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Hippo activation through homodimerization and membrane association for growth inhibition and organ size control

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ABSTRACT

Hippo (Hpo) signaling plays a critical role in restricting tissue growth and organ size in both invertebrate and vertebrate animals. However, how the Hpo kinase is regulated during development has not been clearly understood. Using a Bimolecular Fluorescence Complementation assay, we have investigated the functional significance of Hpo homo-dimer formation and subcellular localization in living cells. We found that Hpo dimerization and membrane association are critical for its activation in growth inhibition. As dimerization facilitates Hpo to access its binding partner, Hpo kinases in the homo-dimer trans-phosphorylate each other to increase their enzymatic activity. Moreover, loss- and gain-of-function studies indicate that upstream regulators, Expanded, Merlin and Kibra, play a critical role in promoting Hpo dimerization as well as association to the cortical F-actin beneath the plasma membrane. Enforced Hpo localization to the plasma membrane increases Hpo dimerization and activity. Therefore, homo-dimerization and plasma membrane association are two important mechanisms for Hpo activation in growth control during animal development.

Introduction

Tissue growth and organ size control is a fundamental issue in developmental biology. Discovery of the evolutionarily conserved Hippo (Hpo) signaling pathway provides an important entry point to address this issue. The Hpo pathway regulates tissue growth via restricting cell proliferation and growth and promoting apoptosis (recently reviewed by Pan, 2010; Halder and Johnson, 2011; Zhao et al., 2011; Staley and Irvine, 2012).

Over the past decade, many factors have been identified to operate through the Hpo pathway. The central part of the Hpo pathway comprises a kinase cascade: the Ste20-like kinase Hpo (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003), the NDR family protein kinase Warts (Wts) (Justice et al., 1995; Xu et al., 1995), the WW domain scaffold protein Salvador (Sav) (Kango-Singh et al., 2002; Tapon et al., 2002) and Mob as tumor suppressor (Mats) (Lai et al., 2005). These core components of Hpo pathway repress tissue growth largely by phosphorylating and thereby inhibiting a growth-promoting protein Yorkie (Yki)/YAP (Dong et al., 2007; Wei et al., 2007; Zhao et al., 2007; Hao et al., 2008; Oh and Irvine, 2008; Zhang et al., 2008). Yki/YAP act through their binding partners such as Scalloped (Sd) transcription factor to regulate the expression of downstream target genes important for cell proliferation and apoptosis (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008).

For factors acting in the upstream of the Hpo pathway, they could be classified into several clusters (reviewed by Grusche et al., 2010): proteins that function via atypical cadherin transmembrane receptor, Fat (Silva et al., 2006); Kibra–Expanded–Merlin complex, which provides a direct link from the apical membrane to core components of the Hpo pathway (Hamaratoglu et al., 2006; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010); several apical-basal polarity proteins, including Lgl, aPKC, Crumb (Grzeschik et al., 2010); and kinase Tao-1 which could directly phosphorylate Hpo (Boggiano et al., 2011; Poon et al., 2011). Although the core components of Hpo pathway are invariant and conserved from fly to human, the upstream regulators are much more diverse and, in many cases, partially redundant. These upstream factors regulate Hpo pathway via complicated network and have been shown to exhibit spatial and temporal specificity. Thus, understanding the mechanism by which Hpo kinase is regulated is a key for us to learn how the

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Hpo pathway is involved in tissue growth and organ size control during development.

Apical Ex–Mer–Kibra complex is uniquely identified as a platform for signal integration among upstream molecules. However, how these proteins transduce signal to Hpo core components are still unknown. Ex and Mer locate at the sub-apical region in *Drosophila* epithelia cells and are proposed to provide input into the Hpo pathway. Kibra, which interacts with both Mer and Ex serves as a linker between Mer and Ex. Several studies suggest that the recruitment of the core complex to the apical membrane is important for activation, but the mechanism by which the Ex–Mer–Kibra complex activates the core complex is currently unclear (recently reviewed by Boggiano and Fehon, 2012).

In this study, we show that dimerization is necessary for Hpo trans-phosphorylation and activation. We further investigated the subcellular localization of Hpo dimer and demonstrated that only membrane targeted Hpo dimer has higher activity. Importantly, we found that Ex–Mer–Kibra complex regulates Hpo activity by influencing Hpo dimer formation and its association to the cortical F-actin beneath the cell membrane. Therefore, homo-dimerization and plasma membrane association are two mechanisms important for Hpo activation.

Results

Hpo kinase dimerization is directly observed in living cells

The Hpo kinase plays an important role in growth inhibition during animal development but how Hpo activity is regulated has not been fully understood. To better investigate Hpo regulation in living cells, we have applied the Bimolecular Fluorescence Complementation (BiFC) technique to directly visualize protein–protein interaction in developing tissues. In BiFC, a fluorescent protein Venus is split into Venus N-terminus (VN) and C-terminus (VC) (Kerppola, 2006; Hu, 2009). These two polypeptides alone cannot produce fluorescent signal. When fused to two proteins A and B separately, however, they may complement each other and generate fluorescence only if proteins A and B can interact (Fig. 1A). In this work, the full-length Hpo protein is fused with VN and VC, respectively. The Hpo BiFC assay was performed both in *Drosophila* S2 cells and developing tissues. This assay allows a direct observation of Hpo–Hpo interaction in living cells and provides an opportunity to examine subcellular localization and regulation of dimerized Hpo protein.

To visualize Hpo dimerization, HpoVC together with HpoVN were transfected into S2 cells or expressed in transgenic flies. bFosVC was used as a negative control. We found that HpoVC and HpoVN exhibited positive BiFC signal both in S2 cells (Fig. 1B) and third-instar larva wing discs (Fig. 1D), whereas bFosVC and HpoVN showed no BiFC signal (Fig. 1C and E), indicating the specificity of the BiFC assay.

To investigate whether Venus-tagging disrupts Hpo protein activity, we performed adult wing analysis. Driven by a wing-specific Gal4 transcription activator, *MS1096-Gal4*, transgenic flies carrying HpoVC or HpoVN had their wings greatly reduced in size similar to that of Hpo transgenic flies free of Venus-tagging (Fig. S1) (e.g. Wu et al., 2003). Thus, Venus-tagging does not interfere with Hpo activity in growth inhibition.

