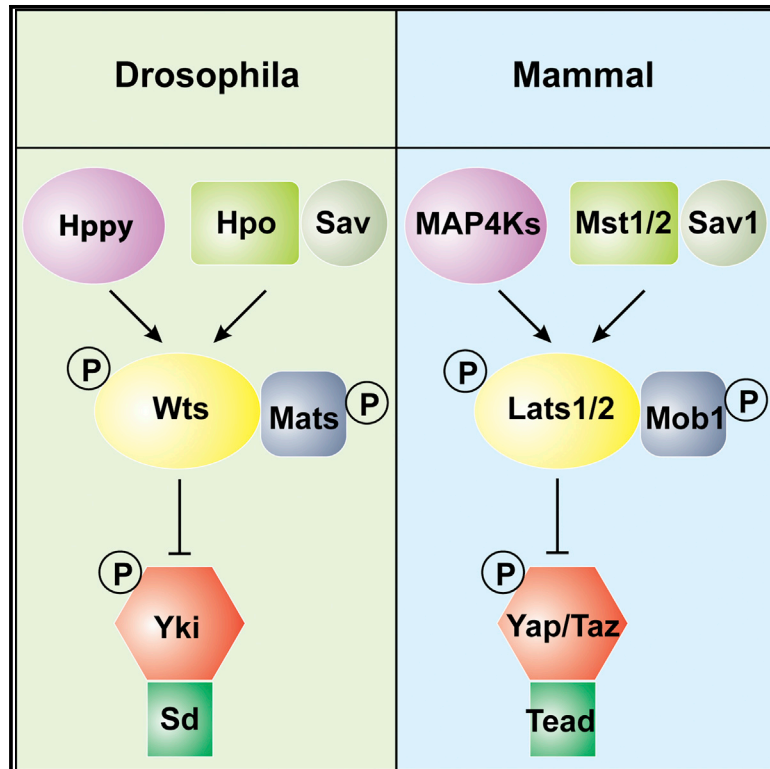


Developmental Cell

Identification of Happyhour/MAP4K as Alternative Hpo/Mst-like Kinases in the Hippo Kinase Cascade

Graphical Abstract



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In Brief

Although the Hippo pathway was named after the protein kinase Hpo, increasing evidence also suggests Hpo-independent activation of downstream signaling. Zheng et al. identify Happyhour and its mammalian counterpart as alternative Hpo-like kinases. Happyhour and Hpo function redundantly in Hippo pathway activation in response to cytoskeletal disruption or contact inhibition.

Highlights

- Hppy/MAP4K directly phosphorylates the hydrophobic motif of Wts/Lats
- *hppy* functions genetically in parallel with *hpo* and upstream of *wts*
- Hppy/MAP4K functions redundantly with Hpo/Mst in F-actin-mediated Hippo signaling
- MAP4K mediates Mst-independent contact inhibition of YAP nuclear localization



Identification of Happyhour/MAP4K as Alternative Hpo/Mst-like Kinases in the Hippo Kinase Cascade

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<http://dx.doi.org/10.1016/j.devcel.2015.08.014>

SUMMARY

In *Drosophila* and mammals, the canonical Hippo kinase cascade is mediated by Hpo/Mst acting through the intermediary kinase Wts/Lats to phosphorylate the transcriptional coactivator Yki/YAP/TAZ. Despite recent reports linking Yki/YAP/TAZ activity to the actin cytoskeleton, the underlying mechanisms are poorly understood and/or controversial. Using *Drosophila* imaginal discs as an *in vivo* model, we show that Wts, but not Hpo, is genetically indispensable for cytoskeleton-mediated subcellular localization of Yki. Through a systematic screen, we identify the Ste-20 kinase Happyhour (Hppy) and its mammalian counterpart MAP4K1/2/3/5 as an alternative kinase that phosphorylates the hydrophobic motif of Wts/Lats in a similar manner as Hpo/Mst. Consistent with their redundant function as activating kinases of Wts/Lats, combined loss of Hpo/Mst and Hppy/MAP4K abolishes cytoskeleton-mediated regulation of Yki/YAP subcellular localization, as well as YAP cytoplasmic translocation induced by contact inhibition. These Hpo/Mst-like kinases provide an expanded view of the Hippo kinase cascade in development and physiology.

INTRODUCTION

The Hippo signaling pathway was initially delineated in the model organism *Drosophila* as a critical mechanism that restricts the growth of imaginal discs (Halder and Johnson, 2011; Harvey and Tapon, 2007; Pan, 2007). This pathway is conserved in mammals and has been implicated in diverse physiological processes such as organ size control, cell fate determination, tissue regeneration, and stem cell renewal (Barry and Camargo, 2013; Harvey et al., 2013; Johnson and Halder, 2014; Pan, 2010; Zhao et al., 2008). Central to the Hippo signaling pathway is a core kinase cascade wherein the Ste-20 family kinase Hippo (Hpo, Mst1/2 in mammals), in conjunction with the scaffold protein Salvador (Sav, Sav1 in mammals), phosphorylates and activates the NDR family kinase Warts (Wts, Lats1/2 in mammals) and its

cofactor Mob as a tumor suppressor (Mats, Mob1A/B in mammals). Activated Wts/Lats then phosphorylates the transcriptional coactivator Yorkie (Yki, YAP/TAZ in mammals), leading to the phosphorylation-dependent nuclear exclusion and inactivation Yki/YAP/TAZ. In the nucleus, the Yki/YAP/TAZ coactivator normally forms a complex with its major DNA-binding partner Sd (TEAD1/2/3/4 in mammals) to promote normal tissue growth. At least in *Drosophila*, a major function of the Sd-Yki complex is to antagonize default transcriptional repression mediated by Sd and its co-repressor Tgi (Koontz et al., 2013).

There has been longstanding interest in understanding how the Hippo kinase cascade is regulated in development and physiology (Boggiano and Fehon, 2012; Enderle and McNeill, 2013; Yu and Guan, 2013). An emerging paradigm from studies in multiple model systems indicates that the Hippo kinase cascade is a signaling module that integrates multiple biological inputs such as cell polarity, adhesion, mechanical forces, and secreted ligands. As the kinase that directly phosphorylates Yki/YAP/TAZ, Wts/Lats represents a converging point of many (if not all) of these upstream inputs. For example, multiple upstream tumor suppressors of the Hippo pathway, such as Hpo/Mst, Merlin/NF2, and Fat, all act on Wts/Lats to control the activity of Yki/YAP. Hpo/Mst directly phosphorylates the hydrophobic motif of Wts/Lats (Chan et al., 2005; Wu et al., 2003; Yin et al., 2013; Yu et al., 2010). Merlin/NF2 targets Wts/Lats to cell membrane to facilitate its phosphorylation by Hpo/Mst (Yin et al., 2013). NF2 and Fat stabilize Lats protein in mammals and Wts protein in *Drosophila*, respectively (Cho et al., 2006; Li et al., 2014b).

Among the multilayers of regulation on Wts/Lats, phosphorylation represents an essential step for Wts/Lats activation. As a NDR family kinase, full activation of Wts/Lats requires two phosphorylation events: one in the activation loop and the other in the hydrophobic motif (Hergovich et al., 2006). The activation loop is autophosphorylated, while the hydrophobic motif is phosphorylated by an upstream kinase. The essentiality of the hydrophobic site has been well demonstrated as mutation of this site abolishes the kinase activity (Chan et al., 2005; Hergovich et al., 2006; Yin et al., 2013). Although Hpo/Mst is the only known kinase that directly phosphorylates the hydrophobic motif of Wts/Lats, the hydrophobic motif of Wts/Lats was still phosphorylated in Mst1/2 null mouse embryonic fibroblasts (MEFs) or Mst1/2 null hepatocellular carcinoma cells (HCCs), suggesting the existence of alternative Hpo-like kinase(s) targeting this phosphorylation site (Zhou et al., 2009). In agreement with this

hypothesis, in the many recent studies that have implicated the actin cytoskeleton as an regulator of Yki/YAP/TAZ activity, a consistent finding was that F-actin reorganization, whether triggered by cell detachment, certain GPCR ligands, or actin polymerization inhibitors, can regulate YAP(TAZ) activity in Mst1/2 null cells (Kim et al., 2013; Yu and Guan, 2013; Yu et al., 2012; Zhao et al., 2012). These studies, however, yielded inconsistent results on the requirement of Wts/Lats—while some studies suggested that cytoskeleton-mediated regulation of YAP/TAZ is Lats dependent, others argued for a Lats-independent mechanism (Dupont et al., 2011; Low et al., 2014; Zhao et al., 2012). Notably, since much of these studies were carried out in cultured mammalian cells, the specific requirement of Mst1/2 or Lats1/2 in cytoskeleton-mediated regulation of Hippo signaling in intact tissues remains to be defined.

Here we use *Drosophila* imaginal discs as an *in vivo* system to assess the requirement of Hpo and Wts in cytoskeleton-mediated regulation of Yki. We show that Wts, but not Hpo, is genetically indispensable for F-actin-mediated regulation of Yki subcellular localization. Through a systematic screen, we identify the Ste-20 kinase Happyhour (Hppy) and its mammalian counterpart MAP4K1/2/3/5 as an alternative kinase that phosphorylates the hydrophobic motif of Wts/Lats in a similar manner as Hpo/Mst. Consistent with their redundant function as activating kinases of Wts/Lats, a combined loss of Hpo/Mst and Hppy/MAP4K abolishes F-actin-mediated regulation of Yki/YAP subcellular localization, as well as nuclear exclusion of YAP induced by contact inhibition. Our identification of these Hpo/Mst-like kinases provides an expanded view of the Hippo kinase cascade and offers another entry point to understanding the regulation of Hippo signaling in development and physiology.

