

# Spatial Organization of Hippo Signaling at the Plasma Membrane Mediated by the Tumor Suppressor Merlin/NF2

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## SUMMARY

Although Merlin/NF2 was discovered two decades ago as a tumor suppressor underlying Neurofibromatosis type II, its precise molecular mechanism remains poorly understood. Recent studies in *Drosophila* revealed a potential link between Merlin and the Hippo pathway by placing Merlin genetically upstream of the kinase Hpo/Mst. In contrast to the commonly depicted linear model of Merlin functioning through Hpo/Mst, here we show that in both *Drosophila* and mammals, Merlin promotes downstream Hippo signaling without activating the intrinsic kinase activity of Hpo/Mst. Instead, Merlin directly binds and recruits the effector kinase Wts/Lats to the plasma membrane. Membrane recruitment, in turn, promotes Wts phosphorylation by the Hpo-Sav kinase complex. We further show that disruption of the actin cytoskeleton promotes Merlin-Wts interactions, which implicates Merlin in actin-mediated regulation of Hippo signaling. Our findings elucidate an important molecular function of Merlin and highlight the plasma membrane as a critical subcellular compartment for Hippo signal transduction.

## INTRODUCTION

The tumor suppressor Merlin encodes a member of the ezrin-radixin-moesin (ERM) family of membrane-cytoskeleton adaptor proteins (Rouleau et al., 1993; Trofatter et al., 1993). It is inactivated not only in the familial cancer syndrome Neurofibromatosis type II (NF2) but also at varying frequency in sporadic tumors outside the nervous system, such as mesotheliomas and bladder, thyroid, and skin cancer (<http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=gene&ln=NF2>). Although it is generally believed that Merlin impacts cell signaling as a membrane-cytoskeleton scaffold, the precise mechanisms by which Merlin functions as a tumor suppressor are poorly understood and remain an active area of research (Li et al., 2012). In

this study, we use a combination of *Drosophila* and mammalian models to investigate the molecular function of Merlin. This combinatorial approach is rooted in the remarkable conservation of Merlin function between *Drosophila* and mammals, as illustrated by the ability of the human *Merlin* gene to rescue *Drosophila mer* mutations (LaJeunesse et al., 1998). For purposes of clarity, we shall use “Merlin” as a non-species-specific reference to this tumor-suppressor protein; “*mer*/Mer” and “*Nf2*/NF2” will be used to refer to the corresponding *Drosophila* and mammalian gene/protein, respectively.

The Hippo signaling pathway regulates organ size in *Drosophila* and mammals, and its dysfunction contributes to human cancers (Halder and Johnson, 2011; Harvey and Tapon, 2007; Pan, 2010). Central to the Hippo pathway is a kinase cascade whereby the Ste20-like kinase Hippo (Hpo) (Mst1/2 in mammals) activates the nuclear Dbf2-related (NDR) family kinase Warts (Wts) (Lats1/2 in mammals) by phosphorylating the latter on its hydrophobic motif; activated Wts/Lats, in turn, phosphorylates and inactivates the transcriptional coactivator Yorkie (Yki) (YAP/TAZ in mammals). Compared to the core kinase cascade leading from Hpo to Yki, signaling events upstream of the Hippo kinase cascade are less well understood. In searching for upstream components of the Hippo pathway, Hamaratoglu et al. analyzed mutant cells lacking Mer and the related FERM protein Expanded (Ex) and provided the first evidence placing *mer* genetically upstream of *hpo* (Hamaratoglu et al., 2006). A Merlin-Hippo connection is also supported by mouse genetics, wherein heterozygosity of *Yap* greatly suppresses the *Nf2*-deficient phenotypes in multiple tissues (Zhang et al., 2010). Consistent with these genetic findings, Mer overexpression promotes Yki phosphorylation and Wts hydrophobic motif phosphorylation in *Drosophila* cells (Hamaratoglu et al., 2006; Yu et al., 2010), and conversely, loss of NF2 leads to reduced YAP and Lats phosphorylation (Zhang et al., 2010). In principle, these genetic and biochemical data are compatible with at least two possibilities. First, Merlin may directly or indirectly activate Hpo/Mst, which in turn phosphorylates Wts/Lats at its hydrophobic motif. Alternatively, Merlin may directly or indirectly translocate Wts/Lats to a subcellular compartment where Wts/Lats undergoes hydrophobic motif phosphorylation by Hpo/Mst. For simplicity, these models will be referred to as linear and parallel models, respectively. Interestingly, although the existing data cannot formally distinguish between the two models, the

linear model is widely assumed in the field (Halder and Johnson, 2011; Harvey and Tapon, 2007; Pan, 2010).

Here we show that in both *Drosophila* and mammals, Merlin does not promote Hippo signaling through Hpo/Mst in a linear pathway. Instead, Merlin directly binds and helps recruit Wts/Lats to the plasma membrane, where it is activated by the Hpo-Sav kinase complex. Our studies therefore uncover a critical function of Merlin in spatial organization of Hippo signaling at the plasma membrane.

## RESULTS

### Merlin Promotes Wts/Lats Phosphorylation without Stimulating the Intrinsic Kinase Activity of Hpo/Mst in *Drosophila* and Mammalian Cells

To distinguish between the linear and parallel models discussed above, we first examined the effect of Mer on Hpo activation in *Drosophila* S2R<sup>+</sup> cells. To facilitate this analysis, we took advantage of Mer<sup>1-600</sup>, an activated form of Mer lacking 35 aa at its C terminus (LaJeunesse et al., 1998). Mer<sup>1-600</sup> was previously identified from an unbiased structure-function analysis, but the underlying molecular mechanism for its gain-of-function activity, including its relevance to Hippo signaling, was unknown (LaJeunesse et al., 1998). As reported before, overexpression of Mer<sup>1-600</sup>, but not the wild-type (WT) Mer, by the wing-specific nub-Gal4 driver resulted in a significant reduction in wing size (Figure S1A available online). In S2R<sup>+</sup> cells, Mer<sup>1-600</sup> showed stronger activities than WT Mer in inducing Wts T1077 (hydrophobic motif) phosphorylation (Figures 1A and S1B) and Yki S168 phosphorylation (Figure 1B), suggesting that the gain of function of Mer<sup>1-600</sup> may be attributed to its enhanced activity in promoting Hippo signaling. Interestingly, under these conditions, neither Mer nor Mer<sup>1-600</sup> enhanced Hpo kinase activity, as revealed by a phosphospecific antibody against Hpo's autophosphorylation site (T195) (Figure 1C). This was further confirmed by in vitro kinase assays that used immunoprecipitated Hpo against bacterially purified GST-Wts as substrate (Figure 1D). Even though Mer or Mer<sup>1-600</sup> did not enhance Hpo's kinase activity per se, Mer- or Mer<sup>1-600</sup>-mediated Wts T1077 phosphorylation required Hpo (Figure 1E), consistent with Hpo being a hydrophobic motif kinase of Wts. Conversely, RNA interference (RNAi) knockdown of Mer resulted in decreased Yki and Wts phosphorylation without a concomitant decrease in Hpo activity—to the contrary, we consistently observed a slight increase in Hpo activity upon Mer RNAi (Figure 1F) (see later for further discussion). Thus, although Mer promotes Wts phosphorylation at its Hpo-responsive hydrophobic motif, it does so without activating the intrinsic kinase activity of Hpo. These results therefore contradict a simple linear model that places Mer biochemically upstream of Hpo activation.

After demonstrating that Mer promotes Wts and Yki phosphorylation without activating the Hpo kinase activity in *Drosophila*, we examined whether a similar mechanism also operates in mammals. Indeed, overexpression of NF2 in HEK293 cells promoted Lats1/2 phosphorylation without enhancing Mst1/2 kinase activity (Figures 1G and S1C). These results were further corroborated with loss-of-function studies. We recently reported that *Nf2*-deficient livers (*Alb-Cre*;