Hpo dimerization is necessary for its trans-phosphorylation and activation, but such phosphorlylation is not required for Hpo dimer formation

During the last stage of our work, Jin et al. (2012) showed that Hpo activation can be mediated by homo-dimerization. Inter-molecular interaction between Hpo proteins is mediated by two distinct mechanisms, one that uses the N-terminal region including the kinase domain and the other one that is mediated by the C-terminal SARAH domain (Jin et al., 2012). We also have independently observed that Hpo without its SARAH domain was still able to form homo-dimer in our BiFC assay (data not shown). To test the idea that N-terminal and C-terminal regions are essential for Hpo dimerization in living cells, $Hpo^{M242E,I634D}$ was generated and expressed in *Drosophila* S2 cells. While the Met242 to Glu mutation is expected to abolish the N-terminal interaction, the Ile634 to Asp mutation would prevent the C-terminal SARAH domain from associating to each other (Jin et al., 2012). In our BiFC assay, $Hpo^{M242E,I634D}$ indeed failed to dimerize (compare Fig. 1G–G′ with F–F′). Western blot analysis revealed that Hpo can no longer undergo auto-phosphorylation and activation when it is unable to dimerize (Fig. 1H).

To further test whether Hpo phosphorylation can only occur in Hpo homo-dimer, we carried out cell sorting experiment (Fig. 1I). HpoVC and HpoVN were co-transfected into S2 cells. After 40 h of culture, the cells were sorted based on their production of BiFC fluorescence into BiFC-positive and BiFC-negative groups. These two groups of cells were used to perform western blot analysis. The results showed that BiFC-positive cells had more phosphorylated Hpo compared with BiFC-negative cells (Fig. 1J). This result supports the idea that Hpo dimerization facilitates its autophosphorylation and activation within the Hpo dimer complex.

We also investigated whether Hpo phosphorylation plays any role in mediating its dimerization, we mutated two evolutionarily conserved Hpo autophosphorylation sites (Glantschnig et al., 2002), Thr189 and Thr195 to Ala or Glu. The Hpo mutants were fused with VC or VN and were tested in the BiFC assay to detect their interaction. We found that mutations of the phosphorylation sites had no obvious influence on Hpo BiFC signal (Fig. S2), suggesting that the phosphorylation is not a prerequisite for Hpo dimerization.

Hpo inter-molecular phosphorylation was then examined. Hpo kinase-dead (Hpo^{K71R} or Hpo^{KD}) mutation was co-transfected with Hpo^{T195E} or Hpo^{T195A}. Although Hpo^{T195E} has partial kinase activity, it cannot be detected by a phospho-specific Hpo antibody towards Thr195 site, which is a residue essential for Hpo activity (Glantschnig et al., 2002; Jin et al., 2012). Western blot data showed that Hpo^{KD} could be phosphorylated by Hpo^{T195E}, but not Hpo^{T195A} (Fig. 1K), suggesting that the phosphorylation normally occurs between two interacting Hpo kinase proteins in the complex.

Hpo kinase-dead mutant acts dominant-negatively due to its ability to dimerize with wild-type Hpo protein

Unlike wild-type Hpo, Hpo^{KD}-VC and Hpo^{KD}-VN were unable to produce BiFC signal, suggesting that they might fail to associate with each other or they did associate but VN and VC failed to complement (Fig. 2, compare A with B). However, Hpo^{KD} can clearly interact with wild-type Hpo (Fig. 2C and D). This interaction allows Hpo^{KD} to inactivate wild-type Hpo through Hpo^{KD}–Hpo^{WT} complex formation, which would explain the dominant-negative effect of the Hpo^{KD} protein (Fig. 2E–H).

Subcellular localization of the Hpo dimer is critical for its activity

To investigate where Hpo is localized at the subcellular level, we immunostained wing discs of third-instar larva from wild-type flies or transgenic flies. Both endogenous Hpo and a Flag-tagged Hpo showed a portion of protein associated with plasma membrane since it co-localizes with adherent junction marker DE-cadherin, whereas most Hpo protein was detected in the