RESULTS

Wts, but Not Hpo, Is Genetically Indispensable for Nuclear Exclusion of Yki Induced by Disruption of F-Actin Cytoskeleton in Intact *Drosophila* Tissues

In mammalian cells, F-actin disruption by the actin polymerization inhibitor latrunculin B (LatB) activates Hippo signaling and promotes YAP phosphorylation (Kim et al., 2013; Zhao et al., 2012). LatB treatment also promotes Yki phosphorylation in *Drosophila* S2R+ cells (Yin et al., 2013) and triggers Yki nuclear exclusion as shown by subcellular fractionation (Figure S1A). Since previous studies examining cytoskeleton-mediated regulation of YAP/TAZ were based on cultured cells, we wished to investigate this question in intact tissues. *Drosophila* imaginal discs are ideal for this purpose since one can use genetic mosaics, in which the localization of Yki in mutant clones of genetically defined mutations can be compared with neighboring wild-type cells in the same tissue. As in cultured mammalian cells, treatment with the actin polymerization inhibitor latrunculin B (LatB) efficiently disrupted actin polymerization in *Drosophila* imaginal discs (Figures S1B and S1C). To test whether F-actin disruption can induce Yki cytoplasmic sequestration in a manner that bypasses Hpo, we generated GFP-positive *hpo* clones in wing imaginal discs using the MARCM technique. We focused on the wing pouch region because this area is less folded, thus facilitating the analysis of Yki localization by confocal microscopy. Without LatB treatment, Yki was largely

excluded from the nucleus in the wild-type cells but showed obvious nuclear staining in the *hpo* mutant clones (Figures 1A–1D and S1E). Consistent with previous observation in Mst1/2 null mammalian cells, LatB treatment induced nuclear exclusion of Yki in *hpo* mutant cells in the wing discs (Figures 1E, 1F, S1F, and S1G). We noted that cells located in the peripheral region of the posterior ventral quadrant of the wing pouch are most sensitive to LatB treatment, as LatB consistently induced nuclear exclusion of Yki in *hpo* mutant cells residing in this region (Figures S1D–S1G). For this reason, we focused our analysis of Yki localization on this region of the wing pouch in all the subsequent studies.

In contrast to *hpo* mutant clones, we found that LatB did not induce nuclear exclusion of Yki in *wts* clones in the wing imaginal discs (Figures 1G–1J), despite that LatB efficiently disrupted F-actin in *wts* mutant tissues (Figures S1B and S1C). Taken together, these findings suggest that Wts, but not Hpo, is genetically indispensable for nuclear exclusion of Yki induced by disruption of F-actin cytoskeleton in intact *Drosophila* tissues.

Identification of Hppy and the KHS Subfamily Kinases MAP4K1/2/3/5 as Activators of Hippo Signaling

The genetic requirement of Wts, but not Hpo, in LatB-induced nuclear exclusion of Yki in *Drosophila*, together with the dispensability of Mst1/2 in LatB-induced nuclear exclusion of YAP in mammalian cells, suggest that an unknown kinase(s) can phosphorylate and activate Wts/Lats in the absence of Hpo.

In order to identify this putative Wts/Lats kinase, we turned to *Drosophila* because it has a much smaller genome and thus less genetic redundancy than mammals. We reasoned that the hypothetical Hpo-like kinase is most likely a member of the Ste20 kinase family, which includes 12 members in *Drosophila*. We therefore conducted an overexpression screen in *Drosophila* S2R+ cells by expressing each Ste20 family member (except for the pseudokinase Stlk), followed by the examination of Yki S168 phosphorylation or Wts hydrophobic motif (T1077) phosphorylation as specific readout for the Hippo pathway (Dong et al., 2007; Yu et al., 2010). Three kinases, including Hpo, Tao-1, and Happyhour (Hppy), were found to induce Yki S168 phosphorylation (Figure 2A). RNAi of Wts greatly attenuated the increased Yki phosphorylation induced by Hpo, Tao-1, or Hppy, consistent with Wts being the kinase for Yki S168 phosphorylation (Figure 2B). Interestingly, these three kinases were the only Ste-20 family members that induced Wts hydrophobic motif (T1077) phosphorylation in our assay (Figure 2C). Hppy also promoted phosphorylation of endogenous Wts (Figure 2D). Since Mats phosphorylation by Hpo can increase its affinity toward Wts as well as Wts kinase activity (Wei et al., 2007), we also systematically assessed all Ste20-like kinases on Mats phosphorylation. Consistent with the results of Yki and Wts phosphorylation, Hpo, Tao-1, and Hppy were the only Ste-20 kinases that induced Mats phosphorylation in our assay (Figure 2E). These results suggest that Hpo, Tao-1, and Hppy all act upstream of the Wts-Mats complex to promote Yki phosphorylation.

Hpo is a canonical Hippo pathway component that directly phosphorylates Wts and Mats (Wei et al., 2007; Wu et al., 2003; Yin et al., 2013; Yu et al., 2010). Tao-1 has been implicated

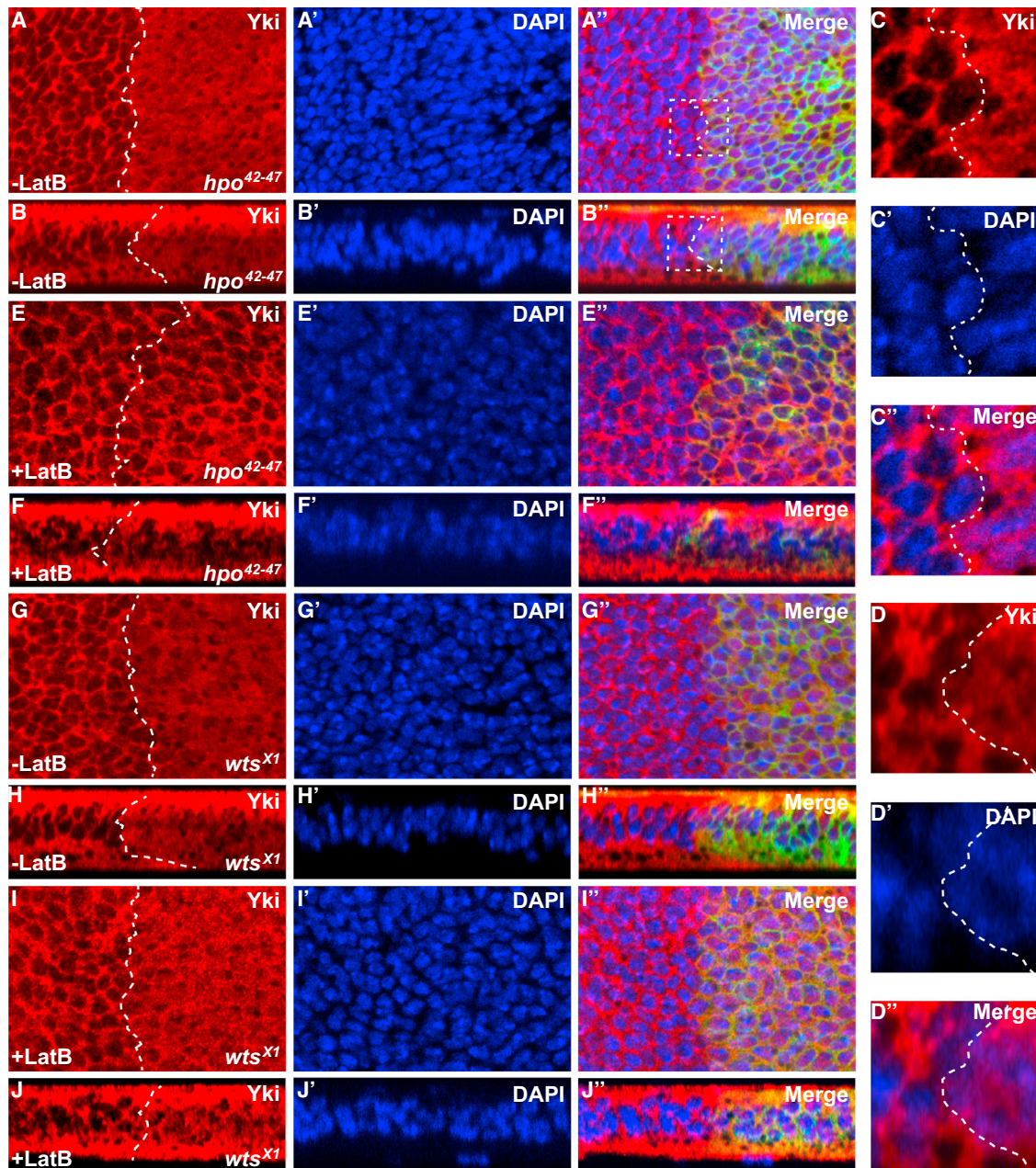


Figure 1. F-Actin Disruption Induces Nuclear Exclusion of Yki in *hpo*, but Not *wts*, Mutant Clones in *Drosophila* Wing Discs

(A–J) Third instar larval wing discs containing GFP-positive mutant clones of the indicated genotypes were cultured in Schneider medium supplemented with (+LatB) or without (–LatB) 4 μ g/ml LatB for 1 hr and then stained with Yki antibody (Red) and DAPI (Blue). Both horizontal (A, E, G, and I) and vertical (B, F, H, and J) confocal sections are shown. Enlarged views of the boxed area in (A'') and (B'') are shown in (C)–(C'') and (D)–(D''), respectively. Without LatB treatment, both *hpo* and *wts* mutant clones showed clear nuclear staining of Yki (A, B, G, and H). LatB treatment induced nuclear exclusion of Yki in *hpo* mutant clones (E and F), but not in *wts* mutant clones (I and J). See also Figure S1.

as a kinase that functions upstream of Hpo (Boggiano et al., 2011; Poon et al., 2011). In contrast, neither Hppy nor its human counterpart has been linked to the Hippo pathway. The kinase activity of Hppy is required for stimulating Hippo signaling, as a kinase dead mutant of Hppy failed to promote Wts phosphorylation (Figure S2A). The human counterpart of Hppy is the KHS subfamily, which includes four kinases, namely MAP4K1, MAP4K2, MAP4K3, and MAP4K5 (Manning et al., 2002). Of

note, MAP4K4 belongs to a different kinase subfamily called the MSN subfamily (Manning et al., 2002). In keeping with the results in *Drosophila*, MAP4K1/2/3/5 markedly induced YAP S127 and S381 phosphorylation when expressed in 293T cells (Figure 2F). Of note, although MAP4K3 was expressed at much lower levels, it induced YAP phosphorylation to a similar extent as the other KHS kinases (Figure 2F). In contrast, Mst3, a Ste20-like kinase more closely related to Mst1/2 than the KHS

