Supplemental Figures

Figure S1. F-actin disruption induces nuclear exclusion of Yki in *hpo* mutant clones in *Drosophila* wing discs, related to Figure 1.

(A) Nuclear/cytoplasmic fractionation of S2R+ cells expressing HA-tagged Yki showing increased cytoplasmic Yki upon LatB treatment.

(B-C) LatB efficiently disrupts F-actin in *Drosophila* wing discs. Wing discs containing GFP-negative *wts* mutant clones were cultured in Schneider medium without (B) or with (C) 4μg/mL LatB for 1 hr, and stained with phalloidin.

(D) A schematic diagram showing the wing area sensitive to LatB-induced nuclear exclusion of Yki in *hpo* mutant clones. The big oval shape includes the whole wing pouch and the small oval shape includes the central region of the pouch. Two straight lines divide the wing pouch into dorsal/ventral and anterior/posterior compartments. The yellow region represents the peripheral region of the posterior/ventral quadrant of the wing pouch which consistently shows Yki nuclear exclusion after LatB treatment, while the blue region represents the less sensitive center area which sometimes still shows nuclear localization of Yki after LatB treatment. The yellow region was used for all the subsequent analysis of Yki localization in response to LatB treatment.

(E-H) LatB treatment induces nuclear exclusion of Yki in *hpo* clones. Wing discs containing GFP-positive *hpo* clones were treated as in (C) and stained with anti-Yki antibody and DAPI. The wing discs are demarcated as in (D). *hpo* mutant clones are outlined by yellow lines. Note the robust nuclear staining of Yki in *hpo* mutant clones in the absence of LatB treatment (E). LatB treatment consistently induced Yki cytoplasmic localization in *hpo* mutant cells located in the peripheral area (yellow arrow in F and G). In the more central area, some discs showed Yki cytoplasmic localization (blue arrow in F) but others still showed nuclear Yki localization (blue arrow in G). A vertical confocal section taken along the dash line in (G) confirmed more nuclear Yki localization in the central area versus the cytoplasmic Yki localization in the peripheral area (compare blue and yellow arrows in H). A *hpo* mutant clone located in the anterior compartment still showed nuclear Yki localization after LatB treatment (marked by an asterisk in F), indicating that area is less sensitive to LatB-induced nuclear exclusion of Yki.