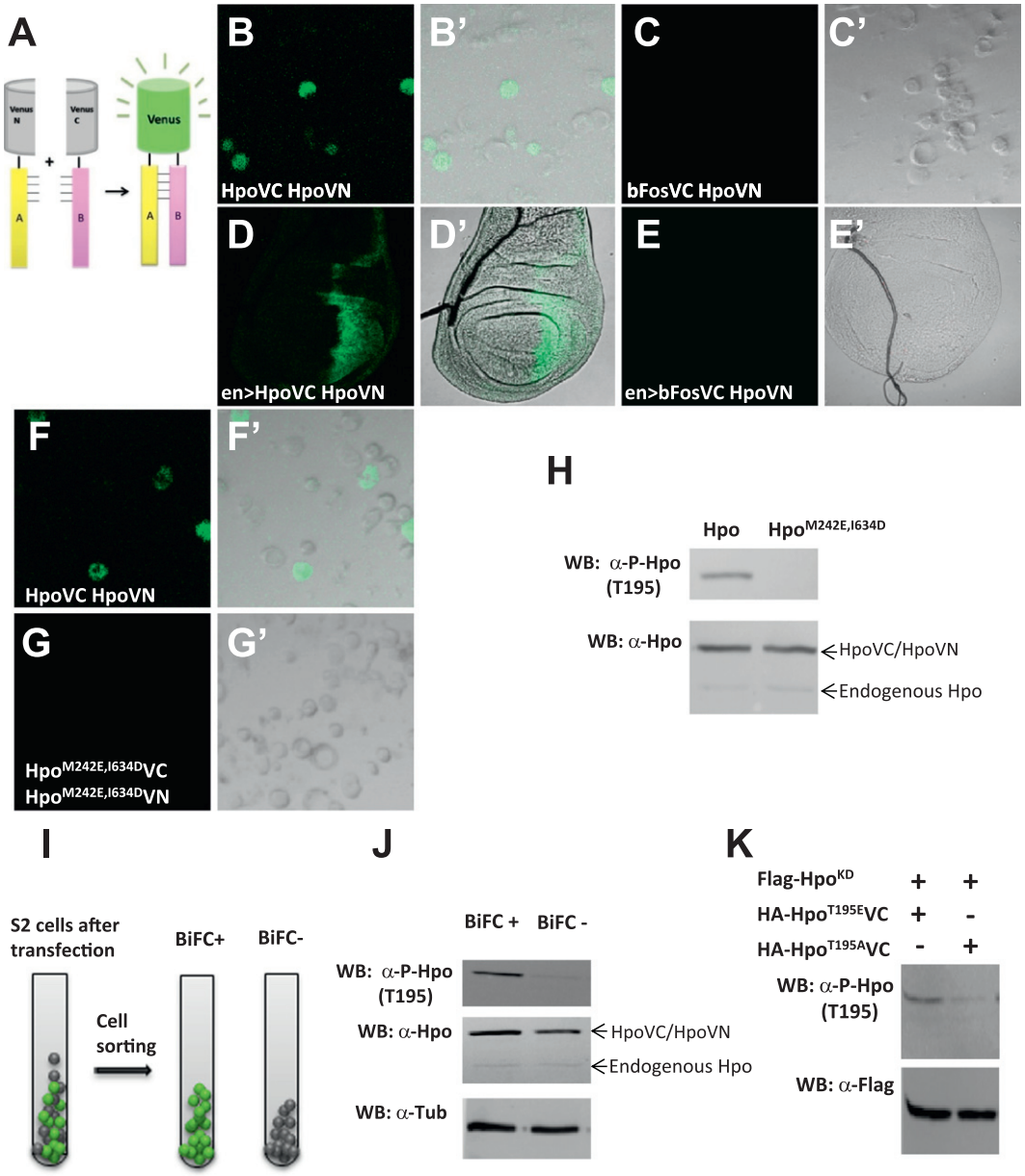

Fig. 1. *Visualization of Hpo dimerization via the BiFC assay in living cells and impact of the dimerization on Hpo autophosphorylation and activation.* (A) Diagram of the BiFC assay. When proteins A and B form a complex, it may allow their fusion partners Venus N- and C-terminal polypeptides to complement to exhibit fluorescence. (B–B′ & F–F′) Direct visualization of Hpo homo-dimer in transiently transfected S2 cells. (C–C′) bFosVC/HpoVN was used as a negative control. (D–D′) Direct visualization of Hpo homo-dimer in fruit fly third-instar larval wing discs. *en-Gal4* drives gene expression in the posterior compartment of wing discs (the compartment on the right side). (E–E′) *en-Gal4/UAS- bFosVC/HpoVN* wing discs were used for a negative control. No BiFC signal was detected. (G–G′) Mutations of Met242 and Ile634 residues abolished Hpo dimerization in S2 cells. (H) Hpo autophosphorylation at Thr195 cannot occur without dimerization. Hpo and $Hpo^{M242E,I634D}$ were expressed in S2 cells. Through western blot analysis, the total Hpo protein was detected with an Hpo antibody and activated Hpo protein was monitored by a phosphor-specific Hpo antibody (Thr195). (I–J) Cell sorting experiment shows BiFC-positive cells had more phosphorylated Hpo compared to BiFC-negative cells. (K) Hpo^{KD} was trans-phosphorylated by Hpo^{T195E} and endogenous Hpo proteins. After cotransfection of Flag-HpoKD/HA-HpoT195E-VC or Flag-HpoKD/HA-HpoT195A-VC to S2 cells, total protein extracts were used for western blot and immune-staining with a Flag antibody to detect total Flag-Hpo^{KD} protein and a phospho-specific Hpo antibody (Thr195) to detect phosphorylated Flag-Hpo^{KD} protein. BiFC signal is shown in green in all panels.

nucleus and cytoplasm (Fig. S3A–F). Then, we examined Hpo dimer localization. Interestingly, a significant fraction of Hpo dimer was found to be associated with the cortical F-actin cytoskeleton close to the plasma membrane in the apical region (Fig. 3A–C; Fig. S3G–J). It can be also detected in the nucleus and cytoplasm (Fig. 3D–I; Fig. S3G–N). Therefore, the Hpo dimer is broadly distributed in major subcellular locations.

To further investigate how the Hpo dimer activity is correlated with its subcellular localization, we did immunostaining against phosphorylated Histone 3 (PH3) to highlight mitotic cells. Our data shows that Hpo dimer was found in nuclei of all PH3-positive cells ($n=89$) (Fig. 3D–I). Hpo dimer in the nucleus should be non-functional in growth inhibition because the cells were actively involved in proliferation. It is also possible that Hpo dimer is directly involved in regulating some mitotic events.

Membrane targeting facilitates Hpo dimerization and phosphorylation

We have previously found that the plasma membrane is an important site for activation of a Hpo pathway component, Mats (Ho et al., 2010). To test the idea that plasma membrane is a

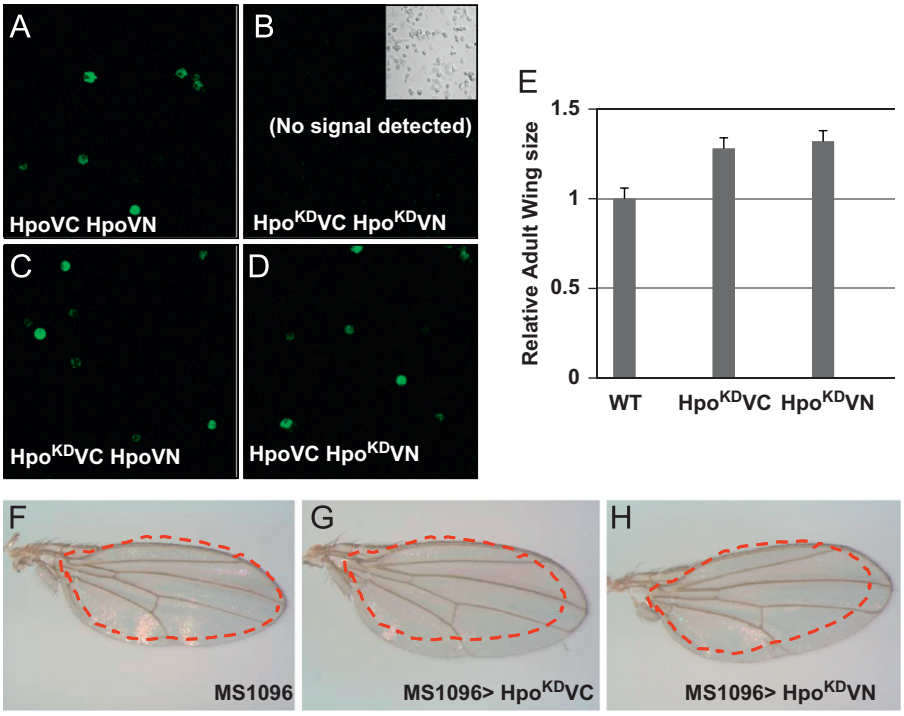

Fig. 2. *Hpo kinase-dead mutant protein is still able to associate with wild-type Hpo protein.* (A–D) BiFC assay revealed that Hpo^{KD} fails to allow VC–VN complementation, but is able to bind the wild-type Hpo protein. (E) Adult wing analysis shows that Hpo^{KD} has a dominant-negative effect to increase the wing size ($N > 30$ wings for each genotype). (F–H) Representative adult wings with various genotypes as indicated are shown. *MS1096 Gal4* driver promotes expression of *UAS* transgenes in larval wing imaginal discs.

