Mars, a *Drosophila* protein related to vertebrate HURP, is required for the attachment of centrosomes to the mitotic spindle during syncytial nuclear divisions

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Summary

The formation of the mitotic spindle is controlled by the microtubule organizing activity of the centrosomes and by the effects of chromatin-associated Ran-GTP on the activities of spindle assembly factors. In this study we show that Mars, a *Drosophila* protein with sequence similarity to vertebrate hepatoma upregulated protein (HURP), is required for the attachment of the centrosome to the mitotic spindle. More than 80% of embryos derived from *mars* mutant females do not develop properly due to severe mitotic defects during the rapid nuclear divisions in early embryogenesis. Centrosomes frequently detach from spindles and from the nuclear envelope and nucleate astral microtubules in ectopic positions. Consistent with its function in spindle organization, Mars localizes to nuclei in interphase and associates with the mitotic spindle, in particular with the spindle poles, during mitosis. We propose that Mars is an important linker between the spindle and the centrosomes that is required for proper spindle organization during the rapid mitotic cycles in early embryogenesis.

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Introduction

The proper establishment and maintenance of the bipolar mitotic spindle is essential for the equal segregation of genetic material into the daughter cells. Any defect in this process can result in aneuploidy, which is often associated with tumorigenesis (Nasmyth, 2002; Weaver and Cleveland, 2005). Currently, two mechanisms have been proposed for the formation of the bipolar mitotic spindle in eukaryotic cells. The stochastic ‘search and capture’ model proposes that the centrosomes nucleate microtubules, which capture the kinetochores of chromosomes from both ends to establish the bipolar spindle (Hill, 1985; Kirschner and Mitchison, 1984; Holy and Leibler, 1994). The second mechanism is microtubule nucleation and growth in the vicinity of condensed chromatin, in which Ran-GTP is required as a crucial regulator (Wilde and Zheng, 1999; Khodjakov et al., 2000; Clarke and Zhang, 2008). These two mechanisms may operate in parallel to different extents in different types of cells (Gruss and Verros, 2004; O’Connell and Khodjakov, 2007).

Several microtubule-associated proteins have been identified in vertebrate cells that are required for the efficient assembly of the spindle (Manning and Compton, 2008). The nuclear mitotic apparatus protein (NuMA), together with cytoplasmic dynein and dynactin, accumulates at spindle poles at mitosis, focuses microtubule minus ends and tethers centrosomes to the body of the spindle (Meredes et al., 1996; Meredes et al., 2000). The targeting protein for *Xenopus* kinesin-like protein 2 (TPX2), targets Xklp2 to microtubule minus ends during mitosis and the kinase Aurora A to the spindle (Kufer et al., 2002). TPX2 is also involved in spindle pole organization and centrosome integrity (Wittmann et al., 2000; Garrett et al., 2002). Hepatoma upregulated protein (HURP), localized to kinetochore microtubules in immediate proximity to the chromosomes, increases the efficiency of chromosome capture by microtubule stabilization during mitosis (Koffa et al., 2006; Siljé et al., 2006; Wong and Fang, 2006). The activities of NuMa, TPX2 and HURP are all regulated by high Ran-GTP concentration around chromosomes, which liberates these factors from inhibition by binding to members of the importin β superfamily (Gruss et al., 2001; Wiese et al., 2001; Koffa et al., 2006; Siljé et al., 2006; Wong and Fang, 2006; Clarke and Zhang, 2008).

In *Drosophila*, the minus end directed microtubule motor cytoplasmic dynein is required for spindle pole organization and centrosome attachment to the nuclear envelope and to the mitotic spindle, as in vertebrate cells (Robinson et al., 1999; Morales-Mulia and Scholey, 2005). By contrast, neither NuMa nor TPX2 have obvious structural homologs in *Drosophila*. The Mushroom body defect (Mud) protein shows limited sequence similarity to NuMa and was shown to bind Pins, the fly homolog of the NuMa binding partner Lgn (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Mud is required for correct spindle orientation in neuroblasts (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006) and for meiosis II in female flies (Yu et al., 2006), but a function in spindle pole organization has not been demonstrated so far. The protein encoded by abnormal spindle (asp) localizes to microtubule minus ends at metaphase spindle poles and is required for focussing of spindle poles (Saunders et al., 1997; do Carmo Avides and Glover,
1999; Wakefield et al., 2001; Morales-Mulia and Scholey, 2005). Due to these properties, Asp has been discussed as a functional Drosophila homolog of vertebrate NuMa and TPX2 (Manning and Compton, 2008).

In order to get a more comprehensive picture of the microtubule-associated factors that are required for the proper execution of mitosis in Drosophila, we focused on Mars, the closest relative of vertebrate HURP (Bennett and Alphey, 2004). Previous studies showed that Mars is enriched in mitotic cells and that overexpression of Mars in the eye imaginal disc caused mitotic defects (Yang et al., 2005). However, the precise subcellular localization and function of Mars were unknown. Here we show that Mars is a microtubule-associated protein that translocates from the nucleus to the mitotic spindle in mitosis and is enriched at spindle poles. Loss-of-function mutants of mars are viable and fertile. However, more than 80% of embryos laid by mars homozygous mutant females show severe mitotic defects during the synchronous nuclear divisions at early blastoderm stages. Based on our results, we propose that Mars is required for centrosome attachment to the mitotic spindle and to the nuclear envelope.

