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The *mob as tumor suppressor* Gene Is Essential for Early Development and Regulates Tissue Growth in Drosophila

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ABSTRACT

Studies in Drosophila have defined a new growth inhibitory pathway mediated by Fat (Ft), Merlin (Mer), Expanded (Ex), Hippo (Hpo), Salvador (Sav)/Shar-pei, Warts (Wts)/Large tumor suppressor (Lats), and Mob as tumor suppressor (Mats), which are all evolutionarily conserved in vertebrate animals. We previously found that the Mob family protein Mats functions as a coactivator of Wts kinase. Here we show that *mats* is essential for early development and is required for proper chromosomal segregation in developing embryos. Mats is expressed at low levels ubiquitously, which is consistent with the role of Mats as a general growth regulator. Like mammalian Mats, Drosophila Mats colocalizes with Wts/Lats kinase and cyclin E proteins at the centrosome. This raises the possibility that Mats may function together with Wts/Lats to regulate cyclin E activity in the centrosome for mitotic control. While Hpo/Wts signaling has been implicated in the control of *cyclin E* and *diap1* expression, we found that it also modulates the expression of *cyclin A* and *cyclin B*. Although *mats* depletion leads to aberrant mitoses, this does not seem to be due to compromised mitotic spindle checkpoint function.

CANCER arises from defective regulation in diverse cellular activities such as cell cycle, apoptosis, signal transduction, maintenance of cell polarity, and cell adhesion. Recent research in Drosophila has contributed to characterizing the Hippo (Hpo) and Warts/Large tumor suppressor (Wts/Lats) signaling pathway that controls both cell proliferation and apoptosis (reviewed in Edgar 2006; Hariharan and Bilder 2006; Harvey and Tapon 2007; Pan 2007). Components in Hpo/Wts signaling are evolutionarily conserved as Drosophila mutants can be functionally rescued by their respective human homologs (Tao *et al.* 1999; Wu *et al.* 2003; Lai *et al.* 2005).

Two upstream components implicated in the Hpo/Wts signaling are the FERM-domain-containing membrane-associated factors, Merlin/Expanded, whose activity can increase the kinase function of Hpo (Hamaratoglu *et al.* 2006). The Fat (Ft) protein may function as a receptor farther upstream or in parallel with Hpo/Wts signaling (Harvey and Tapon 2007; Pan 2007). Hpo associates with an adaptor protein Salvador (Sav), and this interaction is shown to increase Hpo phosphorylation to another kinase Wts/Lats (Kango-Singh *et al.* 2002; Tapon *et al.* 2002; Wu *et al.* 2003; Colombani *et al.* 2006). We have previously identified Mob as tumor suppressor (Mats) as a coactivator for Wts/Lats kinase (Lai *et al.* 2005) and have shown that Hpo enhances Mats function via phosphorylation (Wei *et al.* 2007). Loss of function in any of these genes results in upregulation of cyclin E and Drosophila inhibitor of apoptosis (DIAP1), causing cell overproliferation and defective cell death in mosaic tissues (Justice *et al.* 1995; Xu *et al.* 1995; Kango-Singh *et al.* 2002; Tapon *et al.* 2002; Harvey *et al.* 2003; Jia *et al.* 2003; Pantalacci *et al.* 2003; Udan *et al.* 2003; Wu *et al.* 2003; Lai *et al.* 2005). As a major downstream target, the growth-promoting transcriptional cofactor Yorkie (Yki) is negatively regulated by the Hpo/Wts growth inhibitory pathway (Huang *et al.* 2005).

Studies of homologs in yeast and human cells have shown potential cellular activities of factors involved in the Hpo/Wts pathway. Budding yeast homologs of Wts and Mats (Dbf2 and Mob1, respectively) are components of the mitotic exit network (Komarnitsky *et al.* 1998; Luca and Winey 1998; Luca *et al.* 2001). Intracellular localization of these proteins are regulated during the cell cycle such that, until anaphase, they are localized at the spindle pole bodies but they move to the bud neck prior to actin ring assembly in a functionally interdependent manner (Frenz *et al.* 2000; Luca *et al.* 2001; Yoshida and Toh-e 2001). In mammalian cells, both Wts homologs, LATS1 and LATS2, were found to regulate G_2/M-phase and G_1/S-phase transitions, respectively (Tao *et al.* 1999; Yang *et al.* 2001; Xia *et al.* 2002; Li *et al.* 2003). Both LATS1 and LATS2 are found in the centrosome, and their loss of function results in multinucleation, centrosomal amplification, and genomic instability, suggesting that they

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are involved in some aspects of cell cycle progression (Nishiyama *et al.* 1999; Morisaki *et al.* 2002; McPherson *et al.* 2004; Toji *et al.* 2004).

Although mutations in *wts* or *mats* result in obvious overgrowth, mitotic defects associated with mutations in these factors have not been reported in Drosophila. Here we show that *mats* is an essential gene that is required for early embryonic development. The Mats protein is a centrosomal component that appears to be critical for maintaining genome stability and the disruption of *mats* function results in aberrant mitoses. However, this does not seem to be due to compromised mitotic spindle checkpoint function. Moreover, our data suggest that Mats regulates expression not only of cyclin E, but also of cyclin A and cyclin B, which are key regulators of cell cycle progression in both invertebrate and vertebrate animals.

MATERIALS AND METHODS

Analysis of homozygous *mats* mutant: To assess the lethal stage of homozygous $mats^{-}$ mutants, w^{1118}; *FRT82B* $mats^{-}$/*CyO·TM3*$_{2xhs\text{-}GFP}$ was crossed with w^{1118}; *Df(3R)17D1/CyO·TM3*$_{2xhs\text{-}GFP}$ to generate $mats^{-}$*/Df(3R)17D1* larvae, which are equivalent to the homozygous *mats* mutant. Expression of green fluorescent proteins (GFP) was induced by heat treatment of larvae at 37° for 15 min. Larvae of genotypes $mats^{-}$/*Df(3R)17D1* and $mats^{-}$/*CyO·TM3*$_{2xhs\text{-}GFP}$ [or *Df(3R)17D1/CyO·TM3*$_{2xhs\text{-}GFP}$] were placed on a microscope slide. Both $mats^{e235}$ and $mats^{roo}$ alleles were used. Images were taken with the Nikon Coolpix990 digital camera mounted on the Nikon Eclipse TS100 inverted scope.