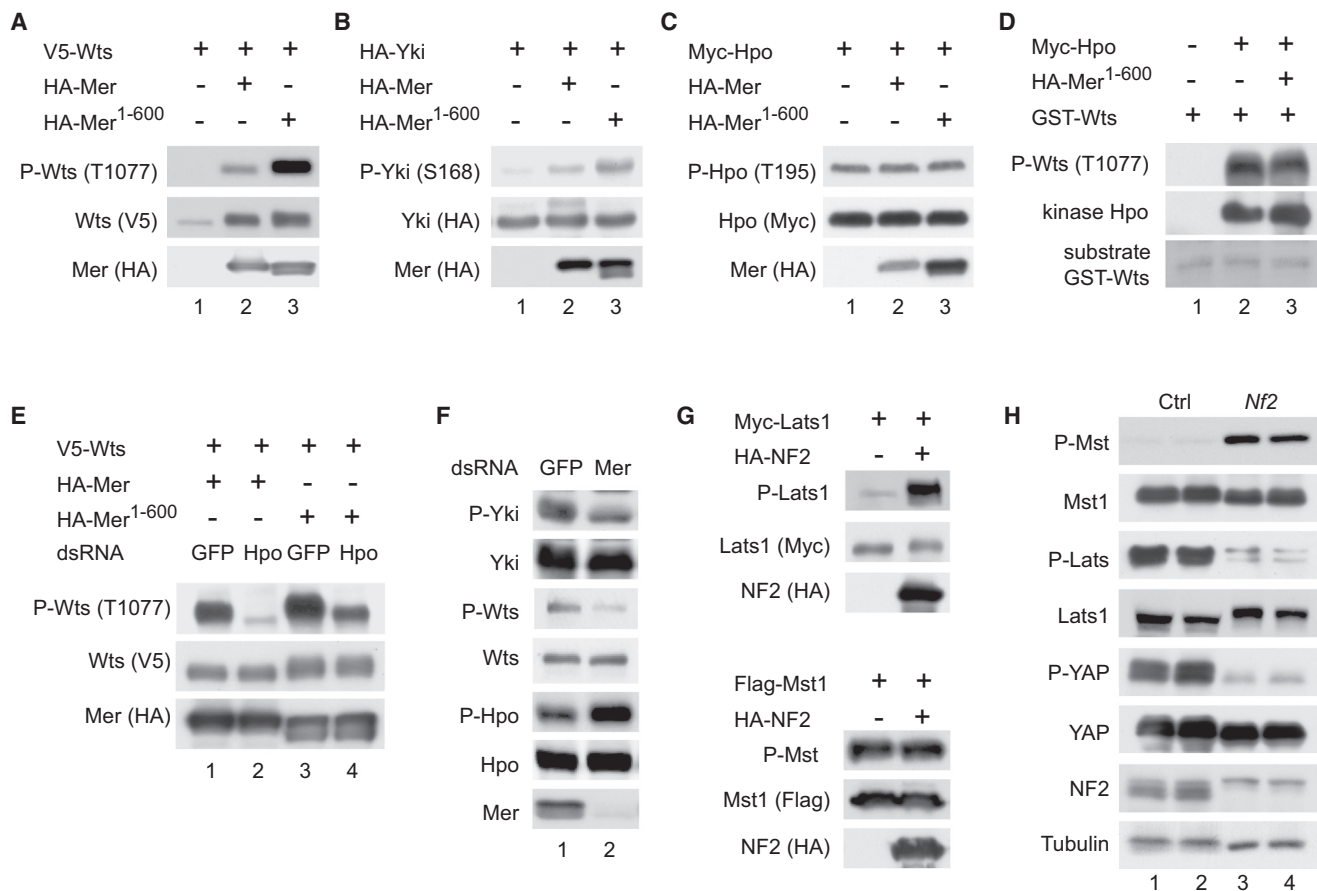
*Nf2*<sup>flox2/flox2</sup>) display reduced Lats1/2 and YAP phosphorylation, supporting the view that NF2 is normally required for Hippo signaling in mammals (Zhang et al., 2010). If NF2 activates downstream Hippo signaling by stimulating the intrinsic kinase activity of Mst1/2, one would expect to see a corresponding decrease in Mst1/2 activity in *Nf2*-deficient livers. Contrary to this prediction, we found that concurrent with a decrease in Lats1/2 and YAP phosphorylation, the *Nf2*-deficient livers instead showed a significant increase in Mst1/2 activity (Figure 1H). The opposite changes in Lats/YAP and Mst1/2 phosphorylation upon loss of NF2 therefore argue against the generally assumed linear model in which NF2 signals through activation of Mst1/2.

The increase in Hpo/Mst activity upon loss of Merlin in S2R<sup>+</sup> cells or mouse livers is likely due to negative feedback in Hippo signaling, whereby increased Yki/YAP activity leads to elevated transcription of multiple upstream components such as Ex and Kibra (Hamaratoglu et al., 2006; Genevet et al., 2010; Xiao et al., 2011), which in turn may promote Hpo/Mst activation. Although the molecular details of this feedback regulation warrant further investigation, our results clearly demonstrate that in the absence of Merlin, even enhanced Hpo/Mst kinase activity cannot stimulate its downstream kinase Wts/Lats. Taken together, these results indicate that in both *Drosophila* and mammals, Merlin activates Wts/Lats phosphorylation without stimulating the intrinsic kinase activity of Hpo/Mst.

### Merlin Promotes Plasma-Membrane Association of Wts/Lats in *Drosophila* and Mammalian Cells

Having excluded the linear model, we next explored the possibility that Mer, a predominantly membrane-associated protein (LaJeunesse et al., 1998), may play a role in targeting Wts to the plasma membrane. Strikingly, coexpression of Mer<sup>1-600</sup>, but not the WT Mer or the related protein Ex, resulted in a dramatic translocation of Wts to the plasma membrane, as revealed by subcellular fractionation (Figures 2A and 2B) and immunofluorescent staining (Figure 4G). This translocation is independent of hydrophobic motif phosphorylation or Mats binding, as Wts mutants lacking the hydrophobic motif phosphorylation site (Wts<sup>T1077A</sup>) (Yu et al., 2010) or the Mats-binding site (Wts<sup>R708A</sup>) (Ho et al., 2010) were translocated by Mer<sup>1-600</sup> to the membrane fraction as efficiently as WT Wts (Figures 2B and S2A–S2C). Consistent with these overexpression studies, RNAi knockdown of Mer resulted in a significant increase of endogenous Wts in the cytosol relative to the membrane fraction (Figure 2C).

Next, we examined whether NF2 plays a similar role in mammals. Indeed, coexpression of NF2 in HEK293 cells resulted in a dramatic translocation of Lats1/2 to plasma membrane, as revealed by subcellular fractionation (Figures 2D and S2D) and immunofluorescence (Figure 4E). To complement these gain-of-function studies, we examined the effect of NF2 deficiency on endogenous Lats1/2 localization in mouse Schwann cells, a cell type that is directly involved in the pathogenesis of the NF2 disease. FC-912 and FH-912 are genetically matched Schwann cells derived from primary *Nf2*<sup>flox/flox</sup> mouse Schwann cells with and without in vitro adenoviral Cre infection,



**Figure 1. Merlin Promotes Hippo Signaling without Stimulating the Intrinsic Kinase Activity of Hpo/Mst**

(A–C) Mer/Mer<sup>1-600</sup> promotes Wts and Yki phosphorylation without affecting Hpo phosphorylation. S2R<sup>+</sup> cell lysates expressing the indicated constructs were probed for P-Wts (A), P-Yki (B), or P-Hpo (C). Relative P-Wts and Wts levels were quantified in [Figure S1B](#).

(D) In vitro kinase assay. Myc-Hpo was expressed alone or together with HA-Mer<sup>1-600</sup> in S2R<sup>+</sup> cells, immunoprecipitated, and incubated with GST-Wts, and the reaction products were probed for P-Wts.

(E) S2R<sup>+</sup> cells transfected with the indicated plasmids along with control or *hpo* double-stranded RNA (dsRNA) were probed with the indicated antibodies. Note reduction of P-Wts levels by *hpo* RNAi.

(F) RNAi of *mer* leads to decreased P-Yki and P-Wts but increased P-Hpo levels in S2R<sup>+</sup> cells. Also note comparable levels of total Yki, Wts, or Hpo in control and RNAi<sup>mer</sup> cells.

(G) NF2 promotes Lats1 phosphorylation without affecting Mst1 phosphorylation. HEK293 cells expressing the indicated constructs were probed for P-Lats and P-Mst.

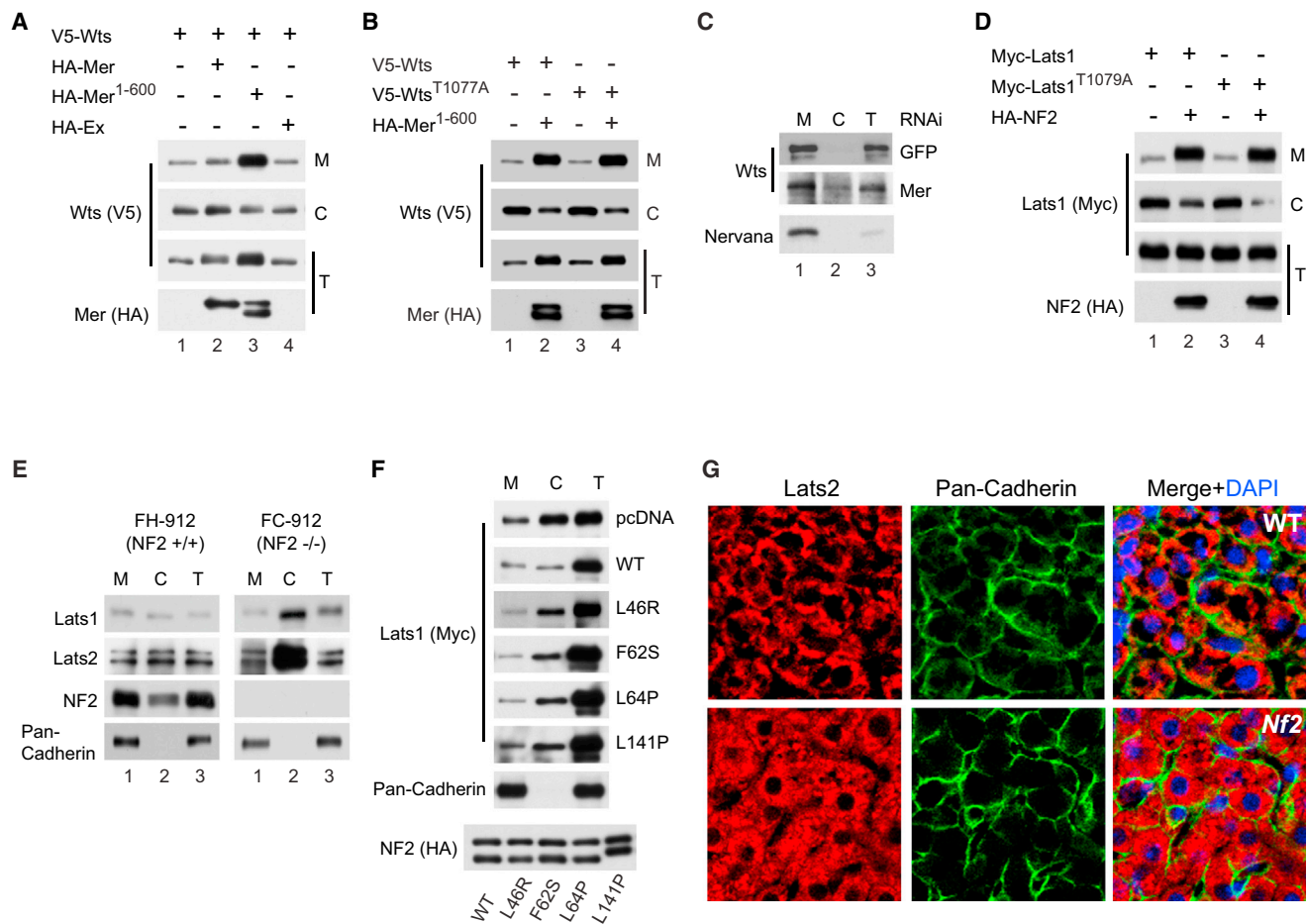
(H) Control (*Nf2*<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup>) and *Nf2* (*Alb-Cre*; *Nf2*<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup>) livers from 2-month-old littermates were probed with the indicated antibodies. Note the comparable levels of Lats1 and YAP but diminished P-Lats and P-YAP levels in *Nf2* mutant livers. Also note the increased P-Mst levels in *Nf2* mutants.