Results
Mars shuttles between the nucleus and the mitotic spindle and is enriched at spindle poles
Mars is a rather basic (pI = 10.0) cytoplasmic protein of 921 aa with a predicted molecular weight of 102 kD. It contains a guanylate kinase-associated protein (GKAP) domain that shows significant homology to the GKAP domain of vertebrate HURP (Tsou et al., 2003; Bennett and Alphey, 2004; Yang et al., 2005). In order to determine the expression pattern and subcellular localization of Mars, we performed whole-mount immunofluorescence stainings of embryos using a specific peptide antibody raised against the C-terminus of Mars. In agreement with RNA in situ data available at the Berkeley Drosophila genome project embryo expression database (http://www.fruitfly.org/cgi-bin/ex/insitu.pl), we found that Mars was maternally contributed and ubiquitously expressed during early embryonic development (data not shown). From gastrulation onwards, when the pattern of mitoses became asynchronous, the staining was much more intense in mitotic cells compared to interphase cells (supplementary material Fig. S1). At the subcellular level, Mars showed punctate staining in interphase nuclei at the syncytial blastoderm stage, but was not associated with interphase microtubules (Fig. 1A). At prometaphase, Mars translocated from the nucleus to the area around the centrosomes (Fig. 1B). At metaphase, Mars staining was restricted to spindle microtubules but not asters and was more intense towards the spindle poles (Fig. 1C). At anaphase, Mars localization was restricted to the minus ends of spindle microtubules (Fig. 1D). At telophase, Mars was recruited to the newly formed nuclei and was absent from the central spindle (Fig. 1E). Similar results were obtained in S2r cells (supplementary material Fig. S2). The subcellular localization of GFP-Mars in living embryos at the syncytial blastoderm stage (Fig. 2; supplementary material Movie 1) was consistent with the data obtained in fixed embryos by immunohistochemistry (Fig. 1A-E).

To check whether Mars is present on centrosomes, we performed stainings of embryos for the centrosome marker γ-tubulin and Mars. Our data show that Mars is absent from centrosomes, both in interphase (Fig. 3A) and in metaphase (Fig. 3B).

Microtubule minus end localization of Mars is not disrupted in cytoplasmic dynein, polo and aurora A hypomorphic mutants
The Dynein-Dynactin complex is required for the transport of microtubule-associated proteins such as NuMA and TPX2 to the minus ends of microtubules (Merdes et al., 2000; Wittmann et al., 2000). To test whether the spindle pole localization of Mars was also dependent on the Dynein-Dynactin complex, we analyzed the localization of Mars in embryos mutant for Dhc64C, the gene encoding the dynein heavy chain. As reported

![Fig. 1. Mars shuttles between the nucleus and the mitotic spindle and is enriched at spindle poles. The subcellular localization of Mars was analyzed in fixed wild-type embryos at the syncytial blastoderm stage. (A) At interphase, Mars (red) localizes to the nucleus and does not co-localize with β-tubulin (green). DNA was stained with DAPI (turquoise). (B) At prometaphase, Mars co-localizes with β-tubulin at microtubule asters in the vicinity to the chromatin. (C) At metaphase, Mars is present on the mitotic spindle and is enriched at the spindle poles. (D) At anaphase, Mars remains localized to the spindle poles and is absent from the central spindle and from aster microtubules nucleated at the centrosomes. (E) At telophase, Mars enters the newly formed nuclei and is absent from the central spindle. (F) In mars91 homozygous mutant embryos, Mars is not detectable with the antibody raised against the C-terminus of Mars. Scale bars: 10 μm.](image-url)
previously, mitotic spindles showed loosely attached centrosomes and unfocused spindle poles upon mutation of Dhc64C (Fig. 4A) (Robinson et al., 1999; Morales-Mulia and Scholey, 2005). However, Mars was still enriched at the minus ends of spindle microtubules in Dhc64C mutant embryos (Fig. 4A).

The proper localization of the microtubule-associated protein DTACC to spindle poles depends on phosphorylation by the mitotic kinase Aurora A, and the localization of γ-tubulin and CP190 to the spindle poles depends on Polo kinase (Donaldson et al., 2001; Giet et al., 2002; Barros et al., 2005). In embryos mutant for hypomorphic alleles of aurora A or polo, Mars was enriched at the minus ends of spindle microtubules (Fig. 4B,C) suggesting that either these two kinases are not required for proper spindle localization of Mars or that the low levels of residual kinase activity still present in the homozygous mutant embryos are sufficient for proper localization of Mars.

