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

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**Running head:** *mats* is an essential gene and regulates tissue growth

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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 HAVE 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 UBQUITOUSLY, WHICH IS CONSISTENT WITH THE ROLE OF MATS AS A GENERAL GROWTH REGULATOR. LIKE MAMMALIAN MATS, DROSOPHILA MATS CAN COLocalIZE 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 ABBRENT 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 homologues (Tao et al. 1999; Wu et al. 2003; Lai et al. 2005).

Two upstream components implicated in the Hpo/Wts signaling are FERM domain containing membrane associated factors, Merlin/Expanded, whose activity can increase the kinase function of Hippo (Hpo) (Hamaratoğlu et al. 2006). The Fat (Ft) protein may function as a receptor in the further 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 Warts/Large tumor suppressor (Wts/Lats) (Tapon et al. 2002; Kangosingh 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 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; Tapon et al. 2002; Kangosingh 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, a growth-promoting transcriptional cofactor
Yorkie (Yki) is negatively regulated by the Hpo/Wts growth inhibitory pathway (Huang et al. 2005).

Studies of homologues in yeast and human cells have shown potential cellular activities of factors involved in the Hpo/Wts pathway. Budding yeast homologues of Wts and Mats, Dbf2 and Mob1 respectively, are components of the mitotic exit network (Luca and Winey 1998; Komarnitsky et al. 1998; Luca et al. 2001). Intracellular localization of these proteins are regulated during the cell cycle such that until anaphase, they are localized to 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 of Wts homologues, LATS1 and LATS2 were found to regulate G2/M phase and G1/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 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 not only regulates expression of Cyclin E but also 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\textsuperscript{\textast} mutants, \textit{w}\textsuperscript{1118}; \textit{FRT82B mats\textsuperscript{\textast}} / \textit{CyO-TM3\textsubscript{2chs-GFP}} was crossed with \textit{w}\textsuperscript{1118}; \textit{Df(3R)17D1}; \textit{CyO-TM3\textsubscript{2chs-GFP}} to generate \textit{mats\textsuperscript{\textast}} / \textit{Df(3R)17D1} larvae, which are equivalent to homozygous mats mutant. Expression of green fluorescent proteins (GFP) was induced by heat treatment of larvae at 37 °C for 15 min. Larvae of genotypes \textit{mats\textsuperscript{\textast}} / \textit{Df(3R)17D1} and \textit{mats\textsuperscript{\textast}} / \textit{CyO-TM3\textsubscript{2chs-GFP}} [or \textit{Df(3R)17D1}; \textit{CyO-TM3\textsubscript{2chs-GFP}}] were placed on a microscope slide. Both \textit{mats}\textsuperscript{e235} and \textit{mats}\textsuperscript{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\textsuperscript{D1} mutation that renders sterility to oocytes (\textsc{Chou} and \textsc{Perrimon} 1996). Thus, in order for heterozygous females carrying ovo\textsuperscript{D1} to be able to lay eggs, somatic recombination needs to happen to generate clones of homozygous oocytes that have eliminated ovo\textsuperscript{D1}. When used in combination with the third chromosome that harbors mats mutation, this technique generates homozygous mats mutant oocytes in heterozygous female, and only these oocytes can generate eggs. Female flies of \textit{y}; \textit{hs-FLP}; \textit{FRT82B mats}\textsuperscript{e235}/\textit{TM6B} were crossed with males of \textit{w}\textsuperscript{1118}; \textit{FRT82B ovo}\textsuperscript{D1}/\textit{TM3}, and embryos were collected for 24 hours. Hatched larvae were heat shocked for 20-30 min at 37 °C in L2 stage, and resulting virgin females of \textit{hs-FLP}; \textit{FRT82B mats}\textsuperscript{e235}/\textit{FRT82B ovo}\textsuperscript{D1} were collected. As ovo\textsuperscript{D1} gives dominant female sterility, in order for females of this genotype to be able to lay eggs, they must undergo somatic recombination in ovary to generate \textit{FRT82B mats}\textsuperscript{e235}/\textit{FRT82B mats}\textsuperscript{e235} cells, eliminating ovo\textsuperscript{D1}.
and functional mats allele from their genome. These flies were crossed with males of w1118, FRT82B mats$^{e235}$/CyO-TM3 2xhe-GFP and resulting embryos were heat shocked at 37 ºC for 15 min. Embryos and larvae were handpicked and placed on the microscope slide and GFP autofluorescence was observed under 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 hours handpicked embryos were washed in PBS, and dechorinated in mild bleach for 2 min. After washes, embryos were fixed in 1:1 mix of paraformaldehyde-lysine-phosphate (PLP: 2% paraformaldehyde, 0.75M 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 BSS Triton 0.3% (ASHBURNER, 1989), and stained with anti-Cnn antibodies (a gift of Thomas Kaufman, MEGRAW et al. 2002) at 4 ºC 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 ºC). 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, Leiscestershire, UK) was used for DNA staining.
Genetic interactions of Wts/Lats signaling: UAS-myc-wts6R on the second chromosome is a relatively strong allele and was recombined with ey-Gal4 to generate w1118; ey-Gal4, UAS-myc-wts6R/SM6·TM6B. Females of this genotype were crossed with w1118, UAS-cycE, UAS-diap1, UAS-cycA, UAS-cycB, cyclin EAr85/TM3, diap1⁴/TM3, cyclin Ac8LR1/TM3, cyclin A⁰₃⁹₄⁶/TM3, cyclin B²/CyO, or cyclin Bkg08886/CyO male flies. UAS transgenic flies were crossed with w1118; 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 w1118 flies to determine their heterozygous phenotypes.