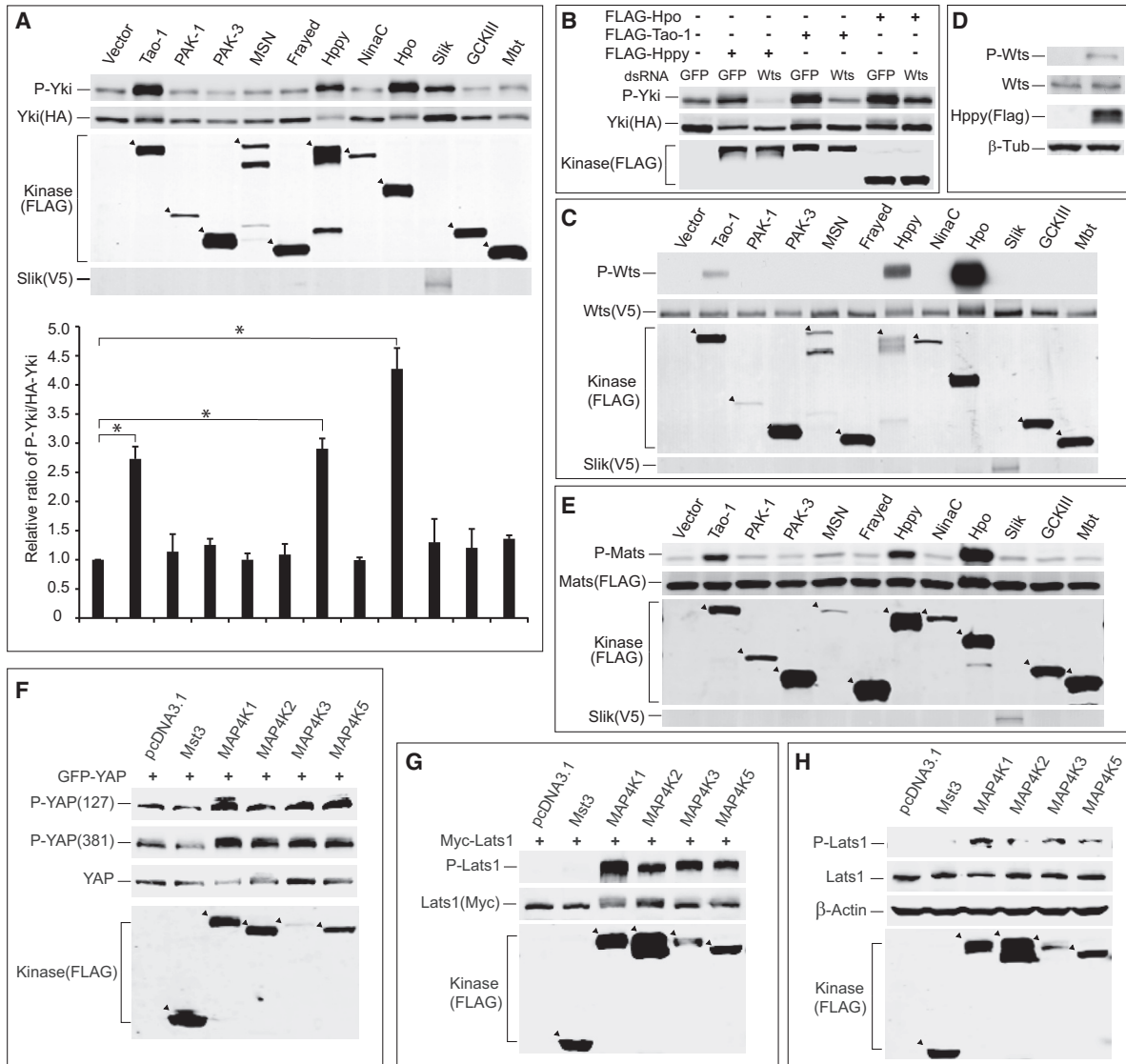


Figure 2. Identification of Hppy/MAP4K(1/2/3/5) as Activators of Hippo Signaling

(A) Eleven *Drosophila* Ste-20 kinases were individually expressed in S2R+ cells with HA-Yki. Cell lysates were probed for P-Yki S168, HA-Yki, FLAG-Ste20 kinases, or V5-Slik (upper panel). The relative ratio of P-Yki versus total Yki was quantified (lower panel; mean \pm SD, n = 3). Tao-1, Hppy, and Hpo increased the relative P-Yki/Yki ratio. Slik increased both Yki and P-Yki levels, but the relative P-Yki/Yki ratio was not increased. *p < 0.05.

(B) *wts* RNAi abolished Yki phosphorylation induced by Hppy, Tao-1, or Hpo in S2R+ cells. Cell lysates expressing the indicated constructs were probed with the indicated antibodies.

(C) Eleven *Drosophila* Ste-20 kinases were individually expressed in S2R+ cells with V5-Wts. Cell lysates were probed for P-Wts T1077, V5-Wts, FLAG-Ste20-like kinases, and V5-Slik. Tao-1, Hppy, and Hpo induced Wts phosphorylation.

(D) Hppy promoted phosphorylation of endogenous Wts in S2R+ cells. Cell lysates expressing FLAG-Hppy were probed for P-Wts T1077.

(E) Eleven *Drosophila* Ste-20 kinases were individually expressed in S2R+ cells with FLAG-Mats. Cell lysates were probed for P-Mob T12, FLAG-Mats, FLAG-Ste20 kinases, and V5-Slik. Tao-1, Hppy, and Hpo induced Mats phosphorylation.

(F) 293T cells expressing the indicated constructs were probed for the phosphorylation of co-transfected GFP-YAP at YAP^{S127} and YAP^{S381} sites.

(G) 293T cells expressing the indicated constructs were probed for the phosphorylation of co-transfected Myc-Lats1.

(H) 293T cells expressing the indicated constructs were probed for the phosphorylation of endogenous Lats1.

See also Figure S2.

kinases based on protein sequence alignment (Manning et al., 2002), had no effect on YAP phosphorylation (Figure 2F). Consistent with their effects on YAP phosphorylation, MAP4K1/2/3/5, but not Mst3, also strongly induced Lats1 phosphorylation in 293T cells (Figure 2G), and a kinase dead mutant of MAP4K1

abolished this activity (Figure S2B). MAP4K1/2/3/5 also induced the phosphorylation of endogenous Lats1 (Figure 2H). Taken together, these findings implicate Hppy and its mammalian counterpart as potential activators of Hippo signaling in both *Drosophila* and mammalian cells.

Hppy/MAP4K Directly Phosphorylate the Hydrophobic Motif of Wts/Lats Independently of Hpo/Mst

Having demonstrated that Hppy/MAP4K1/2/3/5 can stimulate hydrophobic motif phosphorylation of Wts/Lats, we sought to investigate the underlying mechanism. We first explored this in *Drosophila*. Since Hpo is the only known kinase that directly phosphorylates the hydrophobic motif of Wts, we assessed whether Hppy, like Tao-1 (Boggiano et al., 2011; Poon et al., 2011), acts through Hpo to activate Wts in S2R+ cells. Consistent with Tao-1 acting through Hpo (Boggiano et al., 2011; Poon et al., 2011), Tao-1-induced Wts phosphorylation was abolished by *hpo* RNAi (Figure 3A). In contrast, Hppy-induced Wts phosphorylation was largely insensitive to *hpo* RNAi (Figure 3A). These results suggest that Hppy likely functions in parallel with, not upstream of, Hpo to activate Wts.

Since previous studies have implicated Hppy/MAP4K as activators of the JNK and TOR pathways (Bryk et al., 2010; Findlay et al., 2007; Lam et al., 2010; Resnik-Docampo and de Celis, 2011), we examined whether these effector pathways are required for Hppy to promote Hippo signaling. Inhibition of the JNK or TOR pathway did not affect Hppy-induced Wts or Mats phosphorylation (Figures S3A–S3D), suggesting that Hppy may directly phosphorylate and activate Wts. To test whether Hppy is indeed a Wts kinase, we carried out in vitro kinase assay using bacterially purified GST-Wts fusion protein as substrate and FLAG-tagged Hppy purified from S2R+ cells by immunoprecipitation as kinase. Hppy strongly phosphorylated Wts in vitro as detected by an antibody that is specific for phosphorylated T1077 of Wts (Yu et al., 2010) (Figure 3B). FLAG-tagged Hpo also phosphorylated Wts in the same assay (Figure 3B). In contrast, a kinase dead mutant of Hppy did not phosphorylate Wts (Figure 3B). Similar in vitro kinase activity was demonstrated using FLAG-tagged MAP4K1, MA4K2, MAP4K3, or MAP4K5 immunoprecipitated from 293T cells using GST-Wts as substrate (Figure 3C; note that the hydrophobic motif is identical in *Drosophila* and human Wts/Lats). The ability of Hpo/Mst and Hppy/MAP4K to directly phosphorylate the hydrophobic motif of Wts/Lats further supports the notion that they act in parallel with each other to activate Wts/Lats.

To further corroborate that the mammalian counterpart of Hppy can function independently of Mst1/2 to stimulate Lats phosphorylation, we expressed MAP4K1, MA4K2, MAP4K3, and MAP4K5 individually in the Mst1/2 null HCCs and examined their effects on Lats1 hydrophobic motif phosphorylation. MAP4K1 strongly induced Lats1 phosphorylation in Mst1/2 null HCCs, suggesting that MAP4K1 can activate Lats1 independently of Mst1/2 (Figure 3D). MAP4K2 also induced Lats1 phosphorylation, albeit to a lesser extent (Figure 3D). Our analyses of MAP4K3 and MAP4K5 were inconclusive since we could not express these two kinases to detectable levels (Figure 3D). To circumvent this technical difficulty, we expressed MAP4K1/2/3/5 individually in *Drosophila* S2R+ cells. Each of these kinases induced Wts phosphorylation, and such phosphorylation was largely insensitive to *hpo* RNAi (Figure 3E). In contrast, *hpo* RNAi completely abolished Wts phosphorylation induced by Tao-1 or its human homolog TAO3 (Figure 3E). Taken together, these results suggest that Hppy/MAP4K functions in parallel with Hpo/Mst to directly phosphorylate the hydrophobic motif of Wts/Lats.

Hppy Directly Phosphorylates Mats Independently of Hpo

Besides its well characterized role as Wts kinase, Hpo also directly phosphorylates Mats, and this phosphorylation has been shown to further stimulate Wts activity (Wei et al., 2007). Given the strong Mats phosphorylation induced by Hppy in S2R+ cells, we examined whether Hppy also functions as a Mats kinase. An in vitro kinase assay was conducted using FLAG-tagged Hppy or Hpo immunoprecipitated from S2R+ cells as kinase and bacterially purified GST-Mats fusion protein as substrate. Both Hppy and Hpo, but not a kinase dead mutant of Hppy, strongly phosphorylated Mats in vitro as detected by an antibody that specifically recognized T12 phosphorylation of Mats (Praskova et al., 2008) (Figure 3F). Thus, Hppy and Hpo display similar kinase activity toward Mats.