Figure S1

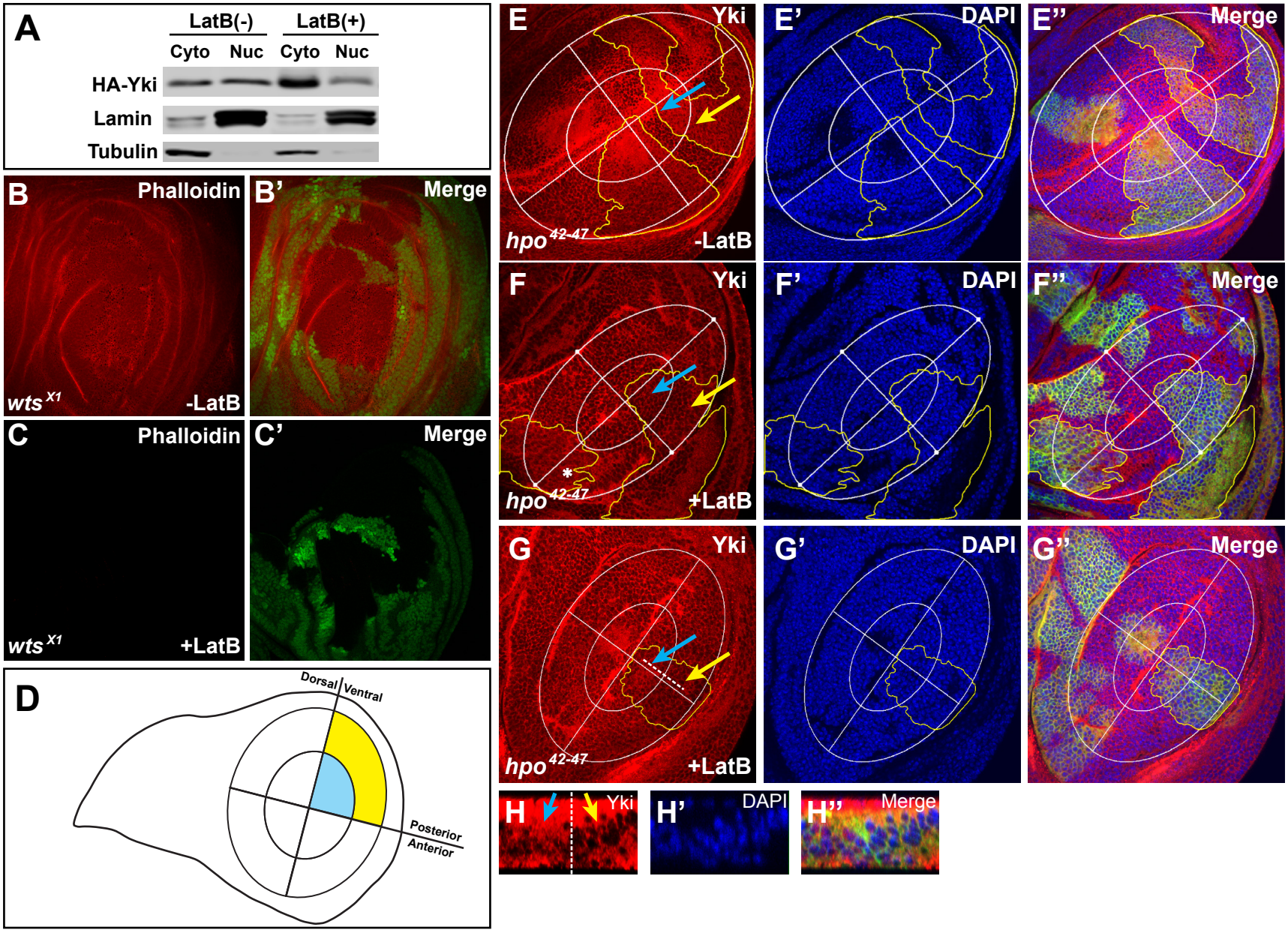

A
LatB(-)
LatB(+)
Cyto
Nuc
Cyto
Nuc
HA-Yki
Lamin
Tubulin
B
Phalloidin
wts X1
-LatB
B'
Merge
C
Phalloidin
wts X1
+LatB
C'
Merge
D
Dorsal
Ventral
Posterior
Anterior
E
Yki
hpo 42-47
-LatB
E'
DAPI
E''
Merge
F
Yki
hpo 42-47
+LatB
F'
DAPI
F''
Merge
G
Yki
hpo 42-47
+LatB
G'
DAPI
G''
Merge
H
Yki
H'
DAPI
H''
Merge

Figure S2. Hppy/MAP4K requires kinase activity to activate Hippo signaling, related to Figure 2.

(A) The kinase activity of Hppy is required to promote Wts phosphorylation in S2R+ cells. S2R+ cells transfected with the indicated plasmids were analyzed by western blotting. HppyN contains the N-terminal half including the kinase domain, while HppyC contains the remaining half of Hppy. $Hppy^{KD}$ is a kinase dead mutant of Hppy containing a K55E mutation.

(B) MAP4K1, but not the kinase dead mutant $MAP4K1^{KD}$ containing a K46E mutation, promotes YAP^{S127} and YAP^{S381} phosphorylation.