critical site for Hpo kinase to be dimerized and activated, we generated HpoVN/HpoVC containing a myristoylation (Myr) signal sequence in the Hpo N-terminus for membrane localization. Interestingly, Myr-HpoVC and Myr-HpoVN failed to produce BiFC signal (data not shown). However, Myr-HpoVN (or Myr-HpoVC) could generate membrane-associated BiFC signal when it is complexed with an unmyritoylated Hpo partner (Fig. 4A) and Hpo dimer was found to be mostly associated with cortical F-actin close to the plasma membrane (Fig. 4A′). Furthermore, BiFC assay showed that Myr-HpoVN/HpoVC have higher BiFC efficiency than HpoVN/HpoVC, suggesting that membrane-targeting facilitates Hpo dimerization (Fig. 4B). Through western blot analysis, we found that membrane-targeted Hpo (Myr-HpoVN) was more phosphorylated than wild-type Hpo at Thr195 (Fig. 4C). Finally, a luciferase reporter assay revealed that membrane-targeted Hpo (Myr-HpoVN) was more potent to reduce Yki/Sd transcription activity compared to unmyritoylated Hpo (Fig. 4D). Therefore, cell membrane association is critical for Hpo to form dimer and become activated.

Merlin, Expanded and Kibra act together to regulate Hpo subcellular localization and dimerization

Mer, Ex and Kibra function together and act in a partially redundant manner to regulate Hpo signaling (Hamaratoglu et al., 2006; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). However, how Hpo is regulated by these upstream regulators is not clearly understood. To investigate the hypothesis that they regulate Hpo activity by influencing Hpo dimerization, RNA interference (RNAi) treatment was done to knock down endogenous Mer, Ex and Kibra. Western blot analysis indicates that the RNAi was effective in reducing expression of these genes dramatically (Fig. S4). Through these RNAi experiments, we found that Hpo dimerization efficiency decreased when the expression of Mer, Ex and Kibra proteins was reduced (Fig. 5A, left panel). In contrast, Hpo-Sav interaction was not dramatically influenced by Mer–Ex–Kibra triple knocking-down (Fig. 5A, right panel). Through a gain-of-function approach, we found that the Hpo BiFC signal was increased by Mer and Ex co-expression (Fig. 5B, left panel), whereas the Hpo-Sav BiFC signal did not show any significant change under the same condition (Fig. 5B, right panel). These results suggest that Mer, Ex and Kibra act together to facilitate Hpo dimer formation in living cells.

Furthermore, we tested the influence of Mer/Ex/Kibra complex on Hpo dimer subcellular localization in S2 cells. We arbitrarily classified the distribution of Hpo BiFC signal into three patterns (membrane-associated, cytoplasm, and cytoplasm+nucleus) (Fig. 5C). Then we examined the Hpo dimer distribution under different genetic background. The results showed that when Mer, Ex and Kibra were knocked down by RNAi, the portion of membrane-associated Hpo dimer was decreased from 37% to 19% and cytoplasm/nucleus-localized Hpo dimer was increased (Fig. 5D, $n=432$). On the other hand, the membrane-associated Hpo dimer was increased from 21% to 59% when Mer, Ex and Kibra were over-expressed, whereas the nucleus/cytoplasm localized Hpo dimer was decreased (Fig. 5E, $n=523$). Next, the Hpo dimer distribution in developing tissues was examined. Compared with wild-type control, we observed that more Hpo dimers were localized to the nucleus when *mer* and *ex* were knocked down by RNAi (Fig. 5, compare F–F′ with G–G′). These results support the idea that Mer, Ex and Kibra facilitate Hpo membrane association and dimerization.

Discussion

The *Drosophila* Hpo kinase plays a key role in tissue growth inhibition and organ size control, but how Hpo is regulated is still not fully understood. In this study, we took advantage of direct visualization of protein–protein interaction to observe Hpo

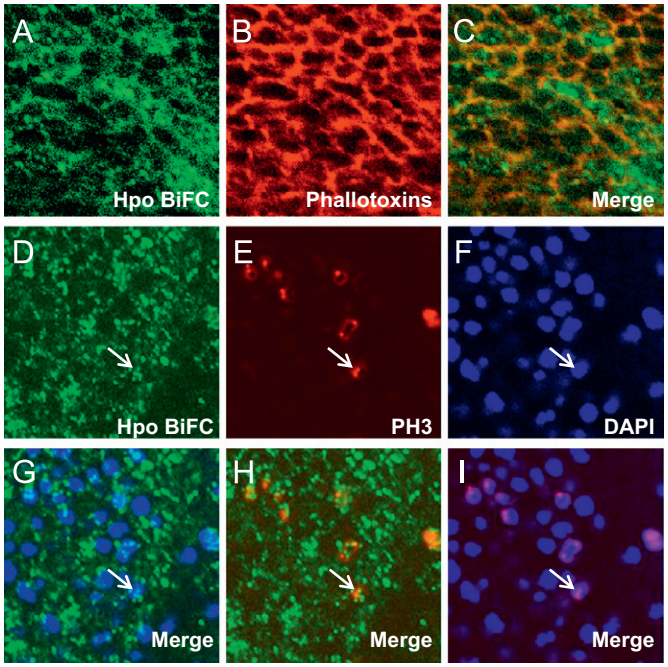

Fig. 3. *Subcellular localization of Hpo homo-dimer in developing tissues.* (A) Hpo dimer localization is shown by Hpo-Hpo BiFC (green) at the apical region of cells in larval wing discs. (B) Phallotoxin was used to highlight the F-actin cytoskeleton (red). (C) A fraction of Hpo dimer was found to associate with cotical F-actin near the plasma membrane. (D–I) In the mitotic cells labeled by PH3 staining (red), Hpo BiFC signal (green) was always found in the nucleus. DAPI staining highlighted the nucleus (blue). Arrows pointed out one example. Expression of VN or VC-tagged *hpo* transgenes was driven by *en-Gal4* in the posterior compartment of late third-instar larval wing discs.

homo-dimerization and activation in living cells. We demonstrate that Hpo can be activated by trans-phosphorylation upon homo-dimer formation. Importantly, this mechanism is evolutionarily conserved. Mammalian homologs of Hpo protein, Mst1 and Mst2 protein kinases, are activated through dimerization and autophosphorylation in response to stress signaling (reviewed by de Souza and Lindsay, 2004).

