MOB AS TUMOR SUPPRESSOR IS ACTIVATED AT THE
PLASMA MEMBRANE TO
CONTROL TISSUE GROWTH AND ORGAN SIZE IN DROSOPHILA

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Integrative Biosciences

by
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ABSTRACT

The control of organ size is a fundamental issue in life. This control can be achieved by coordinating cell proliferation and cell death during development. A newly discovered tumor suppressor pathway, Hippo pathway, has been shown to restrict cell proliferation and promote apoptosis. However, the cellular mechanism of how the core components like Mob as tumor suppressor (Mats) and Warts (Wts) protein kinase are activated is not completely understood. In my thesis studies, I found that the endogenous Mats is able to locate at the plasma membrane in *Drosophila* developing tissues. Membrane-targeting Mats constitutively activates Mats to promote apoptosis and reduce cell proliferation leading to reduction of tissue growth and organ size. In addition, these growth inhibitory effects require the presence of Wts and Wts kinase activity is increased by constitutively active Mats. Furthermore, constitutively active Mats is sufficient to reduce Yorkie nuclear localization. All these data suggest the idea that plasma membrane is a crucial site for the action of Mats tumor suppressor to control tissue growth and organ size.
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Chapter 1

Introduction

1.1 Organ Size Control

The development of organism requires proper growth and patterning. Several pathways, such as Notch, dpp (decapentaplegic), TGF-β, transforming growth factor-α, Wingless, JAK/STAT, nuclear receptor and receptor tyrosine kinase have been shown to be involved in regulating growth and determining fates of cells in metazoans (Barolo and Posakony, 2002). Among all the processes during development, organ size control is one of the interesting questions which has attracted a lot of attentions in recent years. How do different organs sense the limitations of size or availability of space, and then stop growing during development? Why do different organs have variable sizes? How do the extrinsic and intrinsic signals control organ size and how do recipient cells respond to the stimuli?

One of the factors making different organ size results from change in cell sizes (Cook and Tyers, 2007). Previously, cell size control has been demonstrated to be regulated by availability of nutrition. The stimuli transmit growth signals through receptors, and activate Akt/PKB, TOR, Myc, E2F and cyclin D downstream to allow cell size to pass size threshold or check point. However, the cell size control can not explain the entire phenomenon in organ size control. For example, overexpression of E2F in Drosophila wings increases cell size but not organ size because of the compensation effects from reduction in cell numbers (Edgar, 2006). Based on screens done in eye and wing discs in Drosophila, cell number control, polarity, morphogen pathways, endocytosis and growth factors are all involved in organ size control (Edgar, 2006; Jorgensen and Tyers, 2004).

To maintain the homeostasis of organ size, cell number control is crucial. It consists of two main processes: cell proliferation and cell death. Proliferation represents increase in cell numbers while cell death leads to decrease in cell numbers. These two processes need to be tightly coordinated to achieve the certain numbers to reach proper organ sizes. Deregulation of cell number control in multicellular organism leads to pathological diseases, such as cancers. Recently, a novel pathway, Hippo pathway, has been discovered to coordinate both
proliferation and death of cells through genetic screens or spontaneous mutations (Pan, 2007). Loss of function mutations of some components in this pathway result in overgrowth which resemble the loss of function mutation of tumor suppressors in multicellular organism. In the last decade, several molecules have been identified to be involved in the regulation of Hippo pathway from receptor-ligand interaction to downstream target genes expression.

1.2 Drosophila melanogaster as a Cancer Model

*Drosophila melanogaster* has been used as a model organism in addressing developmental processes due to its short reproductive cycle and fertility of progenies. The generation of the balancer chromosome allows a mutant allele to be kept and passed to the progenies. This makes *Drosophila* an organism in genetic studies. With the development of transgenic techniques (Spradling and Rubin, 1982), genetic and fluorescent markers inserted in *Drosophila* genome (Brand and Perrimon 1993; Lee and Luo, 2001), people are able to express transgenes and observe molecules in tissue and developmental stage specific manners. All these developments make *Drosophila* an *in vivo* system to address physiological questions through cell biological means.

In addition to the genetic tools mentioned above, *Drosophila* also has some other advantages for studying disease. Over two thirds of human disease-related genes have homologues in *Drosophila* which makes it a model organism relevant to human disease studies (Bernards and Iariharan, 2001; Bier, 2005). Moreover, tissues in *Drosophila* may have similar characteristics in human. For example, *Drosophila* epithelial tissues have intact structures which resemble epithelial tissues in human. Mutation of genes controlling proliferation and apoptosis in *Drosophila* epithelial tissues results in overgrowth which is similar to solid tumors arised in human epithelia (Vidal and Cagan, 2006).

Cancer, a disease resulting from exposure to genetic mutations, chemical mutagens and various incident factors, has been studied for several decades. One type of typical occurrence is losing tumor suppressor function in tumorigenic cells. Loss of heterozygosity in tumor suppressor heterozygous mutant cells leads to homozygous mutation of tumor suppressors. The complete loss of tumor suppressors leads to deregulation of cell cycle or loss of check point which later causes overgrowth of cancer cells (Fitzgerald et al., 1983; Testa and Hino, 2003). To study cancers, generation of an individual with homozygous mutant genetic backgrounds for a tumor suppressor is sometimes challenging. This is due to the
fact that most of tumor suppressors are required during developmental stages. Therefore, complete loss of these essential genes is mostly lethal to animals (Clarke et al., 1992; Davidson et al., 2008). To circumvent this limitation, clonal generation of homozygous mutant cells within heterozygous background in an individual animal would be an alternative approach. It also mimics the incidence of how cancer occurs in human in the first place.

To achieve this goal, yeast FLP/FRT recombination system (Chou and Perrimon, 1992; Golic, 1991; Golic and Lindquist, 1989) has been introduced into the Drosophila genome to generate genetic mosaic animals (Xu and Rubin, 1993). This allows scientists to identify and study the effects of homozygous mutations of the tumor suppressors at single cell and tissue levels. By taking advantage of genetic mosaics, several genetic screens were carried out to search for mutations of novel genes leading to overgrowth phenotypes. If similar mutant phenotypes from these growth-regulating genes are observed, these genes may function in the same signaling pathway. An example is the Hippo signaling pathway which will be described in more detail in the next section.

1.3 A Brief History of Hippo Signaling Pathway Discovery

In recent years, the components of a novel Hippo signaling pathway have been identified through genetic screen or spontaneous mutations based on overgrowth phenotypes of epithelial tissues without affecting patterning in Drosophila. The first tumor suppressor of the pathway, warts (wts; also called lats), was discovered in 1995 (Xu, et al., 1995; Justice, et al., 1995). It belongs to Nuclear Dbf-2-related (NDR) protein kinase family of the AGC group of Ser/Thr kinases. The mammalian homologs are termed Lats1 and Lats2 (Large tumor suppressor) (Tao et al., 1999; Xia et al., 2002). Loss of wts results in overgrowth in eyes, central nervous system and extra cuticles due to excess cells which indicate that wts acts as a suppressor for cell number control.

A ww-domain containing protein, Salvador (Sav)(also called Shar-Pei), has been discovered to interact with Wts. Both mutants have similar phenotypes. Loss of sav induces cell proliferation and delays cell cycle exit by increasing Cyclin E level and decreasing apoptosis by reducing DIAP-1 (Drosophila Inhibitor of apoptosis-1), an inhibitor of apoptosis stimulated by various signals (Tapon et al., 2002; Kango-Singh et al., 2002). In 2003, Hpo kinase belonging to mammalian Ste-20 (MST) Ser/Thr kinase family has been identified to activate Wts through phosphorylating Wts and Sav. The phosphorylation of Sav by Hpo makes Sav a
better scaffold protein to bring Wts and Hpo together, and potentiates Wts activation (Harvey et al., 2003; Hay and Guo, 2003; Udan et al., 2003; Wu et al., 2003). Tissues containing loss of hpo also have overgrowth phenotypes similar to wts and sav mutants. Cyclin E and DIAP-1 levels are elevated which further support Hpo, Sav and Wts function in the same pathway. In addition, a coactivator called Mats (Mob as a tumor suppressor) was found to interact with Wts kinase to potentiate full kinase activity of Wts (Lai et al., 2005). Mats belongs to a Mob family whose yeast homolog serves as a coactivator of NDR kinase family, a homolog of Wts. Taken together, Wts, Sav, Hpo and Mats form a core complex called Hippo kinase cassette acting as a center of Hpo signaling pathway between upstream and downstream effectors.

The connection between Hpo kinase cassette and transcription targets is now known to be linked through Yorkie (Yki), a transcriptional co-activator that interacts with Wts and upregulates transcription of diap-1. It is a substrate of Wts kinase (Huang et al., 2005). yki encodes the Drosophila ortholog of yes-associated protein (YAP), a transcriptional coactivator in mammalian cells (Yagi et al., 1999; Strano et al., 2001; Vassilev et al., 2001). In clonal studies, yki gain-of-function phenocopies loss of hpo, sav and wts suggesting that yki may function as an oncogene in this pathway. Importantly, loss of yki suppresses overgrowth phenotypes resulting from loss of wts, hpo or sav indicating yki is epistatic to wts, hpo and sav. The discovery of yki provides a missing link between Hippo signaling and transcriptional control.

The question remains for how Hpo signaling activates downstream target genes since Yki does not bind to DNA directly. In search for the DNA binding factors of Hippo signaling pathway, several candidates have been proposed based on the molecular studies of YAP, the mammalian ortholog of Yki. For example, YAP interacts with p73, Runx family proteins and TEAD family proteins. In Drosophila, Scalloped (Sd), which belongs to TEAD family, was reported to interact with Yki and bind to Hippo Responsive Element (HRE) to drive transcription of diap-1 gene. Sd transcriptional ability increases as Sd interacts with Yki. In addition, Sd is required for Yki’s effects on downstream target genes (Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008). However, not every imaginal disc expresses Sd implying that there may be some other binding partners for Yki.

To search for upstream signals activating Hpo pathway is also an important issue. How do cells respond to extracellular signal related to cell number control? Two four point one, ezrin, radixin, moesin (FERM) domain cytoskeleton proteins, Expanded (Ex) and Merlin (Mer) (Hamaratoglu et al., 2006; McCartney et al.,
and a protocadherin transmembrane receptor Fat (Ft) were found to activate Hippo signaling (Feng and Irvine, 2007; Willecke et al., 2006; Silva et al., 2006; Cho et al., 2006; Bennett and Harvey, 2006). Based on the extra cells in pupal interommatidial region, loss of ex, mer, or ft all result in increase in cell numbers; however, the phenotypes are not as strong as those in loss of ex and mer double mutants. In addition, the Cyclin E and Diap-1 level were higher in double mutants than single mutants indicating ex and mer may act redundantly at some levels. Later studies showed that Ex in Drosophila was responsible more in cell cycle exit and Mer plays more important roles apoptotic mechanism (Pellock et al., 2007).

Ft, a receptor with multiple cadherin domains in its extracellular region, was placed upstream of Ex. The receptor upstream of Mer has not been identified yet. Loss of ft not only leads to overgrowth of imaginal discs but also to change of planar cell polarity (PCP). Two distinct mechanisms were reported for ft to regulate Hippo signaling pathway: one is through Ex and then Hippo (Bennett and Harvey, 2006; Silva et al., 2006; Willecke et al., 2006), and the other one is through an atypical myosin, Dachs (D)(Mao et al., 2006) which regulates Wts stability (Cho et al., 2006; Feng and Irvine, 2007). The Ft-Ex-Hpo pathway mainly increases Hpo kinase activity after Ft activates Ex, and increase of Hpo kinase activity results in elevated Wts kinase activity and decreased Yki in the nucleus. Therefore, the downstream target genes were downregulated. In Ft-Dachs-Wts pathway, Dachs acts as a destabilizing molecule to decrease amount of Wts kinase. If the pathway is active, Dachs will be inhibited by Ft and abundant Wts kinase is able to perform its growth inhibitory function. If the pathway is inactive, Dachs will accumulate at the plasma membrane and decrease Wts level which results in inactivation of Hpo kinase cassette. This leads to Yki localization in the nucleus and drive the expression of diap-1(reviewed in Kango-Singh and Singh, 2009).

Three additional molecules were shown to interact with Ft receptor. Dachsous (Ds), another protocadherin transmembrane protein, presumably serves as a ligand on the neighboring cells interacting with the Ft receptor. However, overexpression and loss of ds both cause elevation of downstream target genes suggesting that there are other mechanisms involved. Later, discontinuities of induction were observed (Willecke et al., 2008).

Recently, another mechanism regulating Ft in parallel to Ds has been characterized upstream of Ft. Four-jointed (Fj), a golgi kinase (Ishikawa et al., 2008), phosphorylate extracellular domain of Ft and modulate the interaction between Ds and Ft. An intracellular Casein kinase I, Discovergrown (Dco), is able to phosphorylate cleaved Ft containing intracellular and transmembrane domain.
This Dco-dependent phosphorylation on Ft then activates growth control and PCP signaling downstream of Ft (Sopko et al, 2009; Feng and Irvine, 2009).

1.4 Downstream Target Genes of Hippo pathway

Activation of Hippo pathway results in inhibition of cell proliferation and upregulation of apoptosis. These phenotypes are supported by elevated BrdU signals and reduced TUNEL stainings in clones lacking tumor suppressors in the Hippo pathway. Most downstream target genes in response to Hippo pathway encode proteins related to cell proliferation and apoptosis. In the regulation of cell

Figure 1-1: Components of Hippo signaling pathway
proliferation, elevation of Cyclin E (Kango-Singh et al., 2002; Tapon et al., 2002), Cyclin B (Tyler and Baker, 2007), E2F (Nicolay and Frolov, 2008) have been observed in the Hippo pathway mutant cells. In addition, Diap-1 (Drosophila inhibitor of apoptosis protein-1) is also enhanced meaning apoptosis is suppressed in cells defecting of Hippo signaling (Tapon et al., 2002).

Furthermore, Hippo pathway also activates downstream target genes including microRNA bantam, which affects cell survival and growth (Nolo et al., 2006), and cytoskeleton protein Ex which regulates cell morphology. Therefore, Ex not only acts upstream of Hpo signaling but also is a target of Hippo pathway which forms a negative feedback loop of the regulation. Genes from other pathways are also regulated by Hippo signaling like four-jointed which modulates planar cell polarity (Fanto et al., 2003; Cho and Irvine, 2004) and wingless which regulates patterning and growth (Cho and Irvine, 2004; reviewed in Edgar, 2006; Reddy and Irvine, 2008).

1.5 Regulation of Hippo Signaling Pathway in Drosophila

Hippo pathway is mainly regulated in three different manners including protein-protein interactions, phosphorylation of substrates by kinases and change of subcellular localizations. The activity of Hippo pathway can be determined by Wts kinase activity since two possible activation mechanisms act through Wts to regulate transcription. When Wts is in phosphorylated state indicating Hippo pathway is active, Wts stability and level should be maintained because D is inhibited by activated Ft and undetected at the plasma membrane. Phosphorylation of Ft by Dco and Ds-binding both result in Ft activation which leads to plasma membrane localization and accumulation of Ex. In addition to Ft, Ex and Mer may also be activated by other upstream receptors which lead to Hpo phosphorylation. Phosphorylated Hpo then phosphorylates Sav which brings Wts and Hpo together. Moreover, Wts is also phosphorylated and subsequently activated by Hpo. Wts kinase activity is further potentiated by its binding partner, Mats. Active Wts phosphorylates Yki and blocks Yki from entering into nucleus. This inactive cytosolic Yki is associated with 14-3-3 proteins (Basu et al., 2003; Dong et al., 2007; Hao et al., 2008; Oh and Irvine, 2008; Zhang et al., 2008; Zhao et al., 2007). Therefore, downstream target genes are not transcribed even though Sd may reside in the nucleus (reviewed in Reddy and Irvine, 2008).
When Wts is not phosphorylated meaning Hippo pathway is not active, D accumulates at the plasma membrane reducing the level of Wts and Ex. Mer is phosphorylated by unknown receptors which inactivate Mer. Inactivated Ex and Merlin are not able to phosphorylate Hpo so that components of Hpo kinase cassette are not assembled. Wts and Sav are also in unphosphorylated state; therefore, Wts can not retain Yki in the cytosol. Active nuclear Yki then binds with Sd to form a complex to promote the transcription of downstream target genes which induce proliferation and inhibit apoptosis (Dong et al., 2007; Hao et al., 2008; Oh and Irvine, 2008; Zhang et al., 2008; Zhao et al., 2007; reviewed in Reddy and Irvine, 2008).
1.6 Hippo Pathway Components are Conserved in Mammals

Most components of Hippo pathway found in *Drosophila* have homologs in mammalian cells. These mammalian genes, Lats1, Mst2 and Mob1, YAP, are able to rescue mutant phenotypes caused by loss of their *Drosophila* homologs (Hay and Guo, 2003; Huang et al., 2005; Lai et al., 2005; Tao et al., 1999) and some of them also function as tumor suppressors in mouse or human (St John et al., 1999). Mutations of tumor suppressors in Hippo pathway homologs in mammals result in higher incidence of tumor. In addition, some mutations have been found in mouse or human cancers (Jiang et al., 2006; Lai et al., 2005; Strazisar et al., 2009; Seidel et al., 2007; Tapon et al., 2002; Yoo et al., 2003). The results above show that Hippo pathway is functionally conserved. Table 1 shows the current findings of *Drosophila* and mammalian homologs (reviewed in Saucedo and Edgar, 2007; Kango-Singh and Singh, 2009).
Table 1-1 Mammalian Hippo signaling homologues and their contributions to tumorigenesis

<table>
<thead>
<tr>
<th>D. melanogaster Gene</th>
<th>Mammalian Gene</th>
<th>Implications in cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>fat</td>
<td>FATJ/FAT4</td>
<td>Mutated in mouse and human breast cancer</td>
</tr>
<tr>
<td>dachsous</td>
<td>DCHS1</td>
<td>unknown</td>
</tr>
<tr>
<td>discovergrewn</td>
<td>Unknown</td>
<td>unknown</td>
</tr>
<tr>
<td>dachs</td>
<td>Unknown</td>
<td>unknown</td>
</tr>
<tr>
<td>merlin</td>
<td>Neurofibromatosis type-2 (NF-2)</td>
<td>Inactivated in human tumors of nerve tissue.</td>
</tr>
<tr>
<td>expanded</td>
<td>FERM-domain-containing-6 (FRMD6)</td>
<td>None reported</td>
</tr>
<tr>
<td>dRASFF1</td>
<td>RASSF1</td>
<td>Hypermethylated in lung and kidney cancers</td>
</tr>
<tr>
<td>hippo</td>
<td>Mammalian sterile-20 -like kinases type 1 and 2 (MST1, MST2)</td>
<td>Hypermethylated in soft tissue sarcoma</td>
</tr>
<tr>
<td>salvador</td>
<td>WW45</td>
<td>Mutations have been found in cell lines derived from kidney cancers but no other cancer.</td>
</tr>
<tr>
<td>warts</td>
<td>Large tumor suppressor (Lats)</td>
<td>Inactivation in mice results in tumors. This gene is also downregulated in human sarcomas.</td>
</tr>
<tr>
<td>mats</td>
<td>Mps-one binder kinase activator-1 (MOB1)</td>
<td>Mutations have been found in cancer cells lines from human and mice.</td>
</tr>
<tr>
<td>yorkie</td>
<td>Yes-associated protein (YAP)</td>
<td>Overexpression transforms human cell lines and promotes tumorigenesis in mice</td>
</tr>
<tr>
<td>scalloped</td>
<td>TEAD</td>
<td>unknown</td>
</tr>
</tbody>
</table>
1.7 Regulation of Hippo Pathway in Mammals

In mammalian Hippo pathway, MST, a homolog of Hpo, phosphorylates three other components in Hpo kinase cassette. MST autophosphorylates itself and phosphorylates Lats 1/2. MST also phosphorylates WW45 and Mob1 which later increases interaction between Mob1 and Lats1 (Callus et al., 2006; Chan et al., 2005; Hirabayashi et al., 2008; Lee et al., 2008; Praskova et al., 2008; Wei et al., 2007). Phosphorylated Lats1 then phosphorylates mammalian homologs of Yki, YAP (Yes-associated protein) or TAZ (transcriptional co-activator with PDZ binding motif), keeping phosphorylated YAP/TAZ in the cytosol away from binding with transcription factors, TEAD (TEA domain family members 1/2/3/4). If MST is inactivated, the unphosphorylated Lats1 no longer phosphorylates YAP/TAZ, leading to the nuclear localization of YAP/TAZ. Nuclear localized YAP/TAZ will bind with TEAD transcription factors to transcribe downstream targets (Wang et al., 2009; Zhang et al., 2009; reviewed in Zhao et al., 2009; Zhao et al., 2008).