Analysis of maternally *mats* null embryos: Dominant female sterility (DFS) technique takes advantage of dominant ovo^{D1} mutation that renders sterility to oocytes (Chou and Perrimon 1996). Thus, for heterozygous females carrying ovo^{D1} to be able to lay eggs, somatic recombination needs to happen to generate clones of homozygous oocytes that have eliminated ovo^{D1}. When used in combination with the third chromosome that harbors the *mats* mutation, this technique generates homozygous *mats* mutant oocytes in heterozygous females, and only these oocytes can generate eggs. Female flies of *y, hs-FLP; FRT82B* $mats^{e235}$*/TM6B* were crossed with males of w^{1118}; *FRT82B* ovo^{D1}*/TM3*, and embryos were collected for 24 hr. Hatched larvae were heat-shocked for 20–30 min at 37° in L2 stage, and resulting virgin females of *hs-FLP; FRT82B* $mats^{e235}$*/FRT82B* ovo^{D1} were collected. As ovo^{D1} gives dominant female sterility, for females of this genotype to be able to lay eggs, they must undergo somatic recombination in the ovary to generate *FRT82B* $mats^{e235}$*/FRT82B* $mats^{e235}$ cells, eliminating ovo^{D1} and functional the *mats* allele from their genome. These flies were crossed with males of w^{1118}*; FRT82B* $mats^{e235}$*/CyO·TM3*$_{2xhs\text{-}GFP}$ and resulting embryos were heat-shocked at 37° for 15 min. Embryos and larvae were handpicked and placed on the microscope slide, and GFP autofluorescence was observed under a Zeiss microscope. The animals were categorized by developmental stage (embryo or larva) and the presence or absence of GFP, and their numbers were scored.

For immunostaining, embryos were collected for 2–3 hr, and handpicked embryos were washed in PBS and dechorinated in mild bleach for 2 min. After washes, embryos were fixed in a 1:1 mix of paraformaldehyde-lysine-phosphate (PLP: 2% paraformaldehyde, 0.75 m poly-l-lysine, 0.25% sodium periodate) and heptane on the bench top for 20 min. Then PLP was replaced by methanol, and the samples were vigorously shaken to remove vitelline membrane. Embryos were then rehydrated with balanced salt solution (BSS) Triton 0.3% (Ashburner 1989) and stained with anti-Cnn antibodies (a gift of Thomas Kaufman; Megraw *et al.* 2002) at 4° overnight. The secondary anti-rabbit Alexa Fluor (AF) 488 antibodies (Molecular Probes, Eugene, OR) were used at 1:500 dilutions. Images were taken with an Olympus FluoView 300 confocal microscope.

Cell culture and immunostaining: HEK293T cells were cultured in DMEM containing 10% fetal bovine serum (FBS). Transfection was mediated through Polyfect transfection reagent (QIAGEN, Valencia, CA). Transfected cells were seeded on coverslips coated with FBS and fixed with methanol (−20°). Cells were washed and stained with either anti-Myc antibodies or anti-CycE antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in BSS with 0.3% Triton. Anti-rabbit AF594 (1:500) and anti-mouse AF680 (1:200) antibodies were used for secondary staining. Draq5 (Biostatus, Leicestershire, UK) was used for DNA staining.

Genetic interactions of Wts/Lats signaling: *UAS-myc-*wts_{6R} on the second chromosome is a relatively strong allele and was recombined with *ey-Gal4* to generate w^{1118}*; ey-Gal4, UAS-myc-*wts_{6R}*/SM6·TM6B*. Females of this genotype were crossed with w^{1118}, *UAS-cycE*, *UAS-diap1*, *UAS-cycA*, *UAS-cycB*, *cyclin* E^{AR95}*/TM3*, *diap1*^{4}*/TM3*, *cyclin* A^{C8LR1}*/TM3*, *cyclin* A^{03946}*/TM3*, *cyclin* B^{2}*/CyO*, or *cyclin* $B^{KG08886}$*/CyO* male flies. *UAS* transgenic flies were crossed with w^{1118}*; ey-Gal4/SM6·TM6B* to generate flies for comparison. Flies carrying loss-of-function alleles of *cyclin E*, *diap1*, *cyclin A*, and *cyclin B* were also crossed with w^{1118} flies to determine their heterozygous phenotypes.

Somatic homozygous *mats* mutant cells were generated by crossing w^{1118}*; FRT82B* $mats^{e235}$*/TM6B* males with *y,w, ey-FLP; FRT82B arm-lacZ/TM6B* females. For control, w^{1118} larvae of the same developmental stage were used. The eye discs of Tb^{+} larvae were dissected and fixed with PLP on ice for 45 min and washed with BSS with 0.3% Triton twice. Peripodial membranes were removed, and discs were stained with primary antibodies [mouse anticyclin A (1:5 dilution) and mouse anticyclin B (1:5 dilution) antibodies; Developmental Studies Hybridoma Bank at the University of Iowa] followed by AF 680 fluorescent secondary antibodies (1:200). Clonal expression of Yki in larval discs was achieved using *hsFLP122 actin>CD2>Gal4 UAS-lacZ/UAS-yki-V5* flies, which were treated at the early second instar larval stage for 1 hr at 37° to allow FLP expression. Wing and eye discs were isolated from matured third instar larvae. Antibodies for cyclin A, cyclin B, and β-galactosidase were used for immunostaining of the larval tissues.