Somatic homozygous mats mutant cells were generated by crossing w1118, FRT82B mats⁶²³⁵/TM6B males with y,w, ey-FLP; FRT82B arm-lacZ/TM6B females. For control, w1118 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 BSST twice. Peripodial membranes were removed, and discs were stained with primary antibodies [mouse anti-Cyclin A (1:5 dilution) and mouse anti-Cyclin 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 one hour at 37 °C 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 mpsl or mats homozygous mutant clones were generated by crossing w1118, FRT82B 90E P[w⁺] or w1118, FRT82B mpsl¹/TM6B (a gift of Christian Lehner, FISCHER et al. 2004) or w1118; FRT82B
mats^{235}/TM6B males with UAS-GFP, ey-FLP; Tub-Gal4, FRT82B Tub-Gal80/TM6B females.

Dissected eye discs were incubated for 3 hours in Schneider S2 medium (Invitrogen, Carlsbad, CA) either with or without 1μM colcemid (Sigma, St. Louis, MO) 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 (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. They 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 in order to eliminate this maternal loading of mats to the embryos. Using a dominant female sterile (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. Out 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, which 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 (NEWPORT and KIRSCHNER 1982; EDGAR *et al.* 1986). Therefore, in maternally mats null embryos, the cell divisions up to the sixteenth 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 as well as 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, 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 2A-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 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 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 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 morphogenetic furrow, such that cells in the furrow are in G1 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 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 co-localized 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 \textit{mats-GFP} and \textit{myc-wts} fusion genes under the control of cytomegalovirus (CMV) promoter. We found that Mats-GFP was localized in the cytosol as well as the nucleus, whereas Wts was exclusively cytosolic during interphase (Figure 3). It was also clear that both Mats and Wts were accumulated at the perinuclear region in a dot-like pattern. This dot was duplicated once the mitotic DNA segregation commenced. These accumulations appeared to be centrosomes, as they co-localized with \[\gamma\]-tubulin signals (Figure 3, A-H). This observation is consistent with the centrosomal localization of human LATS1 and MOB1A (MATS2) reported elsewhere (\textit{Nishiyama et al.} 1999; \textit{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 co-localize with endogenous human Cyclin E at the centrosome (Figure 3, I-P). Thus, Mats and Wts proteins appear to function together in subcellular organells such as centrosomes.