The regulation is not clear upstream of MST kinase cassette in mammalian cells. Unlike how Ex regulated by Ft in Drosophila is known, the upstream regulators of Ex are not identified since there are many homologs of Ex and Ft in mammalian cells (Hamaratoglu et al., 2006; Qi et al., 2009; Saburi et al., 2008). In addition, even though NF2, a mammalian homolog of Mer, has been found to inhibit YAP activity in tissue cultured cells (Striedinger et al., 2008), the detailed mechanisms of how NF2 regulates YAP are still not clear. Moreover, RASSF may have different roles in Drosophila and in mammals since RASSF inhibits Hpo activity in Drosophila but activates MST2 leading to YAP activation on p73 in mammalian cells (Hwang et al., 2007; Guo et al., 2007; Polesello et al., 2006).

1.8 Activation Mechanisms of Hpo Kinase Cassette

Phosphorylation of Wts, Sav and Mats by Hpo is critical for Hippo pathway activation. However, the phosphorylation mechanism alone is not sufficient to explain the entire phenomenon; the availability and interactions between Hpo kinase components also affect Hpo pathway activity.

Based on in vivo studies, we noticed that overexpression of mats alone did not show reduction in organ sizes (Lai et al., 2005) although coexpression of wts and mats led to smaller organ sizes than expression of wts alone. Overexpression
of *wts* or *hpo* both led to reduction in organ sizes while loss of *wts, sav, hpo* and *mats* all cause overgrowth of epithelial tissues. These results suggest that Mats could be tightly regulated by other mechanisms in developing tissues.

### 1.9 Activation Mechanisms of LATS1-MOB1 Complex in Mammalian Cells

In mammalian cells, human NDR kinase, a human homolog of *Drosophila* Wts kinase, has been shown to interact with and be activated by MOB1, a human homolog of Mats protein in *Drosophila* (Bichsel et al., 2004; Devroe et al., 2004). Subcellular localization studies of NDR and MOB1 further support the NDR-MOB1 interaction since NDR kinase and MOB1 co-localize in the cytoplasm and at the plasma membrane (Hergovich et al., 2005). In this study, coexpression of NDR kinase and MOB1 is not sufficient to activate NDR kinase in the absence of okadaic acid (OA), a PP2A phosphotase inhibitor. However, when NDR kinase is targeted to the plasma membrane by adding myristoylation/palmitoylation (mp) motif from Lck tyrosine kinase to the N terminus of NDR1 kinase, its kinase activity was five fold more than wild type NDR1 kinase without OA treatment. This mp-NDR1 kinase activity is further enhanced up to fifteen fold when coexpressing with MOB1. In addition, wild type NDR1 kinase activity is significantly stimulated over fifty fold when coexpressing with membrane-targeting MOB1 compared with coexpression of wild type MOB1 and NDR1 kinase. These evidences indicate that plasma membrane localization of NDR1-MOB1 complex is crucial for kinase activity of NDR1 (Hergovich et al., 2005).

LATS1 kinase is a member of the NDR kinase family and has also been found to interact with MOB1 protein. Its kinase activity was increased by MOB1 in the presence of OA. Similar to wild type NDR1 kinase, LATS1 kinase activity is greatly enhanced by targeting MOB1 to the plasma membrane. If inducible membrane targeting sequence, C1 domain of PKC, is added to N-terminus of MOB1, LATS1 membrane localization and its kinase activity is rapidly increased within minutes after treatment of 12-O-tetradecanoylphorbol 13-acetate (TPA). LATS1 defective in binding with MOB1 is not able to be recruited and activated by plasma membrane-localized MOB1. This not only shows membrane-targeting MOB1 activates LATS1 through LATS1-MOB1 interaction, but also illustrates conservation of the regulatory mechanism of human LATS1 and NDR kinases (Hergovich et al., 2006).
1.10 Regulation of Hippo Pathway at the Plasma Membrane in Mammalian Cells

There are other lines of evidence showing plasma membrane is a key place in the regulation of mammalian Hippo pathway activity. The subcellular localization of MSTs can be correlated with their kinase activity. MSTs are inactive when they are mainly cytosolic. One of the MST activation mechanisms is through binding with a subgroup of RAS adaptor proteins, RASSFs. After RASSF proteins associate with MSTs through SARAH domains on both proteins (Hwang et al., 2007), MSTs are recruited to the proximity of active Ras at the plasma membrane. MSTs then form dimers which results in transphosphorylation on MSTs and kinase activation (Praskova et al., 2004; reviewed in Radu and Chernoff, 2009).

Another proposed mechanism of MST activation at the plasma membrane is through inhibiting AKT, a kinase mediating cell growth and survival. When the MST is inactive, it can not bind with AKT at the plasma membrane and cytoplasm. Following apoptotic stimuli, cytosolic and cholesterol-rich lipid raft membranes residing MST inhibits AKT in an endogenous complex at the plasma membrane and cytoplasm. Upon apoptosis, MST1 is cleaved by caspase 3 and MST1 cleaved products (N-terminus of MST1) can also bind to and inhibit AKT in the cytoplasm and nucleus (Cinar et al., 2007).

Even though localization of MSTs was studied in mammalian cells, how the upstream molecules activate MSTs is still not fully understood. In flies, the membrane receptor Ft and cytoskeleton proteins, Mer and Ex were shown to regulate Hpo and Wts indicating the link between plasma membrane and components of Hpo kinase cassette. Although Ft, Mer and Ex all have counterparts in mammalian cells, the mechanisms on how Ft, Ex, Mer homologs regulate MSTs have not been illustrated in mammals (reviewed in Radu and Chernoff, 2009).

1.11 Regulation of Hippo Pathway Components at the Plasma Membrane in Drosophila

As previously mentioned in 1.3, the membrane receptor Ft regulates Hpo pathway through two mechanisms: Ft-Ex-Hpo or Ft-D-Wts in Drosophila.
Without Ft, Ex no longer localizes at apical junctions complexes suggesting Ft is required for proper Ex localization. Contrast to Ex, Mer and Hpo localization as well as endogenous Hpo level are not obviously affected in ft mutant clones These indicate that the effects of ft on Hippo pathway are not through changing Hpo or Mer localization (Silva et al., 2006; reviewed in Reddy and Irvine 2008).

In Ft-D-Wts pathway, overexpression of ft reduces the level of D at the membrane while loss of ft induces plasma membrane localization of D. In loss of ft clones, Myc-Wts is decreased at the plasma membrane implying plasma membrane is important for Wts availability (Cho et al., 2006). This may further affect Wts activity and target gene transcription downstream of Wts. To detect localization of Wts, Myc-Wts was used while endogenous Wts was not characterized in vivo. Our preliminary studies have shown that Myc-Wts accumulates at the plasma membrane and cytoplasm (Shimizu et al., 2006); however, the relationship between Wts localization and its activity has not been elucidated.

Based on mammalian studies mentioned in 1.9, NDR kinase and human Lats1 kinase can be recruited to the plasma membrane and is significantly activated by targeting Mats to the plasma membrane. Therefore, we wanted to test the hypothesis that membrane targeting of Mats is able to affect Wts localization and activity in vivo. In addition, we wanted to see if there is any physiological correlation between cell numbers and the change of Mats localization. Lastly, we looked into the mechanism of how membrane-targeting Mats inhibits downstream targets of Hippo pathway.
Chapter 2

Materials and Methods

2.1 Construction of Membrane-targeting Mats-GFP

2.1.1 The Myristoylation Mechanism

One way to target protein to the plasma membrane is to make modifications at the certain amino acid in polypeptides. Myristoylation is one of co-translational protein modifications which adds myristoyl group at the N-terminus of nascent polypeptide catalyzed by N-Myristoyltransferase (Figure 2-1). After Methione is removed, a myristic acid is covalently added on alpha-amino group of the second residue, Glycine, via amide bond.

In *Drosophila*, Src42A protein was reported to be myristoylated and targeted to the plasma membrane to perform its function. Its first 16 amino acids encodes a consensus membrane targeting sequence, Met-Gly-X-X-X-Ser/thr, for myristoylation (Figure 2-2). Therefore, this stretch of peptide serves as membrane-targeting sequence in Src42A. Importantly, Gly is the amino acid to which myristic acid is added. After Src42A is myristoylated, it targets to the plasma membrane.
membrane and performs its catalytic activity. If Gly is mutated, myristic acid no longer is attached to the peptide chain so membrane-targeting of Src42A is blocked. This indicates Gly, in the consensus membrane targeting sequence, is essential for membrane localization of Src42A (Roy et al., 2002).

2.1.2 Generation of pMyr-UAST Vector

The oligonucleotide (ATGGGTAACTGCCTCACCAAGAACGCCG) encoding peptide for myristoylation of Drosophila melanogaster Src42A (MGNCLTTQKGEKPDA) was cloned into pUAST vector by replacing 3 copies of hemagglutinin epitope in pHA-UAST vector. EcoR I (5’-gaattc-3’) and Not I (5’-gcggccgc-3’) sites were used as cloning sites at 5’ end and 3’ end flanking the oligonucleotide. Extra nucleotides were added in addition to the restriction sites. The consensus translational start sequence of Drosophila, CAACA, was introduced between the EcoR I site and the oligo sequences encoding signal peptide (Cavener, 1987). The final oligomer used in this cloning was 5’-GAATTCCAAAGAACGGTAAACTGCCTCACCAAGAACGCCG.
AGGGCGAACCCGACAAGCCCGCA
GCGGCCGCTAAACTAT-3' synthesized by Sigma, Inc. The nucleotides underlined are DNA sequences encoding myristoylation signal.

2.1.3 Generation of pMyr\(^{(G2A)}\)-UAST

To generate non-myristoylatable version of Mats-GFP, pMyr\(^{(G2A)}\)-UAST was made by changing the second amino acid from Glycine (GGT) to Alanine (GCT) in pMyr-UAST vector. The sequence of mutant primer was 5'-GAATTCCCAACAATGGCTAACTGCCTCACCAC-3 and the mutation site was underlined.

(A) Membrane targeting Mats-GFP

Myr = \textit{Drosophila} Src42A myristoylation signal (first 16 amino acids)

Amino acid sequence: MGNCLTTQKGEPDKPA

(B) Non-membrane targeting Mats-GFP

Myr (G\(^2\)A) = \textit{Drosophila} Src42A nonmyristoylatable signal

Amino acid sequence: MANCLTTQKGEPDKPA

Figure 2-3: The Myr-Mats-GFP and Myr\(^{(G2A)}\)-Mats-GFP open reading frame in pUAST vector.
2.1.4 Introduction of Mats-GFP into pMyr-UAST and pMyr\textsuperscript{(G2A)}-UAST Vector

The template for \textit{Drosophila mats-gfp} was according to the sequences from Takeshi Shimizu. The PCR cloning was used to introduce mats-gfp sequences into pMyr-UAST and pMyr\textsuperscript{(G2A)}-UAST vector. The first amino acid, Methionine, of \textit{Drosophila} Mats was removed and Not I restriction site was introduced in 5’ end primer. 3’ end primer contains Kpn I downstream of gfp sequences. An additional nucleotide adenosine triphosphate was added between Not I and \textit{mats} sequence to keep Mats-GFP open reading frame in frame. Figure 2-3 shows the open reading frame of Myr-Mats-GFP (A) and Myr\textsuperscript{(G2A)}-Mats-GFP (B).

2.2 Site Directed Mutagenesis

Quick-Change site directed mutagenesis kit from Stratagene was used to introduce point mutations. PfuTurbo\textsuperscript{®} DNA polymerase and two complementary oligonucleotides carrying mutations synthesized from Integrated DNA Technologies were included in PCR reaction mixture. The mutagenic primers should have higher than 40% GC percentage with melting temperature above 78°C.

The total of 50 μl PCR mixture contains 5 μl of 10x reaction buffer, 1 μl of template at 25 ng/μl, 1 μl of forward mutagenic primers (125 ng/μl), 1 μl of reversed mutagenic primers, 1 μl of 10 mM dNTPs, 1 μl of PfuTurbo\textsuperscript{®} DNA polymerase (2.5 Unit/μl ) and 41 μl of ddH\textsubscript{2}O.

To generate pMyr\textsuperscript{(G2A)}-UAST vector, the PCR cycles for the mutagenesis were 95°C for 30 seconds for initial denaturation, followed by 16 cycles of denaturation for 30 seconds at 95°C, annealing for one minute at 55°C and extension for 18 minutes at 68°C.

To generate kinase-dead version of myc-wts in pcDNA3 vector, Lys 743 (AAA) of \textit{Drosophila wts} kinase was changed to Alanine (GCA). The sequence of mutagenic primer was 5’- CCATTGTATGCGATGGCACCCTGCACGAAAGC GGACG-3’. The underlined nucleotides are the mutagenic sites. For the PCR cycle, everything was the same as previously mentioned except the extension time was changed to 16 minutes.
1 μl of Dpn I restriction enzyme (10 unit/μl) was added to the PCR products to digest DNA template at 37°C for 3 hours. 10-20 μl of digested PCR product was used to transformed DH5α competent cells at 42°C heat shock for 90 seconds. Three to five colonies were picked up from the plate around eight to twelve hours after plating. After culturing bacteria at 37°C overnight, the plasmid DNA was purified using SV Wizard DNA purification kit (Promega Inc.), and sequences were confirmed by direct sequencing.

2.3 Microinjection of Drosophila Embryos

UAS-Gal4 system was used to drive the expression of transgenes in tissue specific manners. Microinjection of Drosophila embryos was used to generate flies carrying UAS-transgenes based on P-element transformation (Spradling and Rubin, 1982). pmyr-mats-gfp-UAST and pmyr(G2A)-mats-gfp-UAST constructs were injected into w; Dr'/TMS Δ2-3, Sb' embryos to generate UAS-myr-mats-gfp and UAS-myr(G2A)-mats-gfp transgenic flies. The transposase encoded by Δ2-3 recognized P-elements flanking the UAS-transgenes and the mini white gene, and jumped out sequences between two P-elements. These sequences were inserted into genome randomly. If the UAS-transgenes were inserted into the germline cells, the UAS-transgenes could be kept over generations and identified by red pigments in the eyes of progenies. Tissue specific expression could be done by crossing Gal4 driver lines with UAS-transgenic flies.

To generate UAS-transgenic flies, 250-400 ng/μl of freshly prepared plasmid DNA purified from SV Wizard DNA purification kit should be used in microinjection. 0.5 μl of food green dye could be added in 10 μl of plasmid solution before injection. The dye helps to visualize the injected DNA in the embryos. As for the flies, around 400-500 w; Dr'/TMS Δ2-3, Sb' flies should be transferred to a chamber with molasses plate at the bottom a week before injection. The plates contained yeast paste were changed everyday to ensure the number of egg laying under 25°C. Usually, flies lay better during afternoon. The morning before injection, the plate with a drop of yeast paste should be changed every half an hour at least three times.

The injection was performed in a 20°C constant temperature room and the embryos were collected every 20-30 minutes. After washing and transferring embryos from the plates, 25-30 embryos could be lined up on a coverslip. The embryos were air dried for five minutes and covered with Halocarbon oil mix (9
parts of heavy oil plus 1 part of light oil). The injection was done without dechorionation and the needle was kept close to the posterior end of the embryos.

Once the injection was done for the embryos lined up on one coverslip, the coverslip was put onto an Ampicillin plate and kept under 20°C. To prevent injected embryos from drying out, more halocarbon oil mix could be added everyday. The hatched larvae on the coverslip could be picked up and transferred into fly food under 25°C starting from the second day after injection. The optimal survival rate for injected embryos was 45-50% (usually, 30-40% was sufficient) and around 50% transferred larvae could develop into adult. Among adults, there was 20-30% sterility.

The injected adult flies (G0) were crossed with w flies and the progenies (G1) were screened for red pigments in the eyes. The eye colors may vary from light orange to dark red based on the expression level of transgenes. As a result, twelve UAS-myr-mats-gfp, four UAS-myr(G2A)-mats-gfp and four UAS-myc-wts(K743A) transgenic fly lines were generated.

2.4 Tissue Specific Expression of UAS-transgenes

To express UAS-transgenes in tissue specific manners, the reporter lines (UAS-transgenes) were crossed with Gal4 driver lines (Brand and Perrimon, 1993). The Gal4 transcription factors were able to bind UAS sequences to drive the expression of transgenes depending on where Gal4 is expressed (Figure 2-4). In Drosophila, eyeless-Gal4 and GMR-Gal4 drive expression of UAS-transgenes in the eyes; whereas C5-Gal4 and engrailed-Gal4 express transgenes in different regions of the wings.

2.5 Size Measurement and Statistical Analysis of Adult Wings

The UAS transgenic lines were crossed with C5-Gal4 to express single copy of UAS-transgene in the pouch area of wings. At least 20 male adult wings from each cross were collected and lined up on the slides. After mounting, the pictures of adult wing were taken under a dissection microscopy using SPOT Basic imaging software. The wing sizes were further analyzed by Image J to calculate the pixels of wing regions. The average wing sizes from each genotype were taken and statistical analysis was performed using Microsoft Excel.
2.6 Wing Disc Cells Dissociation and Imaging

To show the Myr-Mats-GFP and Mats-GFP localization in dissociated wing disc cells, *UAS-myr-mats-gfp* and *UAS-mats-gfp* were expressed in the posterior regions of larval wing discs by *engrailed-Gal4*. 30 wing discs from late third instar larvae were washed with 1x PBS for three times and transferred to polystyrene tubes. Wing discs were dissociated in 500 μl of 9x trypsin with three hours of gentle agitation on a shaker under room temperature. The tubes should be tapped by hand every half an hour to ensure full dissociation of wing disc cells (Neufeld et al., 1998).

About 15 minutes before going to the fluorescent microscope, 1 μl of Draq 5 was added into the solutions containing dissociated disc cells to stain the nucleus under room temperature. If the cell clumps were still visible at the time, the tubes should be shaken gently by hand. The wing disc cells were then observed under Olympus 61BX fluorescent microscope.

