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Using *Drosophila* eye as a model system to characterize the function of *mars* gene in cell-cycle regulation

Ching-Po Yang[a], Mei-Shu Chen[a], Gwo-Jen Liaw[b], Shu-Fen Chen[a], Gash Chou[b], Seng-Sheen Fan[a,*]

[a]*Department of Life Science and Life Science Research Center, Tunghai University, Taichung, 407 Taiwan, Republic of China*
[b]*Department of Life Science, National Yang-Ming University, Taipei, 114 Taiwan, Republic of China*

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Abstract

Human hepatoma up-regulated protein (HURP), a cell-cycle regulator, is found consistently overexpressed in human hepatocellular carcinoma. At present, the function of HURP in cell-cycle regulation and carcinogenesis remains unclear. In database mining, we have identified a *mars* gene in *Drosophila*, which encodes a protein with a high similarity to HURP in its guanylate kinase-associated protein (GKAP) motif. Overexpression but not down-regulation of *mars* in eye discs resulted in a higher mitotic index along with a high frequency of mitotic defects, including misalignment of chromosomes and mispositioned centrosomes, at the second mitotic wave (SMW). The consequence of mitotic defects impairs cell-cycle progression, and causes cell death posterior to the furrow. Immunocytochemical studies also have indicated that the expression of Mars is cell cycle regulated, and that its subcellular localization is dynamically changed during cell-cycle progression. Furthermore, we also demonstrated that the first 198 amino acids at the N-terminus of Mars are responsible for the degradation of Mars in non-mitotic cells. Together, we report the use *Drosophila* eye as a model system to characterize the function of the *mars* gene in cell-cycle regulation.

Keywords: *mars* gene; Mitosis; Centrosome; Mitotic spindle; *Drosophila* eye

Introduction

In multicellular organisms, cell-cycle progression is tightly regulated through a multiple step of checkpoints [1]. Defects in the checkpoints usually lead to uncontrolled cell division, chromosomal instability, or inappropriate cell death, which eventually results in tumor formation [2]. In order to identify molecules that participate in cell-cycle regulation and tumorigenesis, a cDNA microarray and bioinformatic analysis were used to identify and isolate such genes in hepatocellular carcinoma tissue. One of the genes identified was hepatoma up-regulated protein (HURP) [3]. Structurally, HURP contains putative motifs including leucine-rich nuclear export signal (NES), destruction box (D box), and KEN box, which are known to be involved in cell-cycle regulation [4,5]. Characterization of HURP in HeLa cells indicates that its expression is elevated in the G2/M phase and declined in the early to middle G1 phase. Immunocytochemical studies also revealed that HURP localizes to the spindle poles during mitosis. Overexpression of HURP in 293T cells enhances cell growth at a lower serum medium [3]. Furthermore, biochemical study also indicated that Fbx7, an E3 ligase, recruits Cdk1-cyclin B-phosphorylated HURP to the core SCF (*S*kp1, *C*dc53/cullin1, *F*-box protein) complex, and mediates HURP degradation through the ubiquitin–proteasome pathway [6]. Although studies have demonstrated the role of HURP in cell-cycle regulation and carcinogenesis,

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* Corresponding author. Department of Life Science, Tunghai University, No. 181, Sec. 3, Taichung-Kan Road, Taichung, 407 Taiwan, Republic of China. Fax: +886 4 2359 0296.
E-mail address: sfan@mail.thu.edu.tw (S.-S. Fan).

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the function of this gene in cell-cycle regulation remains largely unknown.

To further understand the role of HURP in cell-cycle regulation and carcinogenesis, we decided to use *Drosophila* eye as a model system because of its successful application in cancer research [7,8]. *Drosophila* eye is derived from a monolayer epithelium, the imaginal eye disc. Beginning with early third-instar larva, cells at the posterior portion of the eye disc begin to differentiate and organize into ommatidial preclusters. The transition from a proliferative epithelium to differentiating clusters is marked by a dorsal–ventral indentation, the morphogenetic furrow. Anterior to the furrow, cells are unpatterned and divide asynchronously. In the furrow, cell division is transiently arrested in the G1 phase through *dpp* signaling [9]. Posterior to the furrow, cells either begin to differentiate and organize into nascent ommatidial preclusters, or enter another round of synchronous cell division. The narrow band of active cell division posterior to the furrow is referred to as the second mitotic wave (SMW), in which the cell division provides a sufficient amount of cells for further differentiation of the photoreceptors R1, R6, R7, cone cells, and pigment cells [10]. The eye development is tightly regulated between cell division and differentiation [11,12]. Genes that interfere with the cell-cycle regulation may also interrupt cell differentiation, resulting in different adult eye phenotypes [13–16]. Thus, the development of *Drosophila* eye can serve as a model system to study the function of genes in cell-cycle regulation and tumorigenesis.

In this study, we identified the *mars* gene in *Drosophila*, the amino acid sequence of which shares a high similarity to human HURP in its GKAP motif. The *Drosophila mars* gene was cloned and shown to express predominantly in embryos and in the adult germline. RNA in situ hybridization revealed the spatial distribution of *mars* transcripts in the central nervous system, eye discs, and the pre-meiotic germ cells in the testes [17]. To study the function of the *mars* gene, we used genetic manipulation to down-regulate or overexpress *mars* transcripts in developing eyes. We then investigated whether this gene has any effect in cell-cycle regulation. Using the *GMR-Gal4* activator to induce expression of *mars* dsRNA resulted in normal eyes as in the wild type, but the eyes became small and rough in overexpression of full-length *mars*. We further demonstrated that the severe eye phenotype is a consequence of mitotic defects and cell death during eye development. In addition, we found that Mars protein is presented only in mitotic cells and not in the differentiated cells, as expression of the *mars* gene was activated posterior to the furrow using *GMR-Gal4* activator. Using deletion constructs, we were able to show that the first 198 amino acids of the Mars protein are responsible for the degradation of Mars protein in non-mitotic cells. This result suggested that degradation of Mars protein is cell-cycle regulated, and that the N-terminus of Mars is responsible for this regulation. To study the subcellular localization of Mars protein in mitotic cells, we used anti-Flag antibody to detect the exogenous Flag-Mars protein. We found that the expression of Mars is dynamically changed during cell-cycle progression; Mars is located at the nucleoplasm in the prophase, at the mitotic spindles and spindle poles in the metaphase, and then gradually degraded at the anaphase and telophase. Based on these gain-of-function studies, we propose that *Drosophila mars* gene is a novel cell-cycle-related gene, which plays an important role in controlling cell-cycle progression.

