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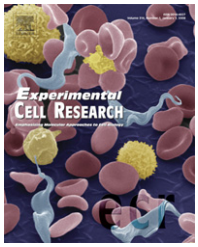

Research Article

Drosophila mars is required for organizing kinetochore microtubules during mitosis

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ABSTRACT

Spindle assembly is essential for the equal distribution of genetic material to the daughter cells during mitosis. The process of spindle assembly is complicated and involves multiple levels of molecular regulation. It is generally accepted that mitotic spindles are emanated from the centrosomes and are assembled in the vicinity of chromosomes. However, the molecular mechanism involved in the spindle assembly during mitosis remains unclear. In this study, we have provided several lines of evidence to show that *Drosophila* Mars is required for the assembly and stabilization of kinetochore microtubules. In an immunocytochemical study, we show that Mars is mainly localized on the kinetochore microtubules during mitosis. Using RNA interference to deplete the Mars expression in *Drosophila* S2 cells resulted in the malformation of mitotic spindle that mainly lacked the kinetochore microtubules. The spindle defect resulted in mitotic delays by increasing the percentage of uncongressed chromosomes both in vitro and in vivo. In summary, this study has extended our previous study of Mars in cell cycle regulation and provided further evidence showing that Mars is required for the assembly of kinetochore microtubules.

Introduction

Accurate distribution of genetic materials to the daughter cells relies on the proper chromosome segregation during mitosis. Chromosome segregation begins by the attachment of sister chromatids to mitotic spindles and is completed by the propagation of chromatids along mitotic spindles to the daughter cells [1]. Thus, the formation of mitotic spindles is required for the chromosome segregation during mitosis. Currently, two models have been proposed to elucidate the mechanisms of spindle assembly. One is the search and capture model, which suggests that all microtubules are nucleated from the centrosomes while the kinetochores simply wait to capture the microtubules to form the typical fusiform mitotic spindle [2–6]. The other model suggests that the chromosome, not the centrosome, drives the mitotic spindle formation [6–8]. Several lines of evidence support the second model; the first evidence is demonstrated by mitotic spindles that are organized perfectly in acentrosomal animal systems [9–11], while the second evidence is shown by small GTPase, Ran, that has been found to nucleate microtubules in the vicinity of chromatin [12–15]. Although extensive studies have uncovered numerous genes that are involved in the spindle assembly during mitosis, the molecular mechanism of spindle formation remains unclear and needs to be further investigated.

The hepatoma upregulated protein (HURP) was initially isolated as a cell cycle regulated gene, which is upregulated in human hepatocellular carcinoma [16]. Peptide analysis indicated that HURP contains a guanylate kinase associated protein (GKAP) domain [16,17]. Biochemical study shows that HURP is the potential target of the Aurora-A kinase, which is a mitotic serine/threonine kinase that is known to regulate mitotic entry, centrosome maturation, and spindle assembly [18–20]. Studies demonstrated

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that HURP is associated with RanGTP and is required for the assembly of the mitotic spindle [21,22]. Wong and Fang further demonstrated that HURP controls the stability of microtubules to ensure spindle formation and thus allows kinetochore to attach to the kinetochore microtubules [23]. In addition, the activity of HURP in controlling the accessibility of its microtubule-binding domain is regulated through Aurora-A kinase [24]. Together, these results suggest that HURP participates in the spindle assembly and regulates chromosome congression during mitosis.

Drosophila Mars, which contains the guanylate kinase associated protein (GKAP) domain, has been found to express in the embryos and adult germline [25]. Overexpression of Mars in the eye disc results in metaphase arrest in the second wave of mitotic cells suggesting that Mars is involved in the cell cycle regulation [26]. In addition, study also finds that Mars promotes dTACC dephosphorylation and ensures spindle stability [27]. In this study we showed that Mars performed similar functions as HURP in forming kinetochore microtubules. Immunocytochemical staining revealed that Mars expression was dynamically changed during the cell cycle progression. It mainly concentrated at the kinetochore microtubules in metaphase cells. In Mars-depleted cells, the kinetochore microtubules were unable to form properly. The consequence of spindle defects impaired the chromosome congression from prometaphase to metaphase both in vitro and in vivo. Together, this study has extended our previous understanding about the role of Mars and provided additional information on the Mars protein in the assembly and stabilization of mitotic spindle.

Materials and methods

Cell culture and dsRNA treatment

Drosophila S2 cells were cultured at 27 °C in Schneider's *Drosophila* media (GIBCO) supplemented with 10% heat inactivated fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin. To deplete Mars expression in S2 cells, two double-stranded RNAs (dsRNAs), *mars* dsRNA-1 (against the base pair 213 to 696 of *mars* RNA) and *mars* dsRNA-2 (against the base pair 1951–2590 of *mars* RNA), that correspond to *mars* transcript were generated by using the PCR. The PCR products, which contain the T7 promoter sequence (5′-TAATACGACTCACTATAG-3′) at each ends, were used to synthesize RNA using a MEGASCRIPT T7 transcription kit (Ambion). The RNAs were annealed to dsRNA by incubation at 65 °C for 30 min followed by slow cooling to room temperature. Fifty micrograms of dsRNA was added to 1.5×10^6 *Drosophila* S2 cells in serum free Schneider's medium for 1 h. After the treatment, cells were grown in serum containing medium for 96 h before being processed for analysis. The dsRNA against GFP was used for the control reaction.

Drosophila stocks and transgenic constructions

Drosophila melanogaster, w^{1118}, was used as the wild type. Transgenic flies that expressed *pUAST-flag-mars* and *pWIZ-mars-RNAi* were described previously [24]. *pUAST-flag-Ran* fly was generated in this study. *GMR-Gal4* and *Tubulin-Gal80^{TS} Tubulin-Gal4/TM6B* were obtained from the Bloomington *Drosophila* stock center. Flies were raised on standard corn meal-agar media at 25 °C.

Generation of anti-Mars antibody

To generate antibodies against the Mars protein, we used PCR to amplify 382 amino acids of N-terminus Mars protein using two primers: 5′-ATCTAGAGATCTCGCCACAAGGAACTGTAC-3′; 5′-GTCCACGTTCTCGCTATGGATATCGTCTAGCGG-3′. The PCR product was cloned to pQE-30 vector (Qiagen, Valencia, CA). After the IPTG induction, a 45 kDa Mars recombinant protein was isolated from *E. coli* and was being used as an antigen. To generate antibody, 500 μg of bacterial expressed Mars protein was mixed with adjuvant and injected into rabbits. After several boosts, the serum was collected and tested for its immunoreactivity using Western blot and immunocytochemistry.

Microtubule sedimentation assay

Purification of embryo extracts was carried out according to Cullen et al. [28] with some modifications. The 0–4 hour old embryos were dechorionated with bleach before being homogenized in BRB80 buffer (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8) containing a cocktail of protease inhibitors including 0.1 mM PMSF, 5 μg/ml Pepstain A, 5 μg/ml Leupetin, 1,0 μg/ml Aprotinin, 20 μM MG132, and 1 mM dithiothreitol. The homogenate was incubated at 4 °C for 10 min and spun at 175,000 g for 1 h at 4 °C. A final concentration of 20 μM of taxol and 1 mM of GTP was added into cell lysate and incubated at room temperature to polymerize microtubules for 30 min. The microtubules and associated proteins were pelleted by spinning at 145,000 g for 1 h at 25 °C through a 30% sucrose cushion in the buffer containing a cocktail of protease inhibitors. The supernatant and pellet were analyzed by Western blotting.