Mitotic checkpoint analysis: Third instar larval eye discs containing control or *mps1* or *mats* homozygous mutant clones were generated by crossing w^{1118}*; FRT82B 90E P[*w^{+}*]* or w^{1118}*; FRT82B* $mps1^{1}$*/TM6B* (a gift of Christian Lehner; Fischer *et al.* 2004) or w^{1118}*; FRT82B* $mats^{e235}$*/TM6B* males with *UAS-GFP, ey-FLP; Tub-Gal4, FRT82B Tub-Gal80/TM6B* females. Dissected eye discs were incubated for 3 hr in Schneider S2 medium (Invitrogen, Carlsbad, CA) either with or without 1 μm colcemid (Sigma, St. Louis) before staining with phosphohistone H3 antibodies (1:200; a gift of Esther Siegfried). Clones were identified by the presence of GFP signals.

RESULTS

Mats is essential for embryonic development: *mats* has been previously shown to be a growth inhibitor and its clonal loss of function promotes tissue outgrowth

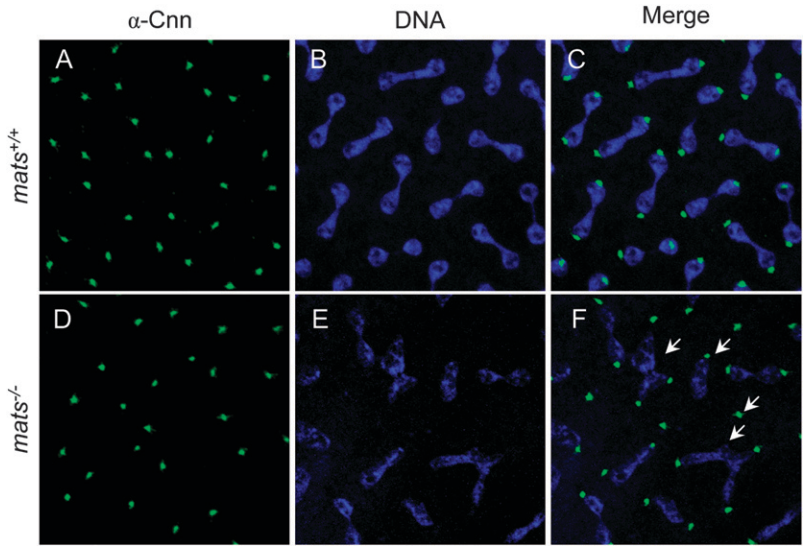

FIGURE 1.—*mats* mutations cause DNA segregation defect. Control (w^{1118}) (A–C) and maternally *mats* null mutant (D–F) embryos were stained with anti-Cnn antibodies and the DNA dye Draq5. Centrosomes were identified by Cnn (A and D) and DNA by Draq5 (B and E). Compared to control embryos (C), maternally *mats*-depleted embryos (F) exhibited DNA segregation defects. Arrows highlight aberrant DNA segregation observed in *mats* mutant embryos.

(LAI *et al.* 2005). *mats* is an essential gene required for normal development, as development of zygotic homozygous *mats* mutant animals did not proceed beyond the second instar larval stage. These animals became sluggish and eventually died. Because *mats* mRNA exists in young embryos (data from Berkeley *Drosophila* Genome Project, http://www.fruitfly.org), it is possible that maternal contribution of *mats* mRNA and/or protein product can rescue the homozygous mutant animals to a certain extent, delaying the lethal stage. To test this possibility, germline clones of *mats* mutant cells were made to eliminate this maternal loading of *mats* to the embryos. Using a DFS technique (CHOU and PERRIMON 1996), maternally and zygotically *mats* null mutants were generated and identified by the absence of GFP expression upon heat shock. On the other hand, *mats* heterozygotes collected after midblastula transition would produce GFP upon heat treatment due to heat-shock-inducible GFP contained in the balancer chromosome. This analysis revealed that maternally and zygotically null embryos did not hatch. Thus, maternal *mats* is indeed critical for viability. Interestingly, maternally null and zygotically heterozygous animals were viable and survived to the adult stage. Of 116 larvae observed, 113 of them were alive and exhibited GFP reporter expression. They grew up to become fertile adults without any obvious morphological defects. The remaining three larvae were dead and had no GFP expression and were probably escapers of *mats* maternal and zygotic null mutants. Alternatively, these may be carcasses of *mats* heterozygotes that lost GFP signals after death. These results suggest that *mats* maternal function can be rescued by zygotic *mats*.

Mats is required for proper chromosomal segregation: Up to the midblastula transition, the cellular function of Drosophila embryos relies on maternally loaded transcripts and protein products (EDGAR *et al.* 1986). Therefore, in maternally *mats* null embryos, the cell divisions up to the 16th cycle occur in the total absence of Mats function. To observe cell division phenotype in the absence of Mats, embryos from the maternally null crosses were collected before midblastula transition (at this point embryos included both maternally and zygotically null and heterozygous zygotes) and the behavior of a centrosomal marker Centrosomin (Cnn) (MEGRAW *et al.* 2002) and DNA (Draq5) was examined. While in wild-type embryos, an equal amount of DNA was segregated to two centrosomal poles (Figure 1, A–C), all embryos generated from maternal null oocytes showed aberrant DNA segregation (Figure 1, D–F). Maternally *mats* null embryos also appeared to have DNA fragmentation in some divisions and occasionally three or more centrosomes were associated with one pool of segregating DNA (Figure 1F). Thus Mats appears to play a role in ensuring the proper chromosomal segregation during mitosis.