See also [Figure S1](#).

respectively (Li et al., 2010). As expected, the *Nf2*<sup>-/-</sup> FC-912 cells were defective in Lats1/2 phosphorylation (Figures S2E and S2F). Subcellular fractionation revealed a significant increase of endogenous Lats1/2 in the cytoplasmic relative to the membrane fraction in the *Nf2*<sup>-/-</sup> FC-912 Schwann cells compared to the *Nf2*<sup>+/+</sup> FH-912 Schwann cells (Figure 2E). This localization defect was rescued by re-expression of NF2 (Figure 2F). Consistent with these findings, examination of *Nf2*-deficient livers revealed a loss of membrane-associated Lats2 localization in hepatocytes (Figure 2G). Taken together, these results indicate that Merlin is required for Wts/Lats membrane association in both *Drosophila* and mammalian cells.

### Active Merlin Recruits Wts/Lats to the Plasma Membrane through Direct Protein-Protein Interaction

To understand the molecular mechanism by which Mer<sup>1-600</sup> promotes membrane association and phosphorylation of Wts, we examined protein interactions between Mer<sup>1-600</sup> and Wts. Consistent with its gain-of-function nature in stimulating Wts/Yki phosphorylation and Wts membrane association, Mer<sup>1-600</sup>, but not the WT Mer or the related protein Ex, coimmunoprecipitated with Wts in *Drosophila* S2R<sup>+</sup> cells (Figure 3A). Thus, the ability of Mer<sup>1-600</sup> to promote Hippo signaling and Wts membrane localization correlates with its ability to physically associate with Wts. The functional significance of Mer<sup>1-600</sup>-Wts interaction was also



**Figure 2. Merlin Promotes Plasma-Membrane Association of Wts/Lats in *Drosophila* and Mammalian Cells**

(A) Mer<sup>1-600</sup>, but not Mer or Ex, induces Wts membrane translocation. S2R<sup>+</sup> cells expressing the indicated constructs were fractionated into membrane (M) and cytosolic (C) fraction and probed with the indicated antibodies. T: total cell lysates.

(B) Mer<sup>1-600</sup> induces membrane association of Wts and Wts<sup>T1077A</sup> in S2R<sup>+</sup> cells. Subcellular fractions were analyzed as in (A).

(C) RNAi of *mer* results in a significant increase of endogenous Wts in the cytoplasmic fraction relative to the membrane fraction. Subcellular fractions were probed with  $\alpha$ -Wts antibody. Nervana, a plasma-membrane-localized Na<sup>+</sup>/K<sup>+</sup> ATPase, serves as a control for fractionation.

(D) NF2 promotes membrane association of Lats1 and Lats1<sup>T1079A</sup> in HEK293 cells, as revealed by subcellular fractionation.

(E) Loss of NF2 results in increased cytosolic accumulation of endogenous Lats1 and Lats2. Subcellular fractions of confluent FH-912 and FC-912 cells were probed.

(F) FC-912 cells were transfected with empty vector (pcDNA), NF2, or NF2 mutants along with Myc-Lats1. Subcellular fractions were probed for Myc-Lats1. FC-912 cells expressing empty vector or NF2 mutants had higher Myc-Lats1 levels in cytosolic than membrane fraction, but FC-912 cells expressing WT NF2 showed similar cytosolic and membrane Myc-Lats1 levels. The cell lysates were also probed for the expression of NF2 mutants (bottom gel).

(G) Liver sections from 8-day-old control and *Nf2*-deficient littermates, stained with Lats2 (red) and the plasma-membrane marker Pan-cadherin (green). Lats2 is concentrated at the cell cortex immediately on the inner face of the plasma membrane in WT hepatocytes but shows a uniform cytoplasmic localization in *Nf2*-deficient hepatocytes.

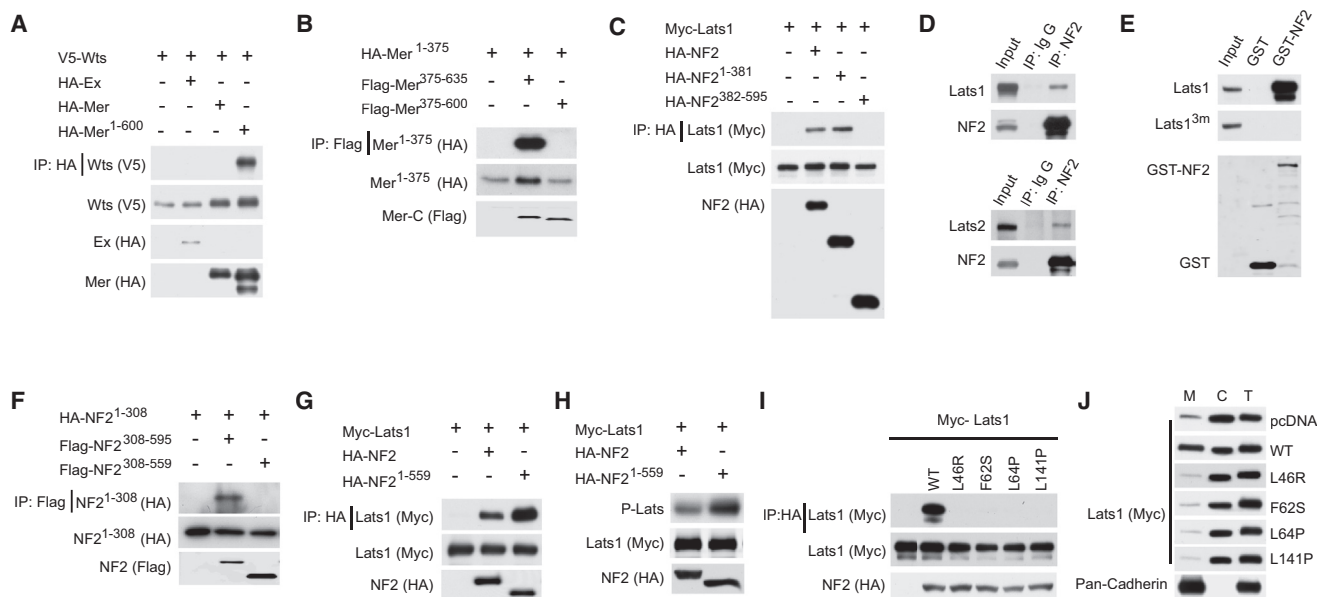
See also Figure S2.

confirmed by genetic interactions in vivo, whereby coexpression of Wts with Mer<sup>1-600</sup>, but not with WT Mer, resulted in a synergistic reduction in eye size (Figure S3A).

Next, we investigated, at a quasi-structural level, why Mer<sup>1-600</sup> confers gain-of-function activity. Merlin, like the related ERM proteins, can exist in two functional/conformational states (Bretscher et al., 2002). In the open and more active conformation, the N-terminal FERM domain of Merlin is free to bind to its effectors. In the more closed conformation, the C-terminal tail (CTT) domain associates with the N-terminal FERM domain,

blocks the accessibility of the FERM domain to its effectors, and therefore results in a less-active state (Sher et al., 2012). Conversion between the two states can be regulated by phosphorylation on a unique CTT residue (S518) in mammalian NF2 (Jin et al., 2006; Shaw et al., 2001; Surace et al., 2004), although this residue is not conserved in *Drosophila* Mer. The ability of Mer<sup>1-600</sup>, but not Mer, to bind Wts suggests that the former, but not the latter, may represent an open conformation. To test this hypothesis, we used coimmunoprecipitation to examine any physical interactions between the C-terminal half of Mer





**Figure 3. Active Merlin Binds to Wts/Lats through Its FERM Domain**

(A) Wts associates with Mer<sup>1-600</sup>, but not Mer or Ex, as revealed by immunoprecipitation in S2R<sup>+</sup> cells as indicated. (B) Mer<sup>1-600</sup> exhibits an open conformation. S2R<sup>+</sup> cells expressing Mer-N fragment (1–375) and WT (375–635) or truncated (375–600) Mer-C fragment were subjected to immunoprecipitation. (C) Lats1 associates with the N-terminal half of NF2, as revealed by immunoprecipitation in HEK293 cells. (D) Coimmunoprecipitation between endogenous NF2 and Lats1/2 in HEK293 cells. Endogenous Lats1 or Lats2 was detected in  $\alpha$ -NF2, but not control, immunoprecipitates. (E) NF2-Lats1 binding in vitro. HEK293 cell lysates containing Myc-Lats1 or Myc-Lats1<sup>3m</sup> were incubated with glutathione Sepharose beads containing GST (as a control) or GST-NF2. GST-NF2 bound to Myc-Lats1 but not Myc-Lats1<sup>3m</sup>. (F–H) C-terminal truncation of NF2 results in a more open conformation and enhances Lats1 binding and phosphorylation. HEK293 cells expressing the indicated constructs were probed for association between NF2-N and NF2-C fragments (F), NF2-Lats1 association (G), and Lats1 hydrophobic motif phosphorylation (H). (I) WT NF2, but not disease-associated missense mutants, associates with Lats1, as revealed by immunoprecipitation in HEK293 cells. (J) WT NF2, but not disease-associated missense mutants, promotes membrane association of Lats1 in HEK293 cells, as revealed by subcellular fractionation. See also Figure S3.