Figure S2

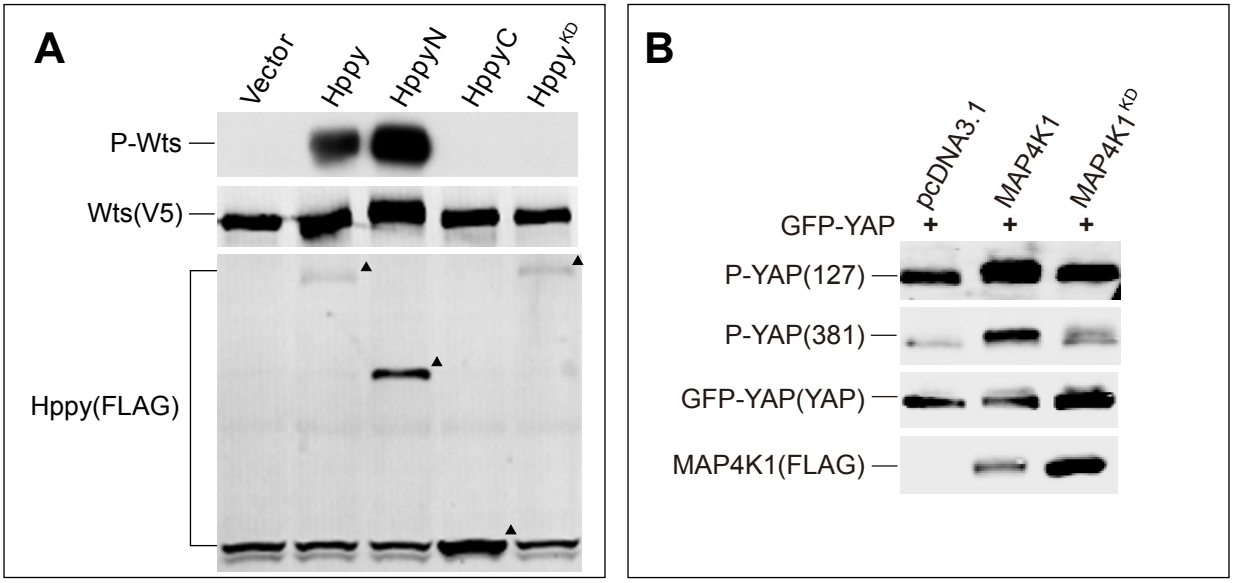

A
Vector
Hppy
HppyN
HppyC
Hppy KD
P-Wts
Wts(V5)
Hppy(FLAG)
B
pcDNA3.1
MAP4K1
MAP4K1 KD
GFP-YAP
+
+
+
P-YAP(127)
P-YAP(381)
GFP-YAP(YAP)
MAP4K1(FLAG)

Figure S3. JNK or TOR signaling is not required for Hppy to activate Hippo signaling, related to Figure 3.

(A) Rapamycin treatment abolished S6K phosphorylation but had no effect on Hppy-induced Wts phosphorylation.

(B) Rapamycin treatment abolished S6K phosphorylation but had no effect on Hppy-induced Mats phosphorylation.

(C) RNAi of the JNK kinase Hemipterous (Hep) abolished Hppy-induced JNK phosphorylation but not Wts phosphorylation.

(D) RNAi of Hep abolished Hppy-induced JNK phosphorylation but not Mats phosphorylation.