Immunoblotting

For immunoblotting, larvae or S2 cells were collected and homogenized with a homogenization buffer (20 mM HEPES, 150 mM NaCl, 10 mM NaF, 10 mM $Na_4P_2O_7$, 10% Glycerol, and 0.5% NP40) with protease inhibitors (0.1 mM PMSF, 5 μg/ml Pepstain A, 5 μg/ml Leupetin, and 10 μg/ml Aprotinin). The cell extract was then centrifuged at 25,000 *g* for 15 min at 4 °C and subjected to SDS-PAGE to separate the protein. After electrophoresis, the proteins were transferred to a PVDF membrane. To process immunoblotting, the membrane was blocked with 5% non-fat milk in TBST. The membrane was then incubated with anti-Mars antiserum (1:5000) at 4 °C overnight. Next day, the membrane was washed three times with TBST and then incubated with peroxidase conjugated goat anti-rabbit IgG (1:10,000). After the secondary antibody, the membrane was washed and processed for chemiluminescent reaction (Pierces, Rockford, IL). The signals were detected with a cool CCD camera (Fuji film, Japan).

Immunocytochemistry

For immunocytochemistry, dissected eye discs or S2 cells were fixed in 4% paraformaldehyde for 15 min. After three washes and blocking, the samples were incubated with primary antibodies overnight at 4 °C. Primary antibodies used in this study included rabbit anti-Mars (1:500), rabbit anti-pH3 (Upstate Biotechnology, Lake Placid, NY), mouse anti-Flag (Stratagene, La Jolla, CA), mouse anti-α-tubulin (Sigma, St. Louis, Mo), mouse anti-γ-tubulin (Sigma, St. Louis, Mo), chicken anti-CID (kindly provided by Dr. Gary Karpen,

University of California, U.S.A.), and goat anti-cyclin B (Santa Cruz Biotechnology, Santa Cruz, CA). The DNA was stained by 1 μg/ml propidium iodide (Sigma, St. Louis, Mo). After the incubation of primary antibodies, cells or eye discs were washed three times with PBST and incubated with secondary antibodies. Secondary antibodies used in this study were conjugated with Alex 488, Alex 543, Alex 647 (Invitrogen Molecular Probes, Carlsbad, CA), and FITC (Jackson ImmunoResearch Lab. West Grove, PA). After three washes, cells and eye discs were mounted in the mounting medium (0.25% *n*-propyl gallate, 50% glycerol in PBS, pH 8.6) and were observed on a Zeiss LSM 510 confocal microscope. Images were processed using the Adobe Photoshop software. For quantitative analysis, the data were analyzed by either Student's *t*-test or Tukey's test. The sampling of individual experiment was stated in the result section.

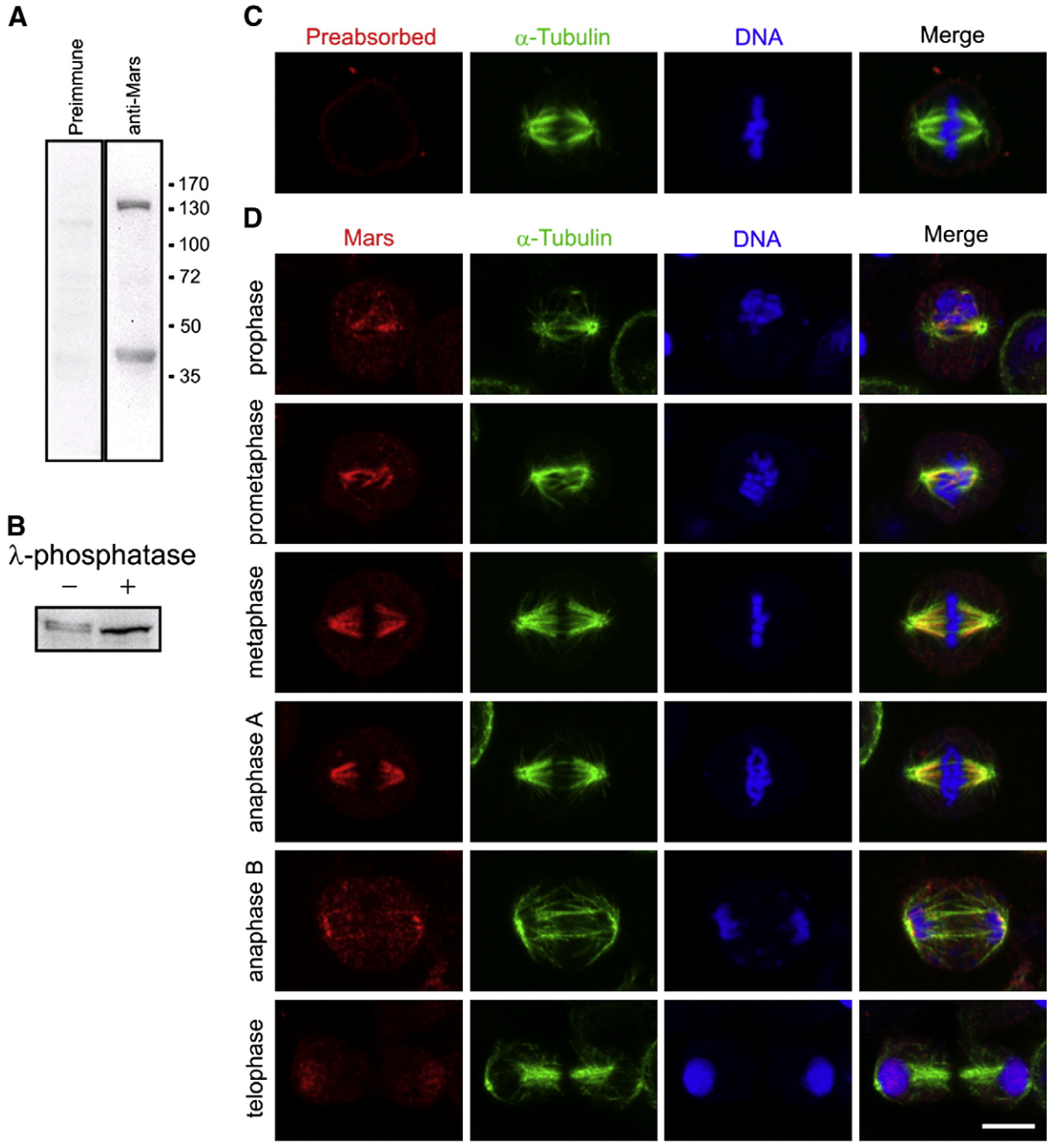

Fig. 1 – Mars was localized at mitotic spindles in mitotic cells. (A) Anti-Mars antiserum specifically detected a 130 kDa Mars protein and an unknown protein at 40 kDa in *Drosophila* S2 cell extract. No signal was detected using preimmune serum in the same condition. (B) The S2 cell extracts were treated with buffer alone or with λ-phosphatase. The treated cell lysate was analyzed by Western blotting with anti-Mars antiserum. The mobility up-shift was abolished after being treated with λ-phosphatase suggesting that Mars is a phosphorylated protein. (C) S2 cells were stained with anti-Mars antibody that was preabsorbed with recombinant Mars protein. No significant signals were detected suggesting that anti-Mars antibody is specific to Mars protein. (D) Confocal images showed the subcellular localization of Mars protein in mitotic S2 cells. Cells were stained as follows: anti-Mars antibody (red), anti α-tubulin antibody for mitotic spindles (green), and propidium iodide for DNA (blue). Scale bar=5 μm.