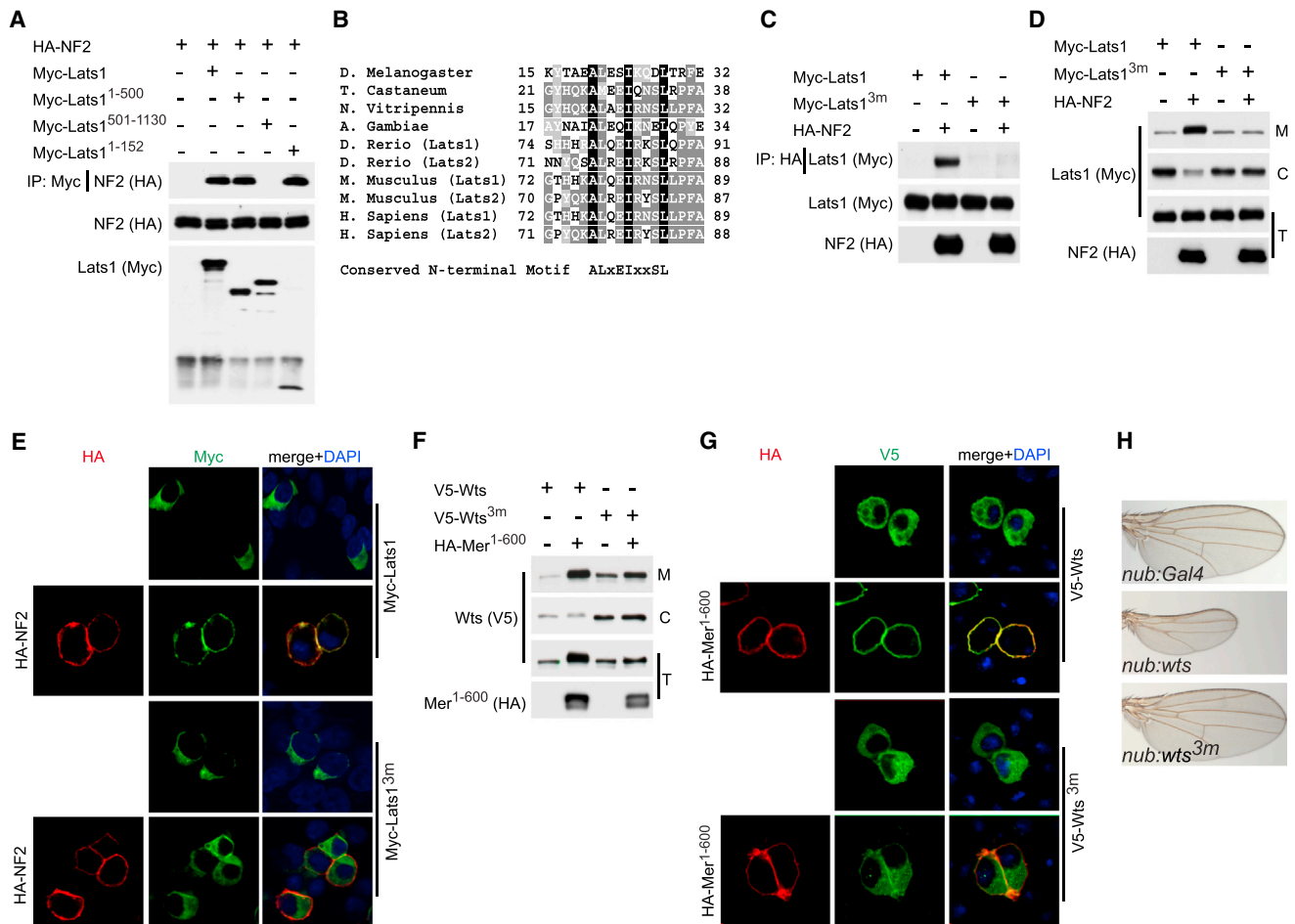
(Mer-C), derived from either WT or truncated Mer, and the N-terminal half of Mer (Mer-N). Of note, a similar assay was used recently to assess the conformational states of mammalian NF2 (Sher et al., 2012). As shown in Figure 3B, the WT Mer-C, but not the truncated Mer-C, strongly interacted with Mer-N. These results suggest that Mer<sup>1-600</sup> displays a more open conformation than the WT Mer, which provides a molecular explanation for the gain-of-function activity of Mer<sup>1-600</sup> in growth suppression, activation of Hippo signaling, Wts binding, and membrane recruitment.

As in *Drosophila*, NF2 and Lats1/2 coimmunoprecipitated with each other in HEK293 cells (Figures 3C and S3B). This interaction was confirmed with endogenous NF2 and Lats1/2 proteins (Figure 3D) and by in vitro GST pull-down assays (Figure 3E). NF2 appeared to be intrinsically more active than Mer, as the full-length NF2 (but not Mer) sufficed to bind and to recruit Lats1/2 to the plasma membrane (Figures 3C and 2D; compare to Figures 3A and 2A). When we used a similar coimmunoprecipitation assay to detect interaction between the N- and C-terminal domains of NF2, this interaction was significantly weaker than that detected between their *Drosophila* counterparts at comparable protein input levels (Figure 3F versus Figure 3B). Thus, the stronger activity of NF2 in binding Lats1/2 correlates with an

intrinsically more open conformation of NF2 than of Mer. Despite such differences, a C-terminal truncation of NF2 (NF2<sup>1-559</sup>, which is analogous to Mer<sup>1-600</sup>) resulted in enhanced binding and activation of Lats1 (Figures 3G and 3H), as well as a more open conformation (Figure 3F). In addition, the phosphodeficient S518A mutant of NF2, which is known to display a more open/active conformation compared to the phosphomimetic S518D mutant (Sher et al., 2012), also showed greater ability to bind Lats1 and to promote Lats1 phosphorylation (Figures S3C–S3D). Thus, despite differences in basal activity and “openness” of the proteins, in both *Drosophila* and mammalian cells, a conformational change in Merlin to a more open form leads to enhanced Wts/Lats binding and Hippo signaling.

### The FERM Domain of Merlin and an Evolutionarily Conserved N-Terminal Motif in Wts/Lats Mediate Wts/Lats-Merlin Binding

We used coimmunoprecipitation assays to map protein domains involved in Mer/NF2-Wts/Lats interactions. The N-terminal halves of Mer and NF2, which correspond to the FERM domain, are required for interaction with Wts (Figures S3E and S3F) and Lats1 (Figure 3C), respectively. Further truncations into the FERM domain abolished this interaction (Figure S3G).



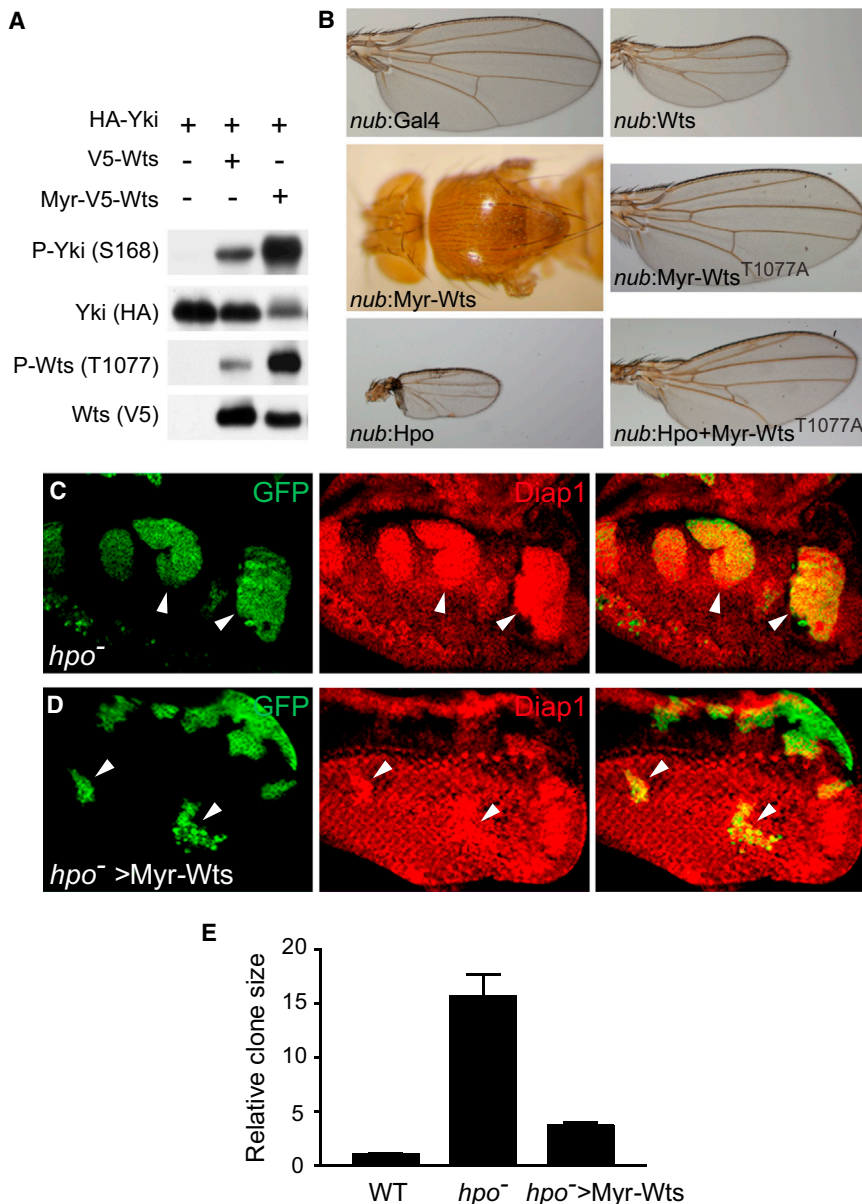
**Figure 4. Identification of a Conserved N-Terminal Motif in Wts/Lats that Mediates Merlin-Wts/Lats Interactions**