Materials and methods

Drosophila stocks and transgenic constructions

Drosophila melanogaster, w^{1118} was used as a wild type. *ap-Gal4*, *dpp-Gal4*, *eyeless-Gal4*, *GMR-Gal4*, *hs-Gal4*, *ptc-Gal4*, and P35 flies were obtained from the Bloomington stock center, Indiana University, U.S.A. The flies were raised on standard corn meal-agar media at 25°C.

To make a double-strand RNA interference transgenic construct of *mars*, a 484-bp DNA fragment corresponding to the position 213 to 696 of *mars* coding sequences was amplified by *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA) from EST clone LD21642 (GeneBank accession no. AI 455178, kindly provided by Dr. Jerry Juang at NHRI, Taiwan) using two sets of primers: (5′-ATCTAGTCTAGAGAATCCACCCAAACTGGAGAGTCC-3′; 5′-ATCTAGACGCGTGCTAGCGTT GTTCTGACGAATTGC-3′; and 5′-ATCTAGTCTAGAGAATCCACCCAAACTG GAGAGTCC-3′; 5′-ATCTAGGCTAGCGTTGTTCTGACGAATTGC-3′). These two PCR products represent identical *mars* DNA sequences with different restriction enzyme sites at the product ends, and they were subcloned, in opposite orientations, into *Eco*RI/*Xho*I and *Nhe*I/*Xba*I sites of *pWIZ* vector (a gift from Dr. Richard Carthew, Northwestern University, U.S.A.) [18]. After the *P-element* mediated germ-line transformation [19], five independent lines were obtained. To test the ability of RNAi to knockdown *mars* expression, we crossed *pWIZ-mars-RNAi* to *hs-Gal4* driver and examined the *mars* expression by immunoblotting.

To make transgenic flies that expressed full-length *mars*, we amplified *mars* cDNA with *PfuTurbo* DNA polymerase using two primers: (5′-ATCTAGAGATCTCCGCCACAAGGAATCGTAC-3′; 5′-ATCTAGTCTAGAGACAATTACTGTCAATAG-3′) from EST clone LD21642. To make transgenic flies that expressed N terminus-deleted Mars, we used two primers: (5′-ATCTAGAGATCTCCCTCCGT CATCCAATACC-3′; 5′-ATCTAGTCTAGAGACAATTACTGTCAATAG-3′) to amplify the DNA fragment that covers amino acids 198 to 921 of the Mars protein. To make transgenic flies that expressed middle-portion-deleted Mars, we used four primers: (5′-ATCTAGAGATCTCCGCCACAAGGAATCGTAC-3′; 5′-GTCCACGTTCTCGCTATGGATATCGTCTAGCGG-3′; 5′-CTAGACGATATCCATAGC GAGAACGTGGACAAG-3′; 5′-ATCTAGTCTAGAGACAATTACTGTCAATAG-3′) to amplify the DNA fragment that encodes Mars protein in which amino acids 407 to 675 were deleted. To make transgenic flies that expressed C terminus-deleted Mars, we used two primers: (5′-ATCTAGAGATCTCCGCCACAAGGAATCGTAC-3′; 5′-CTAGATTCTAGATCAGAACTGCTGCAGATTGC-3′) to amplify the DNA fragment that covers amino acids 1 to 726 of the Mars protein. All PCR fragments were subcloned into *pUAST-Flag* expression vector (Kindly provided by Dr. Henry Sun at IMB, Academia Sinica, Taiwan) to make *pUAST-Flag-mars*, *pUAST-Flag-mars-dN*, *pUAST-Flag-mars-dM*, and *pUAST-Flag-mars-dC*, respectively. All constructions were verified by DNA sequencing before being processed for germ-line transformation. After *P-element* mediated germ-line transformation, more than five independent lines were obtained in each constructs. To detect the expression of *mars* transcripts, we crossed transgenic flies to *hs-Gal4* activator. After three shocks at 37°C, the larvae were harvested and processed for immunoblotting.

Antibody production and Western blotting

To generate antibody against Mars protein, the 20 C-terminal amino acids (SKAKPSSNLQQFLRKAHANM) of the Mars protein were coupled to keyhole limpit hemocyanin (KLH) and injected into rabbit. After boosts, the serum was collected and purified using affinity chromatography. For Western blotting, larvae were collected and homogenized with homogenization buffer (50 mM HEPES, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 10% Glycerol) with protease inhibitors. The cell extract was then centrifuged at 1500 × *g* for 10 min at 4°C and subjected to SDS-PAGE to separate the protein. The SDS-PAGE and Western blotting were performed as previous studies with slight modification [20,21]. After gel electrophoresis, the proteins were transferred to a nylon membrane. To process immunoblotting, the membrane was blocked with 5% non-fat milk in PBST for 2 h. The membrane was then incubated with anti-Mars antiserum (1:500) at 4°C overnight. Next day, the membrane was washed three times with PBST and then incubated with proxidase conjugated goat anti-rabbit IgG (1:5000). After the secondary antibody, the membrane was washed and processed for Chemiluminescent reaction (Pierces, Rockford, IL). The signals were detected with cool CCD camera (Fuji film, Japan).

Immunocytochemistry and histology

For immunocytochemistry, dissected eyes were fixed in 4% paraformaldehyde for 20 min. After three washes and blocking, the eye discs were incubated with primary antibody. Primary antibodies used in this study included mouse anti-BrdU (BD Biosciences, San Diego, CA), goat anti-cyclin B (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-CNN (kindly provided by Dr. Thomas Kaufman, Indiana University, U.S.A.) [22], mouse anti-Flag (Stratagene, La Jolla, CA), and rabbit anti-phospho-histone H3 (Upstate Biotechnology, Lake Placid, NY) antibodies. Stained eye discs were washed three times with PBST, and then incubated with secondary antibodies. Secondary antibodies used in this study were conjugated with FITC, Texas Red, or Cy5 (Jackson ImmunoResearch Lab., West Grove, PA). After three washes, eye discs were mounted in the mounting medium (0.25% *n*-propyl gallate, 50% glycerol in PBS, pH 8.6) and examined on a Zeiss LSM 510 confocal microscope. The images were processed by using Adobe Photoshop 6.0.