Results

Mars is localized to spindle microtubules during mitosis

We have previously shown that Mars was involved in the cell cycle regulation [26]. To further study the function of Mars in cell division, a specific rabbit polyclonal antibody was generated against the amino-terminal of Mars (amino acid 3 to 384). Immunoblotting showed that anti-Mars antibody detected two doublets at 130 kDa in cell lysate of the *Drosophila* Schneider 2 (S2) cells (Fig. 1A) and embryo extracts (data not shown). After treating with λ-phosphatase, the up-migrated band disappeared and only the lower band left, suggesting that Mars is a phosphorylated protein (Fig. 1B). To test the specificity of anti-Mars antibody, we preabsorbed this antibody with recombinant Mars protein. We then used this preabsorbed antibody to stain the S2 cells. The result showed that preabsorbed antibody failed to detect any specific signals in S2 cells suggesting the anti-Mars antibody reported in this study is specific to Mars protein (Fig. 1C).

With the antibody in hand, we then studied the subcellular localization of Mars in the S2 cells using indirect immuno-

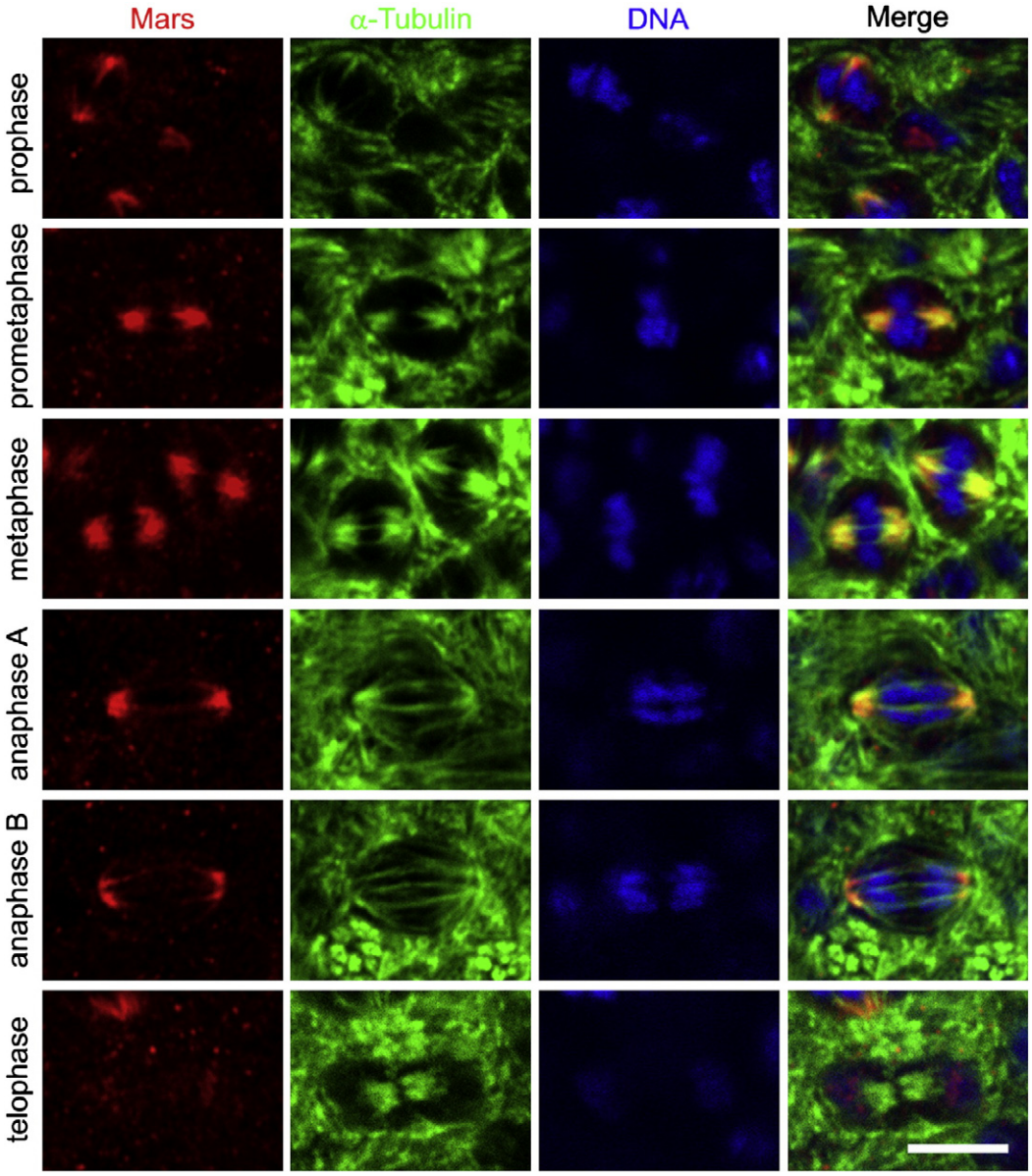

Fig. 2 – Mars specifically localized at the mitotic cells in third instar eye disc. Confocal images showed that subcellular localization of Mars protein in mitotic cells. In the prophase cells, Mars protein was diffused in the cytoplasm. In the prometaphase cells, Mars protein began to concentrate at the mitotic spindles. In the metaphase and the early anaphase cells, the Mars protein was co-localized with a majority of the spindle microtubules. As cells entered the anaphase B, Mars appeared to slightly concentrate at the spindle poles. Mars staining was gradually degraded as cells entered the telophase. Eye discs were stained as follows: anti-Mars antibody (red), anti α-tubulin antibody for mitotic spindles (green), and propidium iodide for DNA (blue). Scale bar=5 μm.

fluorescence microscopy. As cells entered mitosis, the subcellular localization of Mars changed dynamically. In the prophase cells, Mars protein was diffused in the cytoplasm but some began to concentrate at the mitotic spindles (Fig. 1D). In the prometaphase cells, Mars protein began to concentrate at the mitotic spindles (Fig. 1D). In the metaphase and the early anaphase cells, the Mars protein was co-localized with a majority of the spindle microtubules but was excluded from the centrosomes and the astral microtubules (Figs. 1D and 8A). As cells entered the anaphase B, Mars appeared to slightly concentrate at the spindle poles (Fig. 1D). Finally, Mars staining was gradually degraded which appeared as faint staining in the nucleus as cells entered the telophase (Fig. 1D).

In addition to the S2 cells, Mars protein was also expressed in the mitotic cells in vivo. When we used anti-Mars antibody to probe the third instar eye discs in the wild type flies, we found that Mars was only detected in the mitotic cells but not in the differentiated photoreceptor cells. This result is consistent with our previous study using transgenic flies to express exogenous Flag-Mars in developing eye discs [26]. Subcellular localization of Mars in mitotic cells of the third instar eye discs was similar to that shown in the S2 cells (Fig. 2). Together, these results indicated that Mars was predominantly expressed in mitotic cells suggesting its function in regulating mitosis.

Mars is predominantly localized in the kinetochore microtubules

Double immunostaining of Mars and α-tubulin in S2 cells revealed that Mars was only overlapped with a subset of spindle microtubules (Fig. 1D). To further investigate which parts of microtubules that Mars is associated with, we performed the cold treatment to dissociate the non-kinetochore microtubules [22,29]. In normal S2 cells, α-tubulin revealed two subsets of mitotic spindles: one appeared as thick bundles (Fig. 3A, arrowheads) and the other appeared as thin fibers (Fig. 3A, arrows). After the cold treatment, the majority of thin fibers in the S2 cells were disappeared and only thick kinetochore microtubules remained. In this condition, we found that Mars was co-localized with the remained thick kinetochore microtubules (Fig. 3A).