(A) HEK293 cells expressing HA-NF2 and various truncations of Lats1 were subjected to immunoprecipitation as indicated.  
 (B) Alignment of the CNM in Wts/Lats protein from different species.  
 (C) Myc-Lats1, but not Myc-Lats1<sup>3m</sup>, was coimmunoprecipitated with HA-NF2 in HEK293 cells.  
 (D and E) The CNM is required for NF2-induced membrane association of Lats1 in HEK293 cells. Subcellular fractionation (D) and immunofluorescent staining (E) of Myc-Lats1 or Myc-Lats1<sup>3m</sup> in the presence or absence of NF2 are shown.  
 (F and G) The CNM is required for Mer<sup>1-600</sup>-mediated membrane association of Wts in *Drosophila* S2R<sup>+</sup> cells. Subcellular fractionation (F) and immunofluorescent staining (G) of V5-Wts or V5-Wts<sup>3m</sup> in the presence or absence of Mer<sup>1-600</sup> are shown.  
 (H) UAS-Wts and UAS-Wts<sup>3m</sup> transgenes inserted at identical chromosomal loci were crossed to nub-Gal4 driver. Note the small-wing phenotype induced by Wts but not Wts<sup>3m</sup>.  
 See also Figure S4.

Interestingly, most disease-associated missense mutations in NF2 map to its FERM domain (Ahronowitz et al., 2007). We therefore examined a collection of human disease-associated NF2 mutations in the FERM domain (L46R, F62S, L64P, and L141P; mapped to the 3D structure of NF2 FERM domain [Kang et al., 2002] in Figure S3H) for their ability to interact with Lats1 and to recruit Lats1 to the plasma membrane. Unlike WT NF2, these pathological mutants failed to interact with Lats1 (Figure 3I) or to promote membrane association of Lats1 in HEK293 cells (Figure 3J). We also examined these missense mutations for their ability to rescue the Lats1 membrane association defect in the *Nf2*<sup>-/-</sup> FC-912 Schwann cells. Unlike WT NF2, none of the pathological mutations could rescue the Lats1 localization defect in

FC-912 cells (Figure 2F). The impaired ability of pathological NF2 mutations to interact with and recruit Lats1 to the plasma membrane further supports the physiological relevance of NF2-Lats interactions.

In the reverse direction, the N-terminal half of Lats1 (Lats1<sup>1-500</sup>), but not its C-terminal half containing the kinase domain, is required for interaction with NF2 (Figure 4A). The NF2-binding region was further mapped to Lats1<sup>1-152</sup> (Figure 4A) and a similar region in Lats2 (Figure S4A). The overall sequence similarity between *Drosophila* and human Wts/Lats is very low in this region. However, a careful examination of Wts/Lats orthologs from multiple insects and vertebrates revealed a conserved sequence motif (Figure 4B). In fact, this sequence, hereafter



**Figure 5. Constitutive Membrane Targeting Promotes Hpo-Mediated Hydrophobic Motif Phosphorylation and Tumor-Suppressor Activity of Wts**

(A) S2R<sup>+</sup> cells expressing the indicated constructs were probed for P-Yki and P-Wts.

(B) Enhanced growth-suppressive activity of membrane-targeted Wts and dominant-negative activity of membrane-targeted Wts<sup>T1077A</sup>. All UAS-Wts transgenes were inserted at the same locus. Note the complete loss of adult wings in *nub:Myr-Wts* flies and the suppression of the small-wing phenotype of *nub:Hpo* in flies that coexpressed Myr-Wts<sup>T1077A</sup>.

(C–E) Partial rescue of *hpo* mutant clones by Myr-Wts. Eye discs containing clones of the indicated genotype (GFP<sup>+</sup>, arrowheads) were stained for Diap1 (red). Note elevated Diap1 levels in *hpo* clones without or with Myr-Wts expression. Also note the smaller clone size and less rounded shape of the latter genotype. Clone size is quantified in (E). Values are mean ± standard error of the mean (SEM); n = 12 for each genotype. See also Figure S5.

ure S4B). Interestingly, although the CNM and the Mer-C domain bear no sequence similarities, interaction between the CNM and the Mer-N domain could be competed away by the Mer-C domain (Figure S4C). Taken together, these results uncover an evolutionarily conserved sequence motif in Wts/Lats that is crucial for Merlin-binding and growth-suppressive activity.

#### Constitutive Membrane Targeting Promotes Hpo-Mediated Hydrophobic Motif Phosphorylation and Tumor-Suppressor Activity of Wts

The data presented so far support a working model in which active Mer functions by recruiting Wts to the plasma membrane, where Wts undergoes Hpo-

mediated hydrophobic motif phosphorylation. We conducted additional experiments to further corroborate this model.

One prediction of this model is that constitutive targeting of Wts to the plasma membrane may result in enhanced hydrophobic motif phosphorylation and gain-of-function activity for Wts. This was tested by fusing Wts with a well-characterized plasma-membrane-targeting sequence, the myristylation signal (Myr) of *Drosophila* Src (Myr-Wts) (Struhl and Adachi, 1998). As a control, we fused Myr with the hydrophobic motif-defective Wts<sup>T1077A</sup>. As expected, Myr-Wts and Myr-Wts<sup>T1077A</sup> showed exclusive plasma-membrane association (Figures S5A and S5B). In agreement with our prediction, Myr-Wts stimulated Wts and Yki phosphorylation more potently than Wts did (Figure 5A). We also compared the growth-inhibitory activity of

referred to as conserved *N*-terminal motif (CNM), represents the only evolutionarily conserved sequence in the N-terminal half of Wts/Lats. Interestingly, mutation of the three most conserved residues in the CNM (Lats1-A77P-I81T-L85P, abbreviated as Lats1<sup>3m</sup>) abolished NF2-Lats1 interactions in coimmunoprecipitation (Figure 4C) and GST pull-down assays (Figure 3E) and abolished NF2-induced membrane association of Lats1 (Figures 4D and 4E). Introduction of the same mutation into Wts (Wts-A20P-I24T-L28P, abbreviated as Wts<sup>3m</sup>) greatly diminished Mer<sup>1-600</sup>-induced Wts membrane association (Figures 4F and 4G) and abolished the growth-suppressive activity of Wts in *Drosophila* (Figure 4H). When fused to GFP, a minimal fragment containing the CNM (aa 1–50) was sufficient to recruit GFP to the plasma membrane in a Mer<sup>1-600</sup>-dependent manner (Fig-

Myr-Wts and Wts in *Drosophila*. Overexpression of Myr-Wts by the wing-specific nub-Gal4 driver led to a complete loss of wing tissue, whereas overexpression of Wts led to a less severe reduction in wing size (Figure 5B), demonstrating that membrane targeting of Wts promotes its tumor-suppressor activity in vivo.

Another prediction of this model is that hydrophobic motif phosphorylation is required for the gain-of-function activity of Myr-Wts. Indeed, in contrast to Myr-Wts, Myr-Wts<sup>T1077A</sup> showed no activity in vivo (Figure 5B), demonstrating that membrane-targeted Wts requires hydrophobic motif phosphorylation for its activation. In fact, Myr-Wts<sup>T1077A</sup> behaves as a dominant-negative form—it greatly suppresses Hpo-induced undergrowth of eyes and wings and enhances Yki-induced eye overgrowth (Figures 5B and S5C). The ability of a membrane-targeted phosphodeficient Wts mutant to interfere with Hippo signaling further implicates the plasma membrane as a critical subcellular compartment for Wts phosphorylation and activation.

A third prediction of this model is that despite its gain-of-function activity in WT background, Myr-Wts should not rescue *hpo* mutant clones, as membrane-targeted Wts should require Hpo for its activation. To test this prediction, we analyzed *hpo* mutant clones with concurrent Myr-Wts overexpression. Indeed, Myr-Wts cannot rescue *hpo* mutant phenotypes, as *hpo* mutant clones with Myr-Wts overexpression still exhibited elevated *diap1* expression, a hallmark of defective Hippo signaling (Figures 5C and 5D). Interestingly, *hpo* mutant clones expressing Myr-Wts were smaller in size than *hpo* mutant clones (Figures 5C–5E), suggesting that Myr-Wts may partially bypass Hpo. Given that T1077 phosphorylation is absolutely required for Myr-Wts activity in vivo (Figure 5B), the simplest possibility is that in the absence of Hpo, an unknown Hpo-like kinase may phosphorylate the hydrophobic motif of Wts. Although the identity of such Hpo-like kinases requires further investigation, this view is consistent with the stronger overgrowth of *wts* compared to *hpo* null mutant tissues in *Drosophila* (Wu et al., 2003), as well as multiple studies in mammalian cells that unequivocally demonstrated Mst1/2-independent phosphorylation of the Lats1/2 hydrophobic motif (Yu et al., 2012; Zhou et al., 2009).

### Sav and Mer Play Distinct Roles in Spatial Organization of the Hippo Kinase Cassette at the Plasma Membrane

The gain-of-function activity of Myr-Wts and the dominant-negative activity of Myr-Wts<sup>T1077A</sup> suggest that the plasma membrane is an important subcellular compartment for Hippo signal transduction. A comprehensive survey of Hippo pathway components showed that like Merlin, Sav/Sav1, a scaffold protein that has been reported to bind Wts/Lats and Hpo/Mst (Harvey and Tapon, 2007), is localized almost exclusively in the membrane fraction in both *Drosophila* and mammalian cells (Figures S6A and S6B). Given its near-exclusive membrane association, we investigated the possibility that Sav may also play a role in scaffolding the Hippo kinase cassette at the plasma membrane.