If Hpo and Hppy act in parallel to directly phosphorylate Mats, we would expect Hppy-induced Mats phosphorylation to be largely independent of Hpo. Indeed, *hpo* RNAi had little effect on Hppy-induced Mats phosphorylation (Figure 3G). In contrast, Mats phosphorylation induced by Tao-1, a kinase that activates Hpo (Boggiano et al., 2011; Poon et al., 2011), was completely abolished by *hpo* RNAi (Figure 3G). Taken together, these results suggest that Hppy/MAP4K and Hpo/Mst activate Hippo signaling through a similar mechanism, by directly phosphorylating Wts and Mats.

Hppy Overexpression Restricts *Drosophila* Tissue Growth by Activating the Hippo Pathway

After demonstrating that Hppy can act in a similar manner as Hpo to promote Hippo signaling in vitro and in cultured cells, we examined whether Hppy can promote Hippo signaling in *Drosophila* imaginal discs through a similar mechanism. *hppy* was initially identified as homozygous mutant adult flies that display increased resistance to ethanol-induced sedation (Cori et al., 2009). It was later reported that Hppy overexpression can reduce wing size by promoting apoptosis (Lam et al., 2010; Resnik-Docampo and de Celis, 2011). Consistent with the previous studies, overexpression of Hppy resulted in smaller wing size when driven by the poster-compartment-specific *en-Gal4* driver (Figure 4A). Interestingly, Diap1, a well-established Hippo pathway target gene, was decreased by Hppy overexpression in the wing discs (Figures 4B and 4C). Expanded (Ex), another Hippo pathway target gene, was also decreased by Hppy overexpression (Figures 4D and 4E). Hppy overexpression by the eye-specific *GMR-Gal4* driver (*GMR>hppy*) resulted in no obvious phenotype in the eye (Figure 4F). However, when co-expressed with Wts by the *GMR-Gal4* driver, Hppy greatly synergized with Wts to decrease eye size (Figure 4F). Analysis of protein extracts from adult heads revealed increased phosphorylation of Wts and endogenous Yki in flies overexpressing Hppy plus Wts compared with flies overexpressing Wts only (Figure 4G). Thus, as in cultured cells, Hppy can activate the Hippo kinase cascade in *Drosophila* tissues.

If Hppy directly phosphorylates Wts in *Drosophila* tissues independently of Hpo, we would expect the growth suppression induced by Hppy overexpression to be dependent on Wts but not on Hpo. To test this prediction, we used the MARCM technique to generate *hpo* or *wts* mutant clones with or without concomitant Hppy overexpression and quantified clone size.

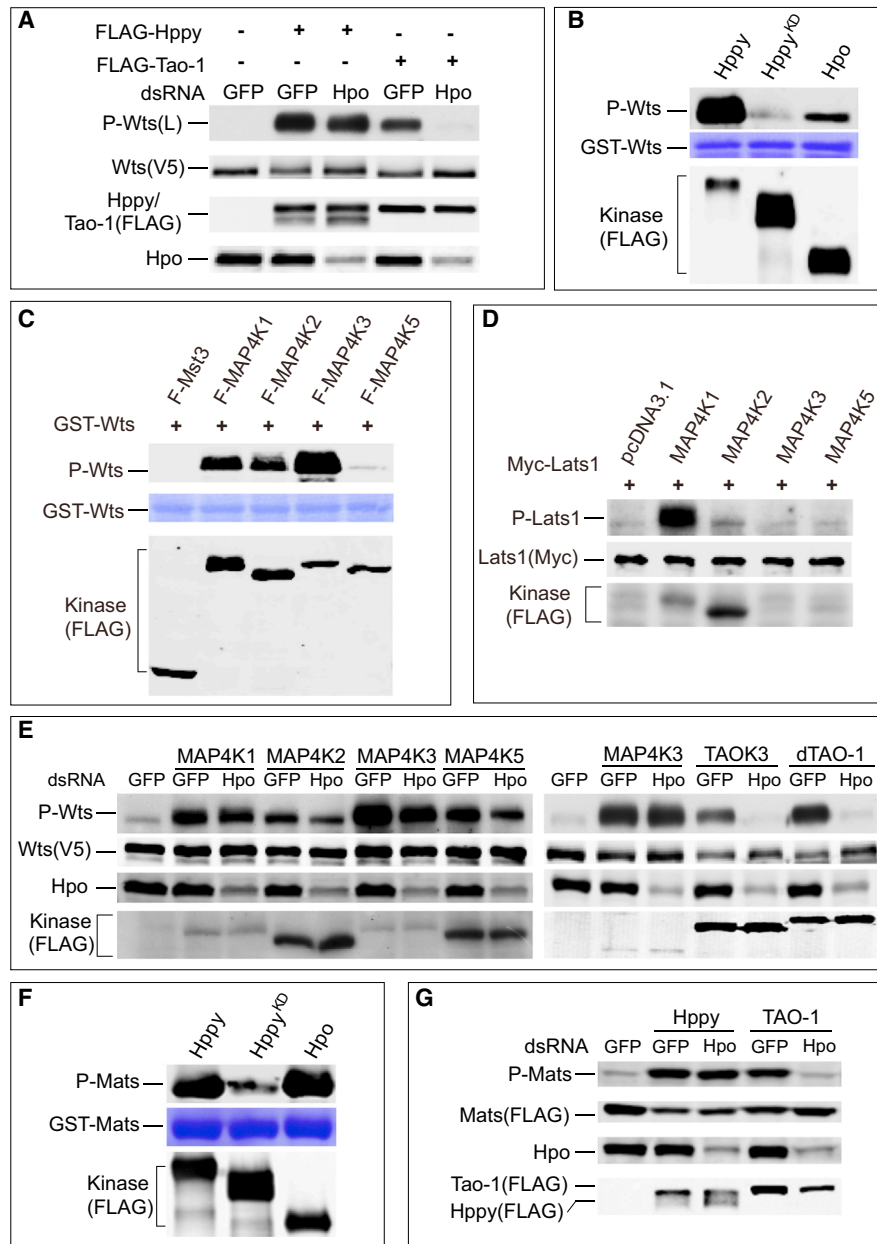


Figure 3. Hppy/MAP4K(1/2/3/5) Directly Phosphorylates the Hydrophobic Motif of Wts/Lats

(A) S2R+ cells treated with the indicated double-stranded RNAs (dsRNAs) were transfected with the indicated plasmids and probed for P-Wts, V5-Wts, FLAG-Tao-1, FLAG-Hppy, or endogenous Hpo. Note that *hpo* RNAi abolished Tao-1-induced Wts phosphorylation, but had little effect on Hppy-induced Wts phosphorylation.

(B) Hppy and Hpo phosphorylate Wts in vitro. Hppy, Hppy^{KD}, and Hpo expressed in S2R+ cells were purified by immunoprecipitation and incubated with GST-Wts, and the phosphorylation products were detected with P-Wts T1077 antibody.

(C) In vitro kinase assay of MAP4K1/2/3/5. MAP4K1/2/3/5 expressed in 293T cells was purified by immunoprecipitation and incubated with GST-Wts, and the reaction products were probed for P-Wts.

(D) MAP4K1 and MAP4K2 induce Lats1 phosphorylation in Mst1/2 null HCCs. MAP4K1, MAP4K2, MAP4K3, and MAP4K5 were individually expressed with Myc-Lats1 in Mst1/2 null HCCs, and were followed by the analysis of Lats1 phosphorylation.

(E) MAP4K1/2/3/5 and TAOK3 induces Wts phosphorylation in S2R+ cells. Note that *hpo* RNAi only slightly reduced Wts phosphorylation induced by MAP4K1/2/3/5 but abolished TAOK3-induced Wts phosphorylation.

(F) Hppy and Hpo phosphorylate GST-Mats in vitro. Hppy, Hppy^{KD}, and Hpo expressed in S2R+ cells were purified by immunoprecipitation and incubated with GST-Mats, and the phosphorylation products were detected with P-Mob T12 antibody.

(G) S2R+ cells treated with the indicated dsRNAs were transfected with the indicated plasmids and probed for P-Mob, FLAG-Mats, FLAG-Tao-1, or FLAG-Hppy. Note that *hpo* RNAi abolished Tao-1-induced Mats phosphorylation, but had little effect on Hppy-induced Mats phosphorylation.

See also Figure S3.