However, dimerization is not the only factor to drive Hpo activation. In our study, we found that Hpo sub-cellular localization also influences its activity. While Hpo dimer exists broadly in both nucleus and cytoplasm, its nuclear form appears to be non-functional or has low activity in inhibiting processes such as cell proliferation (also see Jin et al., 2012). In contrary, mammalian Mst1/2 proteins are cleaved through caspases for nuclear translocation and their nuclear forms are actively involved in promoting apoptosis (reviewed by de Souza and Lindsay, 2004). Moreover, a fraction of Hpo dimer is associated with cortical F-actin beneath the plasma membrane. Such membrane association should have a positive impact on Hpo dimerization, since membrane-targeted Hpo showed higher efficiency of homo-dimer formation. The functional importance of its membrane association is supported by the fact that membrane-targeted Hpo showed higher kinase activity and more potent in inhibiting the transcription activity mediated by Yki and Sd proteins. Similarly, a downstream component of the Hpo pathway, Mats, is constitutively activated to restrict tissue growth and organ size when it is targeted to cell membrane (Ho et al., 2010). Based on these observations, it becomes clear that Hpo localization influences its dimer formation. Hpo dimerization and its subcellular localization work together to regulate Hpo kinase activity.

While membrane-associated proteins that include Ex, Merlin and Kibra act genetically upstream of Hpo (Hamaratoglu et al., 2006;

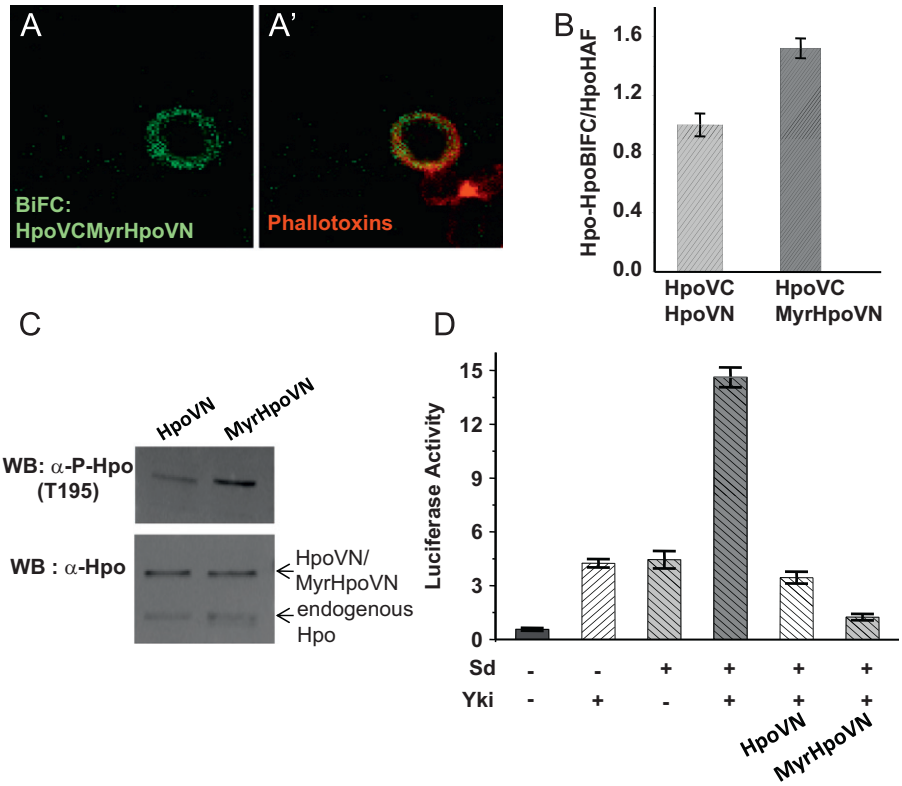

Fig. 4. *Membrane targeting facilitates Hpo dimerization and phosphorylation.* (A–A′) Myristoylation (Myr)-tagged HpoVN can interact with wild-type HpoVC and generate BiFC signal in the plasma membrane region (green) marked by cortical F-actin (red). (B) MyrHpoVN was more effective to form Hpo dimer compared with HpoVN. Through flow cytometry, Hpo dimer formation was measured by BiFC levels and the BiFC signal was normalized with HA-Hpo expression level. (C) MyrHpoVN was more phosphorylated at Thr195 than non-myristoylated Hpo. After transfection, expression of HpoVN and MyrHpoVN were examined with an Hpo antibody as well as a phospho-specific Hpo antibody (Thr195) through western blot analysis. (D) A luciferase reporter assay indicates that myristoylated Hpo was more potent to reduce Yki/Sd transcription activity than non-myristoylated Hpo. The 3x*Sd_luc* reporter is directly activated by the Yki-Sd complex. All the experiments were carried out using *Dropsophila* S2 cells.