Spindle localization of Mars is dependent on microtubules
To investigate whether the spindle localization of Mars depends on microtubules, demecolcine was used to depolymerize microtubules in wild-type embryos. This treatment resulted in the complete disappearance of tubulin staining at mitotic figures in embryos at the syncytial blastoderm stage (Fig. 5A). Concomitantly, Mars staining also vanished, demonstrating that the spindle localization of Mars is dependent on microtubules. Moreover, after depolymerization of microtubules, Mars did not associate with any other cellular structure, e.g. the centrosomes or the chromosomes, showing that its localization strictly depends on microtubules. To test whether Mars is physically associated with microtubules, we performed microtubule spin-down assays using Drosophila embryo extracts. In the absence of taxol and GTP, Mars, α-tubulin and the microtubule-associated protein EB1 (Rogers et al., 2002) were in the supernatant (Fig. 5B). In the presence of taxol and GTP, a significant amount of Mars was detected in the pellet together with α-tubulin and EB1 (Fig. 5B). Thus, Mars is a microtubule-associated protein.

The N-terminal region of Mars is necessary and sufficient for spindle localization
With the exception of the GKAP domain, Mars does not contain any protein domains that are recognized by the SMART (http://smart.embl-heidelberg.de/) search algorithm. In order to find out which portions of Mars are responsible for the spindle localization and for the nuclear localization of the protein, we generated a series of hemagglutinin (HA)-tagged deletion versions of Mars (Fig. 6A) and expressed them in S2r tissue culture cells. HA-tagged full-length Mars (HA-Mars-full) localized to the nucleus in interphase (Fig. 6B) and expressed them in S2r tissue culture cells. HA-tagged full-length Mars (HA-Mars-full) localized to the nucleus in interphase (Fig. 6B) and to the mitotic spindle in metaphase (Fig. 6C), consistent with the localization of endogenous Mars (Fig. 1; supplementary material Fig. S2). HA-Mars-N, corresponding to amino acids 1–430 of Mars, showed essentially the same subcellular localization as HA-Mars-full, both in interphase (Fig. 6D) and in

**Fig. 2.** Subcellular localization of GFP-Mars in living embryos. The subcellular localization of GFP-Mars was analyzed in a living embryo at nuclear cycle 11. The dynamics of GFP-Mars localization during mitosis reflects the subcellular localization of endogenous Mars as described in Fig. 1. The elapsed time (in seconds) after the beginning of the time-lapse recording is given in the lower right corner of each image. pUASP-GFP-Mars was driven by the maternal mat67-GAL4 driver. The corresponding movie can be viewed in the supplementary material to this article (Movie 1).

**Fig. 3.** Mars does not localize to centrosomes.
Wild-type embryos at the syncytial blastoderm stage were simultaneously labeled for the centrosome marker γ-tubulin (green) and Mars (red). DNA was stained with DAPI (turquoise). γ-tubulin and Mars did not colocalize in interphase (A) nor in metaphase (B). Scale bars: 10 μm.
metaphase (Fig. 6E). The middle portion of Mars containing the GKAP domain (HA-Mars-M, aa 431-780) was nuclear in interphase (Fig. 6F) but did not localize to the spindle in metaphase (Fig. 6G). The C-terminal region of Mars (HA-Mars-C, aa 781-921) was cytoplasmic in interphase and metaphase and showed neither nuclear nor spindle localization (Fig. 6H, I). Thus, the N-terminal region of Mars appears to be sufficient for proper localization of Mars in interphase and in mitosis and there appears to be a second nuclear localization signal in the middle portion of the protein.

Generation and molecular analysis of mars mutant alleles
To investigate the function of mars, we generated mars mutant alleles by imprecise excision of the P[EP2477] P-element insertion. In this line, the P-element is inserted in the 5'/H11032 UTR of mars, 20 bp upstream of the predicted translation start site (Fig. 7A). The P-element was mobilized by crossing to the Δ2-3 transposase source (Robertson et al., 1988) and excision events were scored by the loss of the white+ marker. Five excision chromosomes carried deletions of chromosomal DNA that extended into the coding region of mars to different degrees. In the homozygous viable mars91 allele, 531 bp of the first exon including the start codon are deleted (Fig. 7A). The homozygous lethal excision chromosome mars102 carries a larger deletion of 6502 bp that completely removes the coding region of mars and extends into the coding region of the adjacent mip120 and EfTuM loci (Fig. 7A), which is the most likely explanation for the lethality of this allele. mip120 mutants are viable but female sterile (Beall et al., 2007), whereas EfTuM is an essential gene (Spradling et al., 1999).

To check whether the homozygous mutant mars91 and mars102 embryos still expressed the Mars protein, we performed western blot analysis. In wild-type embryonic extracts the antiserum affinity-purified against the C-terminal Mars peptide specifically recognized one band of 145 kD that was absent in homozygous mutant mars91 and mars102 embryos (Fig. 7B). The 145 kD band was also detectable in extracts of S2r cells and disappeared after RNA interference (RNAi) directed against mars (Fig. 7C). Conversely, overexpression of Mars in S2r cells resulted in a significant increase of the 145 kD band (Fig. 7D). Indirect immunofluorescence microscopy was performed to check for the presence of Mars immunoreactivity in embryos and S2r cells. Consistent with the
results of the western blots, no specific staining was detected in homozygous mutant mars\(^{91}\) embryos (Fig. 1F; Fig. 8B) and in S2r cells, in which mars had been knocked down by RNAi (supplementary material Fig. S2F).