To further confirm that Mars was associated with kinetochore microtubules, we stained the S2 cells with antibodies against Mars and CID, the inner kinetochore marker [30,31]. In metaphase and anaphase A cells, all Mars-positive fibers were terminated at

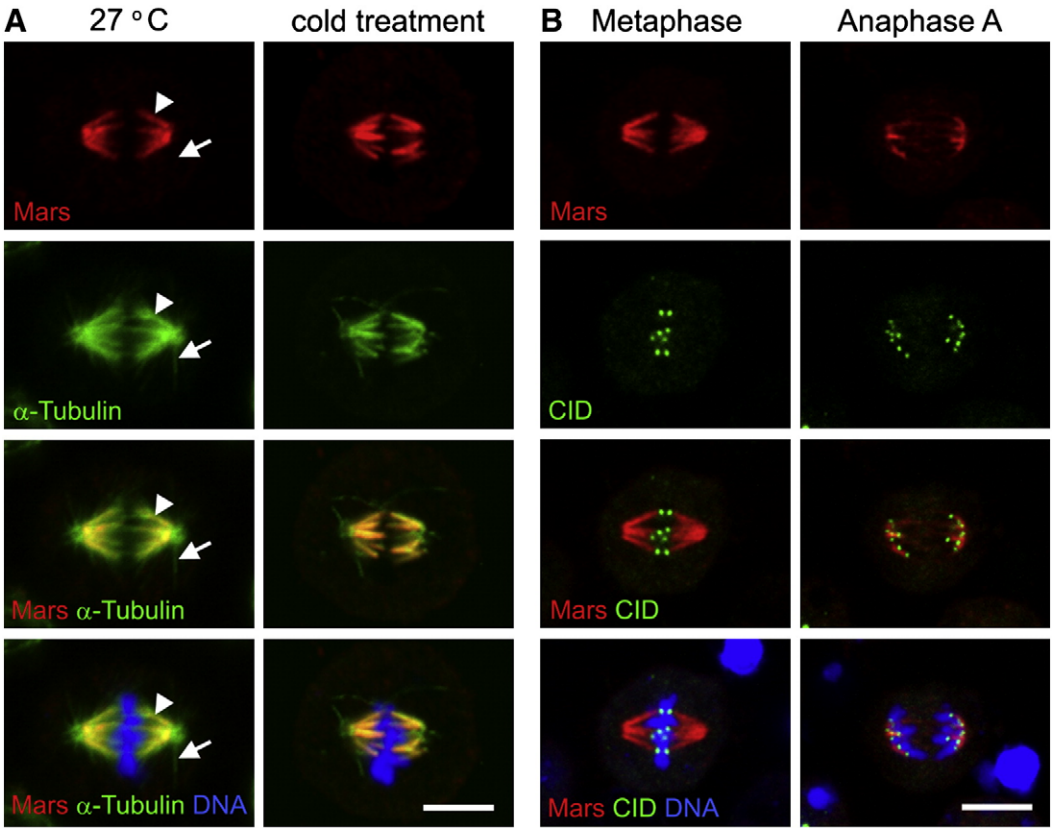

Fig. 3 – Mars was associated with kinetochore microtubules. (A) Using cold treatment for 20 min on ice to disassemble the non-kinetochore microtubules, we found that Mars staining was mainly localized to the kinetochore microtubules. Cells were fixed and probed with anti-Mars antibody (red), anti-α-tubulin antibody (green), and propidium iodide for DNA (blue). (B) Confocal images showed the metaphase and anaphase S2 cells were stained with anti-Mars antibody (red), anti-CID antibody for the kinetochore (green), and propidium iodide for DNA (blue). The arrowheads indicated kinetochore microtubules while the arrows indicated non-kinetochore microtubules. Scale bar=5 μm.

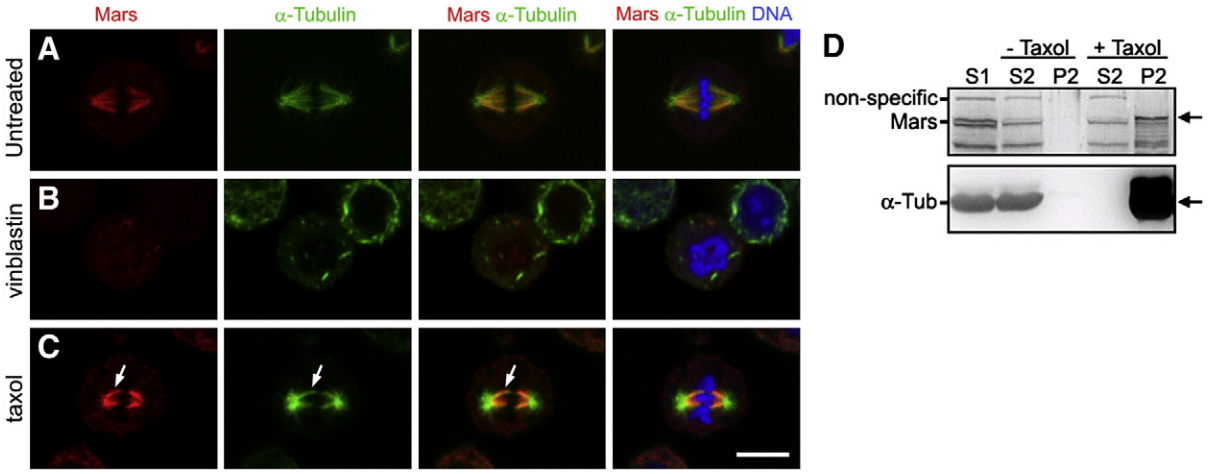

Fig. 4 – Mars localization was microtubule dependent. The S2 cells were (A) untreated or treated with (B) 5 μM vinblastin for 20 min to depolymerize microtubules and (C) 1 μM taxol for 20 min to stabilize microtubules. Treated cells were probed with anti-Mars antibody (red), anti-α-tubulin antibody (green), and propidium iodide for DNA (blue). (D) Crude protein extract from 0 to 4 hour embryos was incubated on ice to depolymerize microtubules. The high speed supernatant (S1) was incubated with taxol and GTP to repolymerize the microtubules. Microtubules and associate proteins (P2) were separated form soluble proteins (S2) by ultracentrifugation and analyzed by Western blotting with antibodies against Mars (top) and α-tubulin (bottom). Arrows indicate that Mars and α-tubulin were present in the microtubule fraction. Scale bar=5 μm.

CID-stained kinetochore (Fig. 3B). This result further demonstrated that Mars was localized predominantly to the kinetochore microtubules.

Spindle localization of Mars is microtubule dependent

We have shown that Mars was localized at the kinetochore microtubules. We then asked whether the localization of Mars protein at the kinetochore microtubules is microtubule dependent. To answer this question, we treated cells with vinblastin and taxol to interfere with the dynamic of microtubules so we could observe the subcellular localization of Mars. In untreated cells, the spindle was a typical fusiform and Mars was co-localized with the kinetochore microtubules (Fig. 4A). When cells were treated with vinblastin, most of microtubules were depolymerized and the spindle pattern of Mars was also missing (Fig. 4B). After using taxol to stabilize microtubules in the S2 cells, we found that Mars was only localized to a subset of microtubules connected to chromosomes, but not in the centrosomes and asters (Fig. 4C, arrows). This result demonstrated that the localization of Mars on kinetochore microtubules was in a microtubule dependent manner.