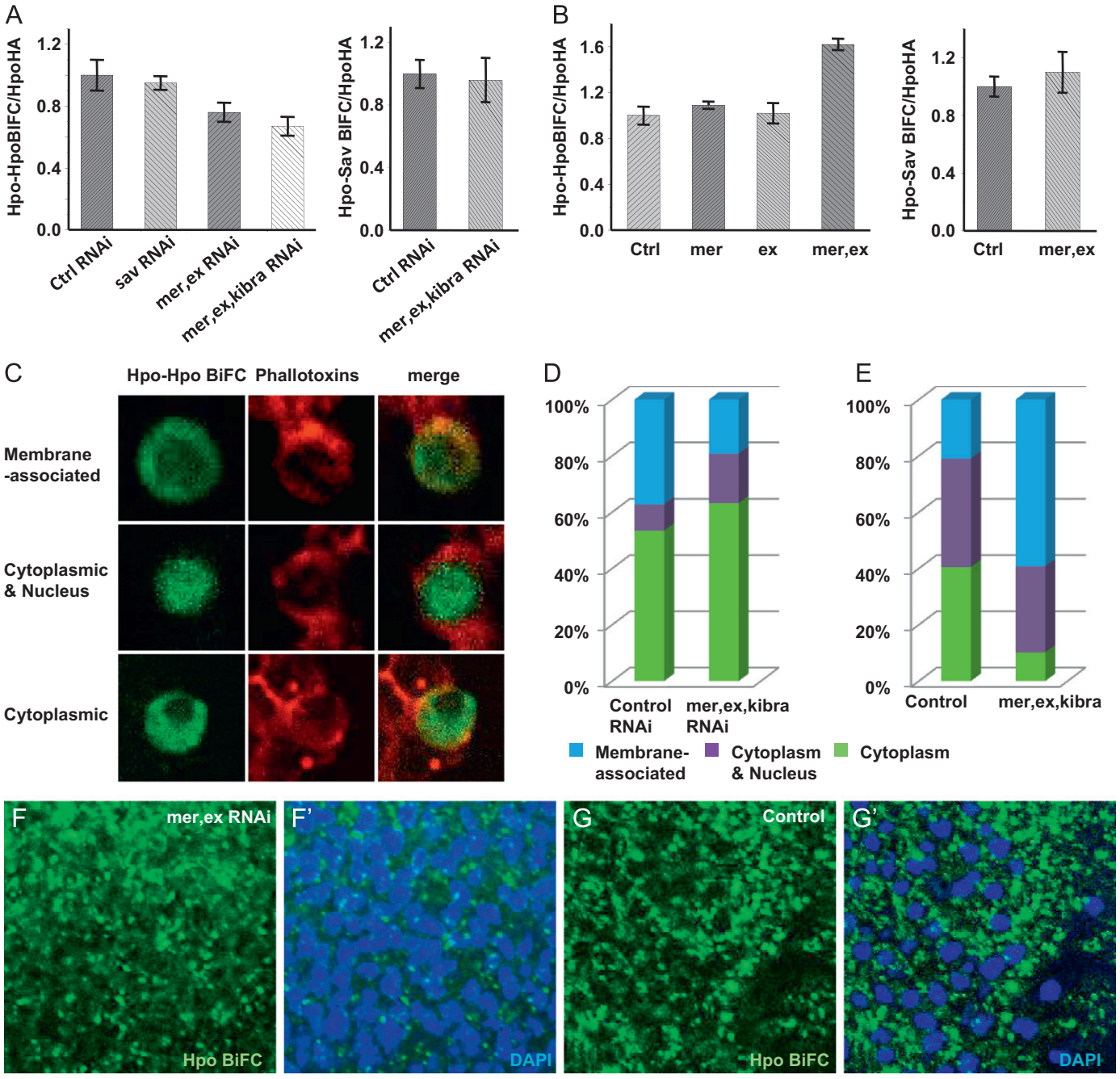

Fig. 5. *Mer, Ex and Kibra act together to regulate Hpo dimer formation and subcellular localization.* (A) Knocking-down Mer, Ex and Kibra decreased Hpo dimerization but had no obvious effect on Hpo-Sav complex formation in *Drosophila* S2 cells. BiFC signal was measured by flow cytometry and BiFC levels were normalized with HA-Hpo expression level. (B) Over-expression of Mer and Ex in S2 cells significantly increased Hpo dimer but not Hpo-Sav complex formation. (C) Classification of Hpo dimer subcellular localization patterns in S2 cells into three groups: membrane-associated, cytoplasmic/nucleus, and cytoplasmic. (D) Statistical analysis to illustrate subcellular localization patterns of Hpo homodimer in S2 cells. Knocking-down Ex, Mer and Kibra decreased membrane-associated Hpo dimer, whereas over-expression of Mer/Ex/Kibra increased Hpo dimerization efficiency in S2 cells (E). (F–F′) Knocking down *ex* and *mer* by RNAi moderately increased nucleus-associated Hpo dimer (green) in larval wing discs. (G–G′) As a control, BiFC signal (green) was found to be less associated with the nucleus in *C5-Gal4/UAS-HpoVC HpoVN* larval wing discs. Hpo dimer in the nucleus was identified by co-localization with DAPI (blue).

Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010), we found that knock-down of Ex, Mer and Kibra function reduces Hpo homo-dimerization and membrane association. Consistently, over-expression of Ex, Mer and Kibra increases Hpo homo-dimerization and membrane association. Therefore, our results validated a hypothesis that these molecules play a critical role in membrane recruitment and dimerization of the Hpo protein. Also supporting this model, Hpo is known to interact with Ex (Formstecher et al., 2005; Yu et al., 2010) as well as Kibra (Yu et al., 2010). Through cell fractionation, the amount of Hpo protein associated with cell membrane was reduced when the function of *ex*, *mer* and *kibra* was knocked down in S2 cells (Yu et al., 2010).

Recently, the Tao-1 protein kinase has been shown to directly activate Hpo via phosphorylation for growth suppression (Boggiano et al., 2011; Poon et al., 2011). However, even without utilizing additional enzymes, Hpo homo-dimerization appears to be sufficient to allow trans-phosphorylation to occur between Hpo binding partners likely due to their proximity. Membrane-associated upstream regulators, such as Mer, Ex and Kibra, help recruit Hpo to cell membrane to locally increase Hpo concentration

to promote Hpo dimerization, autophosphorylation and activation. As mammalian Mst1 kinase activity can be also increased by membrane recruitment (Praskova et al., 2004), cell membrane as well as closely located cortical F-actin appear to be an evolutionarily conserved site of action for Hpo activation for growth inhibition.

Materials and methods

DNA cloning, transgenes and drosophila genetics

A full-length *hpo* cDNA was first cloned into *pBiFC* vectors (Addgene) to make *HA-hpoVC* and *Flag-hpoVN* (also described as *hpoVC* and *hpoVN* for simplicity). *Myr-HA-hpoVC* and *Myr-Flag-hpoVN* were constructed following a strategy described in (Ho et al., 2010). *HA-sav-VC* and *Flag-savVN* were also made. These fusion constructs as well as *bFosVC* and *bJunVN* were then cloned to a *pUAST* vector with attB sequence inserted upstream of the UAS-binding sites. All *hpo* point mutations were generated by PCR-based site-directed mutagenesis. An *hpo* kinase-dead (hpo^{KD}) construct was made by changing the codon for amino acid 71Lys to Arg. Other constructs include $hpo^{M242E,I634D}$, hpo^{T189A}, hpo^{T195A}, $hpo^{T189A,T195A}$, hpo^{T189E}, hpo^{T195E}, $hpo^{T189E,T195E}$, with each of them linked to VC and VN separately. All DNA constructs were verified by sequencing. Primer information is available upon request.

To generate site-directed transgenic flies (Bischof et al., 2007; Fish et al., 2007), phiC31flies were used to make transgenic lines with insertions on the second chromosome (51C) or third chromosome (86F). Transgenic lines generated in this project include *UAS-hpoVN*, *UAS-*hpo^{KD}*VN* and *UAS-*hpo^{KD}*VC*. For *UAS-hpoVC*, *UAS-savVN*, *UAS-bfosVC* and *UAS-bjunVN*, we established transgenic lines after DNA injection done by Rainbow Transgenic Flies Inc. *C5-Gal4*, *en-Gal4* and *MS1096-Gal4* are from the Blooming Drosophila Stock Center. RNAi lines, ex^{RNAi} (#109281) and mer^{RNAi} (#7161), are from the Vienna Drosophila RNAi Center.

