observed for both single and double RNAi experiments suggest that the Mob4 phenotype more closely resembles that observed for Asp than Ncd.

Because the RNAi phenotype of Mob4 most closely resembled that of Asp, we examined whether Mob4 is necessary for the localization of Asp to the minus ends of K fibers, the major site of microtubule crosslinking in the spindle (Morales-Mulia and Scholey, 2005; Saunders et al., 1997). After Mob4 RNAi treatment, we still observed Asp immunofluorescence at K fiber minus ends, as well as a small amount at the centrosome (Fig. 4). It was difficult, however, to compare the signal intensity with that of wild-type cells as the K fibers were unfocused after Mob4 RNAi. We therefore examined the intensity of Asp immunofluorescence after depletion of Ncd, which is required for K fiber focusing but has no known association with Asp. The Asp staining at the minus ends of K fibers in the splayed spindles of Ncd- and Mob4-RNAi-depleted cells was comparable. Therefore, although we cannot rule out a partial effect on Asp localization, Mob4 depletion does not severely disrupt the recruitment of Asp to K-fiber minus ends and the centrosome.

Mob4-GFP localizes to mitotic centrosomes and kinetochores

To learn more about the functions of Mob4, we made a stable S2 cell line expressing Mob4-GFP and visualized cells showing low levels of expression so as to determine best its cellular localization. In interphase cells, the GFP signal was diffuse through the cytoplasm but showed a clear enrichment in the nucleus (Fig. 5A). At prophase before NEB, a GFP signal could be observed at the centrosomes. After NEB, a bright GFP signal was visible at the spindle poles (Fig. 5B), where it colocalized with γ-tubulin (Fig. 5C), and was also visible above the background throughout the spindle. This localization differs from that of Asp, which is at the minus-end of K fibers and does not colocalize with γ-tubulin at the centrosome. We also observed several GFP foci at condensed chromosomes (Fig. 5B), which closely aligned with the centromere/inner kinetochore marker CID (the fly CENP-A homolog) (Fig. 5D). The Mob4-GFP signal was slightly offset away from CID, suggesting that Mob4 accumulates at the outer kinetochore.

To observe Mob4 localization through mitosis, we performed time-lapse imaging of live cells expressing Mob4-GFP (Fig. 6 and supplementary material Movie 5). GFP fluorescence at centrosomes and the low level of signal throughout the spindle remained constant from prometaphase to anaphase. Kinetochore-localized Mob4-GFP, visible as bright pairs of spots adjacent to the silhouetted, condensed chromosomes, was observed before, during and after transport of the chromosomes to the metaphase plate, as exemplified by the mono-oriented chromosome visible in the left two panels of Fig. 6. This indicated that Mob4 is recruited to the kinetochore independent of whether the chromosome is attached to one or both poles. The kinetochore pair on the mono-oriented chromosome in Fig. 6 appeared to have brighter GFP fluorescence before alignment to the metaphase plate than after, suggesting that Mob4 accumulation at the kinetochore might diminish when the spindle checkpoint is satisfied. However, this effect was small and only observed in three of the five cells that were imaged from early prometaphase through anaphase. Thus, Mob4-GFP is found at several locations in the mitotic spindle and does not undergo pronounced changes in its localization at different stages of mitosis.

Discussion

Our mini RNAi screen of centrosomal proteins has uncovered an unreported phenotype associated with depletion of Mob4, consisting of an increased frequency of monopolar spindles and unorganized bipolar spindles that lack proper pole focusing. The other three Mob proteins did not produce a mitotic RNAi phenotype. The founding member of the Mob family was Mob1p from budding yeast, which interacts with Mps1p, a mitotic kinase required for SPB duplication and spindle checkpoint regulation (Luca and Winey, 1998), and Dbf2p, a multifunctional kinase that localizes to the spindle poles and is part of the mitotic exit network (Frenz et al., 2000). Phylogenetic analysis of the four Drosophila Mob proteins reveals that they belong to separate subfamilies of the Mob superfamily.
Each *Drosophila* Mob protein is more similar to homologs in other species than to each other (supplementary material Fig. S4A). Mob4 shares nearly 80% sequence identity with human phocein (Hmob1, accession no. CAE45270) and an uncharacterized mouse protein (supplementary material Fig. S4B) and is very distantly related to *S. cerevisiae* Mob1p (18% identity; Mob1 and Mob3 are more closely related, with 44 and 33% identity to Mob1p, respectively). Phocein appears to function in peripheral ganglia and dendritic spines of many types of neurons, and, in unpolarized HeLa cells, it localizes to the Golgi (Baillat et al., 2002; Baillat et al., 2001; Moreno et al., 2001). The Mob4 branch of the Mob family is otherwise poorly characterized in the literature. Given the high degree of homology between Mob4 and phocein, it is striking that Mob4 does not appear to localize to the Golgi in *Drosophila*.