Mats is ubiquitously expressed at low levels in developing tissues: To better understand the developmental role of Mats, the expression pattern of the endogenous Mats protein has been analyzed. For this purpose, third instar larval imaginal discs were dissected and stained with anti-Mats antibodies (LAI *et al.* 2005). To ensure the specificity of the antibodies, *mats* homozygous null mutant clones were generated by FLP/FRT-mediated somatic recombination (XU and RUBIN 1993). As shown in Figure 2, A and B, the staining level was reduced in the *mats* null mutant clones. In addition, the endogenous staining was compared with a positive control, in which two copies of full-length *mats* transgenes were under the control of *GMR-Gal4* for expression in cells posterior to the morphogenetic furrow. As a result, very strong staining signals were observed posterior to the morphogenetic furrow, while endogenous Mats staining in the anterior region of the eye disc and in other larval tissues such as leg and wing discs was low (Figure 2, C and D; data not shown). Staining level

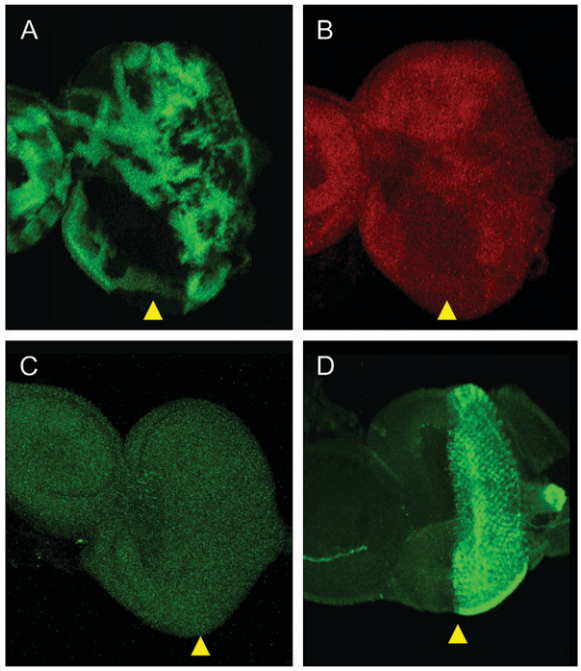

Figure 2.—*mats* is expressed at low levels ubiquitously. A mosaic third instar larval eye disc containing *mats* null clones is shown (A and B). Mutant clones of *mats* were identified by the absence of β-gal staining in A. Anti-Mats antibody staining in B showed lower levels of Mats protein in *mats* mutant cells. (C) Endogenous *mats* staining in the w^{1118} eye disc showed very low signals. (D) When *mats* transgenes were expressed in *GMR-Gal4; UAS-mats*$_{B121B142}$, high levels of Mats staining were observed posterior to the morphogenetic furrow. The anterior half of the eye disc showed very low levels of staining similar to that of endogenous staining observed in C. Arrowheads identify the morphogenetic furrow. Anterior is to the left in A–D.

observed in Figure 2C is comparable to the anterior eye disc staining in Figure 2D. These results suggest that endogenous *mats* is ubiquitously expressed at a low level and that the Mats antibody can specifically recognize the Mats protein.

It is known that cells in an eye disc are synchronized with respect to the progression of the morphogenetic furrow, such that cells in the furrow are in G_1 phase, and as cells emerge from the furrow, they undergo S phase synchronously and complete one cell cycle prior to the terminal differentiation (Baker 2001). Since there is no particular endogenous expression level change adjacent to the morphogenetic furrow, *mats* expression does not appear to fluctuate throughout the cell cycle. Similarly, microarray analysis indicates that mRNA levels of human *MATS* genes do not dramatically alter during the cell cycle (Whitfield *et al.* 2002). These observations suggest that regulation of *mats* may occur mainly through protein modifications.

Mats and Wts are colocalized at the centrosome: To determine subcellular localization of Mats and its binding partner Wts kinase, Mats and Wts proteins were tagged and expressed in cultured cells. Specifically, human embryonic kidney (HEK) 293T cells were used to transfect *mats-GFP* and *myc-wts* fusion genes under the control of the cytomegalovirus promoter. We found that Mats-GFP was localized in the cytosol as well as in the nucleus, whereas Wts was exclusively cytosolic during interphase (Figure 3). It was also clear that both Mats and Wts accumulated at the perinuclear region in a dot-like pattern. This dot-like pattern was duplicated once the mitotic DNA segregation commenced. These accumulations appeared to be centrosomes, as they colocalized with γ-tubulin signals (Figure 3, A–H). This observation is consistent with the centrosomal localization of human LATS1 and MOB1A (MATS2) reported elsewhere (Nishiyama *et al.* 1999; Bothos *et al.* 2005). Mats and Wts are also localized at the midbody area during cytokinesis (Figure 3, B, D, F, and H). Moreover, Mats and Wts colocalize with endogenous human cyclin E at the centrosome (Figure 3, I–P). Thus, Mats and Wts proteins appear to function together in subcellular organelles such as centrosomes.

Genetic interactions among *wts*, *diap1*, and *cyclin E* genes: *cyclin E* and *diap1* are considered to be two downstream targets of the Hpo/Wts pathway (reviewed in Harvey and Tapon 2007; Pan 2007). To further observe functional interactions between Hpo/Wts signaling and its output, *ey-Gal4* was used to express *UAS-myc-wts* in the developing eye. Compared to the previously reported line 16B (Lai *et al.* 2005), the 6R line exhibited stronger phenotypes. Thirty percent of the *ey-Gal4 UAS-myc-wts*$_{6R}$/+ flies died at the late pupal stage and the surviving flies typically had their eyes severely reduced in size and shaped like a cone (Figure 4H). Overexpression of *cyclin E* showed a slightly rough eye (Figure 4B), whereas *ey-Gal4*-driven *diap1* expression showed relatively normal eye morphology (Figure 4C). When combined with *ey-Gal4 UAS-myc-wts*$_{6R}$, expression of *cyclin E* and *diap1* effectively suppressed the cone-shape and small-eye phenotypes caused by *wts* overexpression (compare Figure 4I and 4J with 4H). All of the *ey-Gal4 UAS-myc-wts*$_{6R}$*/UAS-cycE* and *ey-Gal4 UAS-myc-wts*$_{6R}$*/UAS-diap1* flies survived to the adult stage (Table 1). Moreover, the effects of loss-of-function alleles of *cyclin E* and *diap1* on Wts-induced mutant phenotypes were examined. While *cycE*^{AR95} and *diap1*^{4} heterozygotes were phenotypically normal (Figure 4, D and E), Wts-induced mutant phenotypes were strongly enhanced by the reduction of endogenous *cyclin E* or *diap1* function such that animals had underdeveloped head tissue and their eyes were extremely reduced in size with some of them exhibiting a rod-like structure extending from the center of the eye (compare Figure 4K and 4L with 4H). The lethal phenotype was also enhanced as 60% of the *ey-Gal4 UAS-myc-wts*$_{6R}$*/cycE*^{AR95} flies and 85% of the *ey-Gal4 UAS-myc-wts*$_{6R}$*/diap1*^{4} flies died at the pupal stage (Table 1). These results further support *cyclin E* and *diap1* as critical targets of Hpo/Wts signaling.