**Genetic interactions among \textit{wts}, \textit{diap1} and \textit{cyclin E} genes:** \textit{cyclin E} and \textit{diap1} are considered to be two downstream targets of the Hpo/Wts pathway (reviewed in \textit{Harvey} and \textit{Tapon}, 2007; \textit{Pan}, 2007). To further observe functional interactions between Hpo/Wts signaling and its output, \textit{ey-Gal4} was used to express \textit{UAS-myc-wts} in the developing eye. Compared to a previously reported line 16B (\textit{Lai et al.} 2005), the 6R line exhibited stronger phenotypes. Thirty percent of the \textit{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-wts6R*, expression of *cyclin E* and *diap1* effectively suppressed the cone-shaped and small eye phenotypes caused by *wts* overexpression (compare Figure 4I and 4J with Figure 4H). All of the *ey-Gal4 UAS-myc-wts6R/UAS-cycE* and *ey-Gal4 UAS-myc-wts6R/UAS-diap1* flies survived to the adult stage (Table 1). Moreover, effects of loss-of-function alleles of *cyclin E* and *diap1* on Wts-induced mutant phenotypes were examined. While *cycE^{AR95} and diap1^{d4}* heterozygotes were phenotypically normal (Figure 4D and 4E), 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 Figure 4H). The lethal phenotype was also enhanced as 60% of the *ey-Gal4 UAS-myc-wts6R/cycE^{AR95}* flies and 85% of the *ey-Gal4 UAS-myc-wts6R/diap1^{d4}* flies died at the pupal stage (Table 1). These results further support that *cyclin E* and *diap1* are critical targets of Hpo/Wts signaling.

**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-wts6R assay, we have examined how cyclin A and cyclin B genes might genetically interact with wts. To do this, UAS-myc-wts6R 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 wts6R 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-wts6R/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-wts6R flies was observed (data not shown). It appears to be that the eye phenotype of ey-Gal4 UAS-myc-wts6R 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. In order to assess whether Mats plays a role in this cellular response, MARCM system (LEE and LUO, 1999) was used to generate eye discs containing either control, mpsl mutant or mats mutant clones, and the
tissue was dissected and incubated either in the presence or absence of colcemid for two hours and accumulation of M phase cells were 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, - colcemid (n = 7 eye discs), + colcemid (n = 10 eye discs)]. Discs containing mps1 mutant clones, however, showed accumulation of M phase cells only at 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 just as seen in the control group, especially at the 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.

DISCUSSION

Herein, 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 both in the cytosol and 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 \(mats^{lo}\) and \(mats^{e235}\) mutant larvae for DNA sequence analysis (Lai et al. 2005). Using our \(mats^{e235}\) allele and a 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 \(mats\) 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 towards 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 Robert, 2004). Through a Cdk2-dependent mechanism, Cyclin E-Cdk2 plays a critical role to accelerate G1-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 non-degradable 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, 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- as well as 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 upstream signal.

The fact that both Mats and Wts show very similar intracellular localization pattern as in 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 to human (Nishiyama et al. 1999; Yang et al. 2004; 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. Like 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. Europ. Dros. Res. Conf. 19:DR6, 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 shown 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 human, LATS1 was shown to be a negative regulator of Cdc2/Cyclin A (TAO et al. 1999) and functions at the G2/M phase transition (YANG et al. 2001; XIA et al. 2002), while LATS2 affects Cyclin E/Cdk2 activity and regulates G1/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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LITERATURE CITED


TOJI, S., N. YABUTA, T. HOSOMI, S. NISHIHARA, T. KOBAYASHI et al., 2004 The centrosomal protein Lats2 is a phosphorylation target of Aurora-A kinase. Genes Cells **9**: 383-397.


WEI, X., SHIMIZU, T., and Z.-C. LAI, 2007 Mob as tumor suppressor is activated by Hippo kinase in growth inhibition in Drosophila. EMBO J. **26**: 1772-1781.


WU, S., J. HUANG, J. DONG and D. PAN, 2003 *hippo* encodes a Ste-20 family protein kinase that
restricts cell proliferation and promotes apoptosis in conjunction with *salvador* and *warts*.

