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# Mob as tumor suppressor is activated at the cell membrane to control tissue growth and organ size in *Drosophila*

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ABSTRACT

Growth inhibition mediated by Hippo (Hpo) signaling is essential for tissue growth and organ size control in *Drosophila*. However, the cellular mechanism by which the core components like Mob as tumor suppressor (Mats) and Warts (Wts) protein kinase are activated is poorly understood. In this work, we found that the endogenous Mats is located at the plasma membrane in developing tissues. Membrane targeting constitutively activates Mats to promote apoptosis and reduce cell proliferation, which leads to reduced tissue growth and organ size. Moreover, the ability of membrane-targeted Mats to inhibit tissue growth required the *wts* gene activity and Wts kinase activity was increased by the activated Mats in developing tissues. Consistent with the idea that Mats is a key component of the Hpo pathway, Mats is required and sufficient to regulate Yki nuclear localization. These results support a model in which the plasma membrane is an important site of action for Mats tumor suppressor to control tissue growth and organ size.


## Introduction

Growth inhibitory signaling provides an important regulatory mechanism for restricting tissue growth and organ size during development. Genetic and molecular analysis in *Drosophila* has defined a novel growth inhibitory pathway mediated by a number of tumor suppressors; loss of this growth inhibitory signaling leads to tissue overgrowth and tumor development (for reviews, see Hariharan and Bilder, 2006; Harvey and Tapon, 2007; Pan, 2007; Reddy and Irvine, 2008; Saucedo and Edgar, 2007). On the cell surface, growth inhibition is partly mediated by two transmembrane protocadherin proteins, Dachsous (Ds) and Fat (Ft). Two FERM-domain proteins Merlin (Mer) and Expanded (Ex) are proposed to function upstream of four other tumor suppressors: a scaffold protein Salvador (Sav), two serine/threonine protein kinases Hippo (Hpo) and Warts (Wts)/Large tumor suppressor (Lats), and a Mob family protein Mob as tumor suppressor (Mats). Components in this pathway such as Mats are structurally conserved across species from flies to mammals (Ye et al., 2009), and evidence is accumulating to support that functions of these molecules in growth control are evolutionarily conserved as well (recently reviewed in Reddy and Irvine, 2008; Zeng and Hong, 2008; Zhao et al., 2008a).

The Wts protein kinase is a key downstream component of the pathway and is regulated via several different mechanisms. First, Wts is activated by the Hpo protein kinase through phosphorylation (reviewed in Hergovich et al., 2006a). Second, Mats acts as a binding partner and co-activator of Wts kinase to inhibit tissue growth (Lai et al., 2005), while Mats is also phosphorylated by Hpo protein kinase and consequently becomes more potent in up-regulating Wts kinase activity (Wei et al., 2007). Third, the level of Wts protein is negatively regulated by an unconventional myosin, Dachs, which acts downstream of *ft* to promote tissue growth (Cho et al., 2006; Mao et al., 2006). A tagged Dachs protein was shown to locate to the cell membrane in transgenic tissues. Loss of *ft* function elevated the Dachs expression and overexpression of Ft decreased the level of Dachs at the membrane. While expression of the endogenous Wts protein has not been characterized and reported, a Myc-tagged Wts protein was shown to accumulate at the cell membrane (Cho et al., 2006). Ft signaling is critical for recruiting and/or maintaining Myc-Wts at the membrane as loss of *ft* reduced the level of the Wts protein at the cell membrane. Consistent with a negative role of *dachs* in regulating Ft signaling, loss of *dachs* function suppressed the effect of *ft* mutation

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on Wts protein level (Cho et al., 2006). The Dachs protein has been shown to form a complex with Wts in cultured cells, but how Dachs is be involved in regulating Wts level and/or localization remains an open question.

A major signal output of the Hippo pathway in growth control is to target a growth-promoting transcription co-activator Yorkie (Yki) protein for inactivation. Activated Wts kinase directly phosphorylates Yki (Huang et al., 2005; Dong et al., 2007; Oh and Irvine, 2008; Wei et al., 2007; Zhang et al., 2008; Zhao et al., 2007). Consequently, Yki is maintained in the cytoplasm and unable to function as a co-activator of the DNA-binding transcription factor Scalloped (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008b). Thus, regulation of Yki subcellular localization can be achieved by adjusting the level and catalytic activity of Wts protein kinase. However, it is not clear how Wts and Mats proteins are localized inside of the cell and whether their subcellular location is critical for their activation in regulating tissue growth. A previous study using cultured mammalian cells revealed that membrane-targeted expression of human MATS proteins (hMOB1A and hMOB1B) can activate MATS to increase human LATS1 kinase activity (Hergovich et al., 2006b). However, it is not known whether membrane association of MATS has any impact on cell proliferation and apoptosis and whether it is sufficient to affect tissue growth and organ size during development. Moreover, the epistatic relationship between Mats/MATS and Wts/LATS has not been rigorously investigated. Using *Drosophila* as a model, we found that Mats is a membrane-associated protein. We show that membrane-targeted Mats is constitutively activated to promote apoptosis and reduce cell proliferation for restricting tissue growth. We observed that the catalytic activity of Wts kinase can be increased by membrane-targeted Mats in developing tissues. Moreover, we found that *wts* is epistatic to *mats*, which supports a model of Wts kinase acting downstream of Mats. Indeed, the ability of membrane-targeted Mats to inhibit tissue growth required the endogenous *wts* gene activity. Our results indicate that the plasma membrane is a key site for Mats and Wts to mediate Hippo growth inhibitory signaling for tissue growth and organ size control.