To determine the mitotic figures, third-instar eye discs were dissected in Ringer's solution and then fixed in 4% paraformaldehyde in PBS for 20 min. After three washes, eye discs were incubated with phospho-histone H3 antibodies and subsequently with secondary antibody. After three washes, the stained eyes were mounted in mounting medium and examined under a confocal scanning microscope. In some cases, the mitotic figures were labeled by 5 ng/mL of propidium iodide (Sigma-Aldrich, St. Louis, MO). The number of dividing cells at the SMW in wild type, *GMR-Gal4/+*, *GMR > mars-RNAi*, and *GMR > Flag-mars* flies were counted. To determine the percentage of anaphase and telophase cells, we counted all phospho-histone H3 positive mitotic cells in the SMW and used this number as denominator and counted the number of anaphase and telophase cells in the same population of cells and used this number as numerator. We then used percentage to indicate the amount of anaphase and telophase cells within this population. To avoid ambiguous interpretations, we selected the eye discs with similar curvature at the edges and counted all mitotic cells in the SMW except in the curly edges. The results were compared by using one-way ANOVA.

To label S phase cells at the SMW, dissected eyes were incubated in Ringer's solution containing 0.1 mg/mL of Bromodeoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, MO) for 40 min. After incubation, those eye discs were fixed in 4% formaldehyde for 30 min before being treated with 3 N HCl for 15 min. After three washes, the eye discs were incubated with anti-BrdU antibody in PBST containing 5% normal serum at 4°C overnight, and processed for immunostaining as described above.

For scanning electron microscopy, adult eyes were dehydrated using serial ethanol and acetone before being processed for critical point dryer (CPD). After coating with gold, these eyes were examined by a scanning electron microscope (Hitachi S2300). The images were acquired using the Digital Image Acquisition System (GW Electronics, Norcross, GA) and were processed by using Adobe Photoshop 6.0.

Results

Identification of the mars gene

Using Blast search in the NCBI database, we identified the *Drosophila mars* gene, the amino acid sequence of which is highly similar to human HURP in its GKAP motif. The *mars* gene encodes a predicted protein of 921 amino acids with a calculated molecular weight of 100 kDa. A motif search indicated that the Mars protein contains putative dlg1 interacting-repeat, p-rich sequences, and a GKAP homology domain [17]. An overall sequence comparison between *Drosophila* Mars and human HURP proteins revealed 22% identify and 38% similarity, but the homology of sequences reached to 32% identify and 51% similarity at their GKAP domain (Fig. 1). Based on the sequence similarity between these two genes, we decided to investigate if the *Drosophila mars* gene plays a role in cell-cycle regulation.

Targeted expression of mars resulted in severe eye phenotype

To investigate the potential functions of *mars* in cell-cycle regulation, we generated transgenic flies that express *mars* double-strand RNA (dsRNA) or full-length *mars* transcripts. To test whether *mars* dsRNA could down-regulate the expression of the *mars* gene, we crossed *pWIZ-mars-RNAi* to the *heat-shock Gal4* activator. After three heat shocks in third-instar larvae, immunoblotting revealed the down-regulation of *mars* expression (Fig. 2A). However, phenotypic analysis showed that shocked larvae were alive and had no obvious phenotype (data not shown). To induce long-term and spatial expression of dsRNA and full-length *mars* transgene, we crossed transgenic flies to *GMR-Gal4*, an eye-specific activator. For each experiment, we crossed at least three transgenic lines with *GMR-Gal4*. All transgenic lines showed a similar eye phenotype, and thus only one of the lines was selected for further analysis. In *GMR-Gal4/+* and *GMR-Gal4/pWIZ-mars-RNAi* (*GMR > mars-RNAi*) flies, the adult eyes consist of an orderly array of approximately 750 ommatidia (Figs. 2B, C). In *GMR-Gal4/pUAST-Flag-mars* (*GMR > Flag-mars*) fly, the adult eye became small and rough (Fig. 2D). To further examine whether *mars* dsRNA can down-regulate *mars* expression in developing eye, we crossed *pWIZ-mars-RNAi* with *GMR > Flag-mars* fly. The result showed that adult eye phenotype in *GMR > Flag-mars/pWIZ-mars-RNAi* fly became much better than *GMR > Flag-mars fly*, suggesting that *mars* dsRNA could specifically down-regulate *mars* expression in developing eye (Fig. 2E). In addition, we also tested the expression of *pUAST-Flag-mars* in several other Gal4 activators, such as *eyeless-Gal4*, *dpp-Gal4*, *ap-Gal4*, and *ptc-Gal4* in different tissues. All of the combinations resulted in lethal phenotype (data not shown), suggesting that the *mars* gene plays a global role in cell-cycle regulation. In this study, we used *GMR-Gal4* to activate the expression of *mars* gene in developing eye and study its function in cell-cycle regulation.

mars gene regulates cell-cycle progression in the second mitotic wave

To examine whether eye phenotype in *GMR > Flag-mars* fly is a consequence of defect in cell-cycle regulation during eye development, we stained eye discs with anti-phospho-histone H3 antibody for mitotic cells [23]. Our results indicated that the patterns of mitotic cells at the SMW were different in the wild type, *GMR-Gal4/+*, *GMR > mars-RNAi*, and *GMR > Flag-mars* (Figs. 3 A, B, C, D). By systematically counting the number of mitotic cells at the SMW in these flies, we found that the number of mitotic cells were 36.2 ± 8.4 (n = 99) in the wild type, 51.3 ± 9.1 (n = 75) in *GMR-Gal4/+*, 51.7 ± 7.7 (n = 69) in *GMR > mars-RNAi*, and 82.2 ± 15.6 (n = 77) in *GMR > Flag-mars* eye disc. This result indicated that the number of mitotic cells was slightly increased at the SMW in *GMR-Gal4/+*. Thus, the higher mitotic index in *GMR > mars-RNAi* showed no significant difference when compared to *GMR-Gal4/+*, but the number of mitotic cells in *GMR > Flag-mars* eye discs was significantly different from *GMR > mars-RNAi* ($F_{3,316} = 277.53$, $P < 0.0001$) (Fig. 3E).

The increase in the number of mitotic cells at the SMW could be either due to multiple rounds of cell division occurring posterior to the furrow, or because of mitotic defects that occurred in the SMW which led to an accumulation of arrested mitotic cells posterior to the furrow. To distinguish between these two possibilities, we labeled eye discs with bromodideoxyuridine (BrdU) and examined the distribution of S phase cells in the wild type (Fig. 4A), *GMR > mars-RNAi* (Fig. 4B), and *GMR > Flag-mars* (Fig. 4C) eye discs. Our results indicated that the distribution of BrdU-labeling cells was similar in these flies, suggesting that the increase of mitotic cells in *GMR > Flag-mars* eye disc was not due to multiple rounds of cell division posterior to the furrow. Thus, the higher mitotic

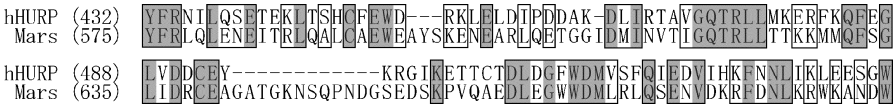

Fig. 1. Amino acid sequence comparison between human HURP and *Drosophila* Mars at their GKAP motifs. Human HURP and *Drosophila* Mars share 32% identity and 53% similarity at their GKAP motifs.