We used subcellular fractionation to examine whether Sav affects the membrane association of Hpo and Wts. Interestingly, coexpression of Sav induced translocation of Hpo but not Wts to the membrane fraction (Figures 6A and 6B). Conversely, RNAi knockdown of Sav led to a significant decrease of endogenous

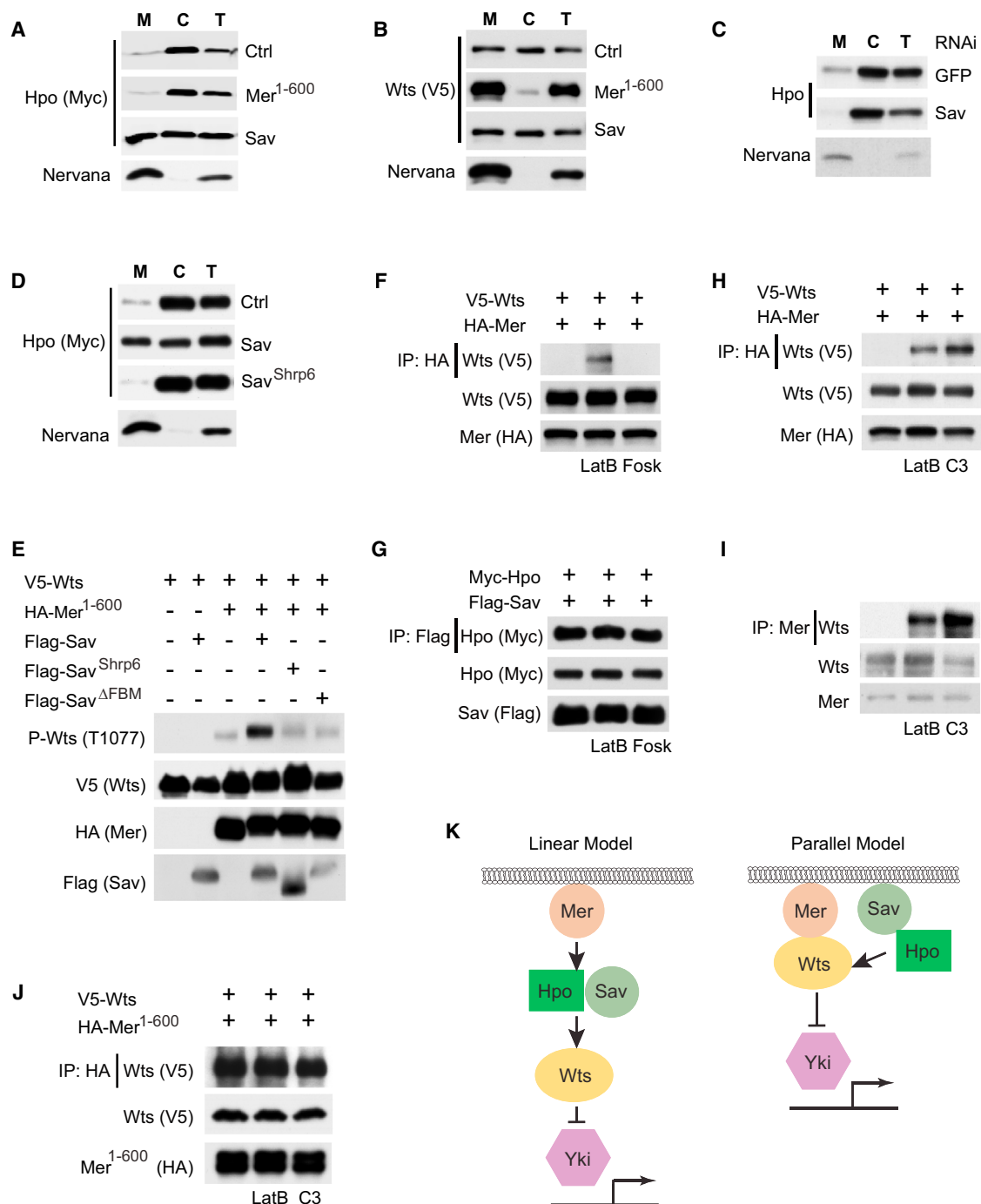
Hpo in the membrane fraction relative to the cytoplasmic fraction (Figure 6C). Sav-induced membrane translocation of Hpo is mediated by direct binding, as it was abolished by mutation of Sav's SARAH domain (Figure 6D), which is known to mediate Sav-Hpo binding (Scheel and Hofmann, 2003). Notably, despite its potent activity in recruiting Wts to plasma membrane, Mer<sup>1-600</sup> had no effect on membrane association of Hpo (Figures 6A and 6B). Unlike Mer and Sav, other components of the Hippo pathway failed to promote Hpo or Wts membrane association in our assays (Figures S6C and S6D). Thus, it appears that Mer and Sav play separable roles in membrane organization of the Hippo kinase cassette by targeting Wts and Hpo, respectively, to the plasma membrane. Similar observations were made in mammalian cells, as Sav1 promoted membrane association of Mst1 but not Lats1, and conversely, NF2 promoted membrane association of Lats1 but not Mst1 (Figures S6E and S6F). Consistent with fractionation, immunofluorescence confirmed that coexpression of Merlin<sup>1-600</sup> and Sav induced translocation of both Wts and Hpo to the plasma membrane in *Drosophila* S2R<sup>+</sup> cells (Figure S6G). Likewise, coexpression of NF2 and Sav promoted membrane translocation of Lats1 and Mst1 in HEK293 cells (Figure S6H).

To investigate the functional role of Sav-induced membrane recruitment of Hpo, we analyzed the effect of Sav on Hippo signaling output in S2R<sup>+</sup> cells. Although overexpression of Sav alone had no visible effect on Wts phosphorylation, coexpression of Sav with Mer<sup>1-600</sup> led to synergistic activation of Wts phosphorylation (Figure 6E). Mutation of the SARAH domain of Sav abolished this synergistic effect (Figure 6E), supporting the importance of Sav-Hpo binding in Hpo membrane recruitment and signal transduction. Sav was recently reported to bind to Mer through an evolutionarily conserved FERM-binding motif (FBM) (Yu et al., 2010). Interestingly, mutation of the FBM in Sav also abolished this synergistic effect (Figure 6E), suggesting that a Mer-Sav interaction may be involved in bringing Hpo and Wts together at the plasma membrane to further facilitate the Hippo kinase cascade.

### Regulation of Merlin-Wts Interaction by the Actin Cytoskeleton

Although WT NF2 suffices to bind Lats1/2 in mammalian cells, our analysis of Mer-Wts interactions in *Drosophila* has so far relied on the use of a constitutive active mutant, Mer<sup>1-600</sup>. Thus it remains an open question whether Wts could interact with WT Merlin under any circumstances. We therefore used the S2R<sup>+</sup> cells to investigate whether physical interactions between Wts and WT Merlin can be detected under conditions that potentially stimulate Hippo signaling. Disruption of actin cytoskeleton has been reported to activate Hippo signaling in both *Drosophila* and mammalian cells (Dupont et al., 2011; Fernández et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011; Zhao et al., 2012). In addition, certain G protein-coupled receptor (GPCR) ligands such as epinephrine and lysophosphatidic acid (LPA) have been reported to activate or inhibit Hippo signaling in mammalian cells (Yu et al., 2012). Given that *Drosophila* lacks LPA receptors (Metpally and Sowdhamini, 2005) and does not produce epinephrine (Roeder, 2005), these particular ligands are unlikely to regulate Hippo signaling in *Drosophila*.





**Figure 6. Sav-Mediated Membrane Association of Hpo and Regulation of Merlin-Wts Binding by the Actin Cytoskeleton**

(A) S2R<sup>+</sup> cells expressing Myc-Hpo together with HA-Mer<sup>1-600</sup> or FLAG-Sav were fractionated into membrane (M) and cytosolic (C) fraction. T: total cell lysates. Sav, but not Mer<sup>1-600</sup>, promoted Hpo membrane association.

(B) S2R<sup>+</sup> cells expressing V5-Wts together with HA-Mer<sup>1-600</sup> or FLAG-Sav were analyzed by subcellular fractionation. Mer<sup>1-600</sup>, but not Sav, promoted Wts membrane association.

(C) RNAi of sav results in decreased membrane localization of endogenous Hpo. Subcellular fractions were probed with  $\alpha$ -Hpo antibody.

(D) Sav<sup>shrp6</sup> mimics a sav allele that deletes just the SARAH domain (Kango-Singh et al., 2002). Unlike WT Sav, Sav<sup>shrp6</sup> failed to induce membrane recruitment of Hpo.

(E) S2R<sup>+</sup> cells expressing the indicated constructs were analyzed for P-Wts. WT Sav, but not Sav<sup>shrp6</sup> (Kango-Singh et al., 2002) or Sav<sup>ΔFBM</sup> (Yu et al., 2010), greatly potentiated Mer<sup>1-600</sup>-induced Wts phosphorylation.

(legend continued on next page)

Nevertheless, we tested protein kinase A (PKA), which was reported to lie downstream of GPCRs in GPCR-Hippo signaling in mammalian cells (Yu et al., 2013). Strikingly, we detected robust interactions between WT Merlin and Wts in S2R<sup>+</sup> cells treated with the actin polymerization inhibitor Latrunculin B (LatB), but not in cells treated with DMSO (control) or the PKA agonist Forskolin (Fosk) (Figure 6F). In contrast, interactions between Sav and Hpo were not modulated by LatB or Fosk (Figure 6G). Thus, it appears that actin cytoskeleton disruption specifically enhanced physical interactions between Merlin and Wts.