\(mars^{91}\) mutant embryos show mitotic defects during cleavage divisions

\(mars^{91}\) homozygous mutant females and males are fertile, but 90.8% of embryos produced by homozygous mutant parents died during embryogenesis. Some 9.2% of embryos hatched as larvae but only 5.5% of embryos survived to adulthood. Of the embryos that died, the majority (92.2%) failed to cellularize properly. Heterozygous \(mars^{91}/\text{Df}(2R)\text{CX1}\) animals were also viable and produced offspring with the same percentage of embryonic defects, supporting the argument that \(mars^{91}\) is a strong hypomorphic or amorphic allele of mars. This interpretation is supported by the molecular analysis of \(mars^{91}\), which shows that the translation start site is deleted in this allele, and by the fact that in homozygous \(mars^{91}\) mutant embryos no staining over background levels is detectable with the anti-Mars antibody. The lethality of \(mars^{91}\) mutant embryos derived from homozygous mutant parents was fully rescued by maternal expression of full-length GFP-Mars protein using the UAS-GAL4 system (see Fig. 2; supplementary material Movie 1), demonstrating that the mutant phenotype was caused by mutation of mars and not by a second site mutation on the same chromosome.

To analyze the function of Mars during early embryogenesis, we stained embryos aged 0-4 hours from homozygous mutant \(mars^{91}\) parents with antibodies against β-tubulin, Mars and DAPI. Unlike in wild-type embryos at the syncytial blastoderm stage (Fig. 8A), nuclei and mitotic figures at the cortex of \(mars^{91}\) mutant embryos were unevenly distributed and the synchrony of nuclear divisions was partially lost (Fig. 8B). Several types of mitotic defects were commonly found in fixed \(mars^{91}\) mutant embryos. From the first mitotic division onward, centrosomes were only loosely attached to the mitotic spindle and spindle poles were poorly focussed (Fig. 8D). This phenotype occurred with very high penetrance at early stages of syncytial development (Table 1) and frequently led to complete separation of centrosomes from the spindle. Most likely as a consequence of this primary defect, additional defects in centrosome attachment and spindle organization were observed.
mitotic abnormalities accumulated in the course of the cleavage divisions. Anastral spindles (Fig. 8E) and monopolar spindles with circular chromosomes (Fig. 8F) were the most common phenotype in embryos at later stages of syncytial development (Table 1). Those monopolar spindles always had one, and sometimes two, centrosomin positive dots in their center (data not shown), demonstrating that the monopolar spindles were associated with a centrosome. Monastral monopolar spindles (Fig. 8G) and multipolar spindles (Fig. 8H) were also frequently found (Table 1). As in wild type, in these abnormal spindles every microtubule aster contained a centrosome at the center. Besides those defects, many microtubule asters that were neither attached to a nucleus nor to a mitotic spindle were present at the embryo cortex (Fig. 8I). Those microtubule asters were nucleated by free centrosomes. Like normal centrosomes in wild-type embryos, these free centrosomes showed staining for the centrosome markers γ-tubulin, Cnn (Centrosomin), DTACC and Aurora A (supplementary material Fig. S3).

To better understand the mitotic defects in the mars91 mutant embryos, we performed live imaging of microtubule and chromosome behavior by confocal microscopy. Transgenes encoding ubiquitin-promoter-driven GFP-α-tubulin and histone-3B-RFP were crossed into the mars91 mutant background, which allowed dual color live recording of microtubules and chromatin. These analyses revealed five ways in which free centrosomes were generated in mars91 mutant embryos. (1) In prophase of mitosis, centrosomes moved away from the nucleus (Fig. 9A; supplementary material Movie 2). (2) Centrosomes detached from the mitotic spindle in meta- or anaphase (Fig. 9B; supplementary material Movie 3). (3) Free centrosomes duplicated and separated, which increased the number of free centrosomes (supplementary material Movie 3C). (4) One centrosome moved away from the mitotic spindle after duplication without attaching to a newly formed nucleus (supplementary material Movie 4). (5) Defective nuclei from aberrant mitotic figures dropped from the cortex into the yolk and the centrosomes originating from such nuclei remained in the cortical layer (Fig. 8I; and data not shown). One characteristic feature of these free centrosomes was the excessive nucleation of very long astral microtubules (Fig. 8I).

In order to study the behavior of the chromosomes during mitosis in living mars mutant embryos, we simultaneously imaged histone 3B-RFP and α-tubulin-GFP. Whereas mitoses occurred almost simultaneously and with even spacing between nuclei in wild-type embryos at the syncytial blastoderm stage (supplementary material Movie 5), many irregular mitoses resulting in nuclei of abnormal size and shape were observed in mars mutant embryos (supplementary material Movie 6). Together, all the defects uncovered by live imaging of mars mutant embryos were highly consistent with the phenotypic analysis based on stainings of fixed embryos.