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**Driver lines**

Gal4

Tissue specific enhancer (eg. eyes, wings)

---

**Reporter lines**

Gal4 ×

**UAS**

mats-gfp

or

myr-mats-gfp

or

myr(G2A)-mats-gfp

---

**Figure 2-4:** *Gal4-UAS* system is used to induce transgenes expression in *Drosophila*. In the driver lines, the *Gal4* gene is linked downstream of genomic enhancer to allow tissue specific expression of *Gal4*. In the reporter line, the transgenes are not expressed due to lack of Gal4 transcription factors. Once the driver line is crossed with the reporter line, Gal4 expression allows binding of Gal4 to *UAS* sequence and drives the transcription of transgenes in tissue or temporal specific manners.
2.7 Clonal Expression of Transgenes using FLPout Method

To show different growth effects between Myr-Mats-GFP, Myr\(^{(G2A)}\)-Mats-GFP and Mats-GFP, FLPout clonal analysis was done to induce the expression of transgenes in clones. The sizes and numbers of clones were used as parameters to demonstrate the growth variability of clones expressing different transgenes.

The flies carrying FLPout allele include several genetic elements, a ubiquitously active actin promoter (Act 5C) upstream of the first FLPase recombination target (FRT) site. A marker gene (\(w^+\)) and a transcriptional stop site (Term) are flanked by FRT sites. A Gal4 element is downstream of the second FRT site. These two FRT sites can be recognized by FLP recombinase (FLPase) (Golic and Lindquist, 1989) and generate DNA recombination within the same chromosome. Without any FLPase present, Gal4 is not expressed due to the transcriptional stop site downstream of actin promoter. Once low level of heat shock is applied to the animals, the heat shock-induced FLP recombinase (hs-FLPase) engineered in flies (Chou and Perrimon, 1992; Golic, 1991; Xu and Rubin, 1993) leads to recombination between two FRT sites. This results in loss of the marker gene and transcriptional stop site (Figure 2-5). Eventually, Gal4 is able to drive expression of UAS-transgenes under the control of a constitutively active actin promoter (Blair, 2003).

In this study, 20 male flies with UAS-myr-mats-gfp, UAS-myr\(^{(G2A)}\)-mats-gfp and UAS-mats-gfp were first crossed with 40 female flies carrying hsFLP, Act>CD2>Gal4; UAS-lacZ/TM6B allele. The embryos were collected every two hours under 25°C. After one day of development, the early first instar larvae were subjected to one hour of heat shock under 37°C and recovered under room temperature for at least five hours. Third instar larvae were collected for dissection after three more days of development at 25°C to observe clone sizes and numbers based on GFP expression (Figure 2-6).
Figure 2-5: Clonal FLPout expression. Low level of heat shock results in recombination between two FRT sites. The marker gene (w+) and transcriptional stop sequences were excised to allow the transcription of Gal4.

Figure 2-6: Generation of clones expressing Myr-Mats-GFP, Myr(G2A-)Mats-GFP and Mats-GFP in larval wing discs. The larvae were treated heat shock to induce the expression of Gal4. Different transgenes tagged with GFP were expressed.
2.8 Detection of expanded-lacZ Expression

To see if ex transcription level is regulated by Myr-Mats-GFP, β-galactosidase expressed by expanded-lacZ (ex-lacZ) was detected. w; ex-lacZ; UAS-myr-mats-gfpB15 / SM6 · TM6B or w; ex-lacZ; UAS-myr(G2A)mats-gfpM38/ SM6 · TM6B male flies were crossed with engrailed-Gal4 female flies to express transgenes in the posterior region of wing discs. The green region marked where transgenes were expressed.

2.9 Clonal Analysis by Negative Labeling

To mark loss of mats clones in eye discs, w; FRT 82B mats e235 / TM6B male flies were crossed with ey-FLP; FRT 82B arm-lacZ/ TM6B female flies (Figure 2-7). The eye discs of Tb+ progenies were stained with anti-β-gal antibody. After mitotic recombination, loss of mats clones were negative while wild type tissues were positive in β-gal staining (Figure 2-8). Peripodium membranes were removed after fixation, and the eye discs were subjected to the incubation of first and secondary antibodies.

Figure 2-7: Generation of cells with heterozygous mats mutation containing FLP and marker genes. The lines containing heat shock promoter (hs-FLP) or eyeless promoter (ey-FLP), and a chromosome with Arm-lacZ marker distal to the FRT
site were crossed with the lines which have loss of mats mutation. The Arm-lacZ and mats mutation were located distal to the FRT sites on homologous chromosomes.

Figure 2-8: Generation of loss of mats clones in the absence of lacZ marker. Flippase induced by heat shock or eyeless promoter recognizes FRT sites and cause the recombination between two FRT sites during mitotic stages. The marker gene, lacZ, was under the control of constitutively active promoter, Arm. The cells containing loss of mats on both alleles are shown in the absence of anti-β-gal staining while those containing one or two copies of marker genes can be visualized by anti-β-gal staining.
2.10 TUNEL Staining of Cell Death

TUNEL assays were performed using the Roche *in situ* cell death detection kit (TMD Red, Catalog number 12 156 792). The staining procedures were modified based on supplier’s instructions. Imaginal discs were fixed with 2.5% formaldehyde containing 1.0% Triton X-100 for 30 min and washed with PBT (PBS, 0.1% Triton X-100) four times. Discs were then washed twice for five minutes with PBT 5×(PBS, 0.5% Triton X-100) and incubated in reaction buffer for 10 min, followed by working-strength reaction buffer containing terminal deoxynucleotide transferase. The discs incubated with the enzyme were kept in PCR machine at 37°C for four hours. Finally, the reactions were stopped by transferring the discs into PBT 5× and discs were rinsed in three changes of PBT. The images were taken by Olympus FV300 laser scanning confocal microscope (Kim et al., 2007).

2.11 BrdU labeling of Proliferating Cells

Wing discs from late third instar larvae were dissected in Ringer’s solution (Schubiger, 1971) and incubated for 45 minutes in 300 μg/ml of BrdU in Ringer’s at room temperature. After three quick washes in PBS with 0.2% Triton X-100, discs were fixed in 2.5% formaldehyde. Discs were incubated in 2 N HCl for 1 hour, and neutralized three times for five minutes each in 0.1 M Borax before staining with anti-BrdU antibody (Johnston and Schubiger, 1996).

2.12 Clonal Analysis by Positive Labeling

Relative to the negative labeling of clones, clones expressing UAS-transgenes under homozygous mutant genetic background can be marked by GFP using MARCM (Mosaic Analysis with a Repressible Cell Marker )(Lee and Luo, 2001). In this study, male flies with a UAS-transgene and a mutation distal to an FRT site on different chromosomes were crossed with female MARCM flies which carry either hs-FLP or eyeless-FLP, a UAS-marker gene, an FRT site, a Gal4 and a Gal80 element under the control of constitutively active tubulin promoter. Gal80 element should locate at distal end of the chromosome where an FRT site locates (Figure 2-9). Without flippase expression, Gal80 protein is able to bind the Gal4 protein to inhibit Gal4 from binding to UAS-transgenes; therefore, the UAS-gfp
and UAS-transgenes are not expressed. After mitotic recombination induced by flippase, Gal80 element was recombined to the homologous chromosome which later would generate one homozygous mutant cells without Gal80 in the genome and the other one with two copies of Gal80. The homozygous mutant cells can be positively marked by expressing UAS-gfp through Gal4 activation. Meanwhile, any other UAS-transgene can also be expressed in the mutant cells (Figure 2-10).

2.13 Epistatic Analysis of wts and myr-mats-gfp

To demonstrate epistatic relationship between myr-mats-gfp and wts, we took MARCM approach, using UAS-GFP, ey-FLP; Tub-Gal4 FRT82B, Tub-Gal80/ TM6B (a gift of J. Treisman, New York University Medical Center). To express Myr-Mats-GFP in loss of wts genetic background w; UAS-myr-mats-gfp
t; FRT82B wts\textsuperscript{x1}/SM6 \cdot TM6B male flies were crossed with MARCM lines. To generate clones expressing Myr-Mats-GFP in wild type genetic background, w; UAS-myr-mats-gfp\textsuperscript{t}; FRT82B P[w+] 90E/SM6 \cdot TM6B male flies were used. w; FRT 82B wts\textsuperscript{x1}/TM6B (a gift of I. Hariharan) and w; FRT82B P[w+] 90E were used to generate loss of wts and wild type clones respectively.

To examine effects of myr-mats-gfp on cell apoptosis in loss of wts or wild type genetic backgrounds, we used w, hs-flp; Tub-Gal4, FRT 82B Tub-Gal80/ TM6B, w; UAS-myr-mats-gfp\textsuperscript{t}; FRT82B wts\textsuperscript{x1}/SM6 TM6B, w; UAS-myr-mats-gfp\textsuperscript{t}; w; FRT82B P[w+] 90E/SM6 TM6B flies.

2.14 Epistatic Analysis of hpo and myr-mats-gfp

To examine epistatic relationship between myr-mats-gfp and hpo, we took MARCM approach, using w, UAS-GFP, hs-flp122(x); FRT 42D Tub-Gal80; Tub-Gal4/TM6B MARCM lines (a gift of J. Treisman). To express myr-mats-gfp in loss of hpo genetic background, male flies of w; FRT 42D hpo\textsuperscript{BF33}; UAS-myr-mats-gfp\textsuperscript{B15}/SM6 \cdot TM6B were crossed with MARCM female flies. w; FRT 42D P[w+]; UAS-myr-mats-gfp\textsuperscript{B15}/SM6 \cdot TM6B male flies were used to generate clones expressing Myr-Mats-GFP. In addition, w; FRT 42D hpo\textsuperscript{BF33}/SM6 \cdot TM6B or w; FRT 42D P[w+] were used to generate loss of hpo clones or wild type clones.
Figure 2-9: Generation of cells for mosaic analysis with a repressible cell marker (MARCM). A fly line which has hs-FLP or ey-FLP, a UAS-GFP marker gene, a Gal4 gene and a Gal80 gene was crossed with the other line carrying another UAS-transgenes and the mutated gene. The Gal80 and the mutated gene both locate distal to an FRT site at the homologous chromosome.
2.15 Antibody Dilutions in Eye and Wing Discs Immunostaining

Primary antibodies listed in Table 2-1 were diluted in BSST containing 0.1% TritonX-100 and 5% Normal goat serum based on Takeshi Shimizu’s
Dissected tissues were incubated with primary antibody at 4°C overnight, and washed with PBS containing 0.3% Triton X-100 for three times. Secondary antibodies were diluted in PBS containing 0.3% Triton X-100 based on the titrations in Table 2-1. Sequential scans were made using the Olympus FV300 laser scanning confocal microscope.

2.16 myr-mats-gfp Transgene engrailed-Gal4 Viability Assay

Late pupae lethality was always observed by crossing UAS-FLAG-hpo^6^ and UAS-myc-wts^6^ with engrailed-Gal4. To show if Myr-Mats-GFP has similar effects, UAS-myr-mats-gfp / SM6 · TM6B flies were crossed with engrailed-Gal4 and the numbers of flies from different genotypes were counted. The numbers of flies carrying myr-mats-gfp were compared with those did not. Individuals with pupae lethality were decided based on the pupae cases still containing pigmented fly tissues four or five days after the expected eclosion date.

2.17 Analysis of the Effects of FLAG-Hpo on Drosophila Mats Localization

Full length Hpo (FLAG-Hpo^6^) and kinase-dead N-terminus FLAG-Hpo (FLAG-Hpo^NK>R^) transgenic flies were gifts from Jin Jiang. We coexpressed full length FLAG-Hpo or kinase-dead N-terminus FLAG-Hpo with Mats-GFP in pupae eye discs using GMR-Gal4. 40-48 hrs old pupae eye disc were collected and stained with rabbit anti-FLAG antibody to label FLAG-Hpo and FLAG-Hpo^NK>R^. Rat anti-DE-Cadherin antibody was used to visualize apical membranes of ommatidial structures.

2.18 Mats Staining in Loss of hpo Clones in Wing Discs

Staining buffers with lower detergent concentration were used in Mats staining to visualize membrane localized Mats in wing discs. The tissues were incubated with anti-Mats antibody diluted in PBS containing 0.05% Triton X-100 at 4°C overnight, and washed with 0.1% Triton X-100 for three times. The discs were then subjected to secondary antibodies incubation at 4°C overnight and washed with PBS containing 0.3% Triton X-100. The secondary antibodies were diluted in PBS containing 0.1% Triton X-100. Sequential scans were made using the Olympus FV300 laser scanning confocal microscope.
Table 2-1 Antibodies or dye dilutions for immunostaining used in this study

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2.19 Analysis of Myc-Wts Localization in Loss of mats/hpo/sav Clones

Using MARCM system, myc-wts is expressed in loss of mats, or loss of hpo, or loss of sav genetic background. For myc-wts expression in wild type clones, w; UAS-myc-wts4A; FRT82B P[w+]/90E (generated by Takeshi Shimizu) male flies were crossed with w; hs-flp; Tub-Gal4, FRT 82B Tub-Gal80/TM6B female flies. For myc-wts expression in loss of mats and sav clones, male flies of w; UAS-myc-wts4A; FRT 82B mats e235/SM6 · TM6B (generated by Takeshi Shimizu), w; UAS-myc-wts4A; FRT 82B sav 1/SM6 · TM6B were crossed with w; hs-flp; Tub-Gal4, FRT 82B Tub-Gal80/TM6B female flies. For myc-wts expression in loss of hpo clones, w; FRT 42D hpo 833 · UAS-myc-wts1D/SM6 · TM6B were crossed with w; UAS-GFP, hs-flp122(x); FRT 42D Tub-Gal80; Tub-Gal4/TM6B female flies.

Embryos from each cross were collected under 25°C for one day and then subjected to 37°C heat shock for one hour after one more day of development. Third instar larvae were collected and dissected. Anti-Myc (rabbit) staining was used to visualize Myc-Wts localization in different genetic backgrounds. The secondary antibody was Alexa 488 when staining Myc-Wts in wild type and loss of sav/mats genetic backgrounds while Alexa 647 was used to stain Myc-Wts in loss of hpo genetic background. Pictures were taken using Olympus FV300 confocal microscopy.

2.20 Armadillo, DE-Cadherin and Notum Staining in Loss of mats/hpo/wts Clones

To detect the change in Wg pathway activity under the influence of Hippo signaling, Armadillo, E-Cadherin and Notum were stained in loss of mats/hpo/wts clones using MARCM. The dilutions of antibodies were listed in Table 2-2. w; UAS-GFP; FRT 82B mats e235/SM6 · TM6B and w; UAS-GFP; FRT 82B wts1/ SM6 · TM6B male flies were crossed with w, hs-flp; Tub-Gal4, FRT 82B Tub-Gal8/TM6B female flies to generate loss of mats and loss of wts clones. Loss of hpo clones were generated as described in 2.13. Clones with desired genotypes were marked by GFP expression.
Higher detergent concentrations and room temperature incubation are crucial to visualize Notum inductions in the tissues. Anti-Notum antibody was diluted in PBS containing 0.3% Triton X-100 and tissues were incubated under room temperature overnight. The tissues were washed with PBS with 0.3% Triton X-100 for three times and subjected to secondary antibody incubation at 4°C overnight.

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### 2.2.1 Detection of DE-Cadherin-lacZ and Notum-lacZ Expression

To detect the change of DE-Cadherin or Notum transcription level, *w; DE-cadherin-lacZ; FRT 82B mats\textsuperscript{235}/SM6·TM6B or w; Notum-lacZ; FRT 82B mats\textsuperscript{235}/SM6·TM6B* male flies were crossed with *w, hs-flp, UAS-GFP; Tub-Gal4, FRT 82B Tub-Gal8/TM6B* female flies to generate loss of *mats* clones. The clones were marked by GFP expression from MARCM line in this study. The first instar larvae were heat shocked for two hours at 37°C. After recovering under room temperature for five hours, the larvae grew under 25°C for three days. Late third instar larvae were dissected and stained with anti-β-gal antibody after fixation.
2.22 Analysis of Genetic Interaction between Components of Hippo signaling and Wg signaling

MARCM was used to demonstrate the genetic interaction between Hippo and Wg pathways. To see if induction of Wg pathway resulting from loss of *mats* could be inhibited by dominant-negative form of TCF transcription factors (dTCFΔN), we crossed male flies of *w; UAS-dTCFΔN; FRT 82B mats^e235^ TM6B* with *y, w, hsflp, UAS-GFP; Tub-Gal4, FRT82B Tub-Gal80/TM6B* female MARCM flies. In addition, *w; FRT 82B mats^e235^ SM6 · TM6B or w; UAS-dTCFΔN; FRT 82B 90E P[w+] male flies were crossed with MARCM female flies to serve as positive and negative controls. Notum level in loss of *mats* clones or clones expressing dominant-negative form of dTCF were detected.

To see if Hippo pathway affects Wg pathway through Shaggy(Sgg)/Zeste-white 3 which is an inhibitory kinase to phosphorylate and destabilize β-catenin, constitutively-active Sgg (Sgg^{394}) was expressed in loss of *mats* clones. To generate such clones, male flies of *w; UAS-sgg S9A; FRT 82B mats^e235^ TM6B* were crossed with *y, w, hsflp, UASGFP; Tub-Gal4, FRT82B Tub-Gal80/ TM6B* female MARCM flies.

To see if Hippo pathway affects Wg pathway through Axin, a negative regulator serving as a scaffold protein to interact with β-catenin and Sgg, was expressed in loss of *mats* clones. The clones were generated by crossing *w; UAS-axin-gfp; FRT 82B mats^e235^ SM6 · TM6B* male flies with *y, w, hsflp, Tub-Gal4, FRT82B tub-Gal80/ TM6B* female MARCM flies.

Two dominant-negative forms of Wg receptors, *frizzled^{DN} and frizzled 2^{DN}, were coexpressed in loss of *hpo* clones to see if induction of Wg downstream targets resulting from loss of Hippo signaling could be blocked. The allele containing dominant-negative forms of *UAS-fz* and *UAS-fz2* were gifts from U. Banerjee. Male flies of *w; FRT 42D hpo^{BF33}; UAS-fz^{DN}, UAS-fz2^{DN} / SM6 · TM6B* were crossed with *w, UAS-GFP, hs-flp122(x); FRT 42D Tub-Gal80; Tub-Gal4/TM6B* female flies.

The embryos or first instar larvae from each cross were heat shocked for two hours at 37°C. After recovering under room temperature for five hours, the larvae grew at 25°C. Late third instar larvae were dissected and subjected to immunostaining.
Chapter 3
Subcellular Localization and Regulation of Mats in Developing *Drosophila* Tissues

3.1 Introduction

Previous studies in our lab have demonstrated that Mats regulates Hippo signaling pathway through activating Wts protein kinase (Lai et al., 2005). In addition, phosphorylation of Mats by Hpo is critical for Mats to activate Wts kinase (Wei et al., 2007). However, the subcellular localization of Mats and how Mats localization is regulated have not been illustrated in *Drosophila*. In this chapter, I will show the subcellular localization of endogenous Mats in different developing tissues and how Mats localization is affected by an upstream Hippo pathway component, Hpo.