Figure S3

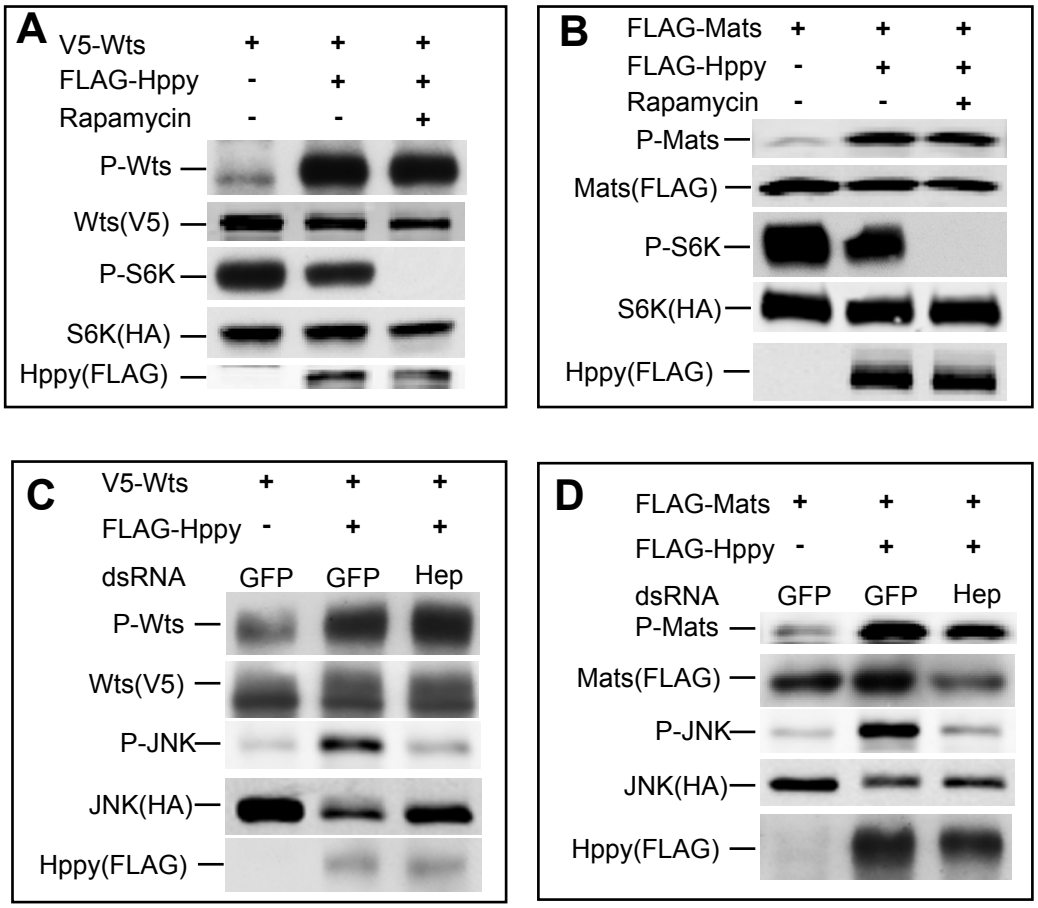

A
V5-Wts + + +
FLAG-Hppy - + +
Rapamycin - - +
P-Wts
Wts(V5)
P-S6K
S6K(HA)
Hppy(FLAG)
B
FLAG-Mats + + +
FLAG-Hppy - + +
Rapamycin - - +
P-Mats
Mats(FLAG)
P-S6K
S6K(HA)
Hppy(FLAG)
C
V5-Wts + + +
FLAG-Hppy - + +
dsRNA GFP GFP Hep
P-Wts
Wts(V5)
P-JNK
JNK(HA)
Hppy(FLAG)
D
FLAG-Mats + + +
FLAG-Hppy - + +
dsRNA GFP GFP Hep
P-Mats
Mats(FLAG)
P-JNK
JNK(HA)
Hppy(FLAG)

Figure S4. Hppy functions redundantly with Hpo to regulate Yki in response to F-actin disruption, related to Figure 5.

(A) Sequencing and western blotting analysis of two *hppy* deletions $hppy^{\Delta 1}$ and $hppy^{\Delta 2}$. $hppy^{\Delta 2}$ deletes exons 4-7 and $hppy^{\Delta 1}$ additionally deletes part of exon 3 (upper panel). Hppy protein was not detected in $hppy^{\Delta 1}$ or $hppy^{\Delta 2}$ by western blotting (lower panel).

(B-C) Third instar larval wing discs containing GFP-negative $hppy^{\Delta 1}$ clones were cultured in Schneider medium supplemented with (+LatB) or without (-LatB) 4μg/mL LatB for 1h and then stained with Yki antibody (Red) and DAPI (Blue). Note similar Yki localization within and outside mutant clones irrespective of LatB treatment.

(D-G) An example of rare *hppy;hpo* double mutant clones that still showed LatB-induced nuclear exclusion of Yki in the LatB-sensitive peripheral region of wing pouch defined in Figure S1D. The area outlined by a square in (D) is enlarged in (E). F and G show two vertical sections through E, in which the positions of the vertical sections are indicated by dotted lines.