Cell culture, transfection, and RNAi analysis

S2R+ cell were cultured in *Drosophila* Schneider's Medium (Sigma) with 10% fetal bovine serum in 24 °C incubator. Plasmid transfection was carried out using Cellfectin II reagent (Invitrogen) according to Manufacturer's instruction. The *pAc-Gal4* construct (Addgene) was co-transfected with all the *pUAST* expression vectors. The plasmids used in this study including *pAc-HA-merlin*, *pAc-HA-expanded* (Gift from Dr. George Halder), *pAc-kibra* (Gift from Dr. Nicolas Tapon). The RNAi protocol with the S2 cells was from the Drosophila RNAi Screening Center (DRSC). The dsRNA design is from public DRSC screening database. RNAi transfection was carried out using Qiagen's Effectene reagent (Qiagen) according to manufacturer's instruction.

Flow cytometry, immunocytochemistry and microscopy

BiFC analysis assay: 40 h after transfection, S2R+ cells were resuspended following flow cytometric analysis (Beckman Coulter FC500). BiFC fluorescence was normalized for expression by analysis of HA-Hpo staining signal through flow cytometry. Each group of data was performed three times.

For general immunocytochemistry, late third-instar larval imaginal discs were fixed in PLP fixative (2.5% paraformaldehyde, 0.075M lysine, 0.25% W/V Na-periodate). In order to maintain the BiFC signal, the tissues were incubated with a primary antibody at room temperature for 2 h, followed by incubation with a secondary antibody for 2 h at room temperature. Antibodies used in this study include: anti-PH3 rabbit (1:100, Cell Signaling), anti-DE-cadherin rat (1:50), anti-HA mouse (1:100, Sigma), anti-Hpo rat (1:200, gift from Dr. Nicolas Tapon), Alexa Fluor (AF) 568, AF594 (1:200), DAPI (1:2000, Invitrogen), phallotoxins (1:200, Invitrogen). Images were collected by sequential scanning using Olympus Fluoview 300 and Olympus Fluoview 1000 Confocal Laser Scanning Microscopes.

Luciferase assay

S2R+ cells were cultured in 24-well plates. All the samples were co-transfected with 10 ng of the *copia-Renilla* luciferase reporter as a normalization control and 250 ng of 3x*Sd_luc* (Gift from Dr. Jin Jiang) firefly luciferase reporter using Cellfectin II. 50 ng of *pAc-ykiV5*, *pUAST-HA-sd* and *pAc-Gal4* were used in each well to promote the firefly Luciferase expression. 48 h incubation after transfection, cell extracts were made and the Dual-Luciferase measurements were performed using the Dual-Glo™ luciferase assay kit (Promega) according to the manufacturer's protocol.

Adult wing size analysis

Adult male fly wings were collected and mounted onto glass slides. Wing images were collected using a dissecting microscope via the SPOT Basic Imaging software. The wing area in pixels was measured using ImageJ software. The wing sizes were normalized to wild-type wings.

Western blot analysis

Western blot was detected by running 10% SDS-PAGE, following standard blotting and immune detection procedures. To detect activated Hpo protein, a phospho-Thr195 specific Hpo antibody was generated in rabbit (Genemed Synthesis, Inc.). The antibodies used in this study include: anti-Hpo rabbit (1:2000, Dr. Helen McNeill), anti-P-Thr195-Hpo rabbit (1:500), anti-Ex rabbit (1:1000, Dr. George Halder), anti-α-Tubulin (1:2000, Sigma), anti-HA mouse, anti-HA rabbit, anti-Flag rabbit, and anti-Myc mouse antibodies.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.12.017.

References

Baumgartner, R., Poernbacher, I., Buser, N., Hafen, E., Stocker, H., 2010. The WW domain protein kibra acts upsteam of hippo in *Drosophila*. Dev. Cell. 18, 309–316.

Bischof, J., Maeda, R.K., Hediger, M., Karch, F., Basler, K., 2007. An optimized transgenesis system for *drosophila* using germ-line-specific phiC31 integrases. Proc. Natl. Acad. Sci. USA 104, 3312–3317.

Boggiano, J.C., Fehon, R.G., 2012. Growth control by committee: intercellular junctions, cell polarity, and the cytoskeleton regulate hippo signaling. Dev. Cell 22, 695–702.