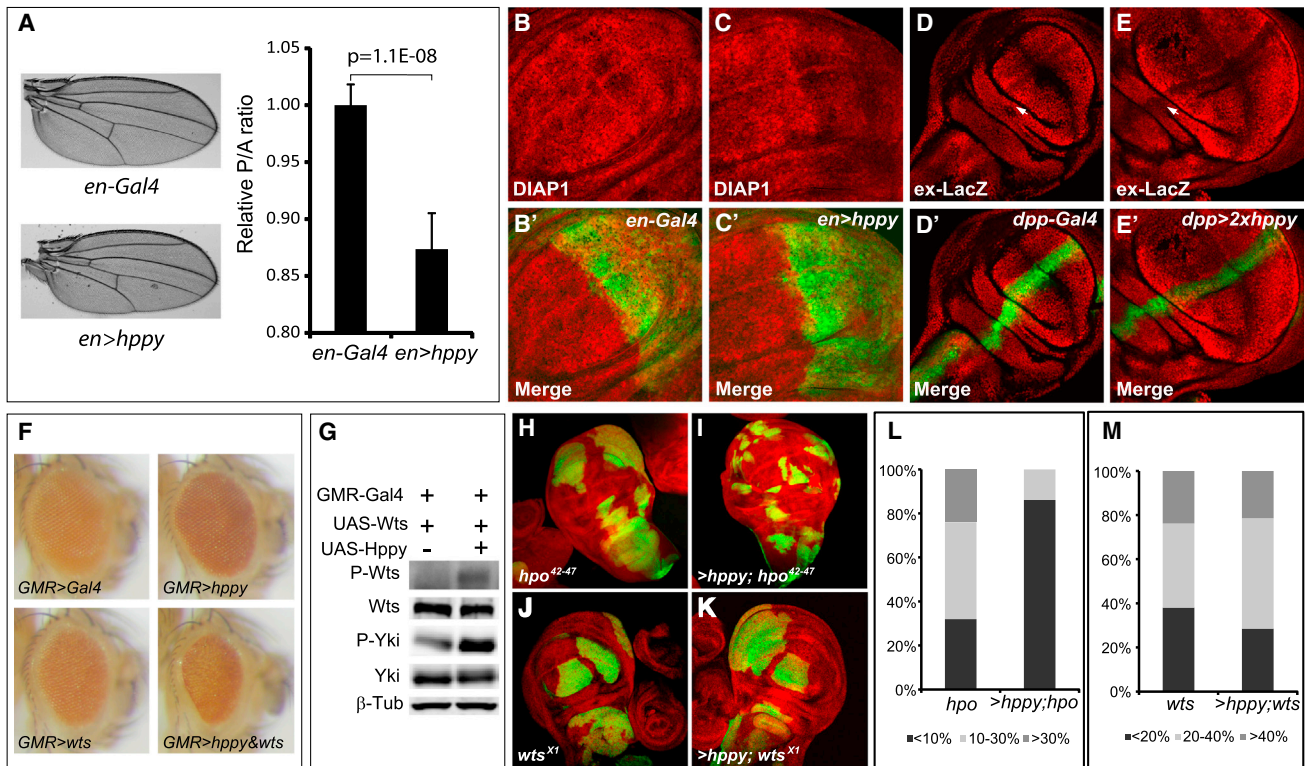


Figure 4. Hppy Overexpression Activates Hippo Signaling In Vivo and Restricts Growth of *hpo*, but Not *wts*, Mutant Clones

(A) Hppy overexpression in the posterior compartment by *en-Gal4* decreases wing size (left), and the relative ratio of posterior to anterior compartment (right; mean \pm SD, $n = 10$) is shown.

(B and C) Wing discs expressing GFP only (B) or GFP plus Hppy (C) by the *en-Gal4* driver were stained for Diap1. Note the downregulation of Diap1 expression in the poster compartment in the wing disc with Hppy overexpression (C).

(D and E) Wing discs expressing GFP only (D) or GFP plus *hppy* (E) by the *dpp-Gal4* driver were stained for the expression of *ex-lacZ*. Note the downregulation of *ex-lacZ* expression upon *hppy* overexpression (arrows). The genotypes are *dpp-Gal4;UAS-GFP* (D) and *dpp-Gal4;UAS-GFP;UAS-hppy/UAS-hppy* (E).

(F) Hppy synergizes with Wts to reduce *Drosophila* eye size. Fly eyes from control (*GMR-Gal4*), Hppy overexpression (*GMR>hppy*), Wts overexpression (*GMR>wts*), or Hppy and Wts co-overexpression (*GMR>hppy&wts*) were imaged under the same magnification. Note that the fly eye overexpressing both Hppy and Wts is smaller than eyes overexpressing either Hppy or Wts.

(G) Hppy promotes Wts and Yki phosphorylation in *Drosophila* eye tissues. Extracts made from fly heads overexpressing the indicated transgenes by the *GMR-Gal4* driver were analyzed by western blotting.

(H–K) Third instar larval wing discs containing GFP-positive clones of the indicated genotypes: *hpo* clones (H), *hpo* clones with Hppy overexpression (I), *wts* clones (J), and *wts* clones with Hppy overexpression (K). Note that Hppy overexpression decreased the size of *hpo* clones.

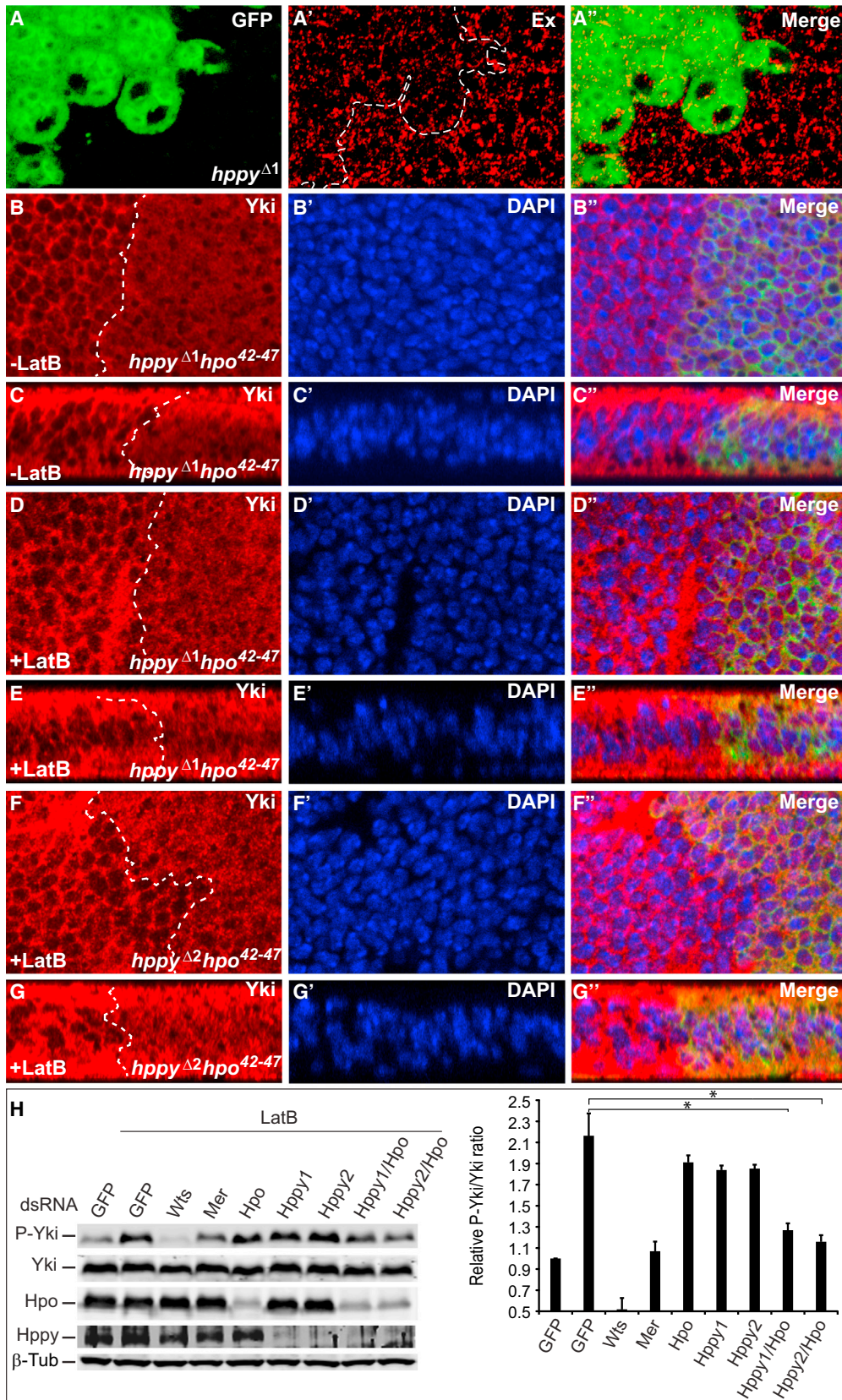
(L and M) Clone size distribution of the indicated genotypes. 20 discs were analyzed for each genotype. Clone size was quantified as the percentage of clone area relative to total wing area. Note that Hppy overexpression significantly decreased the size of *hpo*, but not *wts*, mutant clones.

The majority of *hpo* mutant clones occupied over 10% of the wing area (Figures 4H and 4L). Hppy overexpression restricted the growth of *hpo* mutant clones as the majority of clones had size less than 10% of the wing disc (Figures 4I and 4L). In contrast, Hppy had no effect on the size of *wts* mutant clones, since the size distribution of *wts* mutant clones was similar with or without Hppy overexpression (Figures 4J, 4K, and 4M). These results are consistent with our in vitro characterization implicating Hppy and Hpo as direct, independent activators of Wts.

Hppy and Hpo Play Redundant Roles in Hippo Signaling Induced by F-Actin Disruption

The similar biochemical activity of Hppy and Hpo suggests that these Ste-20 kinases may function together to regulate Hippo signaling, at least in some cellular contexts. Given our observa-

tion that the F-actin inhibitor LatB induced nuclear exclusion of Yki in the wing discs in a Wts-dependent, but Hpo-independent, manner, we considered the possibility that Hppy may play a redundant role with Hpo in this process. To test this hypothesis, we generated *hppy* mutant flies using CRISPR/Cas9 system mediated genome editing (Gratz et al., 2014; Port et al., 2014). Two guide RNAs (gRNAs) were designed and used to delete genomic DNA encompassing exons 4–7 of the *hppy* gene. Multiple independent lines were obtained and the deletions were verified by DNA sequencing and western blotting (Figure S4A). Two sequence-confirmed mutant lines, *hppy* ^{$\Delta 1$} and *hppy* ^{$\Delta 2$} , were used for subsequent analysis. Since exon 4 encodes essential part of the kinase domain, we consider these mutants as null alleles. As reported before (Resnik-Docampo and de Celis, 2011), *hppy* mutant flies are viable and fertile without noticeable morphological



(legend on next page)

malformations. Despite that, *hppy* mutant clones in pupal retina consistently showed a modest increase of the Hippo target gene *Ex* (Figure 5A), consistent with enhanced Yki activity upon loss of *hppy*.

To examine whether Hppy and Hpo play redundant roles in LatB-induced Hippo signaling, we combined *hppy*^{Δ1} or *hppy*^{Δ2} with a null allele of *hpo* to generate two independent *hppy;hpo* double mutants. Loss of *hppy* does not affect the subcellular localization of Yki, regardless of LatB treatment (Figures S4B and S4C). Similar to *hpo* mutant clones, *hppy;hpo* mutant clones showed apparent nuclear accumulation of Yki (Figures 5B and 5C). In contrast to *hpo* mutant clones, however, *hppy;hpo* double mutant clones were largely unresponsive to LatB-induced nuclear exclusion of Yki, as the majority of *hppy*^{Δ1};*hpo* and *hppy*^{Δ2};*hpo* mutant clones (17 out of 20 clones from 20 discs) still showed robust nuclear Yki accumulation after LatB treatment (Figures 5D–5G). We noticed that in occasional *hppy;hpo* clones (3 out of 20 clones from 20 discs), nuclear exclusion of Yki was still observed in the periphery region as defined in Figure S1D (Figures S4D–S4G). These results suggest that Hppy and Hpo play redundant roles in Hippo signaling induced by the disruption of F-actin.