In addition, we also performed a microtubule sedimentation assay in order to demonstrate that Mars is a microtubule associated protein. We isolated high speed supernatant (S1) from embryos (Fig. 4D). After treating microtubules in S1 with Taxol and GTP, the extracts were centrifuged to separate the

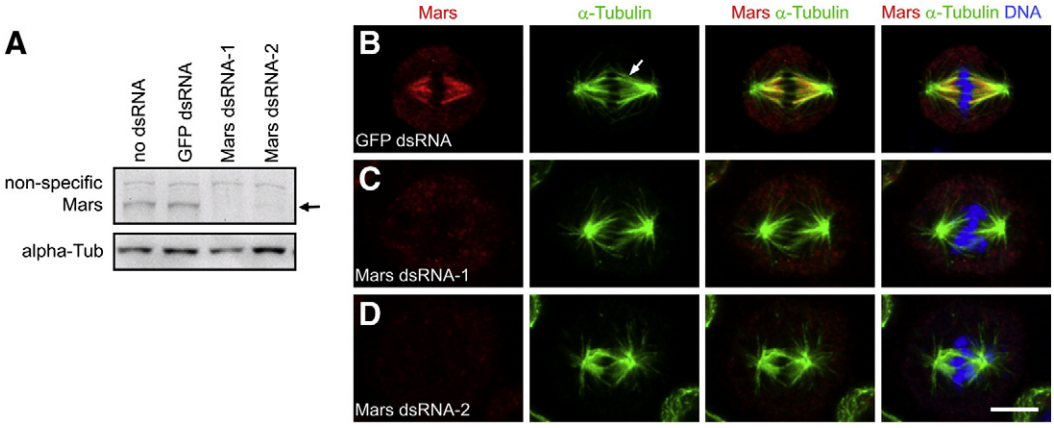

Fig. 5 – The depletion of Mars affected the spindle assembly. (A) Western blot indicated that *mars* dsRNA could specifically downregulate Mars expression in S2 cells. The S2 cells were treated with (B) control dsRNA, (C) *mars* dsRNA-1, and (D) *mars* dsRNA-2. After dsRNA treatment, cells were fixed and stained for anti-Mars antibody (red), anti-α-tubulin for spindles (green), and propidium iodide for DNA (blue). Scale bar=5 μm.

extracts into microtubule fraction (P2) and soluble fraction (S2) (Fig. 4D). After being subjected to electrophoresis and transferred to the membrane, the proteins were probed with anti-Mars and anti-α-tubulin antibodies. The result indicated that both Mars and α-tubulin were found in the microtubule fraction (Fig. 4D, arrows). Interestingly, the majority of Mars, presented in the microtubule fraction, appeared as an up-migrated band suggesting that phosphorylated Mars instead of non-phosphorylated Mars was associated with microtubules. Together, these results demonstrated that Mars is a microtubule associated protein.

Depletion of Mars affects the assembly of kinetochore microtubules

The above studies showed that the spindle localization of Mars was microtubule dependent. We next asked whether Mars is responsible for controlling the microtubule assembly. To perform this experiment, we used RNA interference (RNAi) to deplete the Mars expression in S2 cells and then used anti α-tubulin antibody to detect the array of microtubules in mitotic cells. In this study, we used two dsRNA fragments, which correspond to

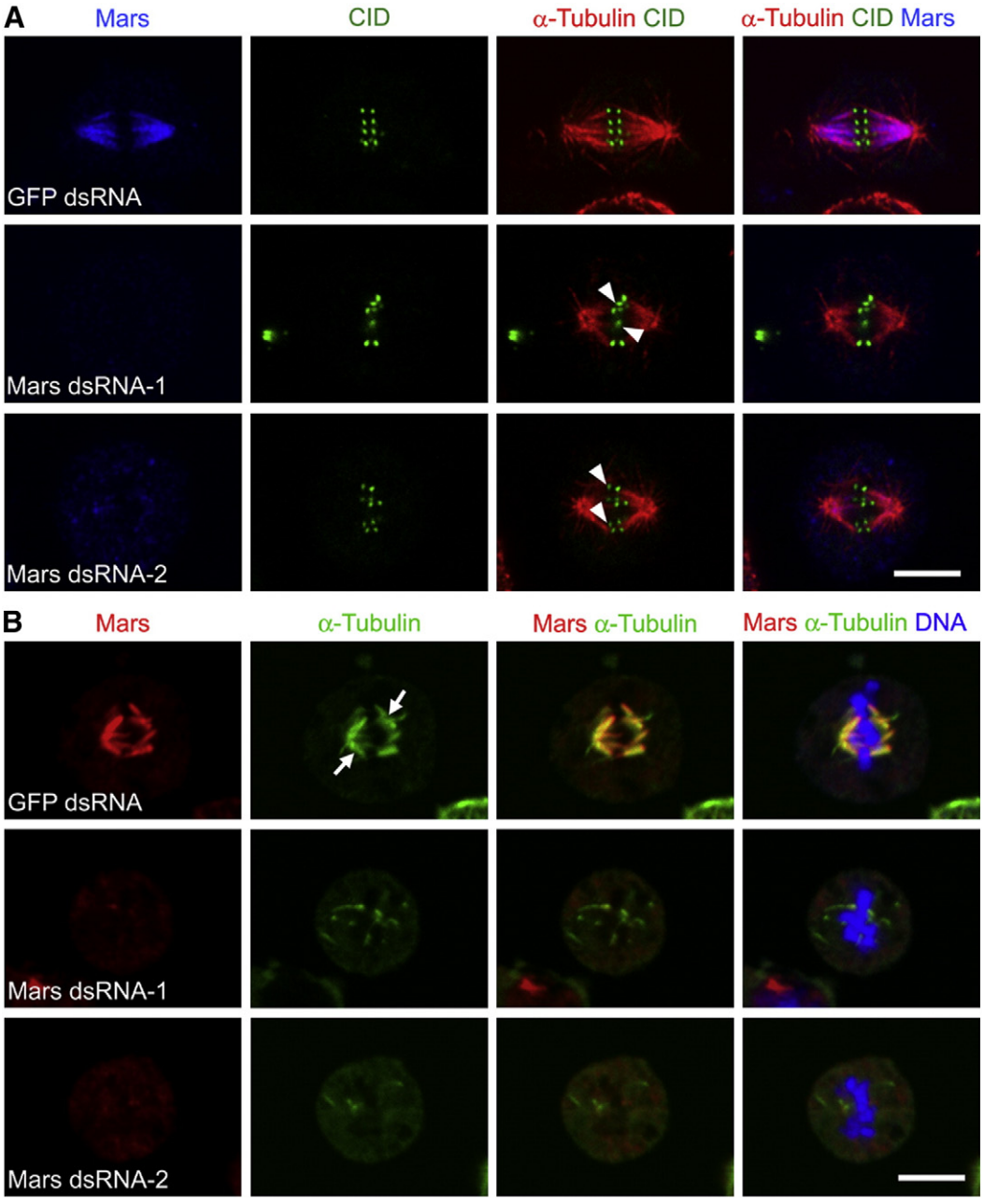

Fig. 6 – Mars was specific for the assembly of kinetochore microtubules. (A) Control and Mars-depleted cells were probed with anti-Mars antibody (blue), anti-CID antibody (green), and anti-α-tubulin antibody (red). (B) S2 cells were treated with control and Mars-specific dsRNA following by the cold treatment. After treatment, cells were stained with Mars (red), α-tubulin (green), and propidium iodide for DNA (blue). Arrowheads indicate the CID spots without being attached to the kinetochore microtubules. Arrows indicate the cold-resistant kinetochore microtubules. Scale bar=5 μm.