Boggiano, J.C., Vanderzalm, P.J., Fehon, R.G., 2011. Tao-1 phosphorylates hippo/MST kinases to regulate the Hippo–Salvador–Warts tumor suppressor pathway. Dev. Cell 21, 888–895.
de Souza, P.M., Lindsay, M.A., 2004. Mammalian sterile20-like kinase 1 and the regulation of apoptosis. Biochem. Soc. Trans. 32, 485–488.
Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S.A., Gayyed, M.F., Anders, R.A., Maitra, A., Pan, D., 2007. Elucidation of a universal size-control mechanism in *drosophila* and mammals. Cell 130, 1120–1133.
Fish, M.P., Groth, A.C., Calos, M.P., Nusse, R., 2007. Creating transgenic *drosophila* by microinjecting the site-specific phiC31 integrase mRNA and a transgene-containing donor plasmid. Nat. Protocols 2, 2325–2331.
Formstecher, E., Aresta, S., Collura, V., Hamburger, A., Meil, A., Trehin, A., Reverdy, C., Betin, V., Maire, S., Brun, C., Jacq, B., Arpin, M., Bellaiche, Y., Bellusci, S., Benaroch, P., Bornens, M., Chanet, R., Chavrier, P., Delattre, O., Doye, V., Fehon, R., Faye, G., Galli, T., Girault, J.A., Goud, B., de Gunzburg, J., Johannes, L., Junier, M.P., Mirouse, V., Mukherjee, A., Papadopoulo, D., Perez, F., Plessis, A., Rossé, C., Saule, S., Stoppa-Lyonnet, D., Vincent, A., White, M., Legrain, P., Wojcik, J., Camonis, J., Daviet, L., 2005. Protein interaction mapping: a *drosophila* case study. Genome Res. 15, 376–384.
Genevet, A., Wehr, M.C., Brain, R., Thompson, B.J., Tapon, N., 2010. Kibra is a regulator of the Salvador/Warts/Hippo signaling network. Dev. Cell 18, 300–308.
Glantschnig, H., Rodan, G.A., Reszka, A.A., 2002. Mapping of MST1 kinase sites of phosphorylation. Activation and autophosphorylation. J. Biol. Chem. 277, 42987–42996.
Goulev, Y., Fauny, J.D., Gonzalez-Marti, B., Flagiello, D., Silber, J., Zider, A., 2008. SCALLOPED interacts with YORKIE, the nuclear effector of the hippo tumor-suppressor pathway in *drosophila*. Curr. Biol. 18, 435–441.
Grusche, F.A., Richardson, H.E., Harvey, K.F., 2010. Upstream regulation of the *hippo* size control pathway. Curr. Biol. 20, R574–582.
Grzeschik, N.A., Parsons, L.M., Allott, M.L., Harvey, K.F., Richardson, H.E., 2010. Lgl, aPKC, and Crumbs regulate the Salvador/Warts/Hippo pathway through two distinct mechanisms. Curr. Biol. 20, 573–581.
Halder, G., Johnson, R.L., 2011. Hippo signaling: growth control and beyond. Development 138, 9–22.
Hamaratoglu, F., Willecke, M., Kango-Singh, M., Nolo, R., Hyun, E., Tao, C., Jafar-Nejad, H., Halder, G., 2006. The tumor-suppressor genes NF2/merlin and expanded act through hippo signaling to regulate cell proliferation and apoptosis. Nat. Cell Biol. 8, 27–36.
Hao, Y., Chun, A., Cheung, K., Rashidi, B., Yang, X., 2008. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. J. Biol. Chem. 283, 5496–5509.
Harvey, K.F., Pfleger, C.M., Hariharan, I.K., 2003. The *drosophila* Mst ortholog, *hippo*, restricts growth and cell proliferation and promotes apoptosis. Cell 114, 457–467.
Ho, L.L., Wei, X., Shimizu, T., Lai, Z.C., 2010. Mob as tumor suppressor is activated at the cell membrane to control tissue growth and organ size in *drosophila*. Dev. Biol. 337, 274–283.
Hu, C.D., 2009. Visualization of molecular interactions using bimolecular fluorescence complementation analysis: characteristics of protein fragment complementation. Chem. Soc. Rev. 38, 2876–2886.
Jia, J., Zhang, W., Wang, B., Trinko, R., Jiang, J., 2003. The *drosophila* Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis. Genes Dev. 17, 2514–2519.
Jin, Y., Dong, L., Lu, Y., Wu, W., Hao, Q., Zhou, Z., Jiang, J., Zhao, Y., Zhang, L., 2012. Dimerization and cytoplasmic localization regulate hippo kinase signaling activity in organ size control. J. Biol. Chem. 287, 5784–5796.
Justice, R.W., Zilian, O., Woods, D.F., Noll, M., Bryant, P.J., 1995. The *drosophila* tumor suppressor gene *warts* encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev. 9, 534–546.
Kerppola, T.K., 2006. Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. Nat. Protocols 1, 1278–1286.
Lai, Z.C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L.L., Li, Y., 2005. Control of cell proliferation and apoptosis by mob as tumor suppressor, *mats*. Cell 120, 675–685.
Oh, H., Irvine, K.D., 2008. In vivo regulation of Yorkie phosphorylation and localization. Development 135, 1081–1088.
Pan, D., 2010. The hippo signaling pathway in development and cancer. Dev. Cell 19, 491–505.
Pantalacci, S., Tapon, N., Léopold, P., 2003. The Salvador partner hippo promotes apoptosis and cell-cycle exit in *drosophila*. Nat. Cell Biol. 5, 921–927.
Poon, C.L., Lin, J.I., Zhang, X., Harvey, K.F., 2011. The sterile 20-like kinase Tao-1 controls tissue growth by regulating the Salvador–Warts–Hippo pathway. Dev. Cell 21, 896–906.
Praskova, M., Khoklatchev, A., Ortiz-Vega, S, Avruch, J., 2004. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. Biochem. J. 381, 453–462.
Kango-Singh, M., Nolo, R., Tao, C., Verstreken, P., Hiesinger, P.R., Bellen, H.J., Halder, G., 2002. *Shar-pei* mediates cell proliferation arrest during imaginal disc growth in *Drosophila*. Development 129, 5719–5730.
Silva, E., Tsatskis, Y., Gardano, L., Tapon, N., McNeill, H., 2006. The tumor-suppressor gene *fat* controls tissue growth upstream of *expanded* in the *hippo* signaling pathway. Curr. Biol. 16, 2081–2089.
Staley, B.K., Irvine, K.D., 2012. Hippo signaling in *drosophila*: recent advances and insights. Dev. Dyn. 241, 3–15.
Tapon, N., Harvey, K.F., Bell, D.W., Wahrer, D.C., Schiripo, T.A., Haber, D.A., Hariharan, I.K., 2002. *Salvador* promotes both cell cycle exit and apoptosis in *drosophila* and is mutated in human cancer cell lines. Cell 110, 467–478.
Udan, R.S., Kango-Singh, M., Nolo, R., Tao, C., Halder, G., 2003. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat. Cell Biol. 5, 914–920.
Wei, X., Shimizu, T., Lai, Z.-C., 2007. Mob as tumor suppressor is activated by hippo kinase in growth inhibition in *drosophila*. EMBO J. 26, 1772–1781.
Wu, S., Huang, J., Dong, J., Pan, D., 2003. *Hippo* encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with *salvador* and *warts*. Cell 114, 445–456.
Wu, S., Liu, Y., Zheng, Y., Dong, J., Pan, D., 2008. The TEAD/TEF family protein scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. Dev. Cell 14, 388–398.
Xu, T., Wang, W., Zhang, S., Stewart, R.A., Yu, W., 1995. Identifying tumor suppressors in genetic mosaics: the *drosophila lats* gene encodes a putative protein kinase. Development 121, 1053–1063.
Yu, J., Zheng, Y., Dong, J., Klusza, S., Deng, W.M., Pan, D., 2010. Kibra functions as tumor suppressor protein that regulates hippo signaling in conjunction with merlin and expanded. Dev. Cell 18, 288–299.
Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., Jiang, J., 2008. The TEAD/TEF family of transcription factor scalloped mediates hippo signaling in organ size control. Dev. Cell 14, 377–387.
Zhao, B., Tumaneng, K., Guan, K.L., 2011. The hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. Nat. Cell Biol. 13, 877–883.
Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., Zheng, P., Ye, K., Chinnaiyan, A., Halder, G., Lai, Z.-C., Guan, K.-L., 2007. Inactivation of YAP oncoprotein by the hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev. 21, 2747–2761.
Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Yu, J., Lin, J.D., Chinnaiyan, A.M., Lai, Z.-C., Guan, K.-L., 2008. TEAD mediates YAP-dependent gene induction and growth control. Genes Dev. 22, 1962–1971.