To further corroborate our findings from imaginal discs, we examined the requirement of Hppy and Hpo in LatB-induced Hippo signaling in cultured cells. We have previously shown that LatB induced Yki phosphorylation in *Drosophila* S2R+ cells (Yin et al., 2013) (Figure 5H). In keeping with the essential role of Wts in LatB-induced nuclear exclusion of Yki in vivo, LatB-induced Yki phosphorylation was reduced to lower than the basal level by Wts RNAi, suggesting that Wts is the major Yki kinase in S2R+ cells (Figure 5H). As reported before (Yin et al., 2013), RNAi of the upstream Hippo pathway regulator Merlin also attenuated Yki phosphorylation elicited by LatB treatment (Figure 5H). In contrast, Hpo or Hppy RNAi had little effect on LatB-induced Yki phosphorylation (Figure 5H). However, co-depletion of Hppy and Hpo resulted in a marked decrease of LatB-induced Yki phosphorylation (Figure 5H). These data further support our model Hppy and Hpo play redundant roles in Hippo signaling induced by F-actin disruption.

MAP4K1/2/3/5 Is Required for Hpo/Mst-Independent Phosphorylation and Nuclear Exclusion of YAP Induced by F-Actin Disruption

Having demonstrated the requirement of Hppy and Hpo in F-actin-mediated regulation of Hippo signaling, we wished to examine whether a similar mechanism operates in mammalian cells. It was previously reported that F-actin disruption by LatB treatment still induced YAP phosphorylation in Mst1/2 null cells

(Kim et al., 2013; Zhao et al., 2012). We confirmed this finding using two different Mst1/2 null cell lines: Mst1/2 null MEF cells and HCCs (Figure S5A). As in *Drosophila*, we hypothesized that the mammalian counterpart of Hppy may contribute to Mst1/2-independent activation of Hippo signaling in the Mst1/2 null cells.

To test this hypothesis, we used RNAi to knockdown the KHS kinases MAP4K1/2/3/5 in Mst1/2 null HCCs. Knocking down each KHS kinase alone had no effect on the expression of three commonly studied YAP target genes *ctgf*, *cyr61*, and *survivin* (Figure 6A), nor did co-depletion of MAP4K1 and MAP4K3 (data not shown). We confirmed that all four KHS kinases were expressed in HCCs and efficiently knocked down by RNAi (Figure S5B). Strikingly, simultaneous knockdown of all four kinases resulted in a dramatic increase in the expression of *ctgf*, *cyr61*, and *survivin* (Figure 6A), as well as increased proliferation of the Mst1/2 null cells (Figure 6B), suggesting that these KHS kinases are required as a whole to suppress YAP activity in the Mst1/2 null cells in steady state.

The Mst1/2 null cells with MAP4K1/2/3/5 knockdown allowed us to test whether the KHS kinases contribute to LatB-induced Hippo signaling. Indeed, co-depletion of all four KHS kinases, but not the depletion of individual kinases, resulted in a marked decrease of YAP (at both S127 and S381) and Lats phosphorylation induced by LatB treatment in Mst1/2 null HCCs (Figure 6C). Consistent with these findings, co-depletion of all four KHS kinases also suppressed LatB-induced nuclear exclusion of YAP (Figures 6D and 6E). Thus, in both *Drosophila* and mammalian cells, Hppy/MAP4K contributes to Hpo/Mst-independent activation of Hippo signaling induced by F-actin disruption.

MAP4K1/2/3/5 Is Required for Hpo/Mst-Independent Cytoplasmic Sequestration of YAP Induced by High Cell Density

After establishing a functional role for Hpo/Mst and Hppy/MAP4K in LatB-induced Hippo signaling, we wished to examine the requirement of these kinases in a more physiological setting. In *Drosophila*, Hippo signaling restricts the growth of imaginal discs. Unlike its requirement in LatB-induced Yki nuclear exclusion, loss of *hppy* did not increase the size of *hpo* mutant clones (Figure S6). The interpretation of this finding is complicated by the fact that Hppy is a pleiotropic kinase involved in multiple pathways, some of which are growth promoting, such as the TOR pathway (Bryk et al., 2010; Resnik-Docampo and de Celis, 2011), and some of which are growth suppressing, such as the Hippo pathway. Thus, the size of double mutant clones does not only reflect defects in Hippo signaling. We therefore turned to mammalian cells for a more physiological context. A hallmark of

Figure 5. Hppy Functions Redundantly with Hpo to Regulate Yki in Response to F-Actin Disruption

(A) Pupal eye discs containing GFP-negative *hppy* clones were stained with *Ex* antibody (Red). The clone boundary is marked by dotted line in (A'). Note the increased *Ex* expression in *hppy* mutant clone compared with the neighboring wild-type cells.

(B–G) Third instar larval wing discs containing GFP-positive mutant clones of the indicated genotypes were cultured in Schneider medium supplemented with 4 μg/ml LatB for 1 hr and then stained with Yki antibody (Red) and DAPI (Blue). Both horizontal (B, D, and F) and vertical (C, E, and G) confocal sections are shown. The *hppy;hpo* mutant clone showed nuclear accumulation of Yki (B and C), and LatB treatment did not trigger Yki nuclear exclusion in *hppy;hpo* mutant clones (D–G). (D) and (E) and (F) and (G) represent two different *hppy* alleles recombined with *hpo*.

(H) LatB-induced Yki phosphorylation in S2R+ cells was reduced by depletion of both *hppy* and *hpo*. S2R+ cells depleted with the indicated genes were treated with 1 μg/ml LatB for 1 hr and then analyzed by western blotting (left). The relative P-Yki/Yki ratio was quantified in the graph (right; mean ± SD, n = 3). Two non-overlapping dsRNAs against *hppy* were used (Hppy1 and Hppy2), alone or in combination with Hpo RNAi. *p < 0.05.

See also Figure S4.

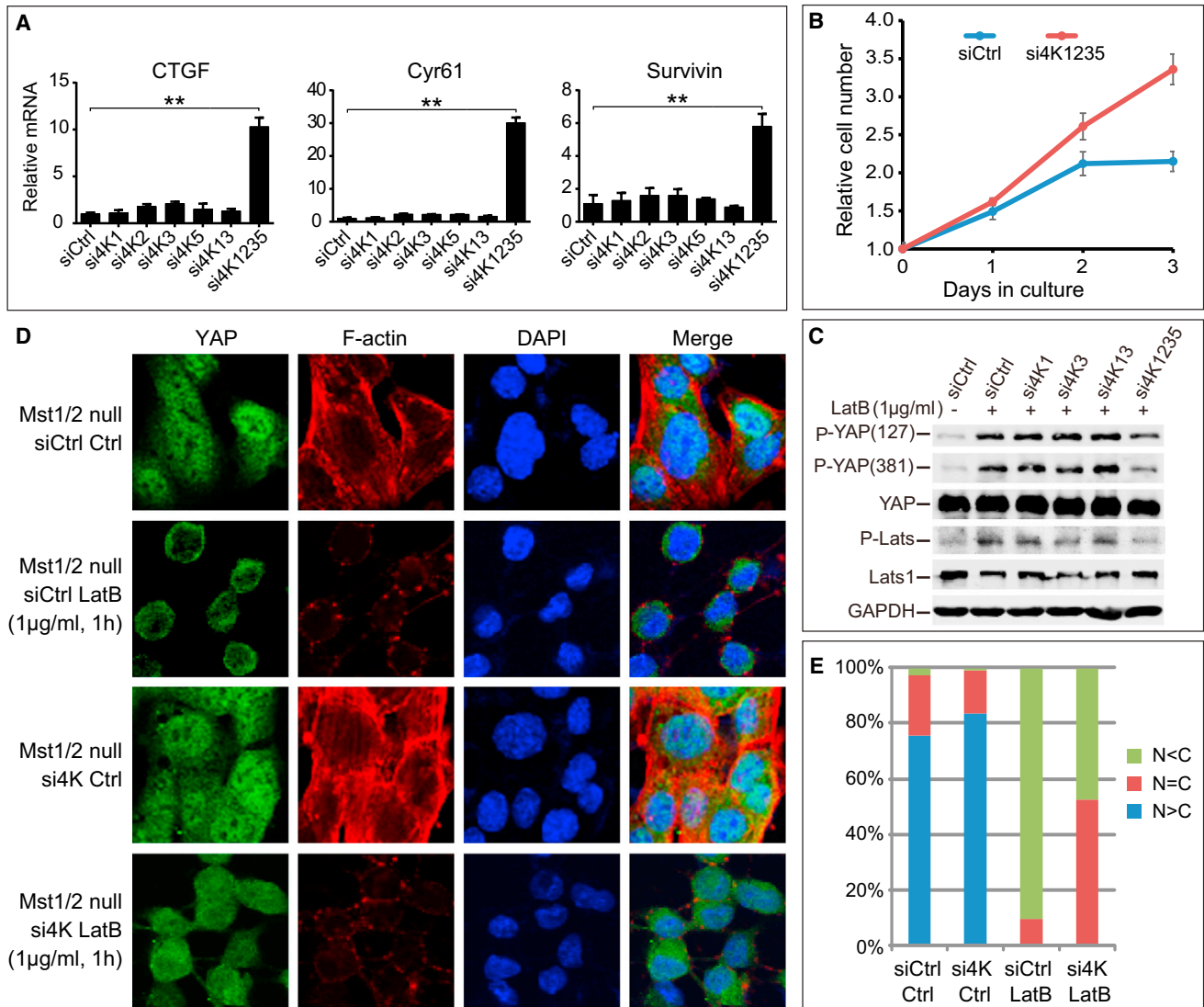


Figure 6. MAP4K1/2/3/5 Is Required for Hpo/Mst-Independent Phosphorylation and Nuclear Exclusion of YAP Induced by F-Actin Disruption

(A) Concomitant depletion of all four MAP4Ks leads to elevation in the expression levels of three Hpo pathway target genes *ctgf*, *cyr61*, and *survivin* in the Mst1/2 null HCCs. Total RNAs were prepared from HCCs transfected with the indicated siRNAs, and the relative mRNA levels of indicated genes were measured by qPCR (mean \pm SD, $n = 3$). Note the significant increase of gene expression when all four kinases were depleted. ** $p < 0.01$.