Mps1p kinase, regulated in budding yeast by Mob1p, is required for duplication of the SPB in yeast and of the centrosome in human cells (Fisk et al., 2003; Luca and Winey, 1998; Winey et al., 1991). The increased incidence of monopolar spindles after Mob4 RNAi might suggest it has a similar function to that of Mob1p, but we observed a normal number of centrosomes in prophase after Mob4 RNAi. Moreover, we could often discern two (or more) centrosomes at the center of a single monopolar aster in Mob4-depleted cells. Thus, our results indicate that Mob4 is involved in centrosome separation, but not duplication. Our results also indicate that Mob4 has a role in K fiber focusing that is separate from its role in centrosome separation. However, it is possible that our depletion of Mob4 protein is incomplete and that other centrosome defects might emerge with greater knockdown. In our fixed-cell screen, we observed an abundance of disorganized bipolar spindles, and time-lapse imaging of live cells revealed that this disorganization was the result of K fiber detachment from the spindle poles. RNAi of Mob4 in cells depleted of functional centrosomes by SAK RNAi also produced a defect in K fiber focusing at acentrosomal poles. Thus, the Mob4 phenotype differs considerably from the only other known mitotic phenotype described for a Mob protein (spindle pole duplication defects associated with Mob1p mutations in yeast).

We also found that Mob4 localizes to kinetochores. However, Mob4-depleted cells did not display a change in mitotic index, normally indicative of a role in the spindle checkpoint or kinetochore assembly. Moreover, Mob4 depletion did not alter the kinetochore localization of either Rod or dynein (data not shown), two proteins that play roles in the spindle checkpoint (Karess, 2005). Dynein depletion also did not change the levels of Mob4-GFP on the kinetochores, which typically occurs with other checkpoint proteins. Therefore, the function of Mob4 at the kinetochore remains unknown.

Because other Mob family members regulate protein kinases, we postulate that Mob4 might similarly regulate one or more kinases that control the activities of spindle-organizing proteins. A possible candidate Mob4 target is *Drosophila* Mps1 kinase, the target of Mob1p in yeast (Castillo et al., 2002; Fischer et al., 2004; Fisk et al., 2004), but the RNAi phenotype that we observed for Mps1 is unlike that of Mob4 (Table 1). Mob1 and Mob2 also can bind to and activate NDR-family kinases in budding and fission yeast (Frenz...
et al., 2000; Hou et al., 2004; Komarintsy et al., 1998; Mah et al., 2001; Weiss et al., 2002), human (hMob1 and hMob2) (Devroe et al., 2004) and flies (Mob1 and Mob2) (Giot et al., 2003; He et al., 2005; Lai et al., 2005). Interactions between Mob2 and the NDR kinases Tricornered (Trc) and Warts (Wts) have been reported, and the kinase-interacting residues in Mob2 are conserved in Mob4 (He et al., 2005), suggesting the possibility of a similar binding interaction. However, cells treated with dsRNA to either Trc or Wts did not show a noticeable spindle phenotype (data not shown). Thus, the target of Mob4 remains unknown and constitutes an important direction for future work. It is also possible that the phenotype is complex and might involve subtle interactions with multiple kinases or other target proteins that are not easily phenocopied by single RNAi knockdowns.

Our experiments suggest that the microtubule organizer Asp could be a downstream target of Mob4. However, the evidence is somewhat circumstantial, being based primarily on similarities in the RNAi phenotypes of Mob4 and Asp. Nevertheless, the Asp phenotype is rather unique even when the entire genome was explored in a large-scale RNAi screen (Goshima et al., 2007). The only other gene product with an RNAi phenotype virtually indistinguishable to that of Asp is calmodulin (Goshima et al., 2007), which probably binds to the multiple IQ motifs in Asp and is essential for Asp function. The human Mob4 homolog phocein also binds to striatin, a calmodulin-binding protein (Baillat et al., 2001), which probably binds to the multiple IQ motifs in Asp and is essential for Asp function. The human Mob4 homolog phocein also binds to striatin, a calmodulin-binding protein (Baillat et al., 2001), which again raises the possibility of some connection between Mob4 and calmodulin.

Identifying binding partners, target kinases and downstream phosphorylated proteins constitutes the next goal for further understanding how Mob4 functions in mitosis. In addition, as the S2 cells constitute an immortalized cell line with a high percentage of abnormal mitoses (Goshima and Vale, 2003), it will be important to investigate the role of Mob4 in an organism by preparing Drosophila mutants.