Figure S4

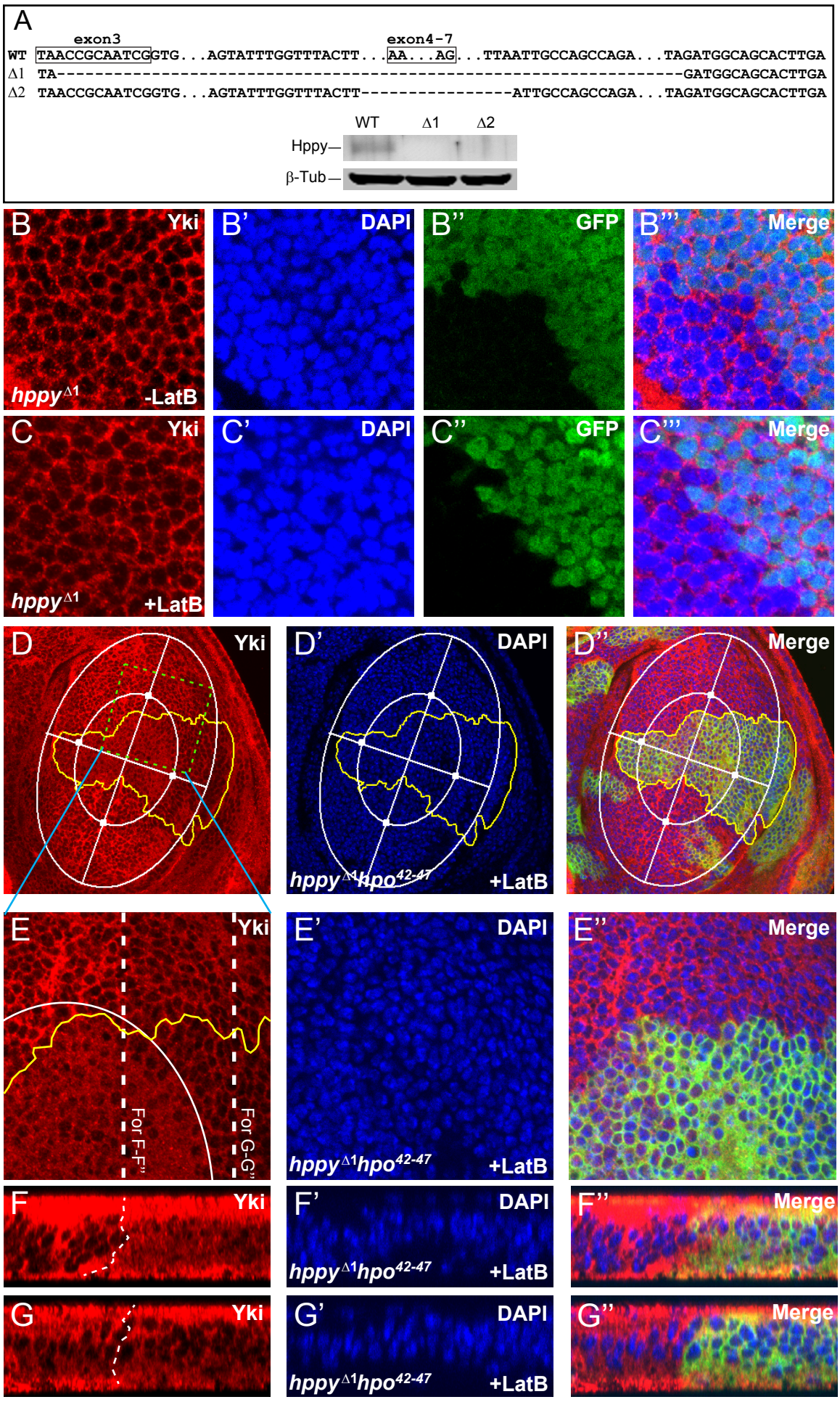

A
exon3
exon4-7
WT TAACCGCAATCGGTG...AGTATTTGGTTTACTT...AA...AG...TTAATTGCCAGCCAGA...TAGATGGCAGCACTTGA
Δ1 TA----------GATGGCAGCACTTGA
Δ2 TAACCGCAATCGGTG...AGTATTTGGTTTACTT----------ATTGCCAGCCAGA...TAGATGGCAGCACTTGA
WT
Δ1
Δ2
Hppy—
β-Tub—
B Yki
B' DAPI
B'' GFP
B''' Merge
hppy Δ1
-LatB
C Yki
C' DAPI
C'' GFP
C''' Merge
hppy Δ1
+LatB
D Yki
D' DAPI
D'' Merge
hppy Δ1 hpo 42-47
+LatB
E Yki
E' DAPI
E'' Merge
For F-F''
For G-G''
hppy Δ1 hpo 42-47
+LatB
F Yki
F' DAPI
F'' Merge