3.2 Endogenous Mats Localization

To detect the localization of endogenous Mats, *Drosophila* Mats antibody is used to stain larval wing discs and pupal eye discs. Wing disc cells from late third instar larval are mostly in proliferating stages while pupae eye discs cells are differentiated. Figure 3-1 shows Mats localizes in the cytosol and at the plasma membrane in wing disc cells (Figure 3-1A to C) and pupae eye discs (Figure 3-1D to F), especially in cone cells. In addition, larval salivary glands are also stained with anti-Mats antibody because cells are bigger in size in salivary glands than those in larval wing discs and pupal eye discs. As shown in Figure 3-2A, Mats localizes in the cytosol and the plasma membrane in salivary glands.
3.3 Endogenous Mats Localization is Affected by Hpo

We further wanted to see how endogenous Mats localization is regulated. In the Hpo kinase cassette, Hpo is upstream of Mats. In addition, human MST has been shown to localize at the plasma membrane, one would predict that Hpo is one of the candidate to regulate Mats localization (Hwang et al., 2007; Praskova et al., 2004; reviewed in Radu and Chernoff, 2009). To detect endogenous Mats localization in loss of hpo genetic background, loss of hpo clones generated by MARCM (Figure 3-3) in wing discs are stained with endogenous anti-Mats antibody (Figure 3-4). As shown in Figure 3-4, loss of hpo clones marked by GFP show more cytosolic Mats staining than wild type tissues identified by the absence of GFP (Figure 3-4B and E). Membrane of wing disc cells is outlined by DE-Cadherin staining (Figure 3-4C and F). In the wild type clones, Mats staining is relatively lower in the cytosol but more abundant close to the plasma membrane. The data suggests that Hpo is required for Mats membrane localization.

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<td>B</td>
<td>C</td>
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</table>

Figure 3-1: Endogenous Mats localizes at the cytoplasm and the plasma membrane in Drosophila wing discs and pupae eye discs. (A) and (D) Endogenous Mats staining in wing discs and pupae eye discs. (B) and (E) DE-Cadherin staining is used as a membrane marker. (C) and (F) Merged picture from (A)(B) and (D)(E).
Figure 3-2: Mats localizes in the cytosol and at the plasma membrane in salivary glands (A) Endogenous Mats staining (B) Draq5 staining is used as a nucleus marker (C) Merged picture from (A) and (B).

Figure 3-3: Generation of loss of hpo clones using MARCM analysis. Loss of hpo clones are marked by GFP expression while wild type clones are not.
In addition to Mats staining in loss of hpo clones, we performed gain-of-function analysis by expressing FLAG-Hpo (gifts from Jin Jiang) (Jia et al., 2003). First, we tested the effectiveness of full-length Hpo, N-terminus Hpo containing kinase domain, C-terminus Hpo containing the regulatory domain which interacts with Sav, and kinase-dead form of N-terminus Hpo driven by C5-Gal4. All forms of Hpo were tagged with FLAG peptide at their N-terminus. At least 20 adult wings were collected and analyzed according to the previously mentioned procedures. The example pictures of adult wings and analysis of wing size ratios from each genotype were shown in Figure 3-5 and Figure 3-6.

**Figure 3-4:** Mats localizes more in the cytosol in loss of hpo clones. Cytosolic Mats was observed in clones absent of hpo marked by GFP. (A) GFP marker is used to label loss of hpo clones. (B) Mats (C) DE-Cadherin stainings. (D) Merged picture from (B)(C). (E),(F) and (G) are the pictures combining (A)(B), (A)(C) and (A)(D).

### 3.4 Expression of FLAG-Hpo Reduces Tissue Sizes

In addition to Mats staining in loss of hpo clones, we performed gain-of-function analysis by expressing FLAG-Hpo (gifts from Jin Jiang) (Jia et al., 2003). First, we tested the effectiveness of full-length Hpo, N-terminus Hpo containing kinase domain, C-terminus Hpo containing the regulatory domain which interacts with Sav, and kinase-dead form of N-terminus Hpo driven by C5-Gal4. All forms of Hpo were tagged with FLAG peptide at their N-terminus. At least 20 adult wings were collected and analyzed according to the previously mentioned procedures. The example pictures of adult wings and analysis of wing size ratios from each genotype were shown in Figure 3-5 and Figure 3-6.
With respect to wild type wings (Figure 3-5A), full-length FLAG-Hpo is the most potent in reducing tissue sizes (Figure 3-5D) compared with N-terminus FLAG-Hpo (Figure 3-5E). The wing size ratio is 0.32 for adult wings expressing full-length FLAG-Hpo while 0.73 for those expressing N-terminus FLAG-Hpo (Figure 3-6). The kinase-dead N-terminus FLAG-Hpo does not show any effect on reduction of tissue size since the wing size ratio is 1 (Figure 3-6). As opposed to full-length FLAG-Hpo and N-terminus FLAG-Hpo, the wings expressing C-terminus FLAG-Hpo show slightly larger wing size ratio (1.18) which is likely due to a dominant-negative effect. These results suggest full-length Hpo is the most effective in growth inhibition.
Figure 3-6: Statistical analysis of adult wings expressing different versions of Hpo. About 20 adult wings expressing different portions of Hpo transgenes were analyzed and average wing sizes were calculated. The average wing sizes were divided by wild type wing size to obtain the wing size ratio for each genotype. The results were listed above each bar. Two asterisk indicate that p<0.001 versus wild type in student t-test.
3.5 The Effects of Hpo on Localization of Mats-GFP in Pupae Eye Discs

Previous studies in mammalian cells have shown that subcellular localization of mammalian MST is related to its kinase activity. MSTs are mainly cytosolic when they are inactive (Hwang et al., 2007). Once MSTs are activated through binding with RASSFs, MSTs are recruited to the plasma membrane. MSTs then form dimers which result in transphosphorylation on MSTs and kinase activation. Due to the reason that *Drosophila* MST, Hpo kinase, functions upstream of Mats; therefore, we tested whether Hpo is sufficient to affect Mats localization *in vivo*.

To show the change of Mats localization in cells, we coexpressed full-length FLAG-Hpo or kinase-dead N-terminus FLAG-Hpo with Mats-GFP in pupal eye discs using GMR-Gal4. Figure 3-7 shows the pupae eye discs expressing full-length FLAG-Hpo (Figure 3-7C) and Mats-GFP (Figure 3-7A) while Figure 3-8 shows ones expressing kinase-dead N-terminus FLAG-Hpo (Figure 3-8C) and Mats-GFP (Figure 3-8A). DE-Cadherin (Figure 3-7B and 3-8B) is used to outline the membrane of cells. Clearly, pupae eye discs are much smaller when expressing full-length Hpo compared with ones expressing kinase-dead N-terminus Hpo. This confirms that full-length Hpo is capable of reducing tissue sizes.

<table>
<thead>
<tr>
<th>Mats-GFP</th>
<th>DE-Cadherin</th>
<th>FLAG-Hpo</th>
<th>DE-Cadherin+FLAG</th>
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<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
</tbody>
</table>

+GFP

| E        | F           | G        |

Figure 3-7: Expression of full-length FLAG-Hpo results in size reduction of pupae eye discs. (A) Mats-GFP (B) DE-Cadherin staining (C)full-length FLAG-Hpo (D) Merged picture from (B)(C). (E),(F) and (G) are the pictures combining (A)(B), (A)(C) and (A)(D).
At the subcellular level (Figure 3-9), Mats-GFP localizes mostly in the cytosol and some are likely associated with the plasma membrane as well. Compared with the cytosolic Mats-GFP signal in Figure 3-9A, the Mats-GFP signal is reduced in cytoplasm in Figure 3-10A. It overlaps more with the plasma membrane marked by DE-Cadherin (Figure 3-10B and E). In addition, FLAG staining also shows distinct membrane signal indicating that FLAG-Hpo possibly

![Mats-GFP, DE-Cadherin, FLAG-Hpo, DE-Cadherin+FLAG](image)

**Figure 3-8:** Expression of kinase-dead N-terminus FLAG-Hpo did not cause size reduction of pupae eye discs. (A) Mats-GFP (B) DE-Cadherin staining (C) kinase-dead N-terminus FLAG-Hpo (D) Merged picture from (B)(C). (E),(F) and (G) are the pictures combining (A)(B), (A)(C) and (A)(D).

At the subcellular level (Figure 3-9), Mats-GFP localizes mostly in the cytosol and some are likely associated with the plasma membrane as well. Compared with the cytosolic Mats-GFP signal in Figure 3-9A, the Mats-GFP signal is reduced in cytoplasm in Figure 3-10A. It overlaps more with the plasma membrane marked by DE-Cadherin (Figure 3-10B and E). In addition, FLAG staining also shows distinct membrane signal indicating that FLAG-Hpo possibly

![Mats-GFP, Dlg, Merge](image)

**Figure 3-9:** Localization of Mats-GFP in the cytosol and at the membrane in pupae eye discs. (A) Mats-GFP (B) Dlg, a plasma membrane marker (C) Merged picture from (A) and (B).
Figure 3-10: Expression of full-length FLAG-Hpo leads to more plasma membrane localization of Mats-GFP at basal and apical side of ommatidium. A more magnified view of ommatidium structures from basal (A-G) to apical (H-N) are shown. (A)(H) Mats-GFP (B)(I) DE-Cadherin and (C)(J) Full length FLAG-Hpo stainings. (D)(K) Merged pictures from (B)(C) and (I)(J). (E)(F)(G) are the pictures combining (A)(B), (A)(C) and (A)(D). (L)(M)(N) are the merged pictures from (H)(I), (H)(J) and (H)(K). DE-Cadherin is a plasma membrane marker.
localizes at the plasma membrane (Figure 3-10C and F). Figure 3-10 (A) to (G) shows the ommatidial cells more basal while Figure 3-10 (H) to (N) shows cells more apical in the pupae eye discs.

As a negative control, we also assayed Mats-GFP localization in the cells expressing kinase-dead N-terminus Hpo. The ommatidial structures and cone cells are very much similar to the wild type ones in Figure 3-9(B). The cells from basal to apical pupae eye discs are shown in Figure 3-11. The cytosolic Mats-GFP was shown in every plane across pupae eye discs and kinase-dead N-terminus Hpo also showed cytosolic stainings. These suggest that Hpo kinase activity is crucial in affecting localizations of Mats-GFP and itself.

3.6 Summary

In summary, endogenous Mats localizes in the cytoplasm as well as plasma membrane in developing epithelial tissues, for example: pupae eye discs and larval wing discs. In addition, similar distributions have been observed in salivary glands. Loss of an upstream kinase, Hpo, results in more cytosolic Mats signal in wing disc cells indicating that endogenous Mats localization is under the control of Hpo kinase. In addition, we used gain-of-function analysis to express FLAG-Hpo with Mats-GFP and observed that Hpo is sufficient to increase membrane localization of Mats-GFP.
Figure 3-11: Expression of kinase-dead N-terminus FLAG-Hpo does not affect Mats-GFP localization in the cytosol either at basal or apical side of ommatidium. A more magnified view of ommatidium structures from basal (A-G) to apical (H-N) are shown. (A)(H) Mats-GFP (B)(I) DE-Cadherin stainings (C)(J) kinase dead N-terminus FLAG-Hpo (D)(K) Merged picture from (B)(C) and (I)(J). (E),(F),(G) are the pictures combining (A)(B), (A)(C) and (A)(D). (L),(M),(N) are the merged pictures from (H)(I), (H)(J) and (H)(K). DE-Cadherin is a plasma membrane marker.
Chapter 4

Activation of Mob as tumor suppressor by Membrane Association for Growth Inhibition

4.1 Introduction

Previous studies have demonstrated that Mob as tumor suppressor protein, Mats, functions as an activating cofactor of Warts (Wts)/large tumor suppressor (Lats) protein kinase in Hippo pathway (Lai et al., 2005). Recently, human Mps1-One binder (MOB1), a human ortholog of Mats, has been shown to increase human LATS 1 kinase activity at the plasma membrane in mammalian cells (Figure 4-1)(Hergovich et al., 2006). To test a hypothesis that Wts protein kinase can be activated by Mats at the plasma membrane during *Drosophila* development, membrane-targeting Mats tagged with GFP (Myr-Mats-GFP) was generated to allow Mats association at the plasma membrane in developing *Drosophila* tissues. As a result, I found that Myr-Mats-GFP is constitutively activated to inhibit tissue growth and capable of reducing organ sizes by regulating cell proliferation and apoptosis.

Figure 4-1: Human LATS 1 is recruited to the plasma membrane by human MOB1 in mammalian cells (Hergovich et al., 2006).
4.2 Localization of Myr-Mats-GFP at the Plasma Membrane

Previously, Mats-GFP has been shown to rescue mats mutant phenotypes as Mats. Both Mats and Mats-GFP over-expression in Drosophila do not disrupt normal development (Shimizu et al., 2006) which indicates that Mats-GFP functions as wild-type Mats in vivo (Lai et al., 2005).

To test the hypothesis that the plasma membrane is a crucial site for Mats activation, I generated transgenic lines expressing Mats-GFP fusion protein with 16 amino acids myristoylation (Myr) signal peptide at its N-terminus. The second amino acid of myristoylation signal was mutated from Gly to Ala to generate Myr(G2A)-Mats-GFP fusion protein to serve as a negative control for Myr-Mats-GFP construct. This Gly to Ala change in the Myr signal peptide disrupts lipid modification and proper membrane localization.

We went on to test if Myr-Mats-GFP localized at the plasma membrane. Myr-Mats-GFP transgene was driven by engrailed-Gal4 in the posterior regions of larval wing discs. As shown in Figure 4-2, the GFP signal from Myr-Mats-GFP (Figure 4-2A) overlapped with Dlg which served as a membrane marker (Figure 4-2B). Draq5 was used as a nuclear marker to visualize individual cells (Figure 4-2C). As predicted, Myr(G2A)-Mats-GFP (Figure 4-3A to C) and Mats-GFP (Figure 4-3D to F) did not show complete overlap with Dlg meaning Myr(G2A)-Mats-GFP and Mats-GFP mainly localized in the cytoplasm and nucleus. In addition, individual cells from larval wing discs were trypsinized and visualized under a fluorescent microscope. Cells expressing Myr-Mats-GFP showed clear membrane GFP signal outside the nucleus stained with Draq5 (Figure 4-4 A and B) while those expressing wild type Mats-GFP had overlapping signals (yellow) from GFP (green) and Draq5 (red) in Figure 4-4 (C) and (D). These data further confirm that Myr-Mats-GFP is stably expressed and localized at the plasma membrane.
Figure 4-2: Myr-Mats-GFP localizes at the plasma membrane in larval wing discs (A) Myr-Mats-GFP (B) Dlg staining is used as a membrane marker (C) Draq 5 staining is used as a nucleus marker (D) merged picture from (A),(B) and (C).

Figure 4-3: Localization of Myr\textsuperscript{(G2A)}-Mats-GFP and Mats-GFP mainly in the cytosol and nucleus in \textit{Drosophila} wing disc cells. Expression of (A) Myr\textsuperscript{(G2A)}-Mats-GFP and (D) Mats-GFP are shown. (B) and (E) Dlg staining outlines the plasma membrane of cells. (C) and (F) are the merged picture from (A)(B) and (D)(E).
4.3 Expression of Myr-Mats-GFP Leads to Reduction in Tissue Sizes

The effects of Myr-Mats-GFP on growth were examined in adult organs using tissue specific drivers. Here I used GMR-Gal4 as an eye specific driver to drive the expression of transgenes posterior to the morphogenic furrow (Figure 4-5). In addition, C5-Gal4 was used as a wing specific driver to express transgenes in the pouch areas (Figure 4-6). In Figure 4-5B, eyes expressing Myr-Mats-GFP are rough and smaller than those expressing Myr(G2A)-Mats-GFP (Figure 4-5C) and wild type eyes (Figure 4-5A). Figure 4-6B shows the expression of Myr-Mats-GFP is able to reduce adult wing sizes compared with wild type ones (Figure 4-6A). The wings expressing Myr(G2A)-Mats-GFP show no difference in wing size (Figure 4-6C) compared with wild type ones meaning membrane localization of Mats activates Mats to reduce organ size.

Since Mats has been shown to activate Wts previously, I wanted to see if membrane-targeting Mats is more potent than the non-membrane targeting Mats in stimulating growth inhibitory effects of Wts kinase. Figure 4-6D shows that the wings expressing Myc-Wts is smaller than wild type ones (Figure 4-6A) meaning Wts has growth suppressive effects. When Myc-Wts was coexpressed with Myr-Mats-GFP, wing sizes are much smaller (Figure 4-6E) compared with those coexpressing Myc-Wts and Myr(G2A)-Mats-GFP (Figure 4-6F). In addition, the wing sizes were larger in expressing Myr-Mats-GFP alone (Figure 4-6B) than

Figure 4-4: Dissociated larval wing disc cells expressing Myr-Mats-GFP or Mats-GFP. Cells expressing Myr-Mats-GFP are shown in (A)(B) while those expressing Mats-GFP are shown in (C)(D) (Green signals). The nucleus of cells was stained with Draq5 shown in red. The yellow regions show the overlapping signals from GFP and Draq 5, meaning that GFP and Draq5 are co-localized.
those coexpressing Myc-Wts and Myr-Mats-GFP (Figure 4-6E) further indicating that membrane-targeting Mats interacts with Wts in reducing organ sizes.

Figure 4-5: Reduction size of adult eyes expressing Myr-Mats-GFP. Scanning Electron Microscopic analysis of adult eyes expressing transgenes using GMR-Gal4 driver. (A) Canton S wild type eyes (B) and (C) shows eyes expressing Myr-Mats-GFP and Myr^{G2A}-Mats-GFP.

Figure 4-6: Expression of Myr-Mats-GFP by C5-Gal4 leads to smaller adult wings. (A) Canton S wings. Adult wings expressing (B) Myr-Mats-GFP (C) Myr^{G2A}-Mats-GFP (D) Myc-Wts (E) Myc-Wts and Myr-Mats-GFP (F) Myc-Wts and Myr^{G2A}-Mats-GFP by C5-Gal4.
In addition to the analysis of adult organ size, I also expressed Myr-Mats-GFP, Myr\textsuperscript{(G2A)}-Mats-GFP or Mats-GFP using *engrailed-Gal4* which drives UAS-transgenes in the posterior regions in larval wing discs. The wing discs were shown in Figure 4-7 and green regions represented cells expressing different forms of Mats tagged with GFP. Compared with posterior regions and whole wing disc sizes expressing Myr\textsuperscript{(G2A)}-Mats-GFP (Figure 4-7D and E) or Mats-GFP (Figure 4-7G and H), those expressing Myr-Mats-GFP are smaller (Figure 4-7 A and B). In conclusion, membrane targeting of Mats is sufficient to reduce organ size during development.