two distinct coding sequences of the *mars* transcript, to treat S2 cells. To avoid the off-target effect, we used blast search to compare the *mars* RNA fragments against the *Drosophila* expressed genome. There was only one twenty-nucleotide fragment in the *mars* dsRNAi-2 fragment that is similar to the *nebbish* gene which encodes a kinesin-like protein, klp38B. Other than this, no significant homology was found between *mars* and any other expressed genes, suggesting the *mars* dsRNA were specific to the *mars* gene. We then treated S2 cells with *mars* dsRNA and the cell lysate was harvested to analyze the Mars expression by using the Western blotting. Immunoblotting showed that S2 cells treated with *mars* dsRNA-1 and *mars* dsRNA-2 almost completely blocked Mars expression (Fig. 5A). After testing the efficiency of the *mars* dsRNA in the down-regulation of Mars expression, we proceeded to determine the effects of Mars-depleted cells in the formation of the mitotic spindle. In the control dsRNA treated cells, a typical fusiform spindle in metaphase cell (Fig. 5B) was detected by the anti-α-tubulin antibody. In Mars-depleted cells, the array of spindle microtubules became irregular; usually the central portion of the thick fibers was missing (Figs. 5C, D).

The missing thick fibers in the central portion of mitotic spindle in Mars-depleted cells made us wonder whether Mars is involved in the formation of kinetochore microtubules. To answer this question, we stained the dsRNA treated cells with anti-α-tubulin and kinetochore marker, CID. In the control metaphase cells, the CID staining appeared as two parallel lines on the equator. Each CID spot matched perfectly to the terminus of the kinetochore microtubules (Fig. 6A). In Mars-depleted cells, the array of CID became irregular (Fig. 6A). There were an increasing number of CID spots that failed to make contact with kinetochore microtubules suggesting a defect in the formation of kinetochore microtubules in Mars-depleted cells (Fig. 6A, arrowheads). Quantitative analysis showed that there were 26.5±5.8% of cells with unattached CID spots in the control cells (five experiments, 280 cells were counted), however, the percentage increased to 53.8±5.0% in *mars* dsRNA1-treated cells (five experiments, 276 cells were counted) and 61.2±3.9% in *mars* dsRNA-2-treated cells (five experiments, 275 cells were counted). Statistical analysis indicated that the number of unattached CID spots was significantly increased in *mars-RNAi* treated cells (Student's *t*-test, $p<0.001$).

In addition, cold treatment can induce depolymerization of the non-kinetochore microtubules but not the kinetochore microtubules. If Mars is required for the formation of kinetochore microtubules, we were unable to detect the kinetochore microtubules in cells simultaneously treated with cold-condition and *mars* dsRNA. Indeed, that was exactly what we found. In untreated and control dsRNA treated cells, most of microtubules were depolymerized, except for some kinetochore microtubules which remained after the cold treatment (Fig. 6B, arrows). In contrast, no cold-resistant kinetochore microtubules were observed in Mars-depleted cells (Fig. 6B). Since RanGTP helps to organize kinetochore microtubule [12, 13, 14], we then investigated whether RanGTP regulates the Mars activities. Using *GMR-Gal4* to activate Ran expression in the eye discs, we found that Mars staining in the mitotic cells became more intensive and condensed as compared to the control cells (Fig 7A). Quantitative analysis showed that the total fluorescence intensity of Mars in *GMR>Flag-Ran* metaphase cells was significantly higher than *GMR-Gal4/+* metaphase cells (Student's *t*-test, $p>0.0001$) (Fig. 7B). Together, these results strongly demonstrated that Mars was required for organizing the kinetochore microtubules.

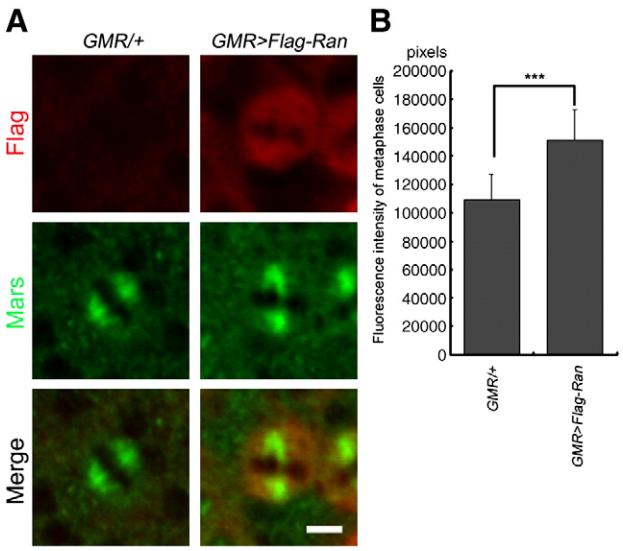

Fig. 7 – Ran regulated Mars expression. (A) The third instar eye discs were dissected from *GMR-Gal4/+* and *GMR>Flag-Ran* transgenic flies and stained with anti-Mars antibodies. (B) The intensity of Mars signal was compared between *GMR-Gal4/+* and *GMR>Flag-Ran* flies using LSM 510 Histo software. The total fluorescence intensity of Mars in *GMR>Flag-Ran* metaphase cells was significantly higher than *GMR-Gal4/+* metaphase cells (Student's *t*-test, $p>0.0001$). Scale bar=5 μm.

Mars affects the localization of γ-Tubulin at spindles but not at the centrosomes

The γ-tubulin, a key regulator for initiating the spindle assembly, is found in both centrosomes and spindles [32]. The importance of Mars in organizing kinetochore microtubules prompted us to investigate whether Mars also affects γ-tubulin localization in mitotic cells. To perform this study, we stained the Mars-depleted S2 cells with anti-γ-tubulin antibodies. In the control metaphase cells, γ-tubulin staining appeared in two populations: the centrosomes and mitotic spindles (Fig. 8A, arrowheads). In Mars-depleted cells, γ-tubulin staining remained at the centrosomes but became diminished in the spindles (Fig. 8A, arrows).

In addition, we also found that the pole to pole distance was slightly reduced in Mars-depleted cells. In untreated and dsRNA control cells, the average pole to pole distance was 6.72±0.93 μm (three experiments, 67 cells were counted) and 6.55±0.77 μm (three experiments, 85 cells were counted), respectively. In Mars-depleted cells, the average of pole to pole distance decreased to 5.95±0.90 μm in *mars* dsRNA-1 (three experiments, 81 cells were counted) and 5.96±0.95 μm in *mars* dsRNA-2 (three experiments, 86 cells were counted) (Fig. 8B). Statistical analysis showed a difference in the average pole to pole distance between the control and Mars-depleted cells (Tukey's test, $F_{3,315}=15.38$, $P<0.0001$). These results suggested that Mars regulates γ-tubulin localization at the mitotic spindle and determine the spindle length.