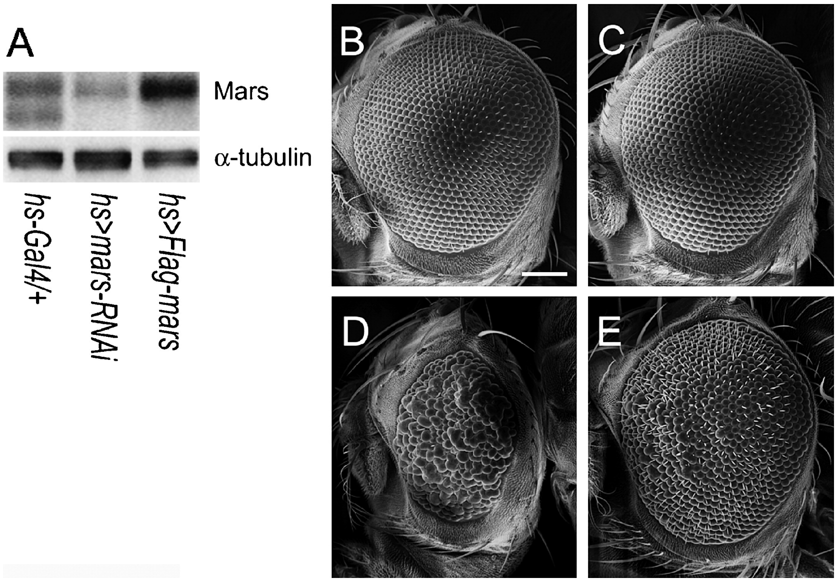

Fig. 2. Manipulation of *mars* expression and the resulting eye phenotypes. (A) Immunoblotting shows the expression of *mars* in control, *mars* RNAi, and *mars* overexpression flies. Scanning electron micrographs reveal adult eye phenotypes in (B) *GMR-Gal4/+* fly, (C) *GMR > mars-RNAi* fly, (D) *GMR > Flag-mars* fly, and (E) *GMR > Flag-mars/pWIZ-mars-RNAi* fly. Scale bar = 100 μm.

index in *GMR* > *Flag-mars* fly could be the consequence of a mitotic defect that resulted in an abnormal accumulation of mitotic cells posterior to the furrow.

To test if the higher mitotic index at the SMW is due to mitotic defects, we labeled the mitotic cells at the SMW with propidium iodide for DNA, anti-centrosomin (CNN) for centrosomes, and anti-Flag for Flag-tagged Mars protein to examine whether any abnormal mitotic figures occurred at SMW. As we expected, about 68.8 ± 9.5% (n = 11) of mitotic cells at the SMW with aberrant chromosomes and centrosomes, such as circular or fragmented chromosomes (Fig. 5B), misalignment of chromosomes (Fig. 5C), aberrant anaphase with lagging chromosomes (Fig. 5E), and incorrect separation of centriole pairs (Fig. 5F) were found in *GMR* > *Flag-mars* eye discs. These defects were not found in the wild type and *GMR* > *mars-RNAi* eye discs (Figs. 5A, D).

To examine whether these abnormal mitotic figures in *GMR* > *Flag-mars* eye discs affected cell-cycle progression and if they could result in accumulation of mitotic cells posterior to the furrow, we analyzed what proportion of mitotic cells were in the anaphase and telophase at the SMW. In the wild type eye discs, there was 21.0 ± 11.0% (n = 29) of the mitotic cells in either the anaphase or the

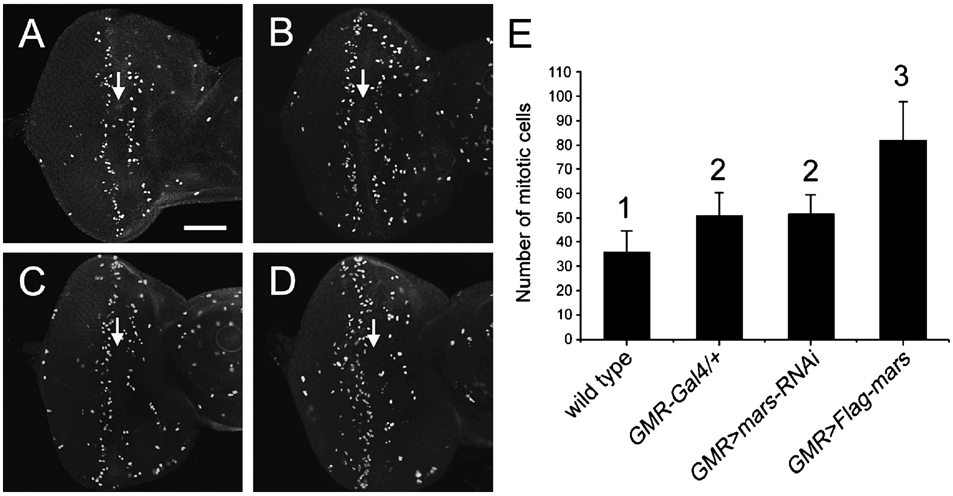

Fig. 3. Targeted expression of *mars* gene in eye disc increases the number of mitotic cells at the SMW. Anti-phospho-histone H3 antibody revealed mitotic cells in (A) wild type, (B) *GMR-Gal4/+*, (C) *GMR > mars-RNAi*, and (D) *GMR > Flag-mars* eye discs. (E) Bar graph showing the number of mitotic cells (mean ± SE) at the SMW in wild type, *GMR-Gal4/+*, *GMR > mars-RNAi*, and *GMR > Flag-mars* eye discs. Bars that do not share letters are significantly different from each other (Tukey's test, SAS 1985, a vs. c, P < 0.0001, b vs. c, P < 0.0001). Arrows indicate the furrow. Scale bar = 50 μm.