<table>
<thead>
<tr>
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<th>Wing Disc</th>
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<tr>
<td>G</td>
<td>H</td>
<td>I</td>
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</table>

Figure 4-7: Expression of Myr-Mats-GFP reduces the posterior regions and sizes of whole wing discs using *engrailed-Gal4*. (A) Myr-Mats-GFP, (D) Myr\textsuperscript{(G2A)}-Mats-GFP and (G) Mats-GFP are expressed in the region as shown in green due to GFP. (B)(E)(H) The resulting wing discs from wings expressing Myr-Mats-GFP, Myr\textsuperscript{(G2A)}-Mats-GFP or Mats-GFP in the posterior regions. (C),(F),(I) Merged pictures from (A)(B), (D)(E), (G)(H).
4.4 Expression of Myr-Mats-GFP by *engrailed-Gal4* Leads to Lethality during Development

Expression of Hippo signaling components (*hpo, yki, wts*) by *engrailed-Gal4* commonly led to late pupae lethality. Table 4-1 shows an example of lethality effects resulted from *myc-wts* overexpression driven by *engrailed-Gal4*. Therefore, we wanted to see if membrane targeting Mats has similar effects. As a result, the lethality effects of membrane-targeting Mats were also observed when expressing it by *engrailed-Gal4*. As shown in Table 4-1, no adult fly eclosed from pupae cases compared with the control genotype (*SM6 TM6B/en-Gal4*) (106). Only 63 individuals develop into late pupae but no eclosed adult was scored with respect to the expected number (106). This indicates that *engrailed-Gal4* driven Myr-Mats-GFP causes lethality issues earlier than pupae stages. Compared with the results from Myr-Mats-GFP, individuals expressing Myr\(^{(G2A)}\)-Mats-GFP all develop into adults without any lethality. These further demonstrate that membrane-targeting Mats is more potent than non membrane-targeting Mats as well as Wts in Hippo signaling pathway.

<table>
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<td>Late Pupae</td>
<td>Adult</td>
</tr>
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<td>SM6 TM6B/en-Gal4</td>
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<tr>
<td>Developmental stages</td>
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<tr>
<td>Numbers</td>
<td>Adult</td>
<td>Late Pupae</td>
<td>Adult</td>
</tr>
<tr>
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<td>myr(^{(G2A)})-mats-gfp(^{M9})/en-Gal4</td>
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<tr>
<td>Numbers</td>
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<td>Late Pupae</td>
<td>Adult</td>
</tr>
</tbody>
</table>

Table 4-1 Lethality Effects of Myr-Mats-GFP or Myc-Wts Driven by *engrailed-Gal4* (*en-Gal4*)
4.5 Clonal Expression of Myr-Mats-GFP Results in Smaller and Fewer Clones

Since Myr-Mats-GFP has been shown to reduce eyes and wings sizes using tissue specific drivers, I wanted to see if randomly induced clones expressing Myr-Mats-GFP in the wings also grew slower than those expressing Mats-GFP or Myr\(^{(G2A)}\)-Mats-GFP. As a result, Myr-Mats-GFP clones were smaller (Figure 4-8A) than those expressing Mats-GFP (Figure 4-8B) and Myr\(^{(G2A)}\)-Mats-GFP clones (Figure 4-8C). This result suggests that cells proliferate slower or die faster when expressing Myr-Mats-GFP compared with Mats-GFP or Myr\(^{(G2A)}\)-Mats-GFP. The clone sizes and numbers are very similar in Figure 4-8B and Figure 4-8C meaning Myr\(^{(G2A)}\)-Mats-GFP and Mats-GFP do not have dramatic functional differences. Moreover, the number of clones expressing Myr-Mats-GFP is significantly fewer than that of Mats-GFP and Myr\(^{(G2A)}\)-Mats-GFP clones in wing discs. This indicates some cells may be eliminated by wild type cells due to cell competitions.

![Figure 4-8: Expression of Myr-Mats-GFP results in smaller and lesser clones in larval wing discs. Clones induced by low level of heat shock expressing (A) Myr-Mats-GFP (B) Mats-GFP (C) Myr\(^{(G2A)}\)-Mats-GFP in Drosophila wing discs.](image)

4.6 Reduction of Cell Numbers in Ommatidial Cells in Pupae Eye Discs

During *Drosophila* development, larval stages are the main periods that cells proliferate. To maintain the homeostasis of tissue size, excessive numbers of cells should be eliminated later in development. In pupae eye discs, there are...
populations of cells called interommatidial cells existing between differentiated cone cells. The interommatidial cells were produced excessively during development and were eliminated by apoptosis in pupal stages. Previous studies have shown that the excessive numbers of cells were observed in loss of *fat, ex, merlin, hpo, sav* and *wts* mutants in the interommatidial cells in pupae eye discs due to the defective of apoptosis in these mutants (Udan et al., 2003; Willecke et al., 2006). Therefore, we wanted to see if reduced number of interommatidial cells can be observed when membrane-targeting Mats is expressed.

The cone cells and interommatidial cells in wild-type pupae eye discs are shown in Figure 4-9(A). The cell boundaries were outlined by a membrane marker, Dlg. Therefore, cone cells and interommatidial cells were identified by repetitive hexagonal units across pupae eye discs. When Myr-Mats-GFP is expressed (Figure 4-9B) using *GMR-Gal4*, the space between cone cells is reduced due to the reduction of ommatidial cells (Figure 4-9C and E).

<table>
<thead>
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<th>Dlg</th>
<th>Merge</th>
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<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>Myr-Mats-GFP expressing pupae eye disc</td>
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</table>

Figure 4-9: The number of interommatidial cells is decreased in pupae eye discs expressing Myr-Mats-GFP using *GMR-Gal4*. (A) Wild type and (B to E) Myr-Mats-GFP expressing pupae eye discs (B) Expression of Myr-Mats-GFP (C) Dlg staining of pupae eye discs (D) Merged picture from (B) and (C). For the purpose of clarity, the Dlg stainings were shown in white again in (E) from (C).
4.7 Expression of Myr-Mats-GFP Inhibits Cell Proliferation and Increases Apoptosis

Reduction of organ sizes and tissue growth by Myr-Mats-GFP could be related to the effects on proliferation or apoptosis. I then examined the cell proliferation by BrdU staining in cells expressing membrane-targeting Mats. BrdU labeling is used to detect cells in S phase through incorporating nucleotide analog in to DNA during DNA replication. Figure 4-10 shows membrane-targeting Mats is able to reduce BrdU signal (Figure 4-10B) in the posterior regions expressing Myr-Mats-GFP (Figure 4-10A).

Other than cell proliferation, slower tissue growth and smaller organ sizes could be due to defects in apoptosis. To test this possibility, I used TUNEL assay to detect cells in apoptotic stage. TUNEL assay marks the apoptotic cells by adding fluorescent labeled dUTP at the ends of fragmented DNA using terminal deoxynucleotidyl transferase. As a result, enhanced TUNEL stainings (Figure 4-11B and E) were observed in regions expressing Myr-Mats-GFP driven by engrailed-Gal4 (Figure 4-11A and D). Compared with TUNEL level from Myr-Mats-GFP expressing regions, TUNEL was not enhanced (Figure 4-11H) in the posterior regions expressing Myr\textsuperscript{(G2A)}-Mats-GFP (Figure 4-11G) meaning membrane-targeting Mats is very potent in inducing apoptosis during development.
In the process of apoptosis, caspase 3 has been shown to be a key apoptotic effector caspase in *Drosophila*. Therefore, I wanted to see if activated caspase 3 was also induced in the posterior region due to the expression of membrane-targeting Mats. As expected, the level of activated caspase 3 (Figure 4-12B and E) was elevated in Myr-Mats-GFP expressing region (Figure 4-12A and D) compared with activated caspase 3 stainings (Figure H and K) in the regions expressing Myr\(^{(G2A)}\)Mats-GFP (Figure 4-12I and L).

Figure 4-11: TUNEL labelings were enhanced in the posterior regions expressing Myr-Mats-GFP. (A) Myr-Mats-GFP (B) TUNEL stainings were used to label apoptotic cells. (C) Merged picture from (A) and (B). (D)(E)(F) are magnified views from (A)(B)(C). (G) Wing discs expressing Myr\(^{(G2A)}\)Mats-GFP using *engrailed-Gal4* driver (H) TUNEL was not enhanced (I) Merged picture from (G) and (H).
Figure 4-12: Activated Caspase 3 staining is enhanced in the posterior region expressing Myr-Mats-GFP (a) Myr-Mats-GFP is expressed using *engrailed-Gal4* driver (B) anti-Activated Caspase 3 antibody is used to label Caspase 3 activated cells (C) Merged picture from (A) and (B). (D)(E)(F) are magnified views from (A)(B)(C). (G) Wing discs expressing Myr<sup>G2A</sup>-Mats-GFP using *engrailed-Gal4* driver (H) No enhancement of activated caspase 3 cells. (I)Merged picture from (G) and (H). (J)(K)(L) are magnified views from (G)(H)(I).
In addition to activated caspase 3, I tested if other molecular markers involved in cell number control in *Drosophila* were affected. *Drosophila* Inhibitor of apoptosis-1 (DIAP-1), a downstream target which was induced during cell survival, was suppressed (Figure 4-13B) due to expression of membrane-targeting Mats (Figure 4-13A). Meanwhile, activated Drice which is induced during apoptosis was elevated in the posterior region (Figure 4-13C) where Myr-Mats-GFP was expressed. These data supported that membrane-targeting Mats reduced organ sizes through upregulating cell death.

<table>
<thead>
<tr>
<th>Myr-Mats-GFP</th>
<th>DIAP-1</th>
<th>Activated Drice</th>
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<tbody>
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<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
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</table>

Figure 4-13: DIAP-1 protein is suppressed and activated Drice is induced in the posterior regions expressing Myr-Mats-GFP. (A) Expression of Myr-Mats-GFP (B) DIAP-1 and (C) Activated Drice stainings (D) Merged picture from (A) to (C).

### 4.8 expanded Transcription is Suppressed by the Expression of Membrane-targeting Mats

Another target gene, *ex*, was reported to be transcriptionally upregulated in the absence of *fat* (Cho et al., 2006). I then took advantage of *ex-lacZ* allele and used anti β-gal staining to monitor the transcriptional activity of *ex* promotor. The results showed that membrane-targeting Mats in the posterior region (Figure 4-14A) reduces β-gal level (Figure 4-14 B) compared with that (Figure 4-14E) within the regions expressing non membrane-targeting version (Figure 4-14D, E). This evidence further indicates membrane-targeting Mats regulates the activity of Hippo pathway.
In this chapter, I have shown that the transgenic flies containing UAS-
myr-mats-gfp does drive the expression of Mats-GFP at the plasma membrane. Membrane-targeting Mats is able to reduce organ sizes. The data indicates that membrane-targeting Mats has constitutive growth suppression activity which resembles the phenotypes of gain-of-function allele of a tumor suppressor. In addition, I have also demonstrated that the growth inhibitory effects are due to decrease in cell numbers resulting from downregulation of cell proliferation and enhancement of cell apoptosis. The level of DIAP-1 is decreased while that of activated caspase 3 and activated Drice is increased further supports the observations above. Lastly, the transcription of ex is suppressed due to the expression of Myr-Mats-GFP. All these evidence indicate that membrane-targeting Mats is acting through Hippo pathway.

Figure 4-14: Transcription of ex is suppressed in the posterior regions expressing Myr-Mats-GFP. Posterior regions expressing (A) Myr-Mats-GFP or (D) Myr(G2A)-
Mats-GFP. (B)(E) ex-lacZ expression levels are detected by anti β-gal stainings. (C) and (F) Merged pictures from (A)(B) and (D)(E), respectively. The arrow indicates the posterior regions which have reduced ex transcription.

4.9 Summary

In this chapter, I have shown that the transgenic flies containing UAS-
myr-mats-gfp does drive the expression of Mats-GFP at the plasma membrane. Membrane-targeting Mats is able to reduce organ sizes. The data indicates that membrane-targeting Mats has constitutive growth suppression activity which resembles the phenotypes of gain-of-function allele of a tumor suppressor. In addition, I have also demonstrated that the growth inhibitory effects are due to decrease in cell numbers resulting from downregulation of cell proliferation and enhancement of cell apoptosis. The level of DIAP-1 is decreased while that of activated caspase 3 and activated Drice is increased further supports the observations above. Lastly, the transcription of ex is suppressed due to the expression of Myr-Mats-GFP. All these evidence indicate that membrane-targeting Mats is acting through Hippo pathway.
Chapter 5
Mats inhibits Tissue Growth through Wts Kinase

5.1 Introduction

In chapter 4, I have shown that membrane-targeting Mats was able to reduce tissue growth and organ size through inhibiting proliferation and inducing apoptosis. However, the mechanisms which Myr-Mats-GFP act through were still not clear. Previously, Mats has been shown to associate with Wts kinase and activate Wts kinase activity. Coexpression of mats and wts further reduces organ size than overexpression of wts or mats alone showing genetic interactions between mats and wts. In this chapter, we wanted to determine how membrane-targeting Mats was more potent in activating Wts kinase. Through in vivo analysis, the effects of membrane-targeting Mats on Wts kinase activity and localization were examined. In addition, MARCM analysis was used to illustrate epistatic relationships between this constitutively active Mats and Wts kinase. Finally, we tested if Yki, a transcriptional co-activator and a key substrate of Wts kinase, is also affected.

5.2 Membrane-targeting Mats Enhances Myc-Wts Activity through Interaction

In Figure 4-6, membrane-targeting Mats has been shown to further reduce organ size and tissue growth when coexpressing with Myc-Wts; however, the mechanisms were not elaborated. To illustrate the mechanism, we coexpressed Mats-binding defective Myc-Wts (Myc-Wts R702E) (generated by Xiaomu Wei) and Myr-Mats-GFP by C5-Gal4 and analyzed size differences of adult wings. The typical examples of each genotype are shown in Figure 5-1. The wings expressing Myc-Wts R702E (Figure 5-1G) is slightly larger than those expressing Myc-wts (Figure 5-1D) suggesting Mats-binding defective Myc-Wts was not as potent as Myc-Wts. Figure 5-1H shows a typical wing expressing Myc-Wts R702E and Myr-Mats-GFP which is larger than that expressing Myc-Wts and Myr-Mats-GFP.
(Figure 5-1E) and similar to that expressing Myr-Mats-GFP alone in Figure 5-1B. This indicates that Wts-Mats interaction is crucial for Myr-Mats-GFP to suppress tissue growth. As a comparison, sizes of wings coexpressing Myc-Wts$^{R702E}$ and Myr$^{(G2A)}$-Mats-GFP (Figure 5-1I) are larger than those expressing Myc-Wts alone (Figure 5-1D) or those coexpressing Myc-Wts and Myr$^{(G2A)}$-Mats-GFP (Figure 5-1F). This is likely due to Myc-Wts$^{R702E}$ is defective in interacting with endogenous Mats.

To see if Wts kinase activity is crucial in membrane-targeting Mats mediated growth inhibition, we coexpressed kinase-dead Myc-Wts mutant (Myc-Wts$^{K743A}$) and Myr-Mats-GFP using C5-Gal4. The wings expressing Myc-Wts$^{K743A}$ (Figure 5-1J) are slightly larger than those expressing Myc-Wts (Figure 5-1D) implying kinase-dead mutant is not as functional as wild type Myc-Wts. As shown in Figure 5-1K, the wing size is larger than that coexpressing Myc-Wts and Myr-Mats-GFP (Figure 5-1E) indicating that kinase activity is critical for Wts to be activated by membrane-targeting Mats.

We further quantified the wing sizes from different mutant genotypes and compared them with those from wild type. Over 20 adult wings from each genotype were collected, photographed and quantified in pixels by image J. Figure 5-2 shows the ratios of mutant wing size with respect to the wild type ones.

Based on the ratios indicated in Figure 5-2, membrane-targeting Mats reduces tissue growth about 64% (ratio=0.36) compared with wild type while non-membrane-targeting Mats (0.98) or wild type Mats (0.94) did not cause apparent organ size reduction. When membrane-targeting-Mats was coexpressed with Myc-Wts, there was a dramatic reduction in wing size (0.11). The ratio was much smaller than those coexpressing Myc-Wts with Myr$^{(G2A)}$-Mats-GFP (0.64) or Mats-GFP (0.60). If Myr-Mats-GFP was coexpressed with Mats-binding defective Myc-Wts$^{R702E}$ or kinase-dead Myc-Wts$^{K743A}$, the ratios were around 0.40 instead of 0.11. The statistical analysis supported that the interaction between membrane-targeting Mats and Wts was critical in reducing tissue growth. Moreover, Myc-Wts kinase activity was important in mediating growth inhibitory effects by constitutively active Mats. Finally, the ratios from Myr$^{(G2A)}$-Mats-GFP and Mats-GFP have similar activities when expressing alone (0.98 vs. 0.94) or coexpressing with wild type Myc-Wts or mutant Myc-Wts suggesting that they were functionally similar.
Figure 5-1: Expression of Myr-Mats-GFP and Myc-Wts further reduces tissue sizes. (A) Canton S and wings expressing (B) Myr-Mats-GFP (C) Myr(G2A)-Mats-GFP (D) Myc-Wts (E) Myr-Mats-GFP and Myc-Wts (F) Myr(G2A)-Mats-GFP and Myc-Wts (G) Myc-Wts R702E (H) Myr-Mats-GFP and Myc-Wts R702E (I) Myr(G2A)-Mats-GFP and Myc-Wts R702E (J) Myc-Wts K743A (K) Myr-Mats-GFP and Myc-Wts K743A (L) Myr(G2A)-Mats-GFP and Myc-Wts K743A by C5-Gal4.
Figure 5-2: Statistical analysis of adult wing sizes. At least 20 adult wings from each genotype listed above were collected and wing size ratios with respect to wild type wings were listed above each bar. The gray bars show wild type and C5-Gal4/+ control wing size ratio. The ratios of wing sizes can be compared within groups and with the bar in the same color. Red bars represent the ratios of average wing size expressing Myr-Mats-GFP or those coexpressing Myr-Mats-GFP with Myc-Wts, Myc-Wts $^{R702E}$, or Myc-Wts $^{K743A}$. Yellow and blue bars indicate the ratios of wings expressing Myr$^{G2A}$-Mats-
To further correlate growth inhibitory effects of constitutively active Mats and Wts kinase activity, an antibody recognizing active form of Wts kinase was used. Third instar larval wing discs expressing Myr-Mats-GFP and mutant Myc-Wts by C5-Gal4 were stained with anti-Myc and anti-phospho Wts antibody. C5-Gal4 driver expresses UAS-transgenes in the pouch regions during larval stages. Figure 5-3C shows that activated Wts kinase was detected in the regions co-expressing Myc-Wts with Myr-Mats-GFP (Figure 5-3A to D) but not Mats-GFP (Figure 5-3, compare A-D with E-H).

Consistent with the adult wing data, phospho-Wts was not detected in the larval wings coexpressing Myc-Wts\textsuperscript{K743A} or Myc-Wts\textsuperscript{R702E} with Myr-Mats-GFP (Figure 5-3K and O) indicating that mutant Wts kinases are not activated even in the presence of constitutively active Mats (Figure 5-3I and M). The wing pouch regions outlining where transgenes expressed are larger in wings coexpressing Myc-Wts\textsuperscript{R702E} or Myc-Wts\textsuperscript{K743A} with Myr-Mats-GFP than in those coexpressing Myc-Wts with Myr-Mats-GFP (Compare Figure 5-3L,P with D). These data support the idea that membrane-targeting Mats activates Myc-Wts by forming a complex and increases Wts kinase activity for tissue growth inhibition.