The vast majority of mars91 mutant embryos did not develop beyond cellularization. In those mutant embryos that looked healthy at later stages of embryonic development, we did not detect major abnormalities in spindle morphology, indicating that Mars is not strictly required for proper spindle formation once the rapid cleavage divisions have been completed. Because the phenotypes of mars mutant embryos were very similar to those reported for asp mutants, we tested whether these two genes might function redundantly. Flies homozygous for mars91 and heterozygous for either aspΔ or aspL1 were viable, showing that one intact copy of asp is sufficient to allow normal development in the complete absence of Mars. Flies transheterozygous for aspΔ and aspL1 that were heterozygous for mars91 were also viable, but we never obtained any doubly mutant flies with the genotype mars91/mars91, aspΔ/aspL1 (n=263), suggesting that the two genes indeed function redundantly.

Discussion

Mars is required for the attachment of centrosomes to the nuclear envelope and to the mitotic spindle

In most cell types, centrosomes are tightly linked to the nuclear envelope in interphase and localize to the spindle poles in mitosis (Kellogg et al., 1988; Gonzalez et al., 1998). The attachment of the centrosome to the nuclear envelope and to the mitotic spindle is generally thought to result from the interaction of microtubules nucleated at the centrosome with microtubule-associated proteins located either at the nuclear envelope or at the minus ends of spindle microtubuli (Robinson et al., 1999; Malone et al., 2003; Kwon and Scholey, 2004; Maiato et al., 2004). In mars mutant embryos at the syncytial blastoderm stage, centrosomes frequently detached from nuclei and from mitotic spindles, pointing to a function of
Mars in linking centrosomal microtubules to the nuclear envelope and to spindle microtubules. Like attached centrosomes in wild type, the free centrosomes in mars mutant embryos showed immunoreactivity for γ-tubulin, Cnn, Aurora A and DTACC. The free centrosomes retained their capacity to nucleate microtubules and continued to duplicate and separate, resulting in numerous microtubule asters detached from nuclei. Similar observations have been reported for other situations that result in the formation of free centrosomes (Raff and Glover, 1988; Gonzalez et al., 1990; Yasuda et al., 1991; Debec et al., 1996). Most likely as a secondary consequence of the centrosome detachment, different types of mitotic defects accumulated in mars mutant embryos, including monopolar spindles with circular condensed chromosomes, multipolar spindles and short anastral spindles that were probably organized by the nucleation of microtubules around chromosomes. Thus, the function of Mars is apparently not strictly required for the normal assembly and microtubule-nucleating activity of centrosomes, but rather for the interaction of the centrosomal microtubules with the nuclear envelope and the spindle microtubules.

A very similar phenotype has been described for Dhc64C mutant embryos (Robinson et al., 1999). In these mutants, centrosomes also detached from the nuclear envelope and from mitotic spindles. The authors proposed that dynein associated with the nuclear envelope might be required for attachment of centrosomal microtubules. During mitosis, dynein at the centrosome could be necessary to link spindle microtubules to astral microtubules (Maiato et al., 2004). We have shown that the spindle pole localization of Mars was unaffected in the hypomorphic allelic combination of Dhc64C mutants that we used. This could either mean that dynein is indeed not required for localization of Mars to the minus ends of microtubules or that the levels of dynein still produced from the hypomorphic Dhc64C alleles are sufficient for proper localization of Mars. Nonetheless, the intriguing similarity of the mars and Dhc64C mutant phenotypes suggests the existence of a functional link between these two proteins.

**Table 1. Quantification of spindle phenotypes in mars⁹¹ homozygous mutant embryos**

| Spindle phenotype                                      | Number before nuclear migration (n=141) | Number after nuclear migration (n=188) |
|--------------------------------------------------------|----------------------------------------|---------------------------------------|
| Bipolar spindle with detached centrosomes              | 40 (28.4)                              | 9 (4.8)                               |
| Monastral monopolar spindle                            | 30 (21.2)                              | 29 (15.4)                             |
| Anastral spindle                                       | 25 (17.7)                              | 47 (25.0)                             |
| Circular monopolar mitotic figure                      | 8 (5.7)                                | 66 (35.1)                             |
| Multipolar fused spindle                               | 0 (0.0)                                | 3 (1.6)                               |
| Normal bipolar spindle                                 | 38 (27.0)                              | 34 (18.1)                             |

Mitotic defects were scored at early stages of syncytial development before nuclei had migrated to the cortex and at later stages of syncytial development after migration of nuclei to the cortex. n, number of nuclei scored for each time point. Representative fields containing 10-20 nuclei of at least ten embryos were scored for each time point. Percentages are shown in parentheses.
Is Mars generally required for proper spindle formation in Drosophila?