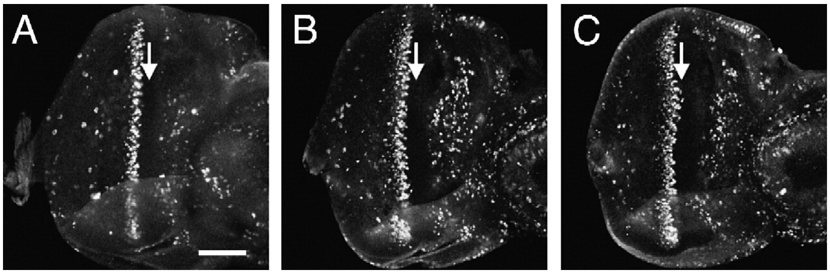

Fig. 4. The higher mitotic index in *GMR* > *Flag-mars* eye disc is not due to multiple rounds of cell division posterior to the furrow. BrdU staining reveals S phase cells at the SMW in (A) wild type, (B) *GMR* > *mars-RNAi*, and (C) *GMR* > *Flag-mars* eye discs. Arrows indicate the furrow. Scale bar = 50 μm.

telophase. In *GMR* > *mars-RNAi* eye discs, the percentage of anaphase and telophase cells was 26.4 ± 7.9% (n = 28). However, the percentage of cells in the anaphase and telophase in the SMW dropped significantly to 2.7 ± 1.8% (n = 27) in the *GMR* > *Flag-mars* eye discs. The results indicated that the percentage of cells in the anaphase and telophase in wild type and *GMR* > *mars-RNAi* were significantly different from the *GMR* > *Flag-mars* eye discs ($F_{2,80} = 61.76$, $P < 0.0001$) (Fig. 6A). Cyclin B staining, a useful marker to label mitotic cells that have passed the G1/S checkpoint but have not yet entered the anaphase [24], also revealed an accumulation of mitotic cells posterior to the furrow in *GMR* > *Flag-mars*, but not in the wild type and *GMR* > *mars-RNAi* eye discs (Figs. 6B, C, D). Because the degradation of cyclin B is a key factor for driving the metaphase–anaphase transition [25], the accumulation of cyclin B at the SMW in *GMR* > *Flag-mars* fly indicated that mitotic cells failed to progress properly to the anaphase or the telophase. This result is consistent with the lower percentage of anaphase and telophase cells in the *GMR* > *Flag-mars* fly, and indicates that targeted expression of Mars interrupts the mitotic progression.

Mitotic defects in GMR > Flag-mars fly cause cell death

Cell proliferation, differentiation, and cell death are highly coordinated in the patterning of *Drosophila* eyes [26]. The conflict of checkpoint arrest and proliferation signals may drive cells to enter apoptosis [27]. To investigate whether mitotic arrest in the targeted expression of Mars could lead to apoptosis, we stained eye discs with Acridine Orange. In the wild type flies (Fig. 7A), scattered apoptotic cells were found behind the furrow. In *GMR* > *mars-RNAi* flies (Fig. 7B), the pattern of apoptotic cells appeared to be similar to those in the wild type flies. In *GMR* > *Flag-mars* flies (Fig. 7C), the apoptotic cells increased significantly posterior to the furrow. We also found that baculovirus-derived p35 survival protein [28] was able to suppress cell death in *GMR* > *Flag-mars* eye discs (Fig. 7D). These results indicated that mitotic defects in targeted expression of

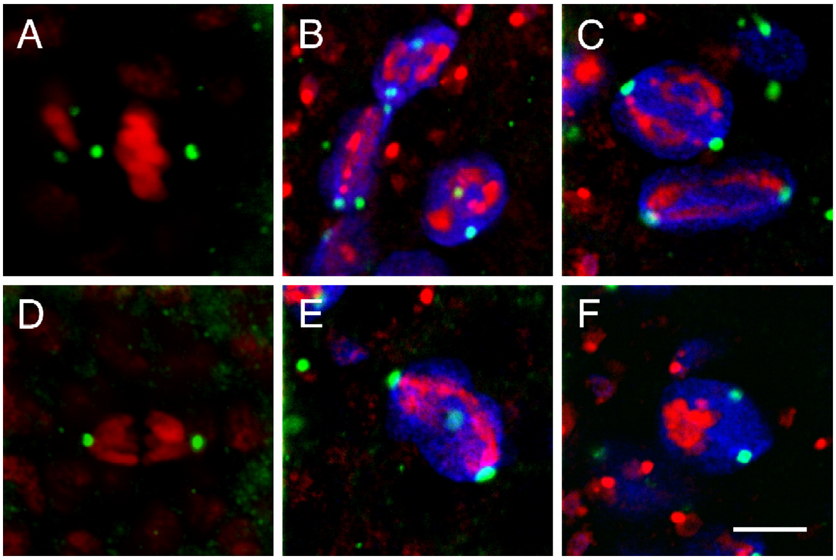

Fig. 5. Targeted expression of Mars resulted in abnormal mitotic figures. (A) The metaphase and (D) the anaphase cells in the wild type eye disc. Using *GMR-Gal4* to activate the Mars expression posterior to the furrow, we found mitotic cells with (B) fragmented and circular chromosomes; (C) misalignment of chromosomes; (E) aberrant anaphase with lagging chromosomes; or (F) mispositioned centriole pairs at the SMW. Blue: anti-Flag antibody indicates expression of Mars; Red: propidium iodine indicates DNA; Green: anti-CNN indicates centrosomes. Scale bar = 2 μm.

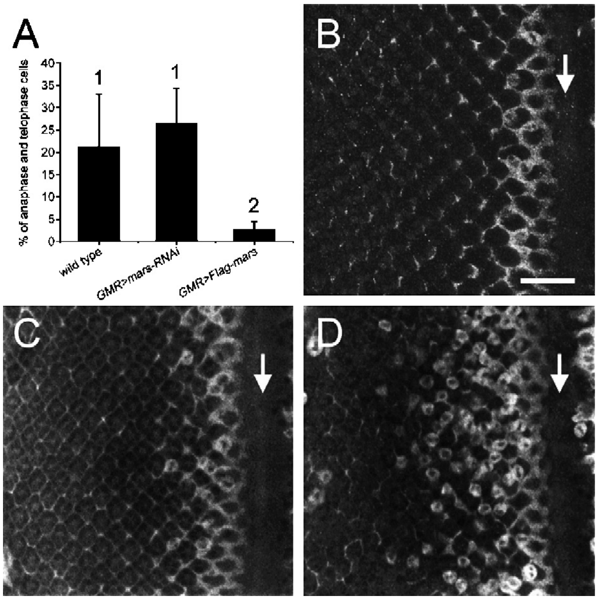

Fig. 6. Targeted expression of Mars in eye disc resulted in mitotic arrest. (A) Bar graph showing the proportion of cells at anaphase and at telophase (mean ± SE) in the wild type, *GMR > mars-RNAi*, and *GMR > Flag-mars* at the SMW. Bars that do not share letters are significantly different from each other (Tukey's test, SAS 1985, $P < 0.0001$). Cyclin B staining further indicates the mitotic arrest in *GMR > Flag-mars* eye disc. In the wild type (B) and *GMR > mars-RNAi* (C) eye discs, cyclin B staining occupies about 0 to 4 rows posterior to the furrow. However, the cyclin B staining accumulates and expands to about 10 rows posterior to the furrow in *GMR > Flag-mars* eye disc (D). Arrows indicate the furrow. Scale bar = 20 μm.