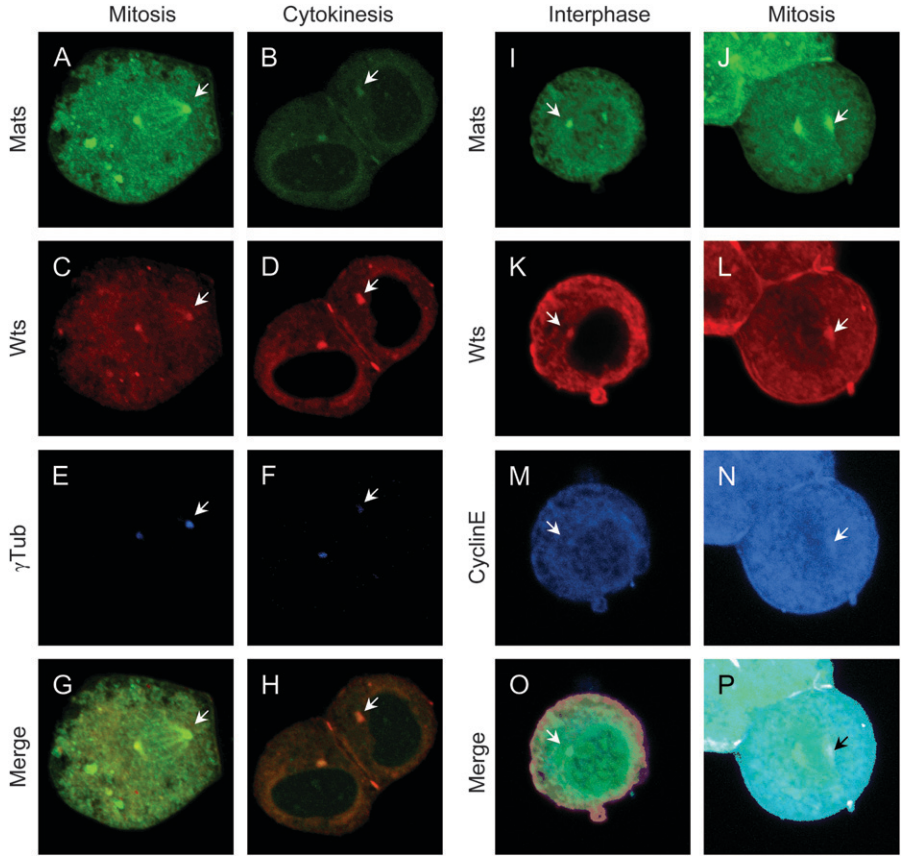

FIGURE 3.—Mats and Wts accumulate at the centrosome throughout the cell cycle. (A–H) In transfected HEK293T cells, Mats and Wts colocalize with γ-tubulin at the centrosome during mitosis (A, C, E, and G) and cytokinesis (B, D, F, and H). After formation of the nuclear membrane, Mats was found in both the cytosol and the nucleus, while Wts was excluded from the nucleus. Both during mitotic phase and after nuclear membrane formation, Mats (A and B) and Wts (C and D) were accumulated at the centrosome together with γ-tubulin (E–H). (I–P) Mats and Wts also colocalized with cyclin E at the centrosome. During interphase, Mats and Wts accumulated at the centrosome in the perinuclear region and this pattern was also seen in the endogenous cyclin E staining (I, K, M, and O). This pattern was maintained throughout mitosis (J, L, N, and P). The centrosome is indicated by arrows.

Expression of *cyclin A* and *cyclin B* is negatively regulated by Hpo/Wts signaling: To examine whether *mats* also regulates expression of other *cyclin* genes such as *cyclin A* and *cyclin B*, immunostaining experiments using cyclin A and cyclin B antibodies were conducted. We found that loss of *mats* function in larval eye discs resulted in elevated levels of both cyclin A (Figure 5, B–B″) and cyclin B proteins (Figure 5, D–D″). Moreover, clonal expression of Yki in developing tissues such as wing discs was able to increase the levels of cyclin A and cyclin B proteins (Figure 5, F–F″ and H–H″). These results support the idea that *cyclin A* and *cyclin B* genes are targets of Hpo/Wts signaling.

Using the *ey-Gal4 UAS-myc-wts$_{6R}$* assay, we have examined how *cyclin A* and *cyclin B* genes might genetically interact with *wts*. To do this, *UAS-myc-wts$_{6R}$* was ectopically expressed in combination with *cyclin A* or *cyclin B*. Fifty-three percent of the *ey-Gal4/UAS-cycA* flies died at the pupal stage (Table 1) and those that survived to the adult stage (18%) showed slightly reduced eyes (Figure 4F). On the contrary, expression of *cyclin B* driven by *ey-Gal4* in the wild-type background had no effect on viability and showed normal-eye phenotypes (Figure 4G). Coexpression of *cyclin B* with *wts$_{6R}$* effectively suppressed Wts-induced pupal lethality as all flies survived to the adult stage (Table 1), and it suppressed cone-shape and small-eye phenotypes as well (Figure 4N). For cyclin A, it was also able to suppress Wts-induced eye phenotypes (Figure 4M), while there were still 63% of the *ey-Gal4 UAS-myc-wts$_{6R}$/UAS-cycA* flies that died at the pupal stage (Table 1). Thus, results from these gain-of-function alleles of *cyclin A* and *cyclin B* are consistent with the model that *cyclin A* and *cyclin B* are targets of the Hpo/Wts signaling pathway. When loss-of-function alleles of *cyclin A* and *cyclin B* genes were tested in this assay, however, no significant modification of the eye phenotype of *ey-Gal4 UAS-myc-wts$_{6R}$* flies was observed (data not shown). It appears to be that the eye phenotype of *ey-Gal4 UAS-myc-wts$_{6R}$* flies is not sensitive to the reduction of *cyclin A* and *cyclin B* function.