**Cell** 114: 445-456.


**Yoshida, S., and A. Toh-e,** 2001 Regulation of the localization of Dbf2 and Mob1 during cell division of *saccharomyces cerevisiae*. Genes Genet Syst 76: 141-147.
Figure 1. mats mutations cause DNA segregation defect.

Control (w^{1118}) (A-A’’) and maternally mats null mutant (B-B’’) embryos were stained with anti-Centrosomin (Cnn) antibodies and a DNA dye Draq5. Centrosomes were identified by Cnn (A, B) and DNA by Draq5 (A’, B’). Compared to control embryos (A-A’’), maternally mats depleted embryos (B-B’’) exhibited DNA segregation defects. Arrows highlight aberrant DNA segregation observed in mats mutant embryos.

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 w^{1118} eye disc showed very low signals. (D) When mats transgenes were expressed in GMR-Gal4; UAS-matsB121B142, high levels of Mats staining was 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 all panels.

Figure 3. Mats and Wts accumulate at the centrosome throughout the cell cycle.

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

Figure 4. wts overexpression phenotype is effectively modified by dosage change of diap1 and cyclin genes.

SEM micrograph of (A) w1118 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 is normal (C, ey-Gal4/+; +/UAS-diap1). Heterozygosity of cyclin E (D, cycE<sup>AR95</sup>/+) or diap1 (E, diap1<sup>4</sup>/+) 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<sub>6R</sub>/+ reduced viability and eyes became smaller and cone-shaped. Coexpression of either cyclin E (I, ey-Gal4 UAS-myc-wts<sub>6R</sub>/ UAS-cycE) or diap1 (J, ey-Gal4 UAS-myc-wts<sub>6R</sub>/+; +/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<sub>6R</sub>/cycE<sup>AR95</sup>) or diap1 (L, ey-Gal4 UAS-myc-wts<sub>6R</sub>/+; +/diap1<sup>4</sup>) 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<sub>6R</sub>/UAS-cyclin A. (N) ey-Gal4 UAS-myc-wts<sub>6R</sub>/UAS-cyclin B. Anterior is to the left in all panels.
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 with the absence of β-Galactosidase expression. The morphogenetic furrow was indicated by yellow arrowheads (A-D’’). Anterior is to the left in panels (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.

Supplementary Figure:

Figure S1. Mitotic checkpoint function is not disrupted in *mats* mutant tissue.

MARCM system was used to generate clones of 90E P[w’] (control, A-F), *mps1*’ (G-L) and *mats*’ (M-R) respectively and the M phase cells were probed with PH3 staining with or without mitotic checkpoint induction by treatment with colcemid. In panels A, D, G, J, M and P, GFP-positive areas represent clones generated by recombination. Panels B, E, H, K, N and Q shows PH3 staining. Colcemid treatment was done as indicated. Yellow arrowheads denote the location of morphogenetic furrow. Anterior is to the left in all panels.
Table 1. *wts* genetically interact with *diap1* and *cyclin* genes.

<table>
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<th>Genotype</th>
<th>% of flies that died at the pupal stage:</th>
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<td><em>ey-Gal4, UAS-myc-wts6R/UAS-cyclin B</em></td>
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Viability of *cyclin* E^{AR95}/+ and *diap14*/+ heterozygotes are normal. Similarly, overexpression of *cyclin E*, *diap1* and *cyclin B* driven by *ey-Gal4* did not affect viability. ND, not determined.
Figure 1

α-Cnn DNA Merge

mats+/

mats−/−

A  B  C

D  E  F

merge
Figure 2
Figure 3
Figure 4

<table>
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<tr>
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<th>UAS-diap1</th>
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<td>I</td>
<td>J</td>
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</table>
Figure 5

Wild-type  ey-FLP/+; FRT82B amlцов FRT82B matB235

Wild-type  hs-FLP22 act>CD2>Gal4 UAS-lacZ/UAS-yki-V5