## Results

### *Association of the endogenous Mats protein with the plasma membrane*

To better understand how Mats regulates tissue growth, subcellular localization of the endogenous Mats protein in developing tissues was characterized. Through immunostaining, Mats was found to be associated with the plasma membrane in differentiated cells such as cone cells in pupal eye discs and larval salivary gland cells (Figs. 1A–C; Fig. S1). In larval wing discs, Mats was colocalized with E-cadherin in the subapical region of the plasma membrane in many cells (Figs. 1D–F and D″–F″). At the basal levels, Mats was mostly localized in the cytoplasm (Figs. 1D′–F′ and D″–F″). These results revealed that Mats can be normally localized at the plasma membrane, where it might play a critical role to control tissue growth and organ size.

### *Membrane localization activates Mats to inhibit tissue growth*

To test a hypothesis that cell membrane is a critical site for Mats activation in growth control, we generated transgenic flies that express a Mats fusion protein containing a 16-amino-acid myristoylation (Myr) signal sequence for membrane localization. As a negative control, a mutant version of Myr with the second residue Gly (G) replaced by Ala (A), $Myr^{G2A}$, was used to generate $Myr^{G2A}$-Mats fusion proteins. The Gly to Ala alteration in the Myr signal sequence abolishes its lipid modification and consequently prevents membrane localization (Roy et al., 2002). To facilitate detection of the fusion proteins, the coding region of green fluorescent protein (GFP) was fused in frame with *mats*. Similar to Mats, Mats-GFP is functional as it can rescue the lethality and tissue overgrowth phenotypes of *mats* mutants (Shimizu, 2006). Moreover, overexpression of Mats-GFP does not interrupt normal development. As predicted, Myr-Mats-GFP was found to associate with the cell membrane in transgenic tissues (Figs. 1G–I). In contrast, $Myr^{G2A}$-Mats-GFP or Mats-GFP was not accumulated at the cell membrane (Fig. S2D; data not shown).

Functional significance of membrane-targeting of Mats was investigated in both eye and wing tissues. Driven by an eye-specific driver GMR-Gal4, membrane-targeted Mats dramatically decreased the eye size (Fig. 1K). Similarly, expression of Myr-Mats-GFP in the developing wing greatly reduced the wing size (Fig. 1N and Table S1). In contrast, $Myr^{G2A}$-Mats-GFP was unable to cause any defect in the eye or wing tissues (Figs. 1L and O; Table S1). In larval wing discs, the posterior compartment that expressed Myr-Mats-GFP but not $Myr^{G2A}$-Mats-GFP was clearly reduced in size (Fig. 2, compare D, G, and J with A). Moreover, clonal expression of Myr-Mats-GFP in wing discs reduced the number of clones as well as the clone size compared to $Myr^{G2A}$-Mats-GFP and Mats-GFP (Figs. 1P–R). Thus, membrane localization of Mats constitutively activated Mats to inhibit tissue growth and reduce organ size.

### *Membrane localization of Mats causes increased apoptosis and decreased cell proliferation*

Reduction of tissue growth and organ size by membrane-targeted Mats could be due to defective apoptosis and/or cell proliferation. To test these possibilities, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling) staining was done to identify apoptotic cells in Myr-Mats-GFP-expressing and control tissues. When expressed in the posterior compartment of larval wing discs, membrane-targeted Mats induced a dramatic increase of apoptosis (Figs. 2G–I). Similarly, expression of an apoptosis-promoting protein, Caspase 3, was also up-regulated by Myr-Mats-GFP but not $Myr^{G2A}$-Mats-GFP (Fig. 2, compare D–F with A–C). Cell proliferation was monitored by examining BrdU incorporation in proliferating cells. We found that BrdU levels were reduced in Myr-Mats-GFP-expressing cells compared to control cells in the anterior compartment (Figs. 2J–L). Thus, membrane targeted-Mats inhibited tissue growth by promoting apoptosis and reducing cell proliferation.

### *Mats functions through Wts kinase to inhibit tissue growth*

Mats functions as a co-activator to increase the catalytic activity of Wts kinase to inhibit tissue growth (Lai et al., 2005; Wei et al., 2007). To further address the importance of *wts* for *mats*-mediated tissue growth inhibition, apoptosis was induced in clones that expressed membrane-targeted Mats in larval wing discs (Figs. 3A, B). In the absence of *wts*, the ability of membrane-targeted Mats to induce apoptosis was greatly reduced (Figs. 3C, D). We have also investigated the epistatic relationship between *mats* and *wts* based on the organ size phenotype. Compared to late third-instar larval eye discs containing wild-type clones (Fig. 3E), eye discs that contain *wts* loss-of-function mutant clones were folded and larger in size (Fig. 3F). In comparison, eye discs that expressed Myr-Mats-GFP were much smaller (Fig. 3G). When *wts* function was abolished in the cells that also expressed Myr-Mats-GFP, the growth inhibitory effect of Myr-Mats-GFP was suppressed as the eye discs exhibited phenotypes similar to that of tissues containing only *wts* mutant clones (Fig. 3H). Thus, *wts* is genetically epistatic to *mats* and is critical for membrane-targeted Mats to inhibit tissue growth. Consistently, Wts expression in *mats* loss-of-function mutant clones induced in eye discs can suppress the tissue overgrowth phenotype caused by *mats* mutation (Shimizu, 2006). Taken together, these results indicate that *wts* is required for *mats*-mediated tissue growth inhibition.