(B) Concomitant depletion of all four MAP4Ks promotes the proliferation of Mst1/2 null HCCs.

(C) Depletion of MAP4K1/2/3/5 by RNAi reduces LatB-induced YAP and Lats phosphorylation in the Mst1/2 null HCCs. HCCs cultured on fibronectin-coated surface at low cell density were treated with 1 μ g/ml LatB for 1 hr and then analyzed by western blotting. Note that LatB induced YAP and Lats phosphorylation (compare lanes 1 and 2). Both inductions were diminished when four kinases were depleted (compare lanes 2 and 6).

(D and E) Depletion of MAP4K1/2/3/5 by RNAi attenuates LatB-induced nuclear exclusion of YAP in the Mst1/2 null HCCs. HCCs transfected with control siRNAs or siRNAs against MAP4K1/2/3/5 were cultured on chamber slides coated with fibronectin at low cell density. Cell were treated with 1 μ g/ml LatB for 1 hr and then fixed and stained with YAP antibody, phalloidin for F-actin, and DAPI for cell nuclei (C). 100 cells were quantified for YAP localization, and the results were shown in (D).

See also Figure S5.

Hippo signaling in cultured mammalian cells is density-dependent subcellular localization of YAP, whereby YAP is nuclear in sparsely cultured cells and translocates to the cytoplasm in confluence cultures (Aragona et al., 2013; Varelas et al., 2010; Zhao et al., 2007). It was suggested that this density-dependent localization of YAP is due to low mechanical stress and low F-actin levels in confluent cultures, as increasing cellular F-actin levels could shuttle YAP to the nucleus at high cell density

(Aragona et al., 2013). Since high cell density still induced cytoplasmic sequestration of YAP in Mst1/2 null cells, it was suggested that Hippo signaling induced by high cell density may also involve an Mst1/2-independent mechanism (Zhou et al., 2009).

To examine whether the KHS kinases contribute to Hippo signaling induced by high cell density, we took advantage of the Mst1/2 null cells with or without MAP4K1/2/3/5 knockdown

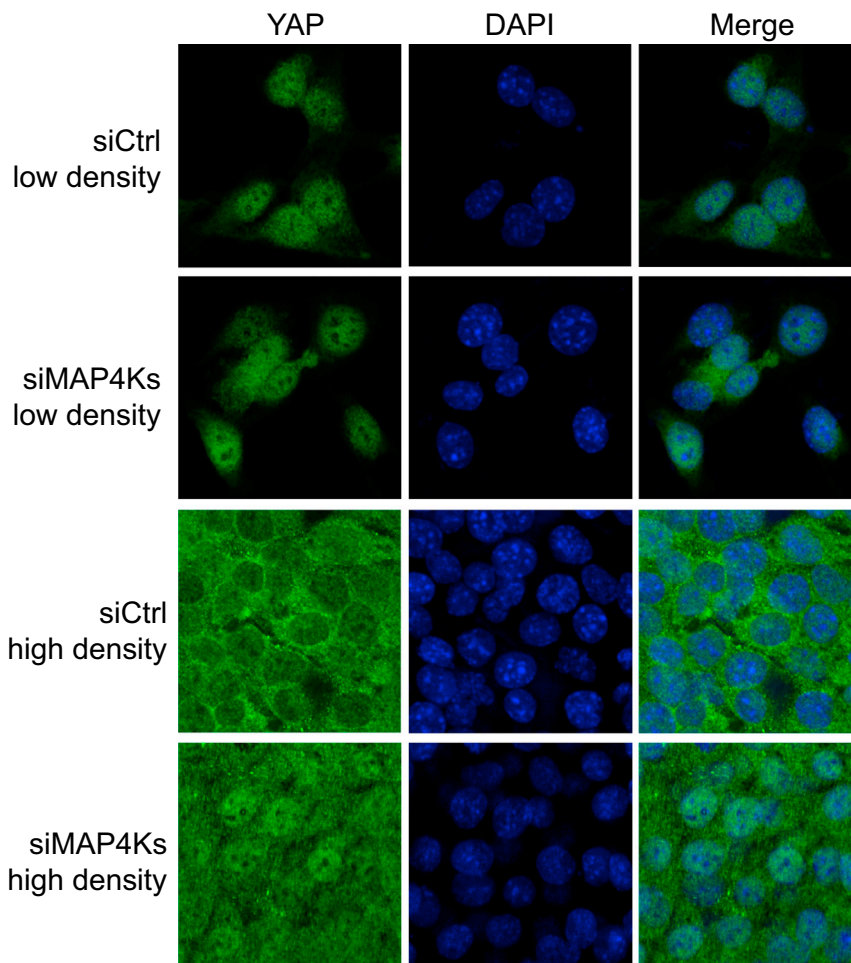


Figure 7. MAP4K1/2/3/5 Is Required for Hpo/Mst-Independent Nuclear Exclusion of YAP Induced by High Cell Density

Mst1/2 null HCCs cultured at low (upper two rows) or high (lower two rows) cell density were transfected with control or MAP4K1/2/3/5 siRNAs for 3 days, fixed, and stained with YAP antibody and DAPI. Both types of cells showed nuclear YAP signal at low density. At high density, cells with control RNAi showed cytoplasmic localization of YAP, while cells with MAP4K1/2/3/5 depletion showed more nuclear localization of YAP. See also Figure S6.

described above by culturing them at low or high density. Consistent with previous suggestion of an Mst1/2-independent mechanism, Mst1/2 null HCCs showed nuclear YAP localization at low cell density and cytoplasmic translocation at high cell density (Figure 7). This nuclear-to-cytoplasmic translocation was greatly diminished in Mst1/2 null cells with MAP4K1/2/3/5 knockdown (Figure 7). Taken together, these results suggest that Hppy/MAP4K contributes to Hpo/Mst-independent activation of Hippo signaling induced by both F-actin disruption and high cell density.

DISCUSSION

Our understanding of the core kinase cascade of the Hippo pathway has been aided by multiple lines of investigation. First, genetic screens for tumor suppressors using mosaic flies have identified Hpo, Sav, Wts, and Mats as main constituents of the core kinase cassette (Harvey et al., 2003; Justice et al., 1995; Kango-Singh et al., 2002; Lai et al., 2005; Pantalacci et al., 2003; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003; Xu et al., 1995). Second, biochemical studies of the activation mechanism of NDR family kinases, which include Lats1/2 and NDR1/2, demonstrate the importance of regulatory phosphorylation sites on the activation loop and the hydrophobic motif

(Hergovich et al., 2006). The realization that the hydrophobic motif of Wts is phosphorylated by Hpo provides a fitting molecular explanation for the linear genetic pathway uncovered by in vivo studies (Chan et al., 2005; Hergovich et al., 2006; Wu et al., 2003; Yu et al., 2010). The simplicity of this linear pathway begun to be challenged based on the observation that Mst1/2 null cells still showed high levels of Lats phosphorylation on the hydrophobic motif (Zhou et al., 2009). Indeed, in many subsequent reports, various signals have been reported to still regulate YAP/TAZ activity in Mst1/2 null cells (Kim et al., 2013; Yu et al., 2012; Zhao et al., 2012; Zhou et al., 2009). While semantically these observations were implied to support the existence of “Mst1/2-independent” mechanisms, the molecular underpinning of this phenomenon

has been elusive. It was also unclear whether this represents a mammalian-specific phenomenon as there has been no evidence to date that a similar mechanism operates in *Drosophila*.

Our current study addresses these issues in several significant ways. We provide definitive evidence supporting an alternative Hpo-independent mechanism of Hippo pathway activation in *Drosophila* by demonstrating the genetic requirement of Wts, but not Hpo, in LatB-induced nuclear exclusion of Yki. Through a systematic screen, we identify the Ste-20 family kinase Hppy/MAP4K as a plausible molecular explanation for Mst1/2-independent regulation of Hippo signaling. Not only does Hppy/MAP4K directly phosphorylate the hydrophobic motif of Wts/Lats in vitro and in cell cultures, but loss of Hppy/MAP4K also abolishes LatB-induced Yki/YAP cytoplasmic translocation in Hpo/Mst null cells in both *Drosophila* tissues and mammalian cell cultures. Our findings support the view that Hpo/Mst and Hppy/MAP4K act as redundant kinases targeting the hydrophobic motif of Wts/Lats. We note that our analysis of Hpo/Mst and Hppy/MAP4K in F-actin-mediated Hippo signaling was largely based on LatB treatment. Thus, it remains to be determined how these kinases cooperate with each other in a more physiological setting of cytoskeleton modulation. Nevertheless, the fact that MAP4K mediates Mst-independent regulation of YAP target gene expression and contact inhibition of YAP nuclear

localization suggests that these kinases co-regulate Wts/Lats in multiple contexts beyond LatB-induced F-actin disruption. Since the hydrophobic motif of NDR1/2 can be phosphorylated by both Mst1 and Mst3 (Hergovich et al., 2009; Stegert et al., 2005), we suggest that phosphorylation of hydrophobic motif by multiple Ste-20 kinases may be a common feature of the NDR family kinases. We note that two other kinases, CK2 and MSN/MAP4K4, were recently reported to promote Wts/Lats activity toward Yki. However, neither kinase was shown to directly phosphorylate the hydrophobic motif of Wts/Lats (Hu et al., 2014; Li et al., 2014a). Furthermore, although MSN was shown to promote Yki phosphorylation when Wts was co-expressed in S2 cells, MSN alone did not affect Yki phosphorylation (Li et al., 2014a), as we observed (Figure 2A). Thus, the mechanisms by which these kinases promote Hippo signaling remain to be determined.

