Figure S1

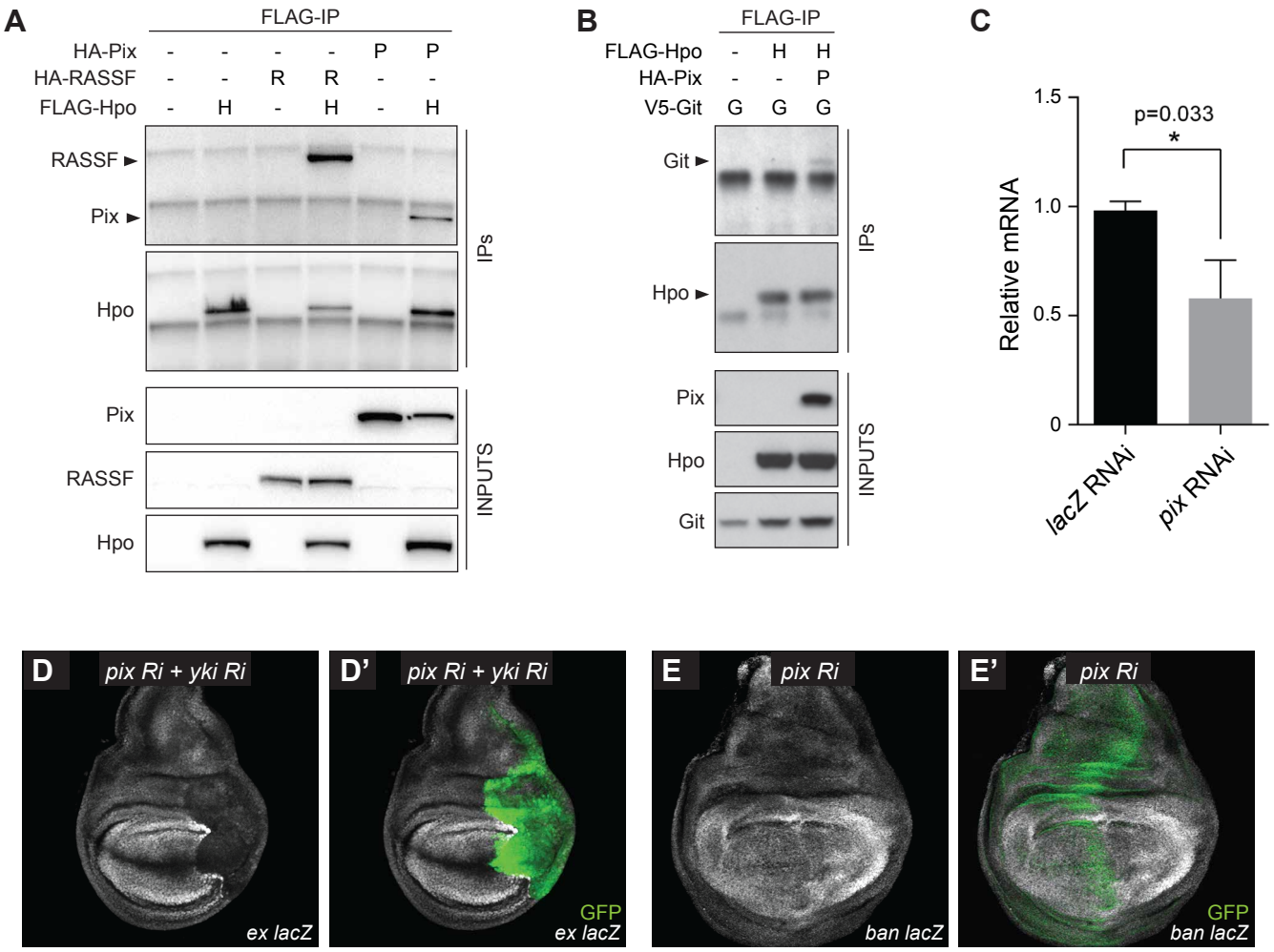

A
FLAG-IP
HA-Pix - - - - P P
HA-RASSF - - R R - -
FLAG-Hpo - H - H - H
RASSF ►
Pix ►
Hpo
IPs
Pix
RASSF
Hpo
INPUTS
B
FLAG-IP
FLAG-Hpo - H H
HA-Pix - - P
V5-Git G G G
Git ►
Hpo ►
IPs
Pix
Hpo
Git
INPUTS
C
Relative mRNA
1.5
1.0
0.5
0
p=0.033
*
lacZ RNAi
pix RNAi
D
pix Ri + yki Ri
ex lacZ
D'
pix Ri + yki Ri
GFP
ex lacZ
E
pix Ri
ban lacZ
E'
pix Ri
GFP
ban lacZ