Materials and Methods

Cell culture and RNAi

Drosophila Schneider cells (S2) were cultured and RNAi was performed as described previously (Goshima and Vale, 2003; Rogers et al., 2002). Templates for in vitro transcription were generated by PCR using specific primers shown in supplementary material Table S1. dsRNA was generated using the MEGAscript® T7 transcription kit (Ambion). The concentration of dsRNA was estimated by agarose gel and dsRNA was added to cell cultures in 96- or 24-well plates (1 or 5 µg per well, respectively). Cells were examined on day 4 or 5 and day 7 after RNAi treatment. Mob4-GFP (in pmT vector, Invitrogen) was expressed in S2 cells 2-3 days after transfection using Cellfectin (Invitrogen) or after hygromycin selection of stably transfected cells by the addition of 10-50 µM CuSO4 to the culture medium 24 hours before fixation and staining and/or visualization. Cells with low levels of GFP expression were selected for analysis. For fixation and staining, cells were plated in Con-A-coated 96-well imaging plates for 2-3 hours before analysis (Rogers et al., 2002).

Immunofluorescence microscopy and mitotic cell analysis

Cells were fixed in 3-6.4% formaldehyde in HL3 buffer (70 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 20 mM MgCl2, 10 mM NaHCO3, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES, pH 7.2), permeabilized with 0.1% Triton X-100 in PBS (PBST) and then blocked with either 5% normal goat serum in PBST or 5% BSA in PBST. For Asp staining, cells were permeabilized in 0.5% SDS in PBS before incubation with PBST. Cells were then stained with DM1αtubulin (antibody against β-tubulin, 1:1000, Sigma-Aldrich) to label tubulin, antibody against phospho-histone H3 (1:300, Upstate) to label mitotic nuclei, and either GTU-88 (antibody against γ-tubulin, 1:1000, Sigma-Aldrich) or antibody against DrpG84 (1:1000) to label centrosomes. After washing in PBST, cells were stained with secondary antibodies linked to Rhodamine Red-X or Cy2 (Jackson Immunoresearch), washed again and mounted in ProLong Gold with DAPI mounting media (Invitrogen) to stain DNA. Specimens were imaged using a cooled CCD camera ( Cooke Semiscan) mounted on an inverted microscope (Zeiss Axioplan 200M; Carl Zeiss Microimaging), or using IXON cooled CCD cameras (Andor) in 16-bit conventional mode coupled to a wide-field fluorescent microscope developed in the Sedat Lab at UCSF. In the latter case, images were processed with constrained iterative deconvolution (Chen et al., 1996) using experimentally determined optical transfer functions specific for the objective used. The mitotic index was determined using the Cellomics Mitotic Index kit and imaged using a Cellomics ArrayScan (Cellomics). The mitotic index of untreated S2 cells varies between experiments (Goshima and Vale, 2003); therefore we included several control samples in every RNAi experiment. K fiber unfocusing and centrosome detachment distances were measured as described previously (Goshima et al., 2005). Briefly, the distance between the minus ends of the outermost K fibers at each pole (K fiber unfocusing distance) and the distance between the centrosome (DrgpG84-positive foci that nucleate astral microtubules) and the minus end of the K fiber lying closest to it (centrosome detachment distance) were measured in image software. RNAi of either Asp or Ncd frequently induces the formation of spindles with multiple asters and severe abnormalities, whereas RNAi of Dhc54C or Mob4 increases the occurrence of monopolar spindles, but we chose cells with an overall bipolar structure (chromosomes aligned on the metaphase plate) and two centrosomes for measurement.

Live imaging of GFP-tubulin

For time-lapse experiments, image sequences of microtubule dynamics were acquired using a previously characterized GFP-tubulin cell line with a constitutively active promoter (Goshima and Vale, 2003; Rogers et al., 2002) or a stable GFP-tubulin cell line with a metallothionein promoter (Mahoney et al., 2006). Cells were adhered on ConA-treated glass-bottom culture dishes (35 mm, Mattek). The majority of movies were collected at 10-second intervals with 50-200 ms exposure times (at room temperature using a cooled CCD camera Orca-ER2 (Hamamatsu) attached to a Yokogawa spinning-disc confocal scanhead (Solamere Technologies) that was mounted on a Zeiss Axiovert inverted microscope equipped with excitation and emission filter wheels (Sutter Instruments). Camera and AOTF were controlled by Metamorph software on a PC computer (Universal Imaging, Mount Pleasant, PA). Live images were also acquired using the Sedat laboratory microscope mentioned above, using 10-50 ms exposure times at intervals of 10 seconds. Images in this case were acquired with IXON CCD cameras in 14-bit electron-multiplier gain mode.

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References