One surprising finding of our work is the fact that homozygous mars91 mutant flies are viable and even fertile, in spite of the dramatic mitotic defects in more than 80% of mutant embryos. This could be most easily explained if mars91 was a hypomorphic and not an amorphic or null allele. For several reasons we think that this is very unlikely: (1) the phenotype of heterozygous mars91/Df(2R)CX1 embryos is indistinguishable from the phenotype of mars91 homozygous mutant embryos, which is a classical genetic criterion for its classification as an amorphic mutation; (2) the mars91 deletion removes the ATG start codon of the gene. Although apparently an N-terminally truncated form of Mars can be translated in this allele starting from an ATG downstream of the 3' breakpoint of the deletion, this truncated form lacks the N-terminal region of Mars required for spindle localization and thus is presumably nonfunctional. Consistent with this, we did not detect any localized staining for Mars in the mars91 homozygous mutant embryos. A second recently published null allele of mars causes phenotypes essentially identical to the ones we report here, but these embryos never develop beyond the fifth nuclear division cycle (Tan et al., 2008). Whether this apparent discrepancy in the lethality of the two alleles is caused by some minor residual function still preserved in the mars91 allele or by some differences in the genetic background of both alleles remains to be shown.

Based on these results we think that Mars is specifically required for spindle organization during the rapid cleavage divisions in the early Drosophila embryo but becomes dispensable later in embryonic, larval and adult development. The same finding was made for centrosomes, which, quite surprisingly, are not essential for mitosis at later developmental stages (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999; Gergely et al., 2000; Stevens et al., 2007). Consistent with this interpretation, we and others (Goshima et al., 2007) did not observe any dramatic increase of mitotic spindle defects after knockdown of Mars by RNAi in S2 cells compared to controls. However, a recent study quantified defects in mitotic spindle formation after RNAi-mediated knockdown of Mars in S2r cells and found a statistically significant increase in spindles with abnormal kinetochore microtubules (Yang and Fan, 2008). Thus, although Mars does not appear to be essential for proper spindle formation after the rapid cleavage divisions, it may contribute to the efficient formation of kinetochore microtubules at later developmental stages.

Is Mars a functional homolog of HURP?

Homology searches using the BLAST algorithm revealed that the closest vertebrate relative of Mars is the spindle-associated protein HURP (Yang et al., 2005). However, by our analysis of Mars localization and mutant phenotype, it appears that those two proteins may have at least partially different functions in spindle organization. HURP was identified as a component of a Ran-dependent complex in Xenopus egg extract, which also contains Eg5, TPX2, XMAP215 and Aurora A (Koffa et al., 2006). Upon depletion of HURP, HeLa cells showed a delayed transition from prophase to anaphase with frequent misalignment of chromosomes at the metaphase plate (Koffa et al., 2006; Sillje et al., 2006; Wong and Fang, 2006). These data indicate that HURP stabilizes K-fibers and is required for the efficient capture of kinetochores by spindle microtubules. Whether Mars has a similar function in chromosome alignment at the metaphase plate is difficult to answer due to the severe mitotic defects resulting from centrosome detachment.

The subcellular localization of HURP is under control of the Ran-GTP gradient originating from the chromosomes. Ran-GTP negatively regulates the binding of HURP to the nuclear import receptor importin β, which in turn prevents its interaction with microtubules (Sillje et al., 2006). In mitosis, HURP is associated with the spindle and is enriched in the part of the spindle that is close to the chromosomes (Koffa et al., 2006; Sillje et al., 2006; Wong and Fang, 2006). During interphase, HURP levels are strongly reduced and the protein is mainly found in the cytosol, with low amounts detectable in the nucleus (Sillje et al., 2006). By contrast, Mars associates with spindle poles, is not enriched in proximity to the chromosomes in mitosis and is localized in the nucleus in interphase. Our results suggest that the subcellular localization of Mars to the spindle poles may be independent from...
Aurora A, in contrast to HURP, for which phosphorylation of its C-terminal region by Aurora A is required for the association with microtubules (Wong et al., 2008). Again, we cannot exclude the possibility that the low levels of Aurora A activity present in embryos homozygous for the hypomorphic aurA287 allele (Giet et al., 2002) are sufficient for proper localization of Mars. In spite of these differences, the microtubule-binding activity of both HURP and Mars resides in the N-terminal region of both proteins (Wong et al., 2008).

Mars may be functionally related to vertebrate TPX2 and NuMa. The subcellular localization and loss-of-function phenotype of Mars shows striking similarities to the vertebrate Ran-GTP-regulated proteins TPX2 and NuMa. Both proteins are required to ensure normal spindle morphology and spindle pole integrity. Upon knockdown of TPX2, mitotic cells form multipolar spindles in HeLa cells (Garrett et al., 2002). In Xenopus egg extract, the depletion of TPX2 causes less compact spindles and a variety of spindle pole defects (Wittmann et al., 2000). The regulation of TPX2 activity occurs via its binding to importin α, which is mutually exclusive with the binding to microtubules and is regulated by Ran-GTP (Gruss et al., 2001). Very interestingly, TPX2 was found in a complex together with Aurora A, Eg5, XMAP215 and HURP (Kofia et al., 2006). TPX2 is required for targeting Aurora A to the spindle (Kufer et al., 2002; Ozlu et al., 2005) and HURP is a phosphorylation target of Aurora A (Yu et al., 2005; Wong et al., 2008), revealing a functional interaction between TPX2 and HURP.