Recent studies have implicated cellular mechanical force as a regulator of Yki/YAP/TAZ activity (Aragona et al., 2013; Dupont et al., 2011; Rauskolb et al., 2014; Wada et al., 2011). Reorganization of F-actin cytoskeleton has been suggested as the common mediator of mechanical forces arising from cell-cell and cell-matrix interactions (Gaspar and Tapon, 2014; Halder et al., 2012). However, the underlying mechanism by which F-actin controls Yki/YAP/TAZ activity remains poorly understood and/or controversial. While some studies suggested that cytoskeleton-mediated regulation of YAP/TAZ is independent of the Hippo kinase cascade (Aragona et al., 2013; Dupont et al., 2011), others suggested that it requires the Hippo kinase cascade (Wada et al., 2011; Yu et al., 2012; Zhao et al., 2012). Our observation that LatB-induced Yki cytoplasmic localization is Wts dependent is more consistent with a Hippo signaling-dependent mechanism. An important modification brought by our current study is that the canonical Hippo kinase cascade should be expanded to include Hppy/MAP4K at the level of Hpo. This expanded Hippo kinase cascade may also include NDR1/2 at the level of Lats1/2, given the recent report of NDR1/2 as Lats1/2-like kinases capable of phosphorylating YAP (Zhang et al., 2015).

Finally, we suggest that the Hippo-signaling-dependent and -independent regulation of YAP/TAZ by F-actin may be potentially reconciled with each other. A major discrepancy between the two models came from the analysis of mutant YAP/TAZ that lacks all the Lats phosphorylation sites (YAP^{5SA} or TAZ^{4SA}) (Zhao et al., 2007). We note, however, that a different readout was used to assay the regulation of these YAP/TAZ mutants in the different studies (Dupont et al., 2011; Zhao et al., 2012). A luciferase reporter assay was used to show that the transcriptional activity of YAP^{5SA} or TAZ^{4SA} still responded to F-actin reorganization (Dupont et al., 2011), whereas subcellular localization was used to show that YAP^{5SA} no longer responded to cytoplasmic localization of YAP induced by F-actin disruption (Zhao et al., 2012). These results may reflect the functionality of different subcellular pools of F-actin—inasmuch as YAP/TAZ localization is regulated by F-actin through the Hippo pathway, F-actin may also play a separate role in regulating the transcriptional activity of YAP/TAZ in the nucleus, especially given the increasing appreciation of a more direct role of nuclear F-actin in transcriptional regulation (Miralles and Visa, 2006; Miyamoto and Gurdon, 2013; Olson and Nordheim, 2010).

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies

HA-Yki, V5-Wts, FLAG-Tao-1, FLAG-Pak1, FLAG-Pak3, GFP-YAP, and Myc-Lats1 expression constructs have been described previously (Boggiano et al., 2011; Dong et al., 2007; Duan et al., 2012; Huang et al., 2005). FLAG-Hpo was generated from the Myc-Hpo construct described before (Wu et al., 2003). FLAG-Hppy (RH10407), FLAG-Msn (LD34191), FLAG-Frayed (RE53265), FLAG-NinaC (GH10824), FLAG-GckIII (RE38276), FLAG-Mbt (LD47563), and FLAG-Mats (LD47553) expression plasmids were generated based on cDNA clones obtained from the Drosophila Genomics Resource Center (DGRC). V5-Slik was made from the cDNA clone LP09626 with the original mutations in the cDNA clone corrected by site-directed mutagenesis. FLAG-Mst3, FLAG-MAP4K1, FLAG-MA4K2, FLAG-MAP4K3, and FLAG-MAP4K5 were obtained from Addgene. FLAG-Hppy^{KD} and FLAG-MAP4K1^{KD} were generated by changing K55 to E and K46 to E using site-directed mutagenesis, respectively.

Primary antibody used in this study include the following: FLAG and HA (Sigma-Aldrich); Myc (Calbiochem); V5 (Invitrogen); YAP (Novus; for immunostaining); Yap (Sigma-Aldrich; for western blotting); Lats1, P-YAP S127, and P-YAP S381 (Cell Signaling Technology); Hpo and Wts (Wu et al., 2003); Yki and P-Yki (Dong et al., 2007); P-Wts T1077 (Yu et al., 2010); P-Mob T12 (Praskova et al., 2008); and β -tubulin and Lamin A/C (Developmental Biology Hybridoma Bank). Rabbit anti-Hppy antibody was raised against the peptide KIGSGTYGDVYKAKRIQS.

Drosophila and Mammalian Cell Culture

Drosophila S2R+ cells were cultured in Schneider's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics at 25°C. 293T, MEF, and HCC cells were maintained at 37°C in DMEM medium supplemented with 10% FBS and antibiotics (Invitrogen). Transfection, immunoprecipitation, and western blotting were carried out as described previously (Yu et al., 2010). RNAi in S2R+ cells was carried out using the bathing protocol obtained from DRSC (<http://www.flyrnai.org/DRSC-PRR.html>). For chemical treatment, HCCs were incubated with Latrunculin B (1 μ g/ml, obtained from Sigma-Aldrich) according to Zhao et al. (2012). Ethanol (0.1%) was used as vehicle control.

Drosophila Genetics

To generate a mutation in *hppy*, two gRNAs were used to delete exons 4–7 of *hppy* by CRISPR/Cas9 according to a described protocol (Port et al., 2014). To facilitate the identification of targeted deletions, a strategy described by Gratz et al. (2014) was adopted and the two gRNAs were designed to flank a P-element insertion *P[GawB]/hppy^{NP3139}* whose removal would generate a white eye phenotype. The two CRISPR target sites (GGCTCCTGAGGTGG CAGCCG and GCGACCCACCATCATATCGG) were selected by CRISPR Optimal Target Finder (<http://tools.flycrispr.molbio.wisc.edu/targetFinder/>). Mutants were first selected on the basis of eye color and then further verified by DNA sequencing. Representative genotypes used in this study are as follows.

GFP+ *hpo*⁻ clones:
 UAS-GFP hs-Flp; tub-Gal80 FRT42D/FRT42 *hpo*⁴²⁻⁴⁷; tub-Gal4.
 GFP+ *hpo*⁻ clones overexpressing Hppy:
 UAS-GFP hs-Flp/UAS-hppy; tub-Gal80 FRT42D/FRT42 *hpo*⁴²⁻⁴⁷; tub-Gal4.
 GFP+ *wts*⁻ clones:
 UAS-GFP hs-Flp; tub-Gal4; tub-Gal80 FRT82B/FRT82B *wts*^{x1}.
 GFP+ *wts*⁻ clones overexpressing Hppy:
 UAS-GFP hs-Flp/UAS-hppy; tub-Gal4; tub-Gal80 FRT42D/FRT42D *wts*^{x1}.
 GFP+ *hppy*⁻ *hpo*⁻ clones:
 UAS-GFP hs-Flp; tub-Gal80 FRT42D/FRT42 *hppy* ^{Δ 1} *hpo*⁴²⁻⁴⁷; tub-Gal4.
 UAS-GFP hs-Flp; tub-Gal80 FRT42D/FRT42 *hppy* ^{Δ 2} *hpo*⁴²⁻⁴⁷; tub-Gal4.

LatB Treatment of *Drosophila* Imaginal Discs

Drosophila wing imaginal discs were dissected in Schneider medium and then cultured in Schneider medium supplemented with 4 μ g/ml LatB for 1 hr on a rocking platform. Ethanol (0.4%) was used as vehicle control. The discs

were then fixed in 4% paraformaldehyde (PFA) and incubated with anti-Yki (1:500) for overnight at 4°C.

Nuclear and Cytoplasmic Fractionation

Fractionation of proteins into nuclear and cytoplasmic fractions was carried out using the Rapid Efficient and Practical (REAP) protocol as described by Suzuki et al. (2010).

In Vitro Kinase Assay

GST-Wts was described previously (Yin et al., 2013). To generate GST-Mats, the ORF of *mats* was PCR amplified from the FLAG-Mats construct and inserted into pGEX-6p-1 and expressed in *BL21-CodonPlus (DE3)-RIPL* cells, and the products were purified. In vitro kinase assay was carried out as previously described, and phosphorylated products were detected using P-Wts T1077 or P-Mob T12 antibody (Yin et al., 2013).

siRNA Transfection and Cell Proliferation Assay

Cells were cultured on Lab-Tek II Chamber Slide (Thermo Scientific) to 80%–85% confluent, and then small interfering RNA (siRNA) transfection was performed using Lipofectamine RNAiMAX. Cells were processed 3 days after transfection. SMARTpool siRNA oligonucleotides toward mouse MAP4K1, MAP4K2, MAP4K3, MAP4K5, and Non-Targeting siRNA Pool #2 (control siRNA) were purchased from GE Dharmacon. The relative cell proliferation rate was calculated on the basis of cell numbers counted in a hemacytometer.

Reverse Transcription and Real-Time PCR

RNA samples (1 µg) were reverse transcribed to cDNA using iScript reverse transcriptase (Bio-Rad). cDNA was then diluted and used for quantification by real-time PCR, which was performed using iQ SYBR Green supermix (Bio-Rad) and the CFX96 real-time system (Bio-Rad). All values were normalized to that of GAPDH, and the normalized values of siCtrl were set as 1. Error bars represent the standard deviations of three independent experiments.

Immunofluorescence Staining

Cells were fixed with 4% PFA for 15 min and then permeabilized with 0.3% Triton X-100. After blocking in 5% goat serum for 1 hr, slides were incubated with anti-YAP antibody (Novus, 1:300 dilution) diluted in 5% goat serum for overnight at 4°C. After washing with PBS, slides were incubated with Alexa Fluor 488-conjugated secondary antibodies (1:500 dilution) for 1 hr, washed, and then incubated with Alexa Fluor 568-conjugated phalloidin for 40 min. The slides were incubated with DAPI (1:1,000 dilution) for 5 min, then washed and mounted.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.devcel.2015.08.014>.

AUTHOR CONTRIBUTIONS

Y.Z. conceived the project; Y.Z. and W.W. designed experiments; Y.Z., W.W., B.L., H.D., and E.U. performed experiments; D.P. supervised the whole study; and Y.Z. and D.P. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Richard Fehon for the pUAST-FLAG-Tao-1 construct and Anti-Ex antibody, Dr. Elizabeth Chen for pUAST-FLAG-PAK1 and pUAST-FLAG-PAK3 constructs, Dr. Joseph Avruch for Mst1/2 null HCCs and P-Mob T12 antibody, and Dr. Shian Wu for advice on CRISPR. We also thank the Bloomington Drosophila Stock Center, the Kyoto Drosophila Genetic Resource Center, and the Drosophila Genomics Resource Center for fly strains and reagents. This study was supported in part by grants from the NIH (EY015708). D.P. is an investigator of the Howard Hughes Medical Institute.

Received: May 1, 2015

Revised: July 14, 2015

Accepted: August 20, 2015

Published: September 10, 2015

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