To further corroborate a link between the actin cytoskeleton and Merlin, we treated S2R<sup>+</sup> cells with the Rho GTPase inhibitor C3. As with LatB, treatment with C3 also promoted Merlin-Wts interaction (Figure 6H), in agreement with findings in mammalian cells implicating the Rho GTPase as a critical regulator of the actin cytoskeleton in modulating Hippo signaling (Dupont et al., 2011; Wada et al., 2011; Zhao et al., 2012). In fact, only when S2R<sup>+</sup> cells were treated with these actin inhibitors could we detect interactions between endogenous Merlin and Wts (Figure 6I). These results suggest that Merlin may be a critical mediator of actin-regulated Hippo signaling. Consistent with this hypothesis, RNAi knockdown of Mer attenuated LatB- or C3-induced Yki phosphorylation in S2R<sup>+</sup> cells (Figure S6I). Significantly, although LatB and C3 promoted interactions between Wts and WT Merlin, neither inhibitor affected physical interactions between Wts and Mer<sup>1-600</sup>, the constitutively active mutant form of Merlin (Figure 6J). Such epistasis further supports a functional link between the actin cytoskeleton and Merlin function.

### A Dual Mechanism of Wts/Lats Activation Is Required for Physiological Hippo Signaling in *Drosophila* and Mammalian Tissues In Vivo

The results presented so far lead to the following model of Merlin-mediated Hippo signaling: Merlin binds and recruits Wts to the plasma membrane, which in effect increases the concentration of Wts at the plasma membrane; furthermore, Sav acts as a membrane-associated scaffold that increases the concentration of Hpo at the plasma membrane. Thus, it appears that full activation of Wts requires a dual mechanism that involves increasing the concentration of both the substrate (Wts) and its activating kinase (Hpo) at the membrane (Figure 6K).

A genetic prediction of this dual mechanism is that double mutants disabling both arms of the regulation may result in stronger phenotypes than disabling each single arm. Indeed, examination of supernumerary interommatidial cells in *Drosophila* pupal retina, a hallmark of defective Hippo signaling, revealed

a massive increase in the number of supernumerary interommatidial cells in *mer; sav* double-mutant eyes compared to *mer* or *sav* single-mutant eyes (Figure 7A). To determine whether this is also true in mammals, we compared mice with liver-specific knockout of *Nf2*, *Sav1*, or both genes (Figures 7B–7F). As shown previously, *Nf2*-deficient livers appeared macroscopically of normal size at 8 days of age (~5% liver/body ratio) and microscopically showed mild focal overproliferation of biliary epithelial cells (BECs). *Sav1*-deficient livers appeared macroscopically of normal size at 8 days of age (~5% liver/body ratio) and microscopically showed normal BECs. Strikingly, age-matched *Nf2; Sav1* double-mutant livers showed massive liver overgrowth (~18% liver/body ratio) (Figure 7E). Microscopically, the double-mutant livers showed massive overproliferation of BECs resembling bile duct hamartoma, in which BECs had overtaken the hepatocytes to become the predominant cell types in the liver (Figures 7B–7D). The double-mutant livers also showed a more dramatic decrease of YAP and Lats1/2 phosphorylation compared to the single-mutant tissues (Figure 7F). Furthermore, in contrast to the life expectancy of > 1 year for each single mutant (Lu et al., 2010; Zhang et al., 2010), all the double-mutant mice died before reaching 3 weeks of age (n = 7). Thus, the dual mechanism of Wts/Lats activation is required for physiological Hippo signaling in both *Drosophila* and mice.

## DISCUSSION

### The Molecular Function of Merlin in Hippo Signaling

Since its initial discovery as a human disease gene underlying NF2, the tumor suppressor Merlin has been the subject of intense investigation. Besides the Hippo pathway, Merlin has been linked to a variety of mechanisms such as transmembrane receptor endocytosis/localization (EGFR and CD44) and signaling by Ras, Rac/PAK, and PI3K pathways (Li et al., 2012). Paradoxically, as a membrane-associated tumor suppressor, Merlin was also reported to suppress tumorigenesis in mammalian cells by translocating to the nucleus to inhibit a specific E3 ubiquitin ligase (Li et al., 2012). Among these proposed targets, the linkage between Merlin and Hippo signaling has attracted much attention given the similarity of the respective mutant phenotypes in *Drosophila* and the dosage-sensitive genetic suppression of Merlin mutant phenotypes by heterozygosity of the Hippo effector YAP in multiple mouse tissues.

Despite the genetic evidence implicating Merlin in Hippo signaling, the molecular basis of this functional link was unknown. Our current study addresses this outstanding issue in

(F and G) S2R<sup>+</sup> cells expressing the indicated constructs were treated with DMSO (0.1%, lane 1), LatB (1  $\mu$ g/ml, lane 2), or Fosk (30  $\mu$ M, lane 3) for 1 hr, followed by immunoprecipitation. LatB, but not Fosk, promoted Mer-Wts interaction (F). Neither drug affected Sav-Hpo interaction (G).

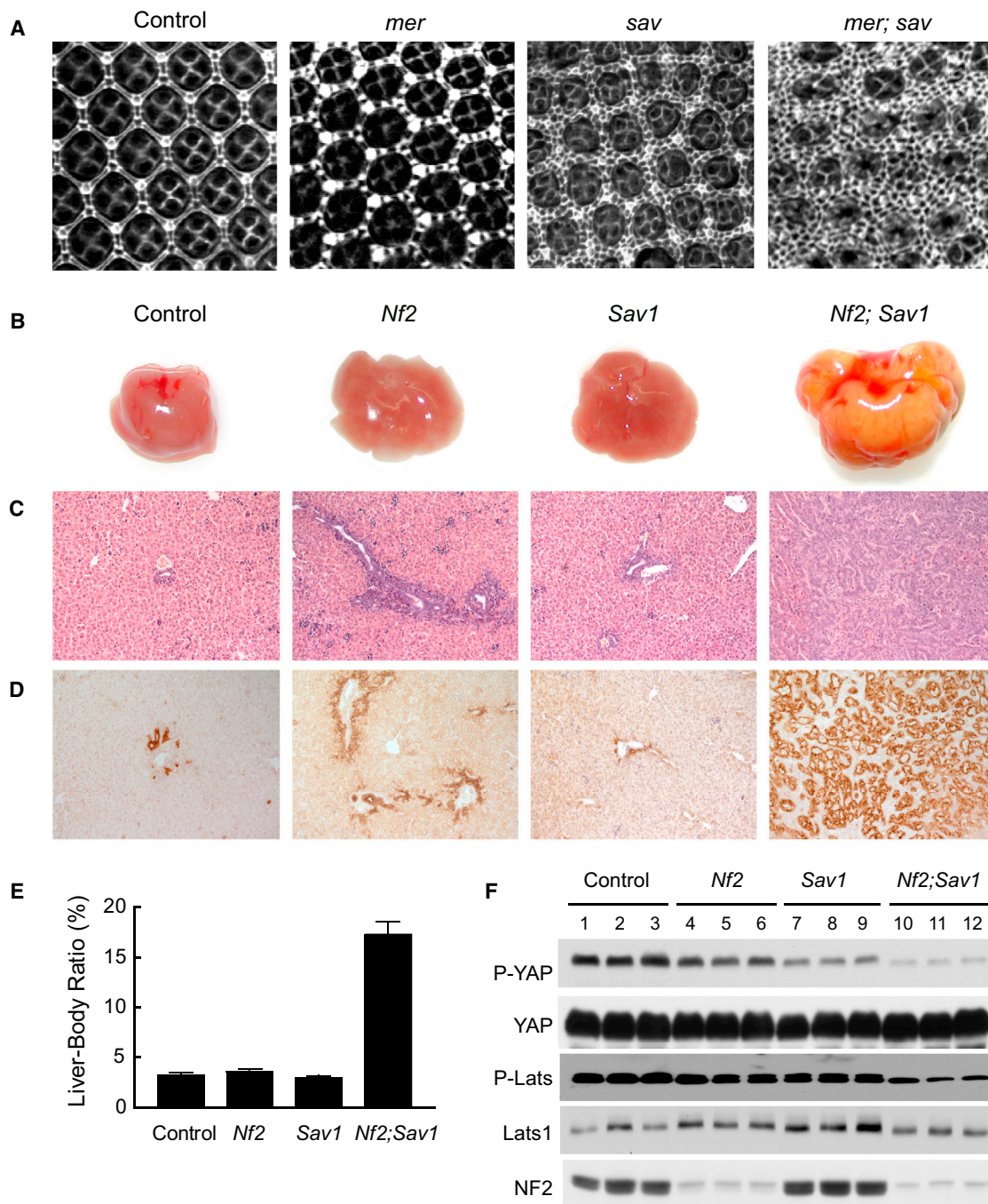
(H) S2R<sup>+</sup> cells expressing the indicated constructs were treated with DMSO (0.1%, lane 1), LatB (1  $\mu$ g/ml, lane 2), or C3 (2  $\mu$ g/ml, lane 3), followed by immunoprecipitation.

(I) S2R<sup>+</sup> cells were treated with DMSO (0.1%, lane 1), LatB (1  $\mu$ g/ml, lane 2), or C3 (2  $\mu$ g/ml, lane 3), followed by immunoprecipitation with anti-Mer antibody and detection with anti-Wts antibody.

(J) S2R<sup>+</sup> cells expressing HA-Mer<sup>1-600</sup> and V5-Wts were treated with DMSO, LatB, or C3 as in (H) and (I), followed by immunoprecipitation. Neither treatment affected interactions between Mer<sup>1-600</sup> and Wts.

(K) Schematic models of Merlin function in Hippo signaling. For simplicity, only proteins relevant to the current study are illustrated in the schematic diagrams. Left: the prevailing linear model placing Merlin upstream of Hpo activation. Right: a new model based on the current study highlighting a dual mechanism in which Merlin and Sav promote the membrane association of Wts and Hpo, respectively.

See also Figure S6.



**Figure 7. Loss of Mer/NF2 and Sav/Sav1 Leads to Synergistic Defects in Hippo Signaling in *Drosophila* and Mice**

(A) Synergistic effect of *mer* and *sav* mutations in *Drosophila*. Mid-pupal retina of the indicated genotypes was stained for Discs-Large to highlight cell outlines. Note the significantly increased number of interommatidial cells in *mer; sav* double-mutant eyes.