The second vertebrate protein that resembles Mars with respect to its subcellular localization and loss-of-function phenotype is NuMa. This protein interacts with the dynein-dynactin complex and is required for the focussing of spindle poles and for the tight attachment of centrosomes to the spindle (Meredes et al., 1996; Meredes et al., 2000). Because the phenotype of mars mutants is very similar to the phenotype of cytoplasmic dynein heavy chain mutants (Robinson et al., 1999) and no function in spindle pole focussing and centrosome attachment has been described for Mud, a potential NuMa homolog in Drosophila (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006), we speculate that Mars may be a Drosophila counterpart to NuMa and TPX2 with respect to its function in spindle organization.

Due to its mutant phenotype and its subcellular localization (Gonzalez et al., 1990; Saunders et al., 1997; do Carmo Avides and Glover, 1999; Wakefield et al., 2001), the Asp protein of Drosophila has been discussed as a potential functional equivalent to NuMa and TPX2 with respect to its interaction with TPX2 (Manning and Compton, 2008). In asp mutant embryos, spindle poles are disorganized and centrosomes frequently detach from the mitotic spindle, leading to the formation of free centrosomes (Gonzalez et al., 1990; do Carmo Avides and Glover, 1999; Wakefield et al., 2001; Morales-Mulia and Scholey, 2005). The subcellular localization of Asp overlaps with Mars at spindle poles, but in contrast to Mars, Asp is also localized to centrosomes in mitosis and is enriched at the site of the centrosome facing the spindle microtubules (Saunders et al., 1997; do Carmo Avides and Glover, 1999; Wakefield et al., 2001). Thus, Mars and Asp may have related and possibly redundant functions in spindle pole focussing and attachment of centrosomes to the spindle. Our genetic interaction studies strongly support this interpretation. We never obtained flies doubly mutant for mars and asp, but one intact copy of either mars or asp is sufficient for development to adulthood.

While this paper was under review, two additional reports on the function of Mars in spindle organization were published (Tan et al., 2008; Yang and Fan, 2008). Fully consistent with our results, both studies show that Mars localizes to spindle microtubules, is enriched at the minus ends of microtubules and is absent from centrosomes and astral microtubules. In one study, null mutants for mars were generated, which showed detachment of centrosomes from the spindle during nuclear divisions at the syncytial blastoderm stage (Tan et al., 2008), the same phenotype that we report here. The latter study furthermore showed that Mars binds to protein phosphatase 1 (PP1) and is required for dephosphorylation of dTACC by PP1 on the spindle.

Conclusions

In this work, we have identified Mars as an important regulator of mitotic spindle organization in Drosophila. Although by sequence similarity Mars is the closest homolog to vertebrate HURP, our functional and immunohistochemical analysis suggests that Mars may also be required for functions provided in vertebrate cells by NuMa and TPX2. Future work on the regulation of Mars by Ran, mitotic kinesins and microtubule-dependent motor proteins will shed more light on its function in the assembly of the mitotic spindle.

Materials and Methods

Fly stocks

The P-element insertion P[EP2477] was used for generating deletion mutants of mars by imprecise excision. Df(2RTCX)I extends from 49C1 to 50D1 and removes the whole mars coding region. Stocks of these mutants were kept over the CyO[fwI::GFP] balancer chromosome (gift of Ben-Zion Shilo, Weizmann Institute of Science, Rehovot, Israel). Dhc64C6 and Dhc64C4 were used to generate transheterozygous females, giving rise to embryos with cytoplasmic dynein C-terminal effect phenotypes in syncytial blastoderm embryos as described (Robinson et al., 1999). Embryos mutant for aurA were obtained by crossing homozygous mutant aurA287 females to their male siblings (Glover et al., 1995; Giet et al., 2002). Embryos mutant for polo were obtained by crossing homzygous mutant polo females to their male siblings (Sunkel and Glover, 1988). asp1 and asp2 (Gonzalez et al., 1990) were used to test for genetic interaction with mars. pUASP-GFP-Mars (this work), ubi-tubulin-GFP (gift from Cayetano Gonzalez, IRB Barcelona, Spain) and ubi-histone 3B-RFP (gift from Yohanns Bellaiche, Institute Curie, Paris, France) transgenes were used for live imaging in embryos.

Antibodies and western blotting

Peptide antibodies directed against Mars were raised against the peptides QRIKYLEQESLVS (aa 2-16, at N-terminus) and TLNRRVRLLLLPSFEM (aa 906-921, at C-terminus) in rabbits (Eurogentec, Seraing, Belgium). The final bleed affinity purified against the C-terminal peptide was used for all experiments described in this study.