We further wanted to confirm the localization of Myc-Wts, Myc-Wts\textsuperscript{R702E} or Myc-Wts\textsuperscript{K743A} in the subcellular level when coexpressed with membrane-targeting Mats. In the magnified pictures of wing disc cells from Figure 5-3, we were able to observe GFP and Myc staining representing the localization of Mats and Myc-Wts. As shown in Figure 5-4, Myc-Wts showed distinct line (Figure 5-4B) overlapping with Myr-Mats-GFP (Figure 5-4A) at the plasma membrane compared with more cytosolic Myc staining (Figure 5-4F) when coexpressed with Mats-GFP (Figure 5-4E). The membrane was outlined by Dlg stainings (Figure 5-4D) to show the cell boundaries in wing disc cells expressing Myc-Wts and Mats-GFP. Myc staining in cells expressing Myc-Wts\textsuperscript{R702E} and Myr-Mats-GFP was not dramatically enhanced at the plasma membrane (Figure 5-4I) indicating that the interaction between Myc-Wts and Myr-Mats-GFP was disrupted. Compared with Myc stainings from cells expressing Myc-Wts\textsuperscript{R702E} and Myr-Mats-GFP, Myc stainings in cells expressing Myc-Wts\textsuperscript{K743A} and Myr-Mats-GFP (Figure 5-4L) is similar to those expressing Myc-Wts and Myr-Mats-GFP (Figure 5-4B). This implies that membrane-targeting Mats is able to interact with Myc-Wts\textsuperscript{K743A} but Myc-Wts\textsuperscript{K743A} can not be activated due to the mutation.
Figure 5-3: Myr-Mats-GFP increases activated Myc-Wts level through interaction. Larval wing pouch regions co-expressing Myr-Mats-GFP and Myc-Wts are shown in (A) to (D). The regions co-expressing Mats-GFP and Myc-Wts are shown in (E) to (H). The areas co-expressing Myr-Mats-GFP and Mats-binding mutant Myc-Wts$^{R702E}$ are shown in (I) to (L). The areas co-expressing Myr-Mats-GFP and kinase-dead mutant Myc-Wts$^{K743A}$ are shown in (M) to (P). (A)(I)(M) Myr-Mats-GFP or (E) Mats-GFP expressing regions. (B)(F)(J)(N) Myc staining is used to detect Myc-Wts. (C)(G)(K)(O) Activated Wts staining (p-Wts). (D)(H)(L)(P) Merged pictures from (A)to(C), (E)to(G), (I)to(K), (M)to(O).
Figure 5-4 Myr-Mats-GFP enhances Myc-Wts level at the plasma membrane through interaction. Higher magnifications are used to see the detailed localization of (B)(F) Myc-Wts, (I) Mats-binding mutant Myc-Wts$^{R702E}$ and (L) kinase-dead mutant Myc-Wts$^{K743A}$ coexpressed with (A)(H)(K) Myr-Mats-GFP or (E) Mats-GFP. (D) Dlg is used as a membrane marker. The transgenes were driven by C5-Gal4. (A) to (C) Cells co-expressing Myr-Mats-GFP and Myc-Wts. (D) to (G) Cells co-expressing Mats-GFP and Myc-Wts. (H) to (J) Cells co-expressing Myr-Mats-GFP and Mats-binding mutant Myc-Wts$^{R702E}$. (K) to (M) Cells co-expressing Myr-Mats-GFP and kinase-dead mutant Myc-Wts$^{K743A}$. (C),(G),(J),(M) Merged pictures from (A) to (B), (D) to (F), (H) to (I), (K) to (L).
Based on the data above, we conclude that membrane-targeting Mats is capable of enhancing Myc-Wts by forming a complex which then activating Wts kinase activity. These effects lead to the reduction of tissue growth and organ size.

5.3 Localization of Myc-Wts in Loss of sav/hpo/mats Genetic Background

In the previous section, we have shown constitutively active Mats is sufficient to enhance Myc-Wts at the plasma membrane in wing disc cells. To see if localization of Myc-Wts requires Mats or other components in Hpo kinase cassette, we used MARCM to express Myc-Wts in loss of mats/hpo/sav genetic backgrounds (Figure 5-5).

Figure 5-5: Expression of Myc-Wts in loss of sav/hpo/mats genetic backgrounds using MARCM. The parental cells with Gal4, Gal80, UAS-myc-wts carry a heterozygous mutation distal to an FRT site. After low level of heat shock, the flippase was induced during mitotic recombination to produce one kind of cells expressing Myc-Wts in homozygous mutant genetic backgrounds, and the other kind of cells in the absence of Myc-Wts expression in wing discs. The Myc stainings was used to mark the clones and localization of Myc-Wts.
In loss of *mats* genetic background, Myc stainings showed clear membrane signals (Figure 5-6A) which overlapped well with DE-Cadherin staining (Figure 5-6B and C), an apical junction membrane marker. This indicated that Mats was not essential for Myc-Wts membrane localization. As opposed to Myc-staining in Figure 5-6A, Myc signals were not as sharp in Figure 5-6D in the absence of *hpo* meaning Hpo maybe more crucial in Myc-Wts membrane localization. Another molecule, Sav, which served as a scaffold protein to bring Wts and Hpo together was not required for Myc-Wts membrane localization since Myc-staining was still showing clear membrane signals in loss of *sav* clones.

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**Figure 5-6**: Reduction of Myc-Wts Localization at the plasma membrane in loss of *hpo* clones in wing disc cells. Expression of Myc-Wts in (A) to (C) loss of *mats*, (D) to (F) loss of *hpo*, (G) to (I) loss of *sav* genetic background. (A)(D)(G) Myc staining is used to show Myc-Wts localization. (B)(E)(H) DE-Cadherin is used to mark the plasma membrane of wing disc cells. (C)(F)(I) are merged pictures from (A)(B), (D)(E), (G)(H).
5.4 Membrane-targeting Mats Reduces Yki Localization in the Nucleus

A transcriptional co-activator to promote tissue growth, Yki, has been shown to be phosphorylated and inactivated by Wts kinase (Dong et al., 2007). Inactivation of Yki by Wts leads to cytoplasmic localization of Yki instead of nuclear localization. To see if membrane-targeting Mats also affects the localization of Yki, we coexpressed Myr-Mats-GFP with Yki-V5 (generated by Xiaomu Wei) using \textit{C5-Gal4} driver. Nucleus localized Yki-V5 was shown to overlap with Draq 5 nucleus staining in wing disc cells without the expression of Myr-Mats-GFP (Figure 5-7A to C). The Yki-V5 staining pattern in cells coexpressing Yki-V5 and Myr-Mats-GFP was less in the nucleus (Figure 5-7D to G) than that in those expressing Yki-V5 only (Figure 5-7A). These data suggested that membrane-targeting Mats was able to reduce Yki in the nucleus for tissue growth inhibition.

![Myr-Mats-GFP, Yki-V5, Draq 5, Merge](image)

Figure 5-7: Membrane-targeting Mats driven by \textit{C5-Gal4} decreases nuclear Yki-V5 in wing disc cells. (A) to (C) Cells expressing Yki-V5 and (D) to (G) Cells coexpressing Myr-Mats-GFP and Yki-V5. (D) Myr-Mats-GFP (A)(E) Yki-V5 staining (B)(F) Draq 5 staining (C)(G) Merged picture from (A)(B) and (D)(E)(F).
In addition to the localization of Yki-V5 transgene, we also analyzed the effects of membrane-targeting Mats on the localization of endogenous Yki. The anterior region of the wing discs (Figure 5-8A, regions without GFP) showed endogenous Yki staining overlapped with Draq 5 nucleus staining (Figure 5-8C and D). This indicated that some endogenous Yki localized in the nucleus. On the contrary, endogenous Yki level was reduced in the nucleus (Figure 5-8B) in the posterior wing disc cells expressing membrane-targeting Mats (Figure 5-8A). The results confirmed the conclusion drawn from Figure 5-7.

5.5 Loss of mats Increases the Level of Nuclear Yki

Relative to the expression of constitutively active Mats which results in decrease of nuclear Yki, we generated loss of mats clones and see if nuclear Yki could be increased. Previous studies have shown that nuclear Yki level was enhanced in loss of wts, hpo and sav clones in eye discs (Dong et al., 2007). Similarly, the endogenous Yki was also increased in the nucleus (Figure 5-9 A,E) in clones absent of mats (Figure 5-9 B,F lacking β-gal staining and indicated by white arrows). This result further supported the idea that mats is regulating Hpo pathway and Mats level affects the level of Yki in the nucleus.
5.6 Wts Kinase is Required for Tissue Growth Inhibition Induced by Constitutively Active Mats

Previously, Mats has been shown to function as a co-factor to activate Wts kinase in inhibiting tissue growth (Lai et al., 2005; Wei et al., 2007). However, the epistatic relationship was not vigorously addressed. To resolve this issue, MARCM system was used to demonstrate epistatic relationship of myr-mats-gfp and wts in larval eye discs and wing discs (Figure 5-10).

Figure 5-9: Nuclear Yki is increased in loss of mats clones in larval eye discs. (A) Yki staining (B) Cells in the absence of mats are negative in β-gal staining (C) Draq 5 nucleus staining (D) Merged picture from (A)(B)(C). (E) to (H) Magnified views of (A) to (D) to see the stainings at the subcellular level. The arrows indicate the cells that have more nuclear Yki.
Figure 5-10: Generation of clones expressing Myr-Mats-GFP in wild type background or loss of wts genetic background. The first instar larvae with genetics elements for MARCM, UAS-myr-mats-gfp and heterozygous mutation distal to an FRT site were subjected to low level of heat shock. After flippase was induced, cells expressed Myr-Mats-GFP in the absence of wts were marked by GFP while those still had Gal80 in their genome showed no signal of GFP.

First, we used ey-FLP to generate clones and showed that loss of wts clones in eye discs (Figure 5-11B) were larger than wild type clones (Figure 5-11A) due to the increase of wts mutant cells. On the contrary, clones expressing Myr-Mats-GFP were much smaller (Figure 5-11D). When Myr-Mats-GFP was expressed in loss of wts genetic background (Figure 5-11C), the sizes of clones were more similar to those of loss of wts clones meaning growth inhibitory effects of constitutively active Mats was suppressed by wts mutation. Thus, wts is genetically epistatic to mats.
The epistatic relationship was further confirmed by TUNEL staining in wing discs using MARCM. Instead of ey-FLP, we used hs-FLP to induce clones. As previously described, TUNEL assay was used to label fragmented DNA in apoptotic cells. As shown in Figure 5-12B, the apoptotic cells were induced in clones expressing Myr-Mats-GFP (Figure 5-12A) while there was no apparent change of apoptotic level (Figure 5-11E) in loss of wts clones expressing Myr-Mats-GFP (Figure 5-11D). These results indicated that loss of wts could suppress the apoptotic effects induced by constitutively active Mats. Therefore, the results supported the idea that wts is required for apoptosis induced by constitutively active Mats and wts is epistatic to mats.

Figure 5-11: Sizes of clones expressing Myr-Mats-GFP in the absence of wts are similar to those of loss of wts clones in eye discs. Antenna discs were on the left in each figure and anterior parts of eye discs were pointed by arrows. MARCM was used to generate clones using ey-FLP in (A) wild type (B) Loss of wts (C) Loss of wts expressing Myr-Mats-GFP (D) Myr-Mats-GFP genetic background. Clones are marked in green due to the expression of GFP.
Since constitutively active Mats is able to reduce Yki in the nucleus, and Yki is a substrate of Wts kinase. Therefore, we wanted to see if effects of constitutively active Mats on Yki localization also require Wts. Figure 5-13B shows that Yki staining is reduced in the nucleus in clones expressing Myr-Mats-GFP (Figure 5-13A). However, the decrease in nuclear Yki staining is not observed in loss of wts clones expressing Myr-Mats-GFP (Figure 5-13E). These data suggest that the depletion of nuclear Yki by constitutively active Mats requires Wts.

**Figure 5-12:** TUNEL staining is not induced in loss of wts clones expressing Myr-Mats-GFP in wing discs. (A) to (C) Clones expressing Myr-Mats-GFP (D) to (F) Clones expressing Myr-Mats-GFP in loss of wts background. (A)(D) Myr-Mats-GFP (B)(E) TUNEL staining is used to label apoptotic cells. (C) and (F) Merged pictures from (A)(B) and (D)(E).
5.7 Constitutively Active mats is Epistatic to hpo

Previously, we have shown mats is epistatic to hpo since overgrowth was observed in eyes expressing hpo transgenes in loss of mats genetic background. In addition, Hpo kinase is able to phosphorylate Mats to potentiate Mats to be more active in enhancing Wts kinase activity (Wei et al., 2007). Based on these results, we wanted to see if constitutively active Mats could bypass the requirement of Hpo activity. To address this point, we generated clones of different genotypes in wing discs using MARCM. The wild type clones were shown in Figure 5-14A. The sizes of loss of hpo clones expressing Myr-Mats-GFP (Figure 5-14C) were similar to those expressing Myr-Mats-GFP in wild type genetic background (Figure 5-14D) but not loss of hpo clones (Figure 5-14B). In conclusion, Mats functions downstream of Hpo kinase consistent with previous results.

Figure 5-13: Increase of nuclear Yki in loss of wts clones expressing Myr-Mats-GFP. (A) to (C) Clones expressing Myr-Mats-GFP.(D) to (F)Clones expressing Myr-Mats-GFP in loss of wts background.(A)(D) Clones expressing Myr-Mats-GFP. (B)(E)Yki staining (C)(F) Merged pictures from (A)(B) and (D)(E).
5.8 Summary

Through adult wing size analysis, membrane-targeting Mats is shown to be sufficient to further reduce tissue size when coexpressed with Wts. This shows that Mats is more potent in activating Wts when it localizes at the plasma membrane. However, a Mats-binding mutant Wts was not responsive to this enhancement of reducing organ sizes indicating that the interaction between membrane-targeting Mats and Wts is critical. In addition, a kinase-dead mutant Wts also fail to be further activated by membrane-targeting Mats as expected. The reduction in tissue sizes is further confirmed by the level of activated form of Wts and Myc-Wts localization in the subcellular level.

This constitutively active Mats not only increases membrane localization of Wts, but also reduces activated Yki in the nucleus in developing Drosophila tissues. This suggests that constitutively active Mats is affecting downstream targets of Hippo pathway. To see if Wts is required for mediating reduction of cell numbers by constitutively active Mats, we compared size of clones and analyzed

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**Figure 5-14**: Constitutively active mats is epistatic to hpo. MARCM was used to generate clones using hs-FLP in (A) wild type (B) Loss of hpo (C) Loss of hpo expressing Myr-Mats-GFP (D) Myr-Mats-GFP genetic background in larval wing discs. Clones are marked in green due to the expression of GFP.
apoptotic effects in different genetic backgrounds. The results in eye and wing discs show that clones expressing constitutively active Mats fail to reduce cell numbers in the absence of \textit{wts}. The apoptotic cells are not induced and nuclear Yki is not decreased in loss of \textit{wts} clones expressing Myr-Mats-GFP. All these results support the idea that Wts is required for constitutively active Mats mediated growth inhibitory effects.

Previous studies in mammalian cells suggested that membrane-targeting human MOB1 is sufficient to recruit human LATS1 kinase to the plasma membrane and then dramatically activates kinase activity of LATS1. However, the relationships between membrane-targeting MOB1 induced kinase activity and cell number control were not addressed. In addition, the mechanisms of how membrane-targeting MOB1 regulates cell proliferation and apoptosis were not elucidated.

In our studies, we expressed membrane-targeting Mats in \textit{Drosophila} developing tissues and demonstrated that plasma membrane is a crucial place for Mats to be a more potent co-activator for Wts kinase. In addition, we provided evidence that expression of constitutively active Mats is able to downregulate cell proliferation and upregulate cell apoptosis to control tissue and organ size. These growth inhibitory effects of membrane-targeting Mats require the presence of Wts.

Our results also suggest that Mats is not essential for membrane localization of Myc-Wts since Myc-Wts can still be detected at the plasma membrane without coexpressing with membrane-targeting Mats. However, expression of membrane-targeting Mats is able to enhance Myc-Wts level at the plasma membrane through Wts-Mats interaction (Figure 5-15). These findings indicate that the regulation of the localization of Wts and Mats is not simply due to the recruitment of Wts by Mats during development.

It is still not clear that how Mats is recruited to or accumulated at the plasma membrane since overexpression of Mats at the plasma membrane has constitutively growth inhibitory effects. It is likely that the molecules regulating the localization of Mats are limited or subcellular localization of Mats is tightly regulated during development. Based on the data that loss of membrane Mats stainings in \textit{hpo} clones, it is possible that Hpo kinase is critical for recruiting or maintaining Mats at the plasma membrane. There maybe other molecules upstream of Hippo signaling pathway responsible for regulating the localization of Mats.
Figure 5-15: Wts is enhanced at the plasma membrane by membrane-targeting Mats in developing Drosophila tissues.
Chapter 6

Crosstalk between Hippo and Wingless Signaling Pathway

6.1 Introduction

Wingless (Wg)/Wnt signaling pathway is known to have multiple roles in regulating cell proliferation, specification and differentiation during animal development. The elevated amount of transcriptional co-activator β-Catenin/Armadillo (Arm) in the nucleus is the key in activating Wg signaling. In the absence of Wg signaling, β-Catenin/Arm is targeted by a degradation complex with Glycogen Synthase Kinase 3 (GSK3)/Sgg, Adenomatous polyposis coli (APC) and a scaffolding protein Auxin (Axin) in the cytosol. Axin and APC facilitates GSK3/Sgg phosphorylation on β-Catenin /Arm and lead it to the proteosomal degradation pathway. When Wg ligands act through Frizzled (Fz) receptors, the signal transduces through an intracellular protein Dishevelled (Dsh), which stabilizes and release β-Catenin/Arm from APC by inhibiting GSK3/Sgg. The β-Catenin /Arm enters into the nucleus and binds with a transcription factor TCF/Pangolin (Pan) to activate target gene expression. This type of regulation is referred to as a canonical Wnt pathway. The noncanonical Wnt pathway does not involve β-Catenin /Arm and TCF/Pangolin (Pan), instead, Rho family GTPases and the Jun kinase pathway are regulated by Dsh to control planar cell polarity and cell movements (Sato et al, 2006).

Previously, Fat and Wg signaling pathway have been shown to regulate epithelial cell-cell adhesion in Drosophila, and ft acts as a suppressor of Wg signaling. The intersection of fat and Wg signaling is downstream of Wg ligands (Jaiswal et al., 2005). In addition, ex is capable of limiting Wg signaling based on a lac-Z reporter study of a downstream target gene, ndk. Overexpression of a Wg signaling negative regulator, Axin, is able to rescue the ex Mad double mutant phenotype on Wg pathway indicating components of Fat and Wg signaling pathway genetically interact (Tyler and Baker, 2007). However, the mechanisms linking these two pathways are not fully understood.
In this project, we observed that loss of hpo, wts or mats resulted in an increase of Wg signaling. Therefore, we wanted to investigate that how the crosstalk between Fat and Wg signaling pathway could occur downstream of ft and ex by using core complex of Hpo kinase cassette components. Furthermore, induction of Wg signaling downstream target genes expression resulting from loss of Hpo kinase cassette components can be blocked by some but not all of the negative regulators of Wg signaling components. These indicate that the crosstalk between these two pathways intersects between certain molecules in Wg signaling pathway.