hppy Δ1 hpo 42-47
+LatB
G Yki
G' DAPI
G'' Merge
hppy Δ1 hpo 42-47
+LatB

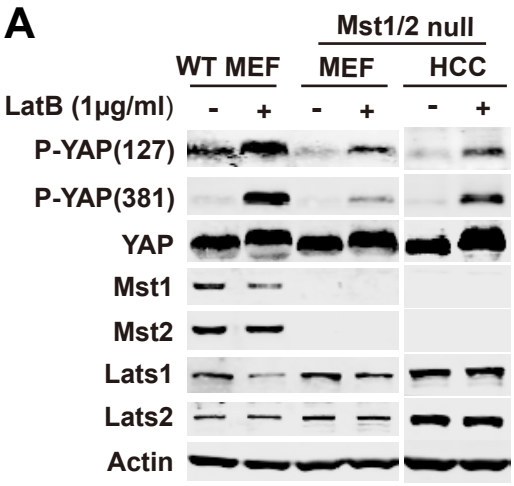
Figure S5. Confirmation of Mst1/2-independent Hippo signaling induced by LatB and characterization of MAP4K1/2/3/5 knockdown efficiency, related to Figure 6.

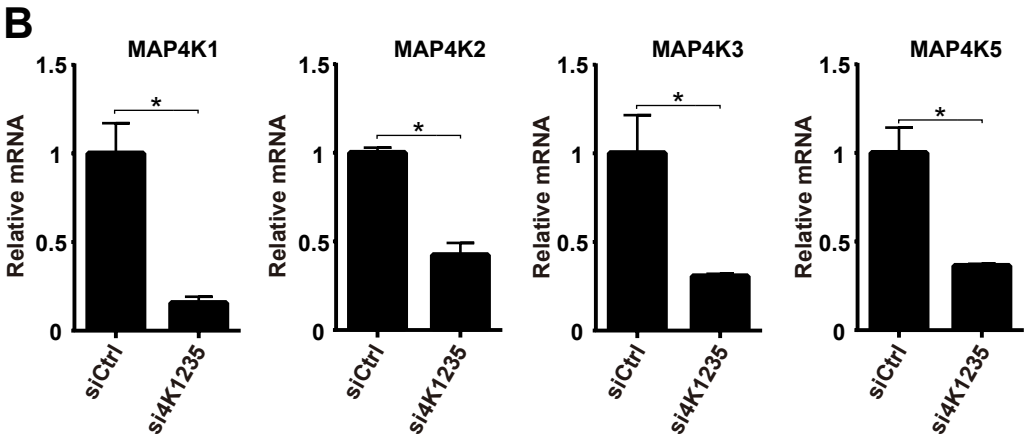
(A) Wildtype MEF cells, Mst1/2 null MEF cells, and Mst1/2 null HCCs were treated with 1μg/mL LatB for 1 hr and then examined by western blotting. LatB induced YAP^{S127} and YAP^{S381} phosphorylation in all three cell lines.