Mats is not involved in mitotic checkpoint: The possibility that *mats* mutation would cause genomic instability suggests its involvement in cell cycle checkpoint function. To assess whether Mats plays a role in this cellular response, the MARCM system (LEE and LUO 1999) was used to generate eye discs containing control, *mps1* mutant, or *mats* mutant clones, and the tissue was dissected and incubated in either the presence or absence of colcemid for 2 hr and the accumulation of M-phase cells was analyzed. Discs containing control clones accumulated M-phase cells in response to colcemid treatment especially at the posterior disc area adjacent to the morphogenetic furrow [Figure S1, A–F, at http://www.genetics.org/supplemental/; − colcemid ($n = 7$ eye discs), + colcemid ($n = 10$ eye discs)]. Discs containing *mps1* mutant clones, however, showed accumulation of M-phase

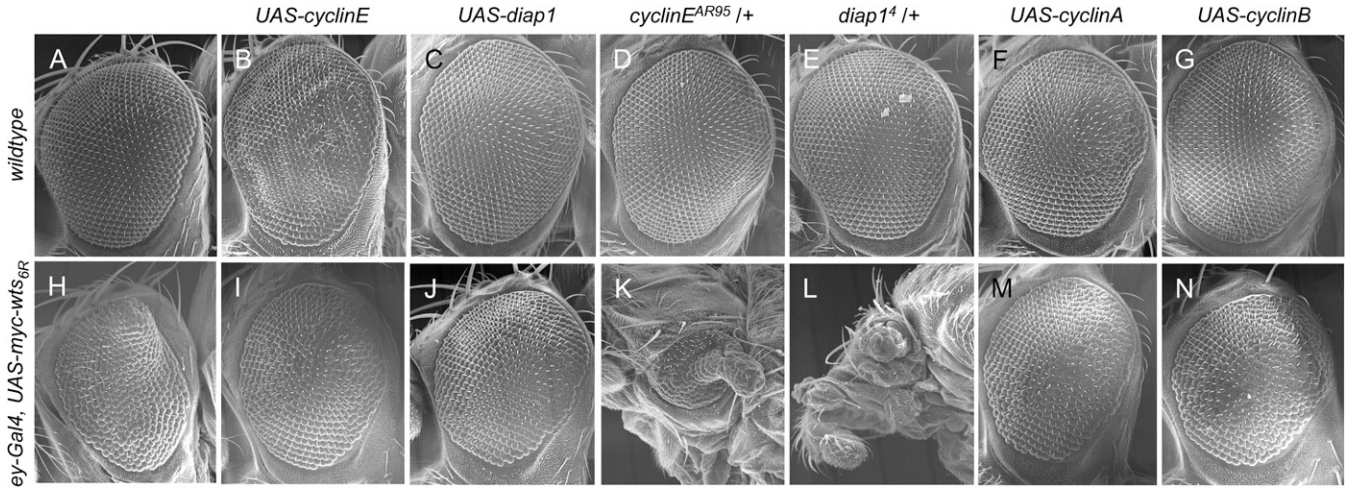

FIGURE 4.—*wts* overexpression phenotype is effectively modified by dosage change of *diap1* and *cyclin* genes. SEM micrograph of (A) w^{1118} adult eye is used as a positive control. Overexpression of *cyclin E* caused a slightly rough-eye phenotype (B, *ey-Gal4/UAS-cycE*), while expression of *diap1* was normal (C, *ey-Gal4/+; +/UAS-diap1*). Heterozygosity of *cyclin E* (D, $cycE^{AR95}$/+) or *diap1* (E, $diap1^4$/+) did not show eye phenotypic change. (F) *ey-Gal4/UAS-cyclin A*. (G) *ey-Gal4/UAS-cyclin B*. (H) Overexpression of *wts* in *ey-Gal4 UAS-myc-wts$_{6R}$*/+ reduced viability and eyes became smaller and cone shaped. Coexpression of either *cyclin E* (I, *ey-Gal4 UAS-myc-wts$_{6R}$/UAS-cycE*) or *diap1* (J, *ey-Gal4 UAS-myc-wts$_{6R}$/+; +/UAS-diap1*) effectively suppressed *wts*-induced small and cone-shaped eye phenotypes. On the other hand, reduction of either *cyclin E* (K, *ey-Gal4 UAS-myc-wts$_{6R}$/$cycE^{AR95}$*) or *diap1* (L, *ey-Gal4 UAS-myc-wts$_{6R}$/+; +/$diap1^4$*) function resulted in strong enhancement of growth inhibition caused by Wts. These flies were lethal at the late pupa stage; thus SEM pictures were taken from flies dissected from the pupa case. (M) *ey-Gal4 UAS-myc-wts$_{6R}$/UAS-cyclin A*. (N) *ey-Gal4 UAS-myc-wts$_{6R}$/UAS-cyclin B*. Anterior is to the left in A–N.

cells only in wild-type tissue (GFP negative) but accumulation was not seen at the *mps1* mutant clones (GFP positive) [Figure S1, G–L; − colcemid ($n = 8$ eye discs), + colcemid ($n = 12$ eye discs)] as previously reported (FISCHER *et al.* 2004). Thus, mutations in *mps1* resulted in failure to initiate mitotic checkpoint response, and consequently *mps1* mutant cells did not arrest at the M phase. Discs containing *mats* mutant clones, on the other hand, accumulated M-phase cells exactly as seen in the control group, especially posterior to the morphogenetic furrow [Figure S1, M–R; − colcemid ($n = 6$ eye discs), + colcemid ($n = 8$ eye discs)]. These results indicate that *mat* is not involved in the spindle checkpoint response.