Figure 6-1: Canonical and non-canonical Wg signaling pathway.
6.2 Generation of Loss of *mats*, Loss of *wts* or Loss of *hpo* Clones

*w; UAS-GFP; FRT82 mats*235/* SM6 · TM6B* or *w; UAS-GFP; FRT82 wts*21/* SM6 · TM6B* were crossed with *w, hs-flp; Tub-Gal4, FRT 82B Tub-Gal8/TM6B* female flies to generate loss of *mats* or *wts* clones. To generate loss of *hpo* clones, *w; FRT 42D hpo BF33 / SM6 · TM6B* were crossed with *UAS-GFP, hsflp122; FRT42D, Tub-Gal80; Tub-Gal4/TM6B*. First instar larvae were subjected to low level of heat shock; clones of desired genotypes were marked by GFP.

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Figure 6-2: Generation of loss of *mats*/wts/hpo clones using MARCM analysis. The cells have *Gal4*, *Gal80* and heterozygous mutation of *mats*/wts/hpo does not express *UAS-GFP*. After low level of heat shock, mitotic recombination is induced to generate loss of *mats*/wts/hpos clones marked by GFP and wild type clones in the absence of GFP.
6.3 Notum, Armadillo and DE-Cadherin are Elevated in Clones Loss of Hippo Signaling

6.3.1 Notum is Elevated in Loss of mat, Loss of wts or Loss of hpo Clones

A secreted repressor of Wingless ligand, Notum, is used to monitor the activity of Wg signaling pathway. Notum encodes a member of the α/β-hydrolase superfamily which modifies the heparin sulfate proteoglycans (HSPG) Dally and Dally-like (Dlp). These two molecules are responsible for mediating ligand-receptor interaction by binding to Wg ligand and stabilizing it at the surface. Meanwhile, Notum expression is under the control of Wg pathway and high levels of Wg signaling induces Notum expression. This suggests that Wg acts through Notum to shape gradient itself (Antonio et. al., 2002).

To see if loss of Hippo signaling affects Wg signaling, Notum staining is used as readout in loss of mats/wts/hpo genetic backgrounds. We observed that Notum levels is elevated in loss of mats (Figure 6-3A-F,) loss of wts clones (Figure6-3 G-I) and loss of hpo clones (Figure 6-3J-L) marked by GFP. These data suggest that loss of Hippo signaling results in induction of Wg signaling.
Figure 6-3: Notum Expression is upregulated in loss of *mat*, loss of *wts* or loss of *hpo* clones. First instar larvae were subjected to heat shock to generate loss of *mats/wts/hpo* clones marked by GFP using MARCM. Tissues were then stained with anti-Notum antibody. (A) to (F) Loss of mats clones. (G) to (I) Loss of wts clones (J) to (L) Loss of hpo clones(b)(e)(H)(K) Notum staining. (C)(F)(I)(L) Merged picture from (A)(B),(D)(E),(G)(H), (J)(K). The clones have higher Notum were indicated by arrows.
6.3.2 Armadillo is Increased in Loss of wts Clones

Previous studies have shown that cytoplasmic pool of transcriptional co-activator β-Catenin/Armadillo (Arm) is negatively correlated with Fat signaling. Elevated level of β-Catenin/Arm has been observed in loss of ft clones (Jaiswal et al., 2005). To see if loss of wts has similar effects on β-Catenin/Arm, we examined Arm level in loss of wts clones. As shown in Figure 6-4, Arm level is upregulated (Figure 6-4B) in loss of wts clones (Figure 6-4A). Therefore, consistent with the results from loss of ft previously, Arm is increased when Hippo signaling is inactive.

![Figure 6-4](image)

Figure 6-4: Armadillo level is increased in loss of wts clones. Loss of wts clones are generated by MARCM and marked by GFP. (A) GFP (B) Armadillo stainings (C) Merged pictures from (A) and (B). The clones have higher Armadillo were indicated by arrows.

6.3.3 DE-Cadherin and Armadillo are Elevated in Loss of mats Clones

In addition to Notum, DE-Cadherin/Shg is also responsive to Wg signaling. In epithelial cells, DE-Cadherin mediates homophilic interaction by assembling adherens junction complex in a sub-apical ring of epithelial cells. DE-Cadherin is elevated along the dorsal-ventral boundary in wing discs due to higher level of Wg signaling. Overexpression of Armadillo also increases transcription of DE-Cadherin autonomously and non-autonomously in clonal analysis. When Wg
signaling is activated, cytoplasmic β-Catenin/Arm is stabilized and associated with cytoplasmic domain of DE-Cadherin to form a complex with DE-Cadherin.

Relative to the upregulation of DE-Cadherin by increased Wg signal, signaling from Fat suppresses DE-Cadherin and cytoplasmic β-Catenin/Arm downstream of Wg ligand. To verify if Hpo kinase cassette components also regulate Wg signaling through increasing DE-Cadherin and β-Catenin/Arm, we generated loss of *mats* clones in wing discs. As shown in Figure 6-5B and 6-5C, DE-Cadherin and β-Catenin/Arm are enhanced in loss of *mats* clones (Figure 6-5A). More magnified views of DE-Cadherin and β-Catenin/Arm level in loss of *mats* clones are shown in Figure 6-5F-J. These data indicate that loss of Hippo signaling results in elevation of DE-Cadherin which is consistent with previous findings (Jaiswal et al., 2005).

### 6.3.4 Transcription of DE-Cadherin is Induced in loss of *mats* Clones

Furthermore, we went on to analyze *DE-Cadherin* transcription level in loss of *mats* genetic background. An enhancer trap allele, *DE-Cadherin-lacZ*, was combined with flies carrying loss of *mats* allele. The lacZ expression level was detected by anti-β-gal staining which represented DE-Cadherin promoter activity. In Figure 6-6C, we observed higher signals of β-gal staining which correlate well with DE-Cadherin stainings (Figure 6-6B) and loss of *mats* clones (Figure 6-6A). This suggests that loss of Hippo signaling induces DE-Cadherin through increasing transcription.
Figure 6-5: Elevated level of DE-Cadherin and Armadillo in loss of mats clones. (B) DE-Cadherin and (C) Armadillo stainings are used to detect DE-Cadherin and Armadillo level in Drosophila wing discs. (A) Loss of mats clones marked in green are generated by MARCM. (D) and (E) are the merged pictures from (A)(B) and (A)(C). (F) to (J) are the magnified pictures from (A) to (E). The clones have higher DE-Cadherin and Armadillo are indicated by arrows.
6.3.5 Armadillo, DE-Cadherin and Notum are Enhanced in Yki Expressing Clones

Next, we tested if Yki is sufficient to induce Wg signaling downstream targets. The transgene Yki-V5 is expressed in the clones in wing discs. Consistent with the results from loss of tumor suppressors in Hippo signaling, we observed induced DE-Cadherin and Notum in the Yki expressing clones (Figure 6-7). This means Yki is able to induce DE-Cadherin and Notum expression. As for the level of Armadillo, we needed to use anti-Notum antibody to mark Yki-V5 clones. This was due to the limitation that anti-V5 and anti-Armadillo are both anti-mouse antibody so double labeling of Yki-V5 and Armadillo is not possible. Figure 6-8 shows Armadillo is elevated in the regions with higher Notum due to Yki-V5 expression. All these evidence show that Yki is capable of inducing Armadillo and downstream targets of Wg signaling, DE-Cadherin and Notum.
Figure 6-7: DE-Cadherin and Notum expression are enhanced in clones expressing Yki. *hs-FLP, act>CD2>Gal4/+; UAS-yki-v5/+* were subjected to heat shock to induce clones marked by V5 staining in larval wing discs. (A) Yki-V5 expressing clones (B) DE-Cadherin and (C) Notum stainings. (D) and (E) are the merged pictures from (A)(B) and (A)(C).

Figure 6-8: Elevation of Armadillo in Yki-V5 expressing clones. (A)(D) Notum staining is used to mark Yki-V5 clones since elevated Notum is observed in Yki-V5 clones in wing discs. (B)(E) Armadillo stainings (C) and (F) Merged pictures
6.4 Armadillo and Notum level are Slightly Suppressed in Regions Expressing Constitutively Active Mats

To see if Mats regulates Wg signaling pathway in addition to loss of mats effects, we expressed constitutively activate Mats (Myr-Mats-GFP) from chapter 3 in the posterior region in wing discs using engrailed-Gal4. As a result, Armadillo and Notum staining are downregulated slightly in Myr-Mats-GFP expressing area (Figure 6-9A to E) compared with those in the regions expressing Mats-GFP (Figure 6-9 F to J). These data suggest that activated Hippo signaling decreases Armadillo and the downstream target gene of Wg signaling, Notum.
Figure 6-9: Slight reduction of Armadillo and Notum in Myr-Mats-GFP expressing regions. (A) Myr-Mats-GFP or (F) Mats-GFP is expressed by *engrailed-Gal4*. (B) Armadillo and (C) Notum stainings are slightly reduced where Myr-Mats-GFP is expressed. (D) and (E) are the merged pictures from (A)(B) and (A)(C). No apparent change of Armadillo and Notum level in Mats-GFP expressing regions. (G) Armadillo and (H) Notum staining is not reduced where Mats-GFP is expressed. (I) and (J) are the merged pictures from (F)(G) and (F)(H).
6.5 Epistatic Analysis of Hpo and Wingless Signaling Components

To demonstrate genetic interaction between Hippo and Wg signaling, negative regulators of Wg signaling are combined with loss of mats or hpo allele. Heat shock was used to induce MARCM clones expressing UAS-transgenes in mutant genetic background.

Figure 6-10: MARCM analysis is used to express components in Wg pathway in loss of mats or loss of hpo genetic backgrounds. Dominant-negative TCF transcription factor (UAS-dTCFΔN), constitutive active GSK3/Sgg (UAS-SggS9A), UAS-Axn-GFP is expressed in clones in the absence of mats. Dominant-negative frizzled and frizzled 2 (UAS-fzΔN, fz2ΔN) were expressed in the absence of hpo. Mitotic recombination was induced by heat shock and the clones are marked in green due to the expression of GFP.
6.5.1 Notum Level is Elevated in Loss of *mats* Clones Expressing Axin, but not Constitutively Active Sgg or Dominant-Negative TCF

Notum is used as readout to monitor the activation level of Wg signaling. Notum stainings in loss of *mats* clones were shown as a positive control (Figure 6-11A to C). Compared with the induction level of Notum in Figure 6-11B, Notum were relatively lower or no apparent upregulation in loss of *mats* clones expressing dominant-negative TCF (Figure 6-11E) or constitutively active Sgg (Figure 6-11H), but not loss of *mats* clones expressing Axin (Figure 6-11K). These current data suggest that loss of *mats* possibly affects Wg signaling upstream of TCF transcription factor and Sgg kinase, but downstream of Axin.

6.5.2 Armadillo Level is Elevated in Loss of *mats* Clones Expressing Axin, but not Constitutively Active Sgg or Dominant-Negative TCF

The similar induction patterns were observed from Arm staining. Arm were relatively lower or no apparent enhancement in loss of *mats* clones expressing dominant negative TCF (Figure 6-12E) or constitutively active Sgg (Figure 6-12H), but not loss of *mats* clones expressing Axin (Figure 6-12K) compared with the induction level of Notum in Figure 6-12B. It is still possible that loss of *mats* affects Arm level through Sgg and downstream of Axin. However, Arm is not a downstream target of Wg signaling so that dominant-negative TCF might downregulate Arm through suppressing the expression of DFz2 receptor upstream. This may lead to the reduction of Wg signaling from the upstream and decrease Arm availability (Candigan et al., 1998).
Figure 6-11: Notum stainings are suppressed in loss of mats clones expressing dominant-negative TCF or constitutively active Sgg, but not Axin. (A) to (C) Loss of mats clones. (D) to (F) Loss of mats clones expressing dominant-negative TCF. (G) to (I) Loss of mats clones expressing constitutively active Sgg. (J) to (L) loss of mats clones expressing Axin. (A)(D)(G)(J) Clones are marked in green due to GFP expression. (B)(E)(H)(K) Notum stainings from each genotype. (C)(F)(I)(L) Merged pictures from (A)(B), (C)(D), (G)(H) and (J)(K).
Figure 6-12: Armadillo stainings are suppressed in loss of *mats* clones expressing dominant-negative TCF or constitutively active Sgg, but not Axin. (A) to (C) Loss of *mats* clones. (D) to (F) Loss of *mats* clones expressing dominant negative TCF. (G) to (I) Loss of *mats* clones expressing constitutively active Sgg. (J) to (L) loss of *mats* clones expressing Axin. (A)(D)(G)(J) Clones are marked in green due to GFP expression. (B)(E)(H)(K) Armadillo stainings from each genotype. (C)(F)(I)(L) Merged pictures from (A)(B), (C)(D), (G)(H) and (J)(K).
6.6 Possible Induction of Notum Independent of Wg Signaling in Loss of hpo Clones

Previously, loss of Fat or Hippo signaling components have been shown to induce Wg expression in the regions which later will develop into proximal wing during larval stages. Here, we wanted to see if the elevation of Notum downstream of Wg signaling is dependent on the induction of Wg ligands caused by loss of Fat or Hpo signaling.

The Wg staining patterns in wild type wing disc are shown in Figure 6-13. The Wg ligands are mainly expressed at the notum and pouch area. The white arrows indicate the Wg expressing regions which will develop into proximal wing while yellow arrowheads indicate those will develop into distal wing. Consistent with the previous findings, loss of mats induces Wg expression in the regions developed into proximal wing (Figure 6-14G and Figure 6-15G, indicated by yellow arrowheads). It is noteworthy that Notum and its transcriptional level is upregulated both in the regions close to (Figure 6-14F and Figure 6-15F, indicated by yellow arrowheads) or distant from (Figure 6-14F and Figure 6-15F, indicated by white arrows) where Wg expresses. This implies that as a down-stream target of Wg signaling, Notum expression and transcription might be regulated through Wg dependent and Wg independent pathway.

Figure 6-13: Wg staining in wild type larval wings. (A) Wg staining in the regions which will develop into proximal wings (white arrows) and distal wings (yellow arrowheads). (B) Wing disc (C) Merged picture from (A) and (B).
Figure 6-14: Notum is still elevated in loss of mats clones even not in the proximity of Wg expression. The arrows or arrowheads indicate the elevated level of stainings. (A) Loss of mats clones marked by GFP (B) Notum stainings (C) Wg stainings (D) Merged pictures from (A) to (C). Magnified pictures from wing pouch areas from (A) to (D) were shown in (E) to (H).

Figure 6-15: Transcription of Notum is also enhanced in loss of mats clones even not in the proximity of Wg expression. The arrows and arrowheads indicate the elevated level of stainings. (A) Loss of mats clones marked by GFP (B) β-gal
staining of Notum-lacZ expression (C) Wg staining (D) Merged picture from (A) to (C). Magnified pictures from wing pouch areas from (A) to (D) were shown in (E) to (H).

Since Wg ligands are the morphogens which are secreted from cells, it can not be ruled out that Notum inductions away from where Wg expresses are not affected by secreted Wg ligands. To examine if Wg ligands are required for Notum induction in clones without Hippo signaling, we expressed two dominant-negative forms of two Wg receptors, Frizzled and Frizzled 2, in the absence of hpo. It has been shown that Frizzled and Frizzled 2 are the major receptors mediating Wg signaling pathway (Sergey et al., 2009).

As shown in Figure 6-16, Notum is still increased in loss of hpo clones expressing dominant-negative forms of Frizzled and Frizzled 2 (6-16B and F, indicated by white arrows). In addition, loss of hpo induces the elevation of Wg expression in the proximal wings (Figure 6-16C yellow arrowheads). These suggest that Notum elevations resulting from loss of hpo signaling is not dependent on Wg ligands which are also induced due to loss of hpo at the same time.

6.7 Summary

Previous studies have shown that ft receptors acts as a Wg signaling suppressor which reduces DE-Cadherin expression and cytoplasmic β-Catenin/Arm. Loss of ft, in turn, induces DE-Cadherin and β-Catenin/Arm level indicating that ft negatively regulates Wg pathway. The genetic intersections of Fat and Wg pathways are downstream of Wg ligand, upstream of or parallel to its receptors, Frizzled and Frizzled 2 (Jaiswal et al., 2005). However, loss of ft only induces Wg signaling to a modest threshold. It is not clear if there are other molecules downstream of Ft could affect Wg signaling.

In this chapter, we show that loss of Hippo signaling could also induce Wg signaling. Downstream targets, Notum and DE-Cadherin, are elevated in the absence of Hippo signaling. These upregulations are accompanied by increased level of Arm. Our results also show that transcription of DE-Cadherin and Notum is upregulated in the clones lacking Hippo signaling. Moreover, constitutively active Mats inhibits while overexpression of Yki induces Arm and Notum level in
wing discs indicating that Hippo pathway may crosstalk with Wg signaling pathway.

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**To further demonstrate the possible crosstalk between Hippo and Wg signaling pathway, MARCM analysis was used to express dominant-negative TCF or constitutively active Sgg in loss of *mats* genetic background. Our data show that dominant-negative form of Wg pathway transcription factor, TCF, is able to suppress Notum induction resulting from loss of *mats*. A constitutively active negative regulator which destabilizes β-Catenin/Arm, Sgg$^{S9A}$, also blocks the elevation of Notum caused by loss of *mats*. These two evidences show that Hippo pathway affects Wg signaling pathway upstream of GSK and TCF. In addition, overexpression of *axin* is not sufficient to block the elevation of Notum in loss of *mats* clones suggesting that there is a possible crosstalk between Hippo and Wg pathway downstream of Axin. This data needs to be confirmed by further statistical analysis. Furthermore, the detailed crosstalks between the Hippo and Wg pathway should be examined by expressing other negative regulators in the Wg pathway under loss of *mats* genetic backgrounds (Figure 6-17).**

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**Figure 6-16**: Notum is still upregulated in loss of *hpo* clones expressing dominant-negative *frizzled* and *frizzled 2*. The arrows and arrowheads indicate the elevated level of stainings. (A) Clones are marked by GFP (B) Notum staining (C) Wg staining (D) Merged picture from (A) to (C). Magnified pictures from wing pouch areas from (A) to (D) were shown in (E) to (H).
Moreover, we co-expressed dominant-negative forms of receptors, Frizzled and Frizzled 2, to show upregulation of a Wg downstream target, Notum, is not due to the induction of Wg ligands. Instead, there are crosstalks between Hippo and Wg signaling so that loss of hpo signaling still induces Notum in the absence of Wg signaling from upstream receptors. This evidence suggests that the effects of loss of Hippo signaling on downstream targets of Wg signaling can be Wg ligand independent.

Figure 6-17: Possible crosstalk between Hippo and Wg signaling pathway.
Chapter 7
Conclusions and Perspectives

7.1 Conclusions

Within this thesis, I first showed that the localization of endogenous Mats is mainly in the cytoplasmic region and at the plasma membrane in developing tissues, for example: larval wing discs and pupal eye discs. The localization of Mats can be regulated by Hpo kinase. I found Mats is mainly in the cytosol in the absence of hpo while membrane Mats-GFP is increased when coexpressing with Hpo. This gave us the first hint that the plasma membrane may be a crucial place in the activation of Hippo signaling.