Primary antibodies were used for western blotting according to standard procedures (Wodarz, 2008) as follows: rabbit anti-Mars (1:1000), rabbit anti-EB1 [1:200 (Rogers et al., 2002)], mouse anti-α-tubulin 12G10 (1:500; DSHB). For the western blot in Fig. 7, the homozygous mutant mars91 embryos were obtained from homozygous mutant parents, whereas the homozygous mutant mars116 embryos were sorted at late embryonic stages for absence of GFP fluorescence from the CyO[fwI::GFP] balancer chromosome.

Immunohistochemistry

Methanol fixation and strong fixation were used in this study as described before (McCarty et al., 1999; Giet et al., 2002). Generally, for methanol fixation, embryos that were 0- to 4-hours old were collected and dechorionated by 50% methanol at room temperature for 10 minutes. After vigorous shaking for 30 seconds, the embryos that sank to the bottom were rinsed three times in methanol. Then the fixed embryos were rehydrated by successive washing in 70, 50 and 30% methanol/PBS for 5 minutes each followed by another 5 minutes incubation in PBS. For strong fixation, embryos that were 0- to 4-hours old were dechorionated in a mixture of 50% bleach and 50% methanol at room temperature for 10 minutes. After vigorous shaking for 30 seconds, the embryos that sank to the bottom were collected and rinsed with 0.1% Triton X-100, 0.7% NaCl and rinsed with 0.1% Triton X-100, 0.7% NaCl after washes. The embryos were transferred into 2 ml heptane and shaken vigorously for 30 seconds. An equal volume of 33% formaldehyde, 50 mM EGTA, pH 8.0 was added to the heptane and the mixture was incubated with gentle shaking for 5 minutes. The aqueous phase was removed and another 3 ml methanol was added. After 30 seconds of vigorous shaking, the embryos that sank to the bottom were collected and...
washed three times with methanol. Embryos were transferred into PBS after rehydration in 50:50 methanol/PBS.

Incubation of fixed embryos with primary and secondary antibodies was done according to standard procedures (Müller, 2008). The antibodies for immunofluorescence were used as follows: rabbit anti-Mars (1:200), mouse anti-β-tubulin E7 (1:50, DSHB), rabbit anti-centrosomin [1:1000 (Vaizel-Ohayon and Schejter, 1999)], rabbit anti-DTACC [1:1000 (Gergely et al., 2000)], mouse anti-p-tubulin (TU-88; 1:1000, Sigma), rat anti-HA 12CA5 (1:1000; Roche).

RNA interference in S2r cells

RNA interference in S2r cells was done as described previously (Giet et al., 2002). The following primers carrying the minimal T7 promoter sequence (5′-TAATACGACCATGGATCATCAAGA-3′) and an N-terminal epitope tag (pAHW) or the UASP promoter and an N-terminal EGFP tag (pPGW).

CATGTCCACC-3′

were depolymerized on ice for 15 minutes. The extract was centrifuged at 16,000 × g for 10 minutes. The supernatant was incubated at room temperature for 30 min to polymerize cold mitotic spindles from embryos were dechorionated in 50% bleach and rinsed with embryo-washing buffer. The treatment of embryos described in (Lu et al., 1999) was modified as follows: embryos were incubated at room temperature for 12 h in Schneider’s medium containing demecolcine (5 μM). The embryos were then transferred into a cold lysis buffer (0.1 M Pipes, pH 6.6, 5 mM EGTA, 1 mM MgSO4, 0.9 M glycerol, 50 μM chromaffin granule protease inhibitor cocktail [Sigma]) at the 5′ end and used to amplify a 3′ fragment of Mars: 5′-T7-GCAGCAGCTTCCCTCGTATCATATA-3′ (forward) and 5′-T7-GGTTGCGCAAACGCTCTCAGAAA-3′ (reverse). Generation of expression constructs

microtubules from embryos was performed as described (Cavey and Lecuit, 2008). Frames were captured every 10 seconds and avi files were generated with a frame rate of 12 frames per second. Movies were further processed using ImageJ (NIH) software.

In vitro microtubule disassembly assay

The treatment of embryos described in (Lu et al., 1999) was modified as follows: embryos were dechorionated in 50% bleach and rinsed with embryo-washing buffer. Embryos were then transferred into Schneider’s medium containing demecolcine (5 μM). For expression of full-length and partially deleted versions of Mars in S2r cells and subsequent analysis by western blot.

Microtubule co-sedimentation

This assay was based on described procedures (Sisson et al., 1997; Lantz and Miller, 1998), which were modified as follows: embryos were dechorionated in 50% bleach and rinsed with embryo-washing buffer. Embryos were then transferred into Schneider’s medium containing demecolcine (5 μM). Samples were examined using a 63×1.4 NA Zeiss Plan-Neofluar oil immersion objective on a confocal laser-scanning microscope (Carl Zeiss LSM 510 Meta).

Microtubule disassembly

Use of fluorescent proteins. In Drosophila embryos. J. Cell Biol. 134, 103-115. This work was supported by grants from the Deutsche Forschungsgemeinschaft to A.W. (SFB 590, TP A2; WO 584/4-1, 4-2; DFG Research Center Molecular Physiology of the Brain, CMPB).

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