(B–E) Synergistic effect of *Nf2* and *Sav1* mutations in mice. Livers from 8-day-old pups of the following genotypes were analyzed: control (*Alb-Cre*), *Nf2* mutant (*Alb-Cre; Nf2<sup>flox2/flox2</sup>*), *Sav1* mutant (*Alb-Cre; Sav1<sup>flox/flox</sup>*), and double mutant (*Alb-Cre; Nf2<sup>flox2/flox2</sup>; Sav1<sup>flox/flox</sup>*). Whole amount liver images (B), H&E staining (C), and Pan-CK staining (D) are shown, along with quantification of liver weight (E). Values are mean  $\pm$  standard deviation (SD);  $n = 5$  for each genotype. Whereas *Nf2* and *Sav1* livers displayed mild or no overproliferation of the CK-positive BECs, *Nf2; Sav1* double-mutant livers were predominantly occupied by the BECs. Also note the unique external appearance and overgrowth of *Nf2; Sav1* double-mutant livers.

(F) Western blot analysis of liver lysates from mouse livers analyzed in (B)–(E). Liver lysates from three mice of each genotype were probed. Note the further decrease in YAP and Lats phosphorylation in *Nf2; Sav1* double-mutant livers compared to the single mutant. Also note the comparable level of Lats1 in control and *Nf2* mutant livers.

two important ways. First, we provide molecular evidence showing that Merlin promotes downstream Hippo signaling without activating the intrinsic kinase activity of Hpo/Mst. Our studies therefore disprove the prevailing assumption that Merlin functions biochemically upstream of Hpo activation (Figure 6K). Along this line, we note that current models of Hippo signaling are actually a composite of true molecular relationships (such as Hpo acting upstream of Wts or Wts acting upstream Yki) and genetic epistasis relationships (such as Mer acting upstream of Hpo). In light of the current study, we caution that biochemical and epistasis relationships should be clearly distinguished in signaling diagrams because mixing and matching them can be misleading. Second, we elucidate direct physical interactions between Merlin and Wts/Lats and show that such interactions promote Hippo signaling by recruiting Wts/Lats to the plasma membrane. The discovery of physical interactions between Merlin and a key component of the Hippo pathway therefore provides molecular support for a Merlin-Hippo connection that has so far been based largely on genetics and indirect evidence. Interestingly, interactions between Merlin and Wts are regulated by the actin cytoskeleton, underscoring Merlin as a potential mediator of actin-regulated Hippo signaling.

Besides identifying a conserved molecular function for Merlin, our studies also revealed quantitative differences between *Drosophila* and mammalian Merlin. WT Mer normally does not associate with Wts in *Drosophila* S2R<sup>+</sup> cells, yet WT NF2 suffices to bind Lats1/2 in human cells. Such differences correlate with an intrinsically more open conformation of NF2 compared to Mer. These findings agree with previous reports that the intramolecular interaction in NF2 is relatively weak and dynamic (Nguyen et al., 2001). We note that the intrinsically more active/open state of NF2 is consistent with the role of S518 phosphorylation in antagonizing NF2 activity and the absence of this negative regulatory site in *Drosophila* Mer. Obviously, such negative regulation would be of more functional relevance in the context of an intrinsically more active Merlin protein as in mammals.

### The Plasma Membrane as the Key Subcellular Compartment for Hippo Signal Transduction

The plasma membrane is the entry point of diverse environmental stimuli and is intimately involved in spatial organization of signaling proteins (Groves and Kuriyan, 2010). Although many reported upstream regulators of the Hippo pathway in *Drosophila* are transmembrane proteins (e.g., Fat and Crumbs) or are localized in apical membrane domains (e.g., Mer, Ex, and Kibra), how these membrane-associated inputs spatially organize the Hippo kinase cassette was poorly understood. This question is further complicated by the possible evolutionary divergence of upstream inputs into the pathway between *Drosophila* and mammals (Bossuyt et al., 2013). Notably, among these upstream inputs, Merlin is the only protein whose contribution to Hippo signaling has been genetically validated in both flies and mammals (Hamaratoglu et al., 2006; Zhang et al., 2010).

Our study demonstrates that an important and evolutionarily conserved molecular function of Merlin is to promote the membrane association of Wts/Lats. We also implicate Sav as a

membrane-associated scaffold that promotes the membrane association of Hpo/Mst, the upstream kinase of Wts/Lats. Thus, two predominantly membrane-associated proteins, Merlin and Sav, are involved in targeting the two essential kinases of the Hippo kinase cassette to the plasma membrane. It is tempting to speculate that at least some of the other upstream regulators of Hippo signaling may function in a similar manner by promoting the membrane association of the Hippo kinase cassette. We note that a functional role for Sav in membrane association of Hpo does not preclude the other previously described roles for the Sav scaffold in Hippo signaling, such as tethering Hpo and Wts. It is possible that Sav potentiates Hippo signaling both by tethering multiple signaling components and by localizing signaling activity to specific subcellular compartments, as shown in other well-studied scaffold signaling proteins such as Ste5 and KSR (Good et al., 2011). Nevertheless, our study has uncovered a role for Sav in spatial organization of the Hippo pathway.

Wts/Lats is known to be subjected to two modes of regulation, including phosphorylation and protein stability. Our study extends previous studies by showing that the membrane association of Wts represents an additional mode of regulation. In addition, our study suggests that Wts may be activated by alternate upstream kinase(s) besides Hpo. Identifying the kinases that mediate Hpo-independent activation and understanding the regulation of such kinases should greatly expand our knowledge about the physiological regulation of Hippo signaling. With its activity subjected to multiple modes of regulation, it is becoming increasingly clear that Wts/Lats represents as a critical node in the Hippo signaling network. These different modes of regulation are not exclusive of each other and are indeed functionally intertwined, as membrane association of Wts/Lats also enhances its phosphorylation. Understanding how the multiple regulatory inputs into Wts/Lats are coordinated will shed light on the physiological regulation of Hippo signaling in normal development and offer new strategies for therapeutic intervention in pathological conditions such as NF2.

## EXPERIMENTAL PROCEDURES

### Subcellular Fractionation and Cell Staining

To separate membrane and cytoplasm-associated proteins, subcellular fractionation was carried out as described (Hergovich et al., 2005). Briefly, cells were incubated for 20 min on ice in S100/P100 buffer (20 mM Tris, 150 mM NaCl, 2.5 mM EDTA, 1 mM EGTA) supplemented with 1 mM PMSF and protease inhibitors (Roche). Cell lysate was then passed through a 26 G needle 20 times, and nuclei were removed by centrifugation at 1,000 × g for 2 min. The supernatant was then ultracentrifuged for 60 min at 100,000 × g at 4°C to separate cytoplasmic fraction (supernatant) and membranous fraction (pellet).

For immunofluorescence staining, cells were grown on a Lab-Tek II Chamber Slide, washed with PBS, and fixed in 3% paraformaldehyde for 10 min at room temperature. Immunofluorescent staining was performed according to the protocols provided by the manufacturers of the commercial antibodies.

### *Drosophila* Genetics

The myristylation signal from *Drosophila* c-Src (amino acids 1–10) was inserted into the N terminus to generate Myr-V5-Wts, Myr-V5-Wts<sup>T1077A</sup>. These fragments were then inserted into the pUAST-attB vector. Transgenic flies were created by phiC31-mediated site-specific transformation, using the attP2 site at 51C (Bischof et al., 2007). *GMR-Gal4* and *nub-Gal4* were used for



overexpression analysis. All crosses were done at 25°C. The following genotypes were used:

GFP<sup>+</sup> *hpo*<sup>-</sup> clones:

UAS-GFP *hs-Flp*; *tub-Gal80 FRT42D/FRT42hpo*<sup>42-47</sup>; *tub-Gal4*.

GFP<sup>+</sup> *hpo*<sup>-</sup> clones overexpressing Myr-Wts:

UAS-GFP *hs-Flp*; *tub-Gal80 FRT42D/FRT42hpo*<sup>42-47</sup>; *tub-Gal4/UAS-Myr-Wts*.

Control pupal eyes:

*y w ey-flp Ubi-GFP FRT19A/FRT19A*.

*mer* mutant pupal eyes:

*y w ey-flp Ubi-GFP FRT19A/mer<sup>Δ</sup> FRT19A*.

*sav* mutant pupal eyes:

*y w ey-flp; FRT 82B sav<sup>3</sup>/FRT 82B Ubi-GFP*.

*mer sav* double-mutant pupal eyes:

*y w ey-flp, Ubi-GFP FRT19A/mer<sup>Δ</sup> FRT19A; FRT82B sav<sup>3</sup>/FRT82B Ubi-GFP*.

### Mouse Genetics

To achieve liver-specific gene deletion, floxed alleles of *Nf2* (Giovannini et al., 2000) and *Sav1* (Cai et al., 2010) were bred to Albumin-Cre transgenic mice (003574) from the Jackson Laboratory (Bar Harbor, ME, USA). To avoid potential variation related to gender, all experiments were performed in male mice with paternal inheritance of Alb-Cre. Mouse liver tissues were collected and fixed in 10% neutral buffered formalin solution (Sigma), paraffin-embedded, sectioned (at 5 μm), and stained with hematoxylin and eosin (H&E). For immunofluorescence, rabbit anti-Lats2 (Bethyl laboratories, 1:100) and mouse anti-pan-cadherin (Sigma, 1:500) were used, together with Cy3-conjugated goat anti-rabbit (1:250) and Alexa 488-conjugated goat anti-mouse secondary antibodies (1:250) (Molecular Probes). Immunohistochemistry staining was performed on paraffin sections using a Vectastain ABC kit according to manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA), with pan-CK antibody (DAKO, 1:500).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.08.025>.

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