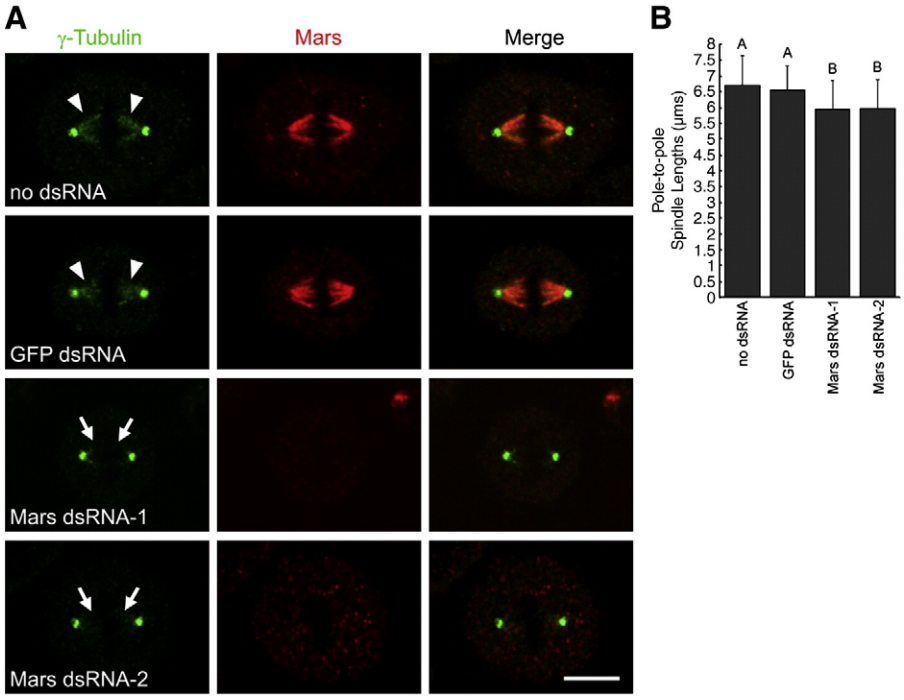

Fig. 8 – Depletion of Mars disturbed spindle localization of γ-tubulin and decreased spindle length. S2 cells were treated with control and Mars-specific dsRNA and probed with anti-Mars antibody (red) and anti-γ-tubulin antibody (green). (A) Confocal images showed the γ-tubulin staining in the control and Mars-depleted cells. (B) Systematic analysis of the pole to pole distance between the control and Mars-depleted cells showed that such distance decreased in Mars-depleted cells (Tukey's test, SAS 1985, A vs. B, $P<0.0001$). Arrows indicate the γ-tubulin staining in the spindles. Arrowheads indicate the missing of γ-tubulin staining in the spindle of Mars-depleted cells. Scale bar=5 μm.

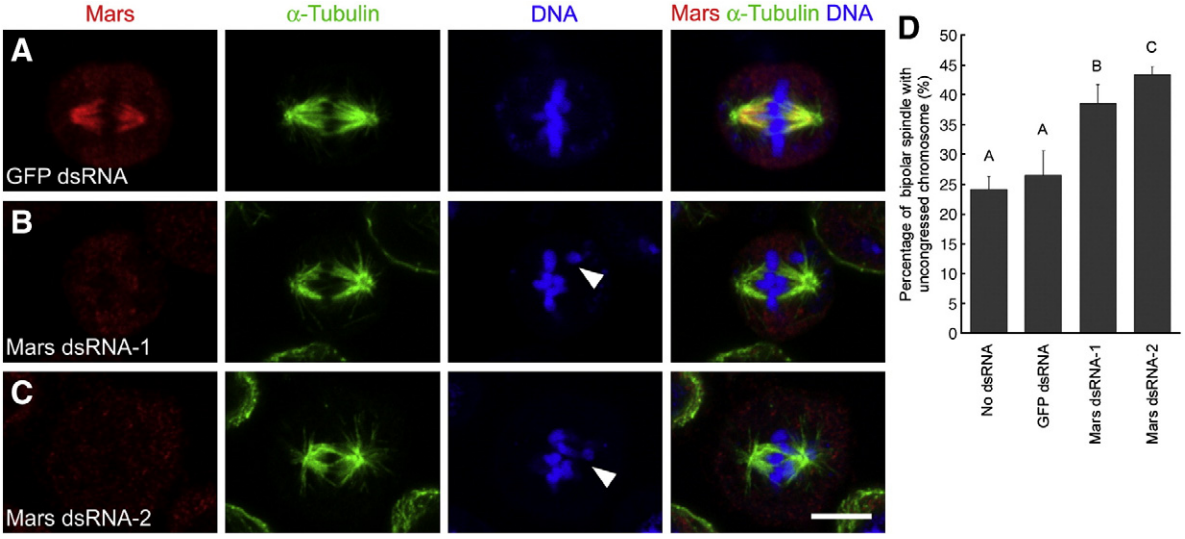

Fig. 9 – Mars depletion increased the number of cells with uncongressed chromosomes. S2 cells were treated with control and Mars-specific dsRNA. After treatment, cells were probed with anti-Mars antibody (red), anti-α-tubulin antibody (green), and propidium iodide for DNA (blue). (A) In control cells, most cells with bipolar spindles have their chromosomes aligned at the equator. (B–C) In Mars-depleted cells, the chromosomes failed to congress properly at the equator in many cells with bipolar spindles. (D) Systematic analysis indicated that the percentage of uncongressed chromosomes increased in the Mars-depleted cells. The bar graph shows the results from five independent experiments (>500 cells in each set of studies) and error bars indicate standard deviations. Bars that do not share letters were significantly different from each other (Tukey's test, SAS 1985, A vs. B, $P<0.0001$, B vs. C $P<0.001$). Arrowheads indicate uncongressed chromosomes. Scale bar=5 μm.

Depletion of Mars affects chromosome congression

We have shown that Mars is required for the assembly of kinetochore microtubules. We then asked whether this defect affects chromosome congression in mitosis. Most of the control cells with bipolar spindles have their chromosomes aligned properly at the equator (Fig. 9A). In Mars-depleted cells, the percentage of bipolar spindle cells with properly aligned chromosomes became less abundant (Figs. 9B, C). After systematically counting the percentage of mitotic cells with uncongressed chromosomes in control and *mars* dsRNA treated cells, we found that depletion of Mars expression increased the percentage of uncongressed chromosomes from 25% to 40% (five independent experiments, >500 cells were counted in each set of studies). Statistical analysis indicated this increase as being significant (Tukey's test, $F_{3,13}=39.35$, $P<0.0001$) (Fig. 9D).