Mars cause apoptosis, and this could be the reason for the severe eye phenotype in adults.

Mars displays a dynamic localization pattern during mitosis

The results in this study pointed out a role of Mars in cell-cycle regulation. We then asked whether the Mars protein is localized at mitotic cells. To find the answer, we raised a polyclonal antibody against C-terminus of Mars peptides. This antibody recognized a 100-kDa band in protein extracts (Fig. 2A) but it failed to detect Mars protein in tissues using indirect immunocytochemistry. In order to localize the Mars protein in mitotic cells, we generated transgenic flies expressing Mars protein with Flag-tag at its N-terminus. Expression of the Flag-Mars protein was detected using anti-Flag antibody. In *GMR > Flag-mars* flies, we found that Mars was presented only in the mitotic cells, but not in the differentiated cells posterior to the furrow (Fig. 8A). Usually, *GMR-Gal4* activator can induce expression of transgenes in most of cells posterior to the furrow [29]. This result raised the question as to why Flag-Mars was present only in mitotic cells. To address this issue, we made three deletion constructs in which the N-terminus, middle portion, and C-terminus of Mars were deleted respectively. We then asked ourselves, what portion of the Mars protein is responsible for its degradation in the differentiated cells posterior to the furrow. Our results indicated that the transgenic fly expressing N-terminus deleted Mars in most of cells posterior to the furrow (Fig. 8B). In the transgenic fly that expresses the middle portion of deleted (Fig. 8C) and the C-terminus-deleted Mars (Fig. 8D), the anti-Flag antibody detected expression patterns similar to those in the expression of full-length of Mars and only presented in mitotic cells. These results suggest that Mars is stabilized only in mitotic cells, and that the first 198 amino acids are required to regulate its stability. In addition, the adult eyes showed variant phenotypes in deletion constructs. In *GMR > Flag-mars-dN* flies (Fig. 8F), the adult eyes were rough, which is similar to *GMR > Flag-mars* flies (Fig. 8E), In *GMR > Flag-mars-dM* and *GMR > Flag-mars-dC* flies (Figs. 8G, H), the adult eyes were normal as in the wild type. This result suggests that both middle portion and the C-terminus of the Mars protein are responsible for its function in cell-cycle regulation.

Immunocytochemical results indicate that Mars is present only in mitotic cells. To further examine the subcellular localization of Mars in mitotic cells, we stained mitotic cells with anti-Flag for Flag-Mars, with anti-CNN for centrosomes, and with propidium iodine for DNA (Fig. 9A). At the prophase, Mars was concentrated at the nucleoplasm. In the metaphase cells, Mars became diffusive in the cytoplasm and some were concentrated at the mitotic spindles and spindle poles. At the anaphase and telophase, the Mars began to degrade. Due to the difficulty in quantifying the

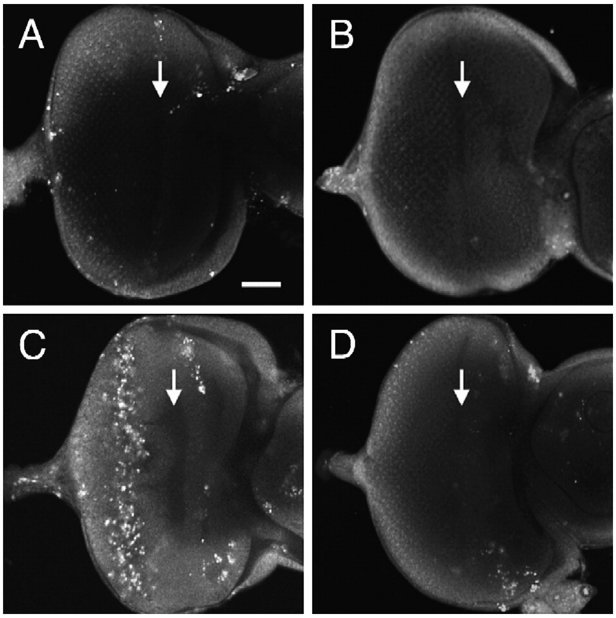

Fig. 7. Targeted expression of Mars using *GMR-Gal4* induced apoptosis in eye disc. Acridine orange staining of third-instar eye discs reveals dying cells posterior to the furrow. (A) In the wild type, there is scattered cell death posterior to the furrow. (B) In *GMR > mars-RNAi* fly, the pattern of the dying cells is similar to those of the wild type. (C) In *GMR > Flag-mars*, the cell death increases significantly posterior to the furrow. (D) Co-expression of P35 with *GMR > Flag-mars* fly reduces the cell death. Arrows indicate the furrow. Scale bar = 50 μm.