TABLE 1

***wts* genetically interact with *diap1*and *cyclin* genes**

Genotype	% of flies that died at the pupal stage
ey-Gal4, UAS-myc-wts$_{6R}$/+	30 ($n = 198$)
ey-Gal4, UAS-myc-wts$_{6R}$/UAS-cyclin E	0 ($n = 65$)
ey-Gal4, UAS-myc-wts$_{6R}$/UAS-diap1	0 ($n = 101$)
ey-Gal4, UAS-myc-wts$_{6R}$/cyclin E^{AR95}	60 ($n = 70$)
ey-Gal4, UAS-myc-wts$_{6R}$/$diap1^4$	85 ($n = 20$)
ey-Gal4/UAS-cyclin A	53 ($n = 66$)
ey-Gal4, UAS-myc-wts$_{6R}$/UAS-cyclin A	63 ($n = 46$)
ey-Gal4, UAS-myc-wts$_{6R}$/UAS-cyclin B	0 (ND)

Viability of *cyclin E^{AR95}*/+ and *$diap1^4$*/+ heterozygotes is normal. Similarly, overexpression of *cyclin E*, *diap1*, and *cyclin B* driven by *ey-Gal4* did not affect viability. ND, not determined.

DISCUSSION

Here we report that *mats* is an essential gene that regulates proper mitotic division. Mats is expressed at low levels ubiquitously, which is consistent with its role as a general regulator of tissue growth. Cellular localization analysis indicated that Mats is present in both the cytosol and the nucleus. Interestingly, Mats and Wts colocalize at the centrosome, suggesting that the centrosome is likely a functional site of the Mats/Wts kinase complex. In addition to *cyclin E* and *diap1*, *cyclin A* and *cyclin B* may also be targets of the Hpo/Wts signaling pathway.

Mats is essential for normal development as *mats* mutants stop their growth at the second instar larval stage and eventually die. In fact, this growth retardation phenotype facilitated identification of $mats^{roo}$ and $mats^{e235}$ mutant larvae for DNA sequence analysis (LAI *et al.* 2005). Using our $mats^{e235}$ allele and the *P*-element-induced allele $mats^{PB}$, HE *et al.* (2005) showed that *mats* homozygotes and hemizygotes grew slowly and their imaginal discs were much smaller than that of wild-type larvae at the same age. *mats* mutant cells in mosaic tissues acquire growth advantage likely through comparison and competition with neighboring wild-type cells. In contrast, the absence of wild-type cells in homozygous *mats* mutant animals renders no competitive growth advantage to mutant cells. The mechanism by which *mats* mutants acquire growth advantage in the context of mosaic tissue still needs to be investigated. *mats* mutant embryos missing both maternal and zygotic