(B) Mst1/2 null HCCs transfected with siRNA against MAP4K1, MAP4K2, MAP4K3 or MAP4K5 were analyzed for the mRNA levels of individual kinase by qPCR (mean±SD, n=3). * denotes a p-value<0.05.

(C) Mst1/2 null HCCs transfected with siRNA against MAP4K1, MAP4K2, MAP4K3 or MAP4K5 individually or their combination were analyzed for the mRNA level of *Yap* by qPCR.

Figure S5


A
Mst1/2 null
WT MEF
MEF
HCC
LatB (1μg/ml)
- + - + - +
P-YAP(127)
P-YAP(381)
YAP
Mst1
Mst2
Lats1
Lats2
Actin


B
MAP4K1
MAP4K2
MAP4K3
MAP4K5
Relative mRNA
1.5
1
0.5
0
*
siCtrl
si4K1235

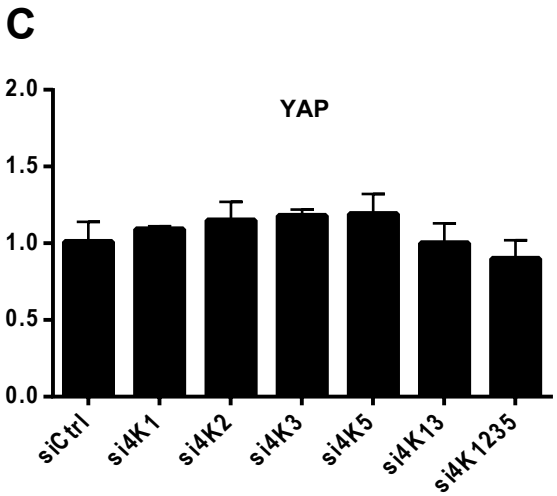
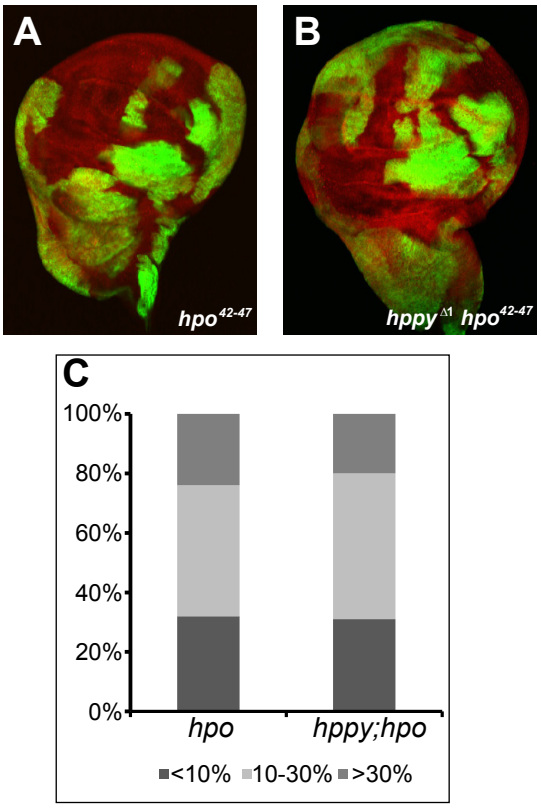

C
YAP
2.0
1.5
1.0
0.5
0.0
siCtrl
si4K1
si4K2
si4K3
si4K5
si4K13
si4K1235

Figure S6. Loss of *hppy* does not alter the size of *hpo* mutant clones, related to Figure 7.

Representative third instar larval wing discs containing GFP-positive clones of the indicated genotypes: *hpo* clones (A), *hppy*; *hpo* clones (B). Clone size distribution of the indicated genotypes was calculated based on 20 discs for each genotype (C). Clone size was quantified as the percentage of clone area relative to total wing area.

Figure S6


A
hpo42-47
B
hppyΔ1 hpo42-47
C
100%
80%
60%
40%
20%
0%
hpo
hppy;hpo
<10%
10-30%
>30%