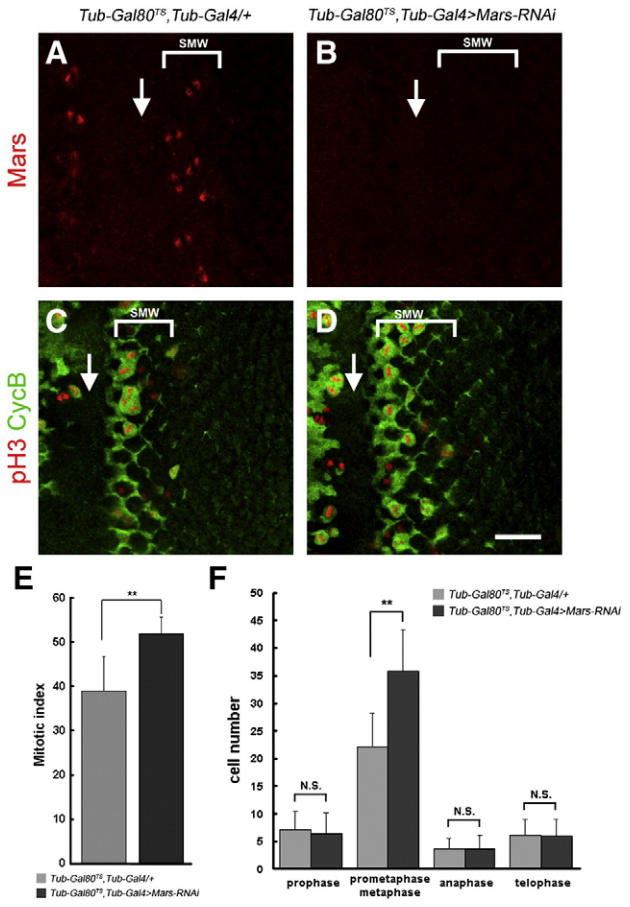

Fig. 10 – Depletion of Mars expression delayed mitotic progression in vivo. (A) The third instar eye discs from (A) control and (B) *Tubulin>mars-RNAi* were stained for anti-Mars antibody to detect the Mars expression. The eye discs were then stained for anti-cyclin B (green) and anti-pH3 (red) antibodies to detect the mitotic cells in the second mitotic wave of (C) control and (D) *Tubulin>mars-RNAi*. (E) Quantitative analysis revealed that the number of mitotic cells in *Tubulin>mars-RNAi* eye discs were significantly higher than in the control. (F) Systematic analysis of the phase of mitotic cells in the second mitotic wave revealed that the number of prometaphase and metaphase cells increased significantly in the *Tubulin>mars-RNAi* eye discs. This result suggested that the occurrence of a mitotic delay was related to the depletion of Mars expression. Arrows indicate the morphogenetic furrow. SMW indicates the second mitotic wave. Scale bar=20 μm.

This in vitro result was further strengthened by observing the mitotic behavior in vivo. In the third instar eye disc, there is a wave of synchronous mitotic cells that is called the second mitotic wave (SMW), which occurred after the morphogenetic furrow. We previously used *GMR-Gal4* activator to drive the expression of *mars-RNAi* in transgenic fly; however, it did not generate any obvious mitotic phenotype [26]. Using anti-Mars antibodies to detect the Mars expression in the eye disc, we found a significant amount of Mars remaining at the SMW (data not shown). In this study, we used the *Tubulin-Gal80^{TS}, Tubulin-Gal4* activator to drive the expression of *mars-RNAi* in transgenic fly. Immunostaining revealed that Mars expression was almost completely blocked in this eye disc (compared Fig. 10A and Fig. 10B). Thus, we stained the control and *Tubulin-Gal80^{TS}, Tubulin-Gal4/pWIZ-mars-RNAi (Tubulin>mars-RNAi)* eye discs with phosphorylated histone H3 and cyclin B antibodies to detect mitotic cells (Figs. 10C, D). After performing systematical counts of the mitotic cells in the second mitotic wave, we found that there were about 39.0±7.7 ($n=20$) and 51.8±8.3 ($n=30$) mitotic cells in the control and Mars-depleted eye discs, respectively (Fig. 10E). Statistical analysis indicated that the mitotic index was significantly different between the control and *Tubulin>mars-RNAi* flies (Student's *t*-test, $p<0.001$).

To investigate the cause of high mitotic index in Mars-depleted eye discs, we compared the population of mitotic cells in the control and *Tubulin>mars-RNAi* flies. A systematic analysis of the stages of mitotic cells in the SMW showed that there were 7.2±3.4 in prophase, 22.1±6.0 in prometaphase and metaphase, 3.6±1.9 in anaphase, and 6.1±2.9 in telophase in control flies ($n=20$ eye discs). However, the number of mitotic cells at SMW were 6.4±3.8 in prophase, 35.7±7.6 in prometaphase and metaphase, 3.8±2.4 in anaphase, and 6.0±3.0 in telophase of *Tubulin>mars-RNAi* flies ($n=30$ eye discs). Statistical analysis indicated that the number of prometaphase and metaphase cells at SMW increased significantly in *Tubulin>mars-RNAi* flies (Student's *t*-test, $p<0.001$) (Fig. 10F). The population of mitotic cells was only different in prometaphase and metaphase cells between the control and the *Tubulin>mars-RNAi* flies. In other words, this mitotic delay implied somehow that the spindle assembly encountered problems in Mars down-regulated cells; thus it took more time for the chromosomes to congress to the metaphase plate. Furthermore, the increasing of uncongressed chromosome number is transient but not permanent. Thus, the eye development in *mars*-RNAi fly is similar to the wild type. Spindle formation is a complicated process that requires the involvement of many genes [33]. Therefore, it is possible that there is a redundant pathway that helps promote spindle formation in Mars-depleted cells.

Discussion

Proper assembly of the mitotic spindle is essential for chromosome segregation in mitosis. In this study, we showed that *Drosophila* Mars protein participated in organizing kinetochore microtubules during mitosis. Immunocytochemical study showed that Mars was specifically localized at the kinetochore microtubules (Figs. 1–3).

Depletion of Mars expression in S2 cells impaired the formation of kinetochore microtubules and the localization of γ-tubulin on the spindle. In addition, we also observed that the pole to pole distance of the metaphase spindle also decreased in Mars-depleted cells (Fig. 8). The fact that spindle defects delayed the chromosome congression (Figs. 9, 10) suggested that Mars is responsible for assembling kinetochore microtubules and regulating chromosome congression during mitosis.

In Mars-depleted cells, the overall structure of the asters and centrosomes appeared normal whereas the kinetochore microtubules showed major defects as compared to the control cells (Figs. 5, 6). This observation suggested that the function of Mars in the assembly of the kinetochore microtubules is through the chromosome-mediated model. Currently, the mechanism of Mars in the assembly of kinetochore microtubules remains unclear. In this report, we found that the depletion of Mars also affected the localization of γ-tubulin on the spindles suggesting that Mars might regulate the spindle assembly through the activity of γ-tubulin (Fig. 8). Studies have shown that the depletion of γ-tubulin results in the misorganized mitotic spindles and the formation of fewer mitotic microtubules in different cell types [34]. Furthermore, the function of γ-tubulin in the spindle assembly is known to be regulated through Ran GTPase [12,14,35,36]. Recent studies further identified that HURP is the target of RanGTP that shuttles HURP between the cytoplasm and nucleus through importin β during the cell cycle [22]. According to their studies, the function of HURP appears to regulate the formation and stabilization of kinetochore microtubules [21–23]. In addition, we also found that Mars staining became intensive and condensed in Ran-overexpressed cells. Together, these results suggest that Mars regulate kinetochore microtubule formation through the Ran pathway.

The misorganized mitotic spindle in Mars-depleted cells resulted in an increase in the number of the uncongressed chromosomes in both the cultured S2 cells and in the eye discs. We had previously shown that an overexpression of Mars almost completely blocked the cell cycle progression from metaphase to anaphase [26]. In this study, we have shown that the downregulation of Mars delayed mitosis by affecting the efficiency of chromosome congression from prometaphase to metaphase (Fig. 10). HURP, the potential homologue of *Drosophila* Mars, is required not only for growing but also for stabilizing the mitotic spindles [21–23]. Thus, the mitotic arrest in Mars-overexpressed cells might be due to the fact that excess Mars has stabilized the spindle microtubules. The consequent microtubule stabilization resulted in the inability of chromosomes to separate during the transition between the metaphase and anaphase, because altering the microtubule stability is essential for the chromosome segregation during such transition [37–39]. Our study showed that the downregulation of Mars expression in the eye discs using the *Tubulin-Gal80^{TS}, Tubulin-Gal4* activator resulted in a mitotic delay but not mitotic arrest (Fig. 10). It is possible that there is still a small amount of Mars left in the cells. Alternatively, it is also possible that there might be one or more factors in addition to Mars that are essential for the spindle formation. In conclusion, this study extended our previous investigation of Mars and further demonstrated that Mars participated in the assembly of mitotic spindles during the cell cycle regulation. More significantly, the sequence-based and functional similarities between *Drosophila* Mars and humans suggest that HURP proteins are conserved evolutionarily.

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