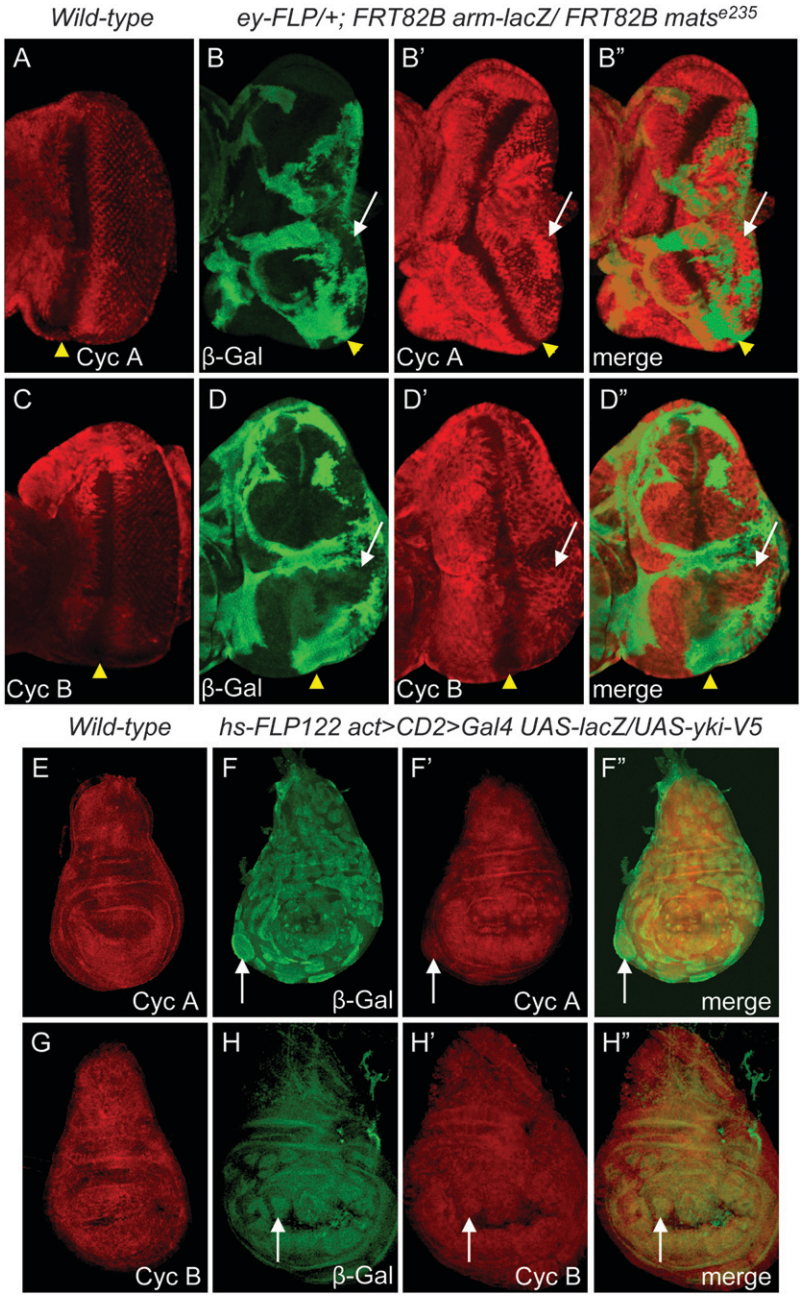

FIGURE 5.—Regulation of *cyclin A* and *cyclin B* expression by *mats* and *yki*. In normal larval eye discs, both cyclin A and cyclin B are upregulated in the second mitotic wave area just posterior to the morphogenetic furrow (A and C, respectively). Probed with cyclin A and cyclin B antibodies, higher levels of cyclin A (B–B″) and cyclin B (D–D″) proteins were found in *mats* mutant clones, which were identified in the absence of β-galactosidase expression. The morphogenetic furrow is indicated by yellow arrowheads (A–D″). Anterior is to the left in A–D″. Expression of endogenous cyclin A (E) and cyclin B (G) in wild-type third instar larval wing discs. (F–F″) Expression of the *yki-V5* transgene was clonally induced, which caused elevated levels of cyclin A (F–F″) and cyclin B (H–H″). Yki-V5 expression clones were identified by the presence of β-galactosidase expression. Examples of *mats* mutant clones and Yki overexpression clones are indicated by white arrows.

mats functions failed to hatch, indicating that *mats* is essential for embryonic development. By analyzing mitotic cells, we found that maternally *mats*-depleted embryos showed aberrant DNA segregation such that uneven amounts of DNA were segregated toward opposing centrosomes. However, this did not appear to be due to the compromised function of mitotic spindle checkpoint, as *mats* mutant tissue still accumulated M-phase cells in response to inhibition of mitotic spindle formation by colcemid treatment. Thus, *mats* is not required for mitotic spindle checkpoint, unlike *mps1*.

Cyclin E is a critical cell cycle regulator (SHERR and ROBERTS 2004). Through a Cdk2-dependent mechanism, cyclin E-Cdk2 plays a critical role in accelerating G_1–S transition in the cell cycle. As a general rule, cyclin E is tightly regulated during the cell cycle by Cdk2 and GSK-mediated phosphorylation and subsequent degradation. A nondegradable cyclin E mutant can cause extra rounds of DNA synthesis and polyploidy, and overexpression of cyclin E is frequently detected in tumor cells exhibiting polyploidy. Intriguingly, cyclin E is a centrosomal protein that functions to promote S-phase entry and DNA synthesis in a Cdk2-independent manner (MATSUMOTO and MALLER 2004). Loss of cyclin E expression in the centrosome inhibits DNA synthesis, whereas ectopic expression of cyclin E in the centrosome accelerates S-phase entry. Thus, the centrosome is

an important subcellular organelle for cyclin E to regulate cell proliferation, and the level and activity of cyclin E in centrosomes must be tightly controlled. The fact that Mats and Wts colocalize with cyclin E at the centrosome raises the possibility that Mats may function together with Wts kinase to regulate cyclin E function in the centrosome for mitotic control. In support of this hypothesis, loss-of-function mutations in *mats* increase the levels of cyclin E protein and both gain- and loss-of-function mutant alleles of *cyclin E* modulate the eye phenotypes caused by Wts overexpression (Lai *et al.* 2005; this study). Although Mats/Wts-mediated inhibition of *cyclin E* could occur through Yki to regulate *cyclin E* transcription, a direct control of cyclin E at the protein level would allow a rapid response to an upstream signal.

The fact that both Mats and Wts show a intracellular localization pattern very similar to that of their respective yeast relatives Mob1 and Dbf2 suggests that their function is conserved. This conservation may extend to mammals, as it has been shown that human LATS1, LATS2, and MOB1A (MATS2) also localize at the centrosome (Nishiyama *et al.* 1999; Morisaki *et al.* 2002; McPherson *et al.* 2004; Toji *et al.* 2004; Bothos *et al.* 2005; Abe *et al.* 2006). In addition, localization at the bud neck/midbody appears to be conserved in humans (Nishiyama *et al.* 1999; Bothos *et al.* 2005). Interestingly, such centrosomal localization of Mats and Wts does not seem to rely on Wts kinase activity as kinase-inactive Wts and Mats can be still localized at the centrosome (our unpublished observation). To examine whether endogenous Mats protein localizes at the centrosome, embryo immunostaining was done with Mats antibodies. As in larval tissues, expression of Mats protein in developing embryos does not exhibit any obvious pattern and Mats expression level is low and ubiquitous. Although we have not been able to show centrosomal localization of endogenous Mats protein, likely due to some technical problems, Mats (CG13852/Mob4) has been recently reported to be a centrosomal protein (Domingues *et al.* 2005).

Both loss- and gain-of-function analysis supports a model in which *cyclin E* and *diap1* are critical downstream targets of Hpo/Wts signaling. Evidence in this report suggests that Hpo/Wts signaling may also target *cyclin A* and *cyclin B*. Consistent with this notion, elevated levels of cyclin B were found in *ex* mutant cells (Pellock *et al.* 2007). In another study, *wts* has been shown to be required for a negative control of cyclin A but not cyclin B expression (Tao *et al.* 1999). In humans, LATS1 was shown to be a negative regulator of Cdc2/cyclin A (Tao *et al.* 1999) and to function at the G_2/M-phase transition (Yang *et al.* 2001; Xia *et al.* 2002), while LATS2 affects cyclin E/Cdk2 activity and regulates G_1/S phase passage (Li *et al.* 2003). Thus, the ability of Hpo/Wts signaling to target *cyclin* genes important for cell cycle progression appears to be evolutionarily conserved.

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