In mammalian cells, it has been demonstrated that human Mps1-One binder (MOB1), a human ortholog of Mats, is able to increase human LATS 1 kinase (LATS1) activity at the plasma membrane (Hergovich et. al., 2006). In Drosophila, I generated myristoylated Mats tagged with GFP (Myr-Mats-GFP) to allow Mats association at the plasma membrane and expressed it in developing tissues using Gal4-UAS system. The results indicated that membrane-targeting Mats is sufficient to reduce organ size through decreasing cell numbers. This effect was mediated by suppressing cell proliferation and inducing cell apoptosis. In addition, the level of DIAP-1 was decreased while that of activated caspase 3 and Drice was increased. These evidences further confirmed the observations that reduction of organ size results from loss in cell numbers.

The targets that mediate growth inhibitory effects of membrane-targeting Mats were also examined. Previously, Mats has been shown to directly interact with Wts kinase and activates Wts kinase activity. In the chapter 5 of this thesis, we showed that membrane-targeting Mats is more potent in activating Wts kinase than wild type Mats and non membrane-targeting Mats. This was due to the enhancement of Wts availability, kinase activity at the plasma membrane and decrease of Yki in the nucleus. In addition, Wts is required for constitutively active Mats induced growth inhibition. All the data suggested that localization of Mats is more crucial than the level of Mats protein in activating Hippo pathway during Drosophila development.
Finally, we examined the crosstalk between Hippo and Wg signaling pathway by examining the response of Wg downstream targets in the absence of Hippo signaling. Previous studies have shown that loss of ft induces one of the targets, DE-Cadherin. In this thesis, we showed another downstream target gene, Notum, was upregulated in the absence of Hippo signaling. We also demonstrated that these inducing effects by loss of Hippo signaling could be suppressed by dominant-negative form of TCF transcription factor and constitutively active Sgg, but not Axin overexpression. These indicated that Hippo signaling possibly crosstalks with Wg pathway upstream of Sgg/GSK but downstream of Axin. These results need to be further confirmed by more extensive analysis. In addition, we expressed dominant-negative forms of Wg receptors, Frizzle and Frizzled 2, in the absence of hpo to show upregulation of Wg downstream targets caused by loss of Hippo signaling could be independent of Wg ligands.

7.2 Perspectives

7.2.1 Regulation of Mats Localization by Other Hippo Signaling Components

It has been shown that plasma membrane is crucial in the activation of Hippo signaling, and loss of hpo results in loss of Mats at the plasma membrane. To further examine how the localization of Mats is regulated in vivo, I can cross exel FRT40A/CyO-GFP; hs-FLP Sb/TM6B male flies with y,w, mer4; P[w+] Ubi-mer+ FRT40A/CyO-GFP female flies to generate mer ex double mutant clones and detect Mats localization. Mer and Ex have been shown to regulate Hpo kinase redundantly since loss of either mer or ex does not give dramatic overgrowth phenotype. In addition, overexpression of ex or mer may also affect Mats localization. Even more upstream of ex and mer, we can also cross y,w; fat8 FRT40A/CyO-GFP male flies with y,w; hs-FLP122; Ubi-GFP FRT40A female flies to generate loss of ft clones or express dominant-negative form of Ft receptor to detect the change of Mats localization.

In addition to Mats localization, Hpo localization maybe also a possible way to regulate activation of Hippo pathway. Endogenous Hpo can be detected by anti-Hpo antibody to show Hpo localization in wild type tissues. Similar strategies from exploring how Mats is localized from upstream can also be applied to Hpo.
The localization of Hpo is worth noting since how Ex and Mer regulate Hpo is not fully understood. Another possibility is that there are other molecules involved upstream of Hpo.

### 7.2.2 The Detailed Mechanisms of Crosstalk between Hippo and Wg Signaling

In chapter 6, we demonstrated that Yki expression in Hippo pathway was able to induce a downstream target of Wg signaling, Notum. To see if Yki is required for mediating effects of Hippo signaling on Wg pathway, we could see if loss of yki suppresses Notum elevation results from loss of hpo. There is a chance that clones with loss of hpo and yki will be too small to be observed; therefore, we can express UAS-ykiRNAi in loss of wts or mats clones as alternatives. If Yki is essential for activating Wg pathway, we would expect downregulation of Notum in loss of mats clones expressing yki-RNAi compared with loss of mats clones.

In addition, we showed that Hippo pathway can possibly crosstalk with Wg signaling upstream of Sgg but must be downstream of Axin; however, more clones need to be analyzed. To further dissect more detailed mechanisms, more negative regulators in Wg pathway can be expressed in the clones lacking Hippo signaling. Two candidates are Apc1 and Apc2, components of “Arm/β-catenin destruction complex”, act redundantly in maintaining turnover of Arm/β-catenin (Benchabane et al., 2008). We could express Apc1/Apc2 in loss of wts/mats/hpo clones and see if downstream targets of Wg signaling are affected.

As for the Wg pathway downstream targets, more molecules can be tested in addition to Notum. Previously, frizzled 3 (fz3) has been shown to be upregulated by Wg signaling. fz3 encodes a Wg receptor whose signal transduction is much less efficient than Fz2. Fz3 act as an attenuator of Wg signaling (Sato et al., 1999). The other target, naked cuticle (nkd), has also been shown to be transcriptionally increased by Wg signaling. It encodes an inducible antagonist for Wg (Zeng et al., 2000). Both Fz3 and Nkd express in embryos and imaginal discs. We can combine fz3-lacZ or nkd-lacZ in loss of wts/mats/hpo clones expressing dominant-negative Fz and Fz2 and then examine the level of lacZ expression. These will further demonstrate if Hippo signaling affects the transcription of fz3 and nkd through Wg pathway independent of Wg ligands.
References


disrupts PCP signaling and oriented cell division and leads to cystic kidney disease. Nat Genet 40, 1010-1015.


Appendix

The *Drosophila* Lines used in the Thesis

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Membrane-targeting Mats Constructs

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<td>medium line</td>
</tr>
<tr>
<td>51</td>
<td>w; UAS myr dmats GFP(^{C45})(^{(II)})/ SM1; Sb(^{-})/TM6</td>
<td>medium line</td>
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<tr>
<td>52</td>
<td>w; UAS myr dmats GFP(^{C14})(^{(II)})/ SM1; Sb(^{-})/TM6 (no (^{-}))</td>
<td>weak line</td>
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<tr>
<td>53</td>
<td>w; SM1/Adv; TM6/ UAS(^{G2A}) myr dmats GFP(^{M38})</td>
<td>non-membrane targeting</td>
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<tr>
<td>54</td>
<td>w; UAS(^{G2A}) myr dmats GFP(^{M2}); Sb(^{-})/TM6</td>
<td>non-membrane targeting</td>
</tr>
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</table>
| 55 | w; UAS\(^{G2A}\) myr dmats GFP\(^{M21}\)/ SM1; Sb\(^{-}\)/TM6               | non-
| 56 w; UAS$^{G2A}$ myr dmats GFP$^{M46}$/SM1; Sb$^{-}$/TM6 |
|---|---|
| 57 w; UAS myc wts$^{6R}$; UAS myr dmats$^{C6}$/SM6TM6B |
| 58 w; GMR-Gal4, UAS myr dmats GFP$^{C45}$; +/+ |
| 59 w; GMR-Gal4, UAS myr dmats GFP$^{C45}$/SM1; Sb$^{-}$/TM6 |
| 60 w; GMR-Gal4, UAS myr dmats GFP$^{C45}$; FRT82B sav$^{5}$/SM6TM6B |
| 61 w; GMR-Gal4, UAS myr dmats GFP$^{C45}$; FRT82B wts$^{X1}$/ST |
| 62 w; GMR-Gal4, UAS myr dmats GFP$^{A15}$/SM1 |
| 63 w; GMR-Gal4, UAS myr dmats GFP$^{A15}$; FRT82B wts$^{X1}$/ST |
| 64 GMR-Gal4, UAS myr mats GFP$^{A15}$; FRT82B90E/ST |
| 65 w; GMR-Gal4, UAS myr mats GFP$^{A15}$; FRT82B Sav$^{3}$/ST |
| 66 w; FRT 82B wts$^{X1}$/TM6 |
| 67 w; FRT82B sav$^{3}$/ST |
| 68 w; UAS myc wts$^{6R}$; Sb$^{-}$/ST |

**Membrane-targeting Mats+ lacZ reporter**

| 69 w; UAS myr dmats GFP$^{A15}$; CycE-lacZ/ST |
| 70 w; UAS$^{G2A}$ myr dmats GFP$^{M46}$; CycE-lacZ/ST |
| 71 w; UAS myr dmats GFP$^{A15}$; th$^{5c8}$/ST |
| 72 w; UAS myr dmats GFP$^{C23}$; th$^{5c8}$/ST (DIAP-1) |
| 73 w; ex-lacZ;UAS myr dmats GFP$^{B15}$/ST |
| 74 w; ex-lacZ;UAS$^{G2A}$ myr dmats GFP$^{M38}$/ST |
| 75 w; fj-lacZ; UAS myr dmats GFP$^{B15}$/ST |
| 76 w; fj-lacZ; UAS$^{G2A}$ myr dmats GFP$^{M38}$/ST |

**Myc-Wts mutant localization**

| 77 w; UAS myc wts$^{R702E(7.2)}$, Sb$^{-}$/TM6 |
| 78 w; UAS myc wts$^{C1(K743A)}$ (I) |
| 79 w; SM1/Adv; UAS myc wts$^{R702E(10.1)}$ |
| 80 w; SM1/Adv; UAS myc wts$^{(K743A)C1(III)}$/TM6 |
| 81 w; SM1/Adv; UAS myc wts$^{(K743A)C10}$/TM6 |
| 82 w; UAS myr dmats GFP$^{A15}$; UAS myc wts$^{(K743A)C1}$/ST |

Xiaomu Wei
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<tr>
<td>83 w; UAS myr dmats GFP&lt;sup&gt;A15&lt;/sup&gt;; UAS myc wts&lt;sup&gt;(K743A) C10&lt;/sup&gt;/ST</td>
<td></td>
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<tr>
<td>84 w; UAS&lt;sup&gt;(G2A)&lt;/sup&gt; myr dmats GFP&lt;sup&gt;M46&lt;/sup&gt;; UAS myc wts&lt;sup&gt;(K743A) C10&lt;/sup&gt;/ST</td>
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<td>85 w; UAS&lt;sup&gt;G2A&lt;/sup&gt; myr dmats GFP&lt;sup&gt;M46&lt;/sup&gt;; UAS myc wts&lt;sup&gt;(K743A) C1&lt;/sup&gt;/ST</td>
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<tr>
<td>86 w; UAS mats GFP&lt;sup&gt;16&lt;/sup&gt;; UAS myc wts&lt;sup&gt;C10(K743A)&lt;/sup&gt;/ST</td>
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<td>87 w; UAS myr dmats GFP&lt;sup&gt;A15&lt;/sup&gt;; UAS myc wts&lt;sup&gt;6L&lt;/sup&gt;/ST</td>
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<td>88 w; UAS&lt;sup&gt;(G2A)&lt;/sup&gt; myr dmats GFP&lt;sup&gt;M46&lt;/sup&gt;; UAS myc wts&lt;sup&gt;6L&lt;/sup&gt;/ST</td>
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<td>89 w; UAS mats GFP&lt;sup&gt;21-1&lt;/sup&gt;; UAS myc wts&lt;sup&gt;6L&lt;/sup&gt;/ST</td>
<td>Takeshi Shmizu</td>
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<td>90 w; UAS myc wts&lt;sup&gt;6R&lt;/sup&gt;; UAS myr dmats GFP&lt;sup&gt;B15&lt;/sup&gt;/ST</td>
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<td>91 w; UAS myc wts&lt;sup&gt;6R&lt;/sup&gt;; UAS&lt;sup&gt;(G2A)&lt;/sup&gt; myr dmats GFP&lt;sup&gt;M38&lt;/sup&gt;/ST</td>
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<td>92 w; UAS myr dmats GFP&lt;sup&gt;A15&lt;/sup&gt;; UAS myc wts&lt;sup&gt;R702E(10.1)&lt;/sup&gt;/ST</td>
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<td>93 w; UAS myc wts&lt;sup&gt;R702E(7.2)&lt;/sup&gt;; UAS myr dmats GFP&lt;sup&gt;B15&lt;/sup&gt;/ST</td>
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<td>95 w; UAS myc wts&lt;sup&gt;R702E(7.2)&lt;/sup&gt;; UAS&lt;sup&gt;(G2A)&lt;/sup&gt; myr dmats GFP&lt;sup&gt;M38&lt;/sup&gt;/ST</td>
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<td>96 w; UAS myr&lt;sup&gt;(G2A)&lt;/sup&gt; dmats GFP&lt;sup&gt;M46&lt;/sup&gt;; UAS myc wts&lt;sup&gt;R702E&lt;/sup&gt;/ST</td>
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**Myc-Wts Localization**

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<td>97 W; FRT42D P&lt;sup&gt;[w+]&lt;/sup&gt;; UAS myc wts /ST</td>
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<td>98 W; UAS myc wts&lt;sup&gt;6R&lt;/sup&gt;; FRT and B sav&lt;sup&gt;/&lt;/sup&gt;/ST</td>
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<td>99 W; FRT42D hpo&lt;sup&gt;MGH2&lt;/sup&gt;; UAS myc wts&lt;sup&gt;6L&lt;/sup&gt;/ST</td>
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Epistatic analysis of **myr-mats-gfp** and **hpo**

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<td>100 w; FRT42D P&lt;sup&gt;[w+]&lt;/sup&gt;; UAS myr dmats GFP&lt;sup&gt;B15&lt;/sup&gt;/ST</td>
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<td>101 w; FRT42D hpo&lt;sup&gt;BF33&lt;/sup&gt;/ST</td>
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<td>102 w; FRT42D hpo&lt;sup&gt;BF33&lt;/sup&gt;; UAS myr dmats GFP&lt;sup&gt;B15&lt;/sup&gt;/ST</td>
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<td>103 w; FRT42D hpo&lt;sup&gt;BF33&lt;/sup&gt;; UAS&lt;sup&gt;G2A&lt;/sup&gt; myr dmats GFP&lt;sup&gt;M38&lt;/sup&gt;/ST</td>
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<tr>
<td>104 w; FRT42D P&lt;sup&gt;[w+]&lt;/sup&gt;; UASmats GFP&lt;sup&gt;15-2&lt;/sup&gt;/ST</td>
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<tr>
<td>105 w; FRT42D hpo&lt;sup&gt;BF33&lt;/sup&gt;; UASmats GFP&lt;sup&gt;15-2&lt;/sup&gt;/ST</td>
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<tr>
<td>106 w; FRT42D yki&lt;sup&gt;B5&lt;/sup&gt;/ST</td>
<td>From D.J. Pan</td>
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<tr>
<td>107 w; FRT42D hpo yki/ST</td>
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<tr>
<td>108 w; FRT42D yki, hpo/CyO</td>
<td>From D. Montell</td>
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**FLAG-Hpo lines**

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<td>109 y;w; UAS FLAG-hpo&lt;sup&gt;F1&lt;/sup&gt;/SM1; Sb&lt;sup&gt;/&lt;/sup&gt;/TM6</td>
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</tr>
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<td>From Jin Jiang</td>
<td>From Jin Jiang</td>
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<tr>
<td>y; w; Sp/CyO; UAS FLAG-dmstF6/TM2</td>
<td>y; w; UAS FLAG dmstN12/CyO; MKRS/TM2</td>
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<td>SM1/Adv; UAS FLAG-dmstN7/TM6</td>
<td>SM1/Adv; UAS FLAG-dmstNKR/TM6</td>
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<td>UAS mats GFP21-3; UAS FLAG-dmstF6/ST</td>
<td>UAS mats GFP21-3; UAS FLAG-dmstNK&gt;R/ST</td>
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<td>UAS mats GFP21-3; UAS FLAG-dmstN7/ST</td>
<td>UAS mats GFP21-3; UAS FLAG-dmstNK&gt;R/ST</td>
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<td>UAS mats GFP21-3; UAS  FLAG-dmstF6/ST</td>
<td>UAS mats GFP21-3; UAS FLAG-dmstNK&gt;R/ST</td>
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<td>UAS myrdmats GFPAT5; UAS FLAG-dmstF6/ST</td>
<td>UAS mats G2GFP46; UAS  FLAG-dmstF6/ST</td>
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<td>UAS myc wts6R; UAS hpo f1, UAS myc wts6R; Sb-/ST</td>
<td>UAS myc wts6R; UAS hpo f6/ST</td>
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<td>UAS myc wts6R; UAS hpo f6/ST</td>
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<td>UAS myc wts6R; UAS hpo f6/ST</td>
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<td>UAS myc wts6R; UAS hpo f6/ST</td>
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Crosstalk with Hpo and Wg signaling

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<tr>
<td>w; UAS-dTCF(N(4)(ll)(4784))</td>
<td>w; UAS-dTCF(N(5)(ll)(4785))</td>
<td>w; UAS-dTCF(24)(cyo)(4837)</td>
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<tr>
<td>w; UAS-dTCF((4)(III)(4838))</td>
<td>w; UAS-arm S2((ll)(4783))</td>
<td>w; UAS-arm S 10_y.w((l)(4782))</td>
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<tr>
<td>w; UAS-SggB((MB5))(III)((GSK)(5361))</td>
<td>w; UAS-SggB((MB14))/(Cyo)((GSK)(5435))</td>
<td>w; Kr((F1-1)/CyO; UAS-Axn GFP/TM3, Sb-((7225))</td>
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<tr>
<td>w; UAS-AxnGFP/ CyO; k; (I)/ TMB ; Sb- ((7224))</td>
<td>UAS-DFz(\text{DN}/\text{SM6.TM6B})</td>
<td>UAS-DFz(\text{DN}/\text{SM6.TM6B})</td>
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<td>#5361</td>
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<td>Banerjee</td>
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UAS-DFz\(\text{DN}/\text{SM6.TM6B}\) Banerjee
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<td>143</td>
<td>UAS-DFz&lt;sup&gt;DN&lt;/sup&gt;, UAS-Dfz2&lt;sup&gt;gpi(DN)&lt;/sup&gt;/TM6,Tb</td>
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<td>144</td>
<td>w; UASEFGP; FRT82B&lt;sup&gt;x1&lt;/sup&gt;/ST</td>
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<td>145</td>
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<td>w; UAS GFP; FRT82B mats&lt;sup&gt;235&lt;/sup&gt;/ST</td>
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<td>w; FRT42D P[W&lt;sup&gt;+&lt;/sup&gt;]; nkd-lacZ/ST</td>
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<td>W; FRT 42D P[w&lt;sup&gt;+&lt;/sup&gt;]; UAS- fzΔN, UAS-fz2ΔN/ST</td>
</tr>
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</table>
VITA

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Pennsylvania State University 2003-2009
Master in molecular biology and biochemistry 1998-2000
Institute of Molecular Biology and Biochemistry,
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Bachelor in chemical engineering 1994-1998
Department of Chemical Engineering, National Taiwan University.

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J. Ben and Helen D. Hill Memorial Fund Award Pennsylvania State University 2005, 2008
Huck Institutes of the Life Sciences Fellowship 2003-2005

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48th Annual Meetings of the American Society of Cell Biology San Francisco, CA. (Poster presentation) 2008

Publications:
Ho L.L., Wei X., Shimizu T., Lai Z.C., Mob as Tumor Suppressor is activated at the cell membrane to control tissue growth and organ size in Drosophila. (accepted) Dev Biol 2009.