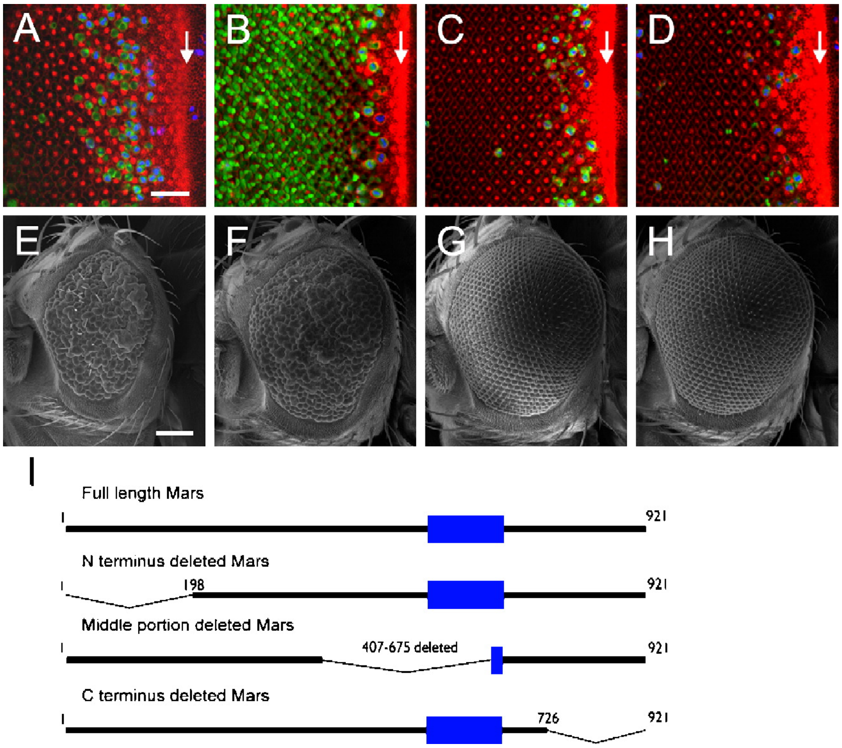

Fig. 8. The first 198 amino acids at N-terminus of Mars protein are responsible for degradation of this protein at non-mitotic cells. (A) Using *GMR-Gal4* to activate the expression of full-length Flag-Mars, the Flag-positive cells are present in rounded-up mitotic cells but not in differentiated photoreceptor cells posterior to the furrow. (B) Activation of N-terminus-deleted Mars protein expression using *GMR-Gal4*, all cells posterior to the furrow are Flag-positive. When activating the middle-portion-deleted (C) or C-terminus-deleted (D) Mars protein, the Flag staining becomes again restricted to mitotic cells. The adult eye phenotypes in expression of Mars deletion constructs. (E) *GMR > Flag-mars* flies, (F) *GMR > Flag-mars-dN* flies, (G) *GMR > Flag-mars-dM* flies, (H) *GMR > Flag-mars-dC* flies. (I) Schematic drawing of the Mars deletion constructs. Green: anti-Flag antibody, Red: phalloidin staining. Blue: anti-phospho-histone H3. Blue blocks at (I) indicate GKAP motif. Arrows indicate the furrow. Scale bar = 20 μm (A–D), 100 μm (E–H).

intensity of Flag signals in an immunocytochemistry, we thus compared the Flag signals of the anaphase and telophase cells (Fig. 9A, arrows) to a neighboring metaphase cells. Based on this observation, we believe that Mars protein is degraded in anaphase and telophase cells. The observation of the Mars degradation at the anaphase and telophase is interesting. Therefore, we investigated whether this degradation could be prevented in N-terminus-deleted Mars. The result showed that N-terminus-deleted Mars protein becomes localized in the cytoplasm and fails to degrade at the anaphase and telophase cells, comparing the anaphase and telophase cells (Fig. 9B, arrows) to neighboring metaphase cells. This result is consistent with Fig. 8 indicating that the N-terminus of Mars protein is responsible for protein stability. Together, this immuncytochemical evidence is consistent with the genetic results, suggesting that the *mars* gene plays an important role in cell-cycle regulation.

Discussion

At the post-genomic era, increasing numbers of cancer-related genes were identified through different screening methods. To facilitate our understanding of the functions of these genes, it is important to develop a model system, which allows us to efficiently study the functions of these genes in vivo. *Drosophila* is one of the ideal model systems, which fits into this category because of the well-established genetic tools that allow us to efficiently manipulate gene expression in flies. In addition, the fundamental cell-cycle machinery of flies and mammals is highly conserved [7,30]. This makes it as an ideal system to study genes involved in cell-cycle regulation and cancer formation [31–33]. In this study, we used *Drosophila* eye as a model system to characterize the function of the *mars* gene in cell-cycle regulation.

To address the function of the *mars* gene in cell-cycle regulation, we generated transgenic flies to express *mars* dsRNA or full-length *mars* during eye development. Our results showed a higher mitotic index at the SMW in *GMR > Flag-mars* flies, but not in *GMR > mars-RNAi* flies (Fig. 3). Using BrdU to label S phase cells, we demonstrated that a higher mitotic index at the SMW of *GMR > Flag-mars* flies is not due to differentiated cells undergoing multiple rounds of cell division (Fig. 4). Using anti-phospho-histone H3 antibody to label the mitotic cells at the SMW, we found widespread mitotic figures at the SMW in *GMR > Flag-mars* eye discs (Fig. 3D). Previous studies have shown that most of the mitotic cells appear between columns 3 to 5 in

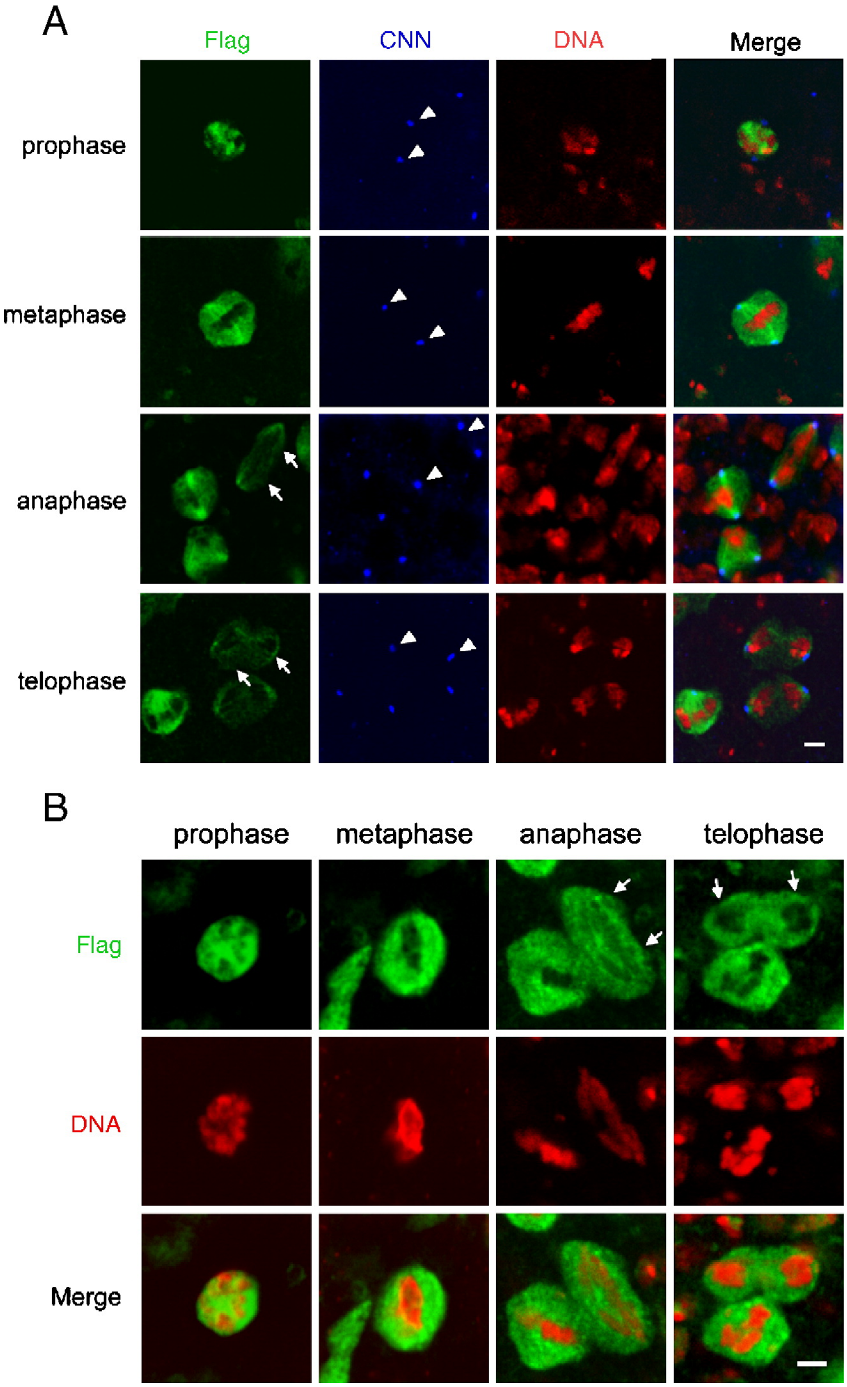

Fig. 9. Subcellular localization of Mars protein in mitotic cells. (A) Localization of Flag-Mars protein in mitotic cells. At the prophase, Mars is localized at the nucleoplasm. At the metaphase, Mars localizes at the cytoplasm and some are concentrated at the mitotic spindle and spindle poles. At the anaphase and telephone, Mars begins to degrade at the cytoplasm, comparing the intensity of Flag-staining in anaphase and telophase cells (arrows) to the neighboring metaphase cell. (B) Localization of N-terminus-deleted Mars protein in mitotic cells. The N-terminus deleted Mars protein localized in the cytoplasm, and failed to degrade at the anaphase and telophase cells, comparing the intensity of Flag-staining in anaphase and telophase cells (arrows) to the neighboring metaphase cell. Green: anti-Flag antibody to reveal the localization of Mars; Blue: anti-CNN antibody for centrosomes; Red: propidium iodine for DNA. Arrowheads indicate centrosomes. Scale bar = 2 μm.

wild type eye discs [11,34,35]. The fact of the mitotic cells being so widespread in *GMR > Flag-mars* eye discs suggested that either mitotic delay or mitotic defects had occurred. To distinguish between these possibilities, we stained the eye discs with anti-cyclin B antibody, because the degradation of cyclin B is a key step for initiating the metaphase–anaphase transition [36,37]. In developing eyes, the cyclin B accumulation began just posterior to column 0, and completely surrounded the five clusters by column 2. Once mitosis began around column 4, in which the cells complete their cell cycle and are ready for further differentiation, the cyclin B-labeled cells reduced progressively [11]. Consistent with the lower percentage of anaphase and telophase cells in *GMR > Flag-mars* eye discs, the accumulation of cyclin B at the SMW in *GMR > Flag-mars* flies (Fig. 6D) indicates that mitosis fails to progress properly. The results in this study also showed a high frequency of abnormal mitotic figures, including circular or fragmented chromosomes, aberrant anaphase with lagging chromosomes, and mispositioned spindle poles in *GMR > Flag-mars* eye discs (Fig. 5). These results suggest that the excess of Mars protein prevents the proper alignment of chromosomes and the separation of centrosomes during mitosis. Our results demonstrated that overexpression, but not down-regulation of *mars* transcripts, impairs the mitosis. These results raise the question of whether *mars* indeed plays a role in cell-cycle regulation. Based on Western blotting (Fig. 2A), and on the eye phenotype in *GMR > Flag-mars/pWIZ-mars-RNAi* flies (Fig. 2E), we believe that *mars* dsRNA can down-regulate the *mars* expression, but cannot block it completely. Therefore, it may be possible that a small amount of Mars is sufficient to regulate its activity. We have found an EP insertion (EP2477) near the *mars* gene and are currently using imprecise excision to generate *mars* mutant. We hope that this hypothesis can be further tested in *mars* null alleles. Alternatively, there is a possibility that *mars* may act as a negative regulator in mitotic cells. Overexpression of *mars* enhances its suppression and results in the prevention of chromosome alignment, chromosome segregation, and centrosomes separation (Fig. 5). In *mars* RNAi flies, the down-regulation of *mars* simply removes this inhibition and results in normal mitosis. The evidence in the present study that supports the function of *mars* in cell-cycle regulation is mainly based on a gain-of-function study; although not physiological, this method can provide a phenotypic suggestion of its physiological function.

The activities of many cell-cycle regulators are regulated through a proteasome-mediated proteolysis [5,38,39]. *GMR-Gal4* activates gene expression in almost every cell posterior to the furrow [29]. In this study, we found the Mars protein was present only in mitotic cells, and not in differentiated cells, posterior to the furrow in *GMR > Flag-mars* fly (Fig. 8A). This result suggests the possibility that the stability of Mars may be cell cycle dependent. Our immunocytochemical results, which indicated that the expression of Mars reduced when cells entered the anaphase (Fig. 9A) support this hypothesis. Using deletion constructs, we further demonstrated that the first 198 amino acids of Mars are responsible for the degradation of Mars protein in most of the cells posterior to the furrow (Fig. 8B). In addition, the fact that degradation of the Mars protein in the anaphase and telophase was prevented in N-terminus-deleted Mars protein (Fig. 9B) further supports this notion. At present, the direct evidence, indicating that the degradation of Mars is caused by proteasome-mediated proteolysis, is missing. The result from the HURP study has shown that the degradation of human HURP is regulated by F-box protein, Fbx7 through proteasome-mediated proteolysis [6]. This supports the concept that the degradation of Mars follows a similar pathway. The variant eye phenotype in the deletion constructs revealed a possible functional domain for Mars in cell-cycle regulation. In the N-terminus-deleted construct, the Mars protein failed to degrade but resulted in an eye phenotype similar to the overexpression of full-length Mars. In the middle portion and C-terminus-deleted constructs, the proteins failed to stabilize in differentiated photoreceptors and had no obvious eye phenotypes, suggesting that these regions may be responsible for the Mars activity in cell-cycle regulation.

The present study suggests a novel function of *mars* gene in cell-cycle regulation. Although the amino acid sequences between *Drosophila* Mars and human HURP are not identical, the subcellular localization and its pattern in degradation do show a significant similarity. With the help of this *Drosophila* model, we will be able to screen for genes interacting with *mars* and study the role of this gene in cell-cycle regulation and tumorigenesis in more detail.

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