Figure S2

Figure S3

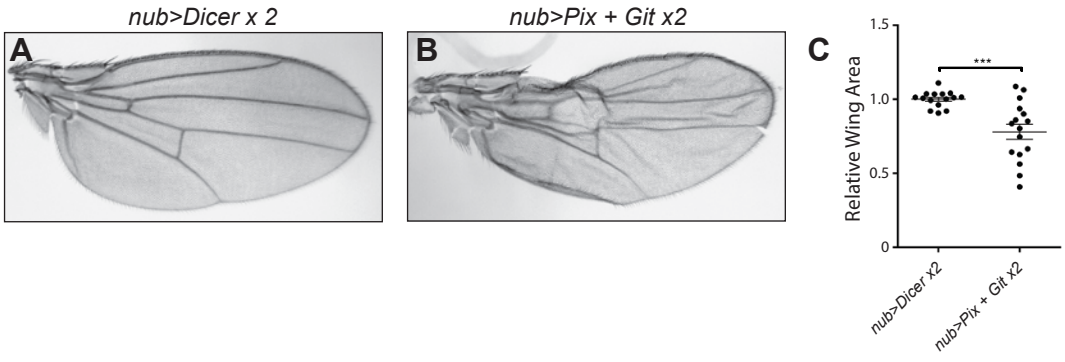
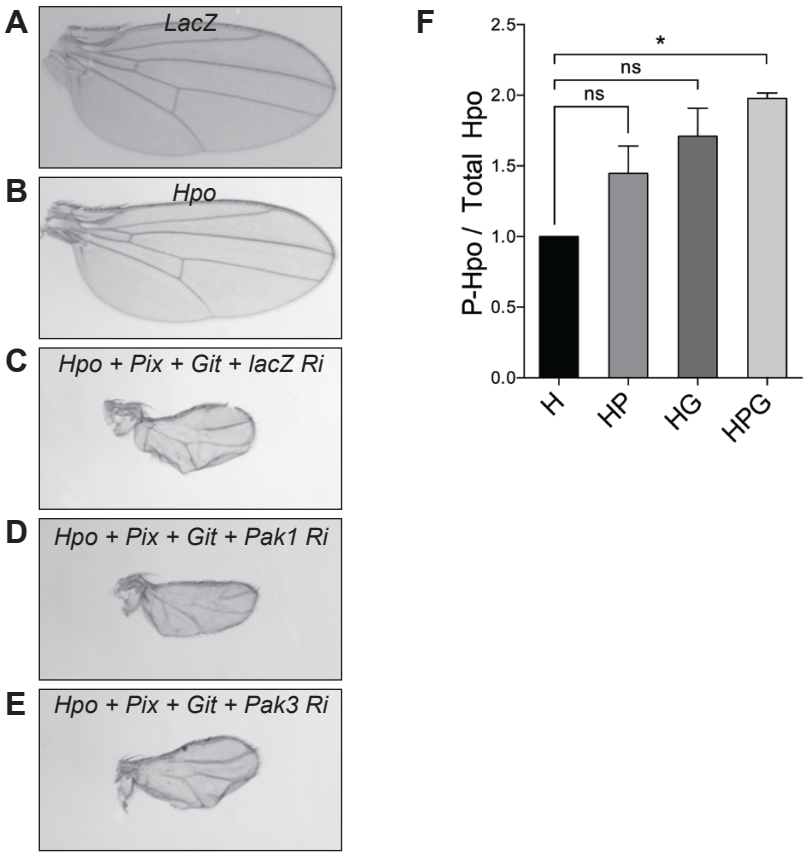

nub>Dicer x 2
nub>Pix + Git x2
A
B
C
Relative Wing Area
1.5
1.0
0.5
0
***
nub>Dicer x2
nub>Pix + Git x2

Figure S4


A
LacZ
B
Hpo
C
Hpo + Pix + Git + lacZ Ri
D
Hpo + Pix + Git + Pak1 Ri
E
Hpo + Pix + Git + Pak3 Ri
F
P-Hpo / Total Hpo
2.5
2.0
1.5
1.0
0.5
0.0
*
ns
ns
H
HP
HG
HPG

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1, related to Figure 1.
(A and B) Co-immunoprecipitation experiments were performed on protein lysates from S2 cells transfected with the indicated plasmids using anti-Flag antibodies to immunoprecipitate Hpo. In (A) Western blot analysis was performed using anti-Flag to reveal Hpo and anti-HA to reveal RASSF and Pix. Hpo immunoprecipitated with both RASSF and Pix. In (B) Western blot analysis was performed using anti-Flag to reveal Hpo, anti-HA to reveal Pix and anti-V5 to detect Git. Hpo immunoprecipitated with both Git, but only when Pix was co-expressed.
(C) The relative levels of *pix* mRNA to *actin* mRNA were assessed in third instar larval wing imaginal discs expressing either *lacZ* RNAi or *pix* RNAi under the control of *nubbin-Gal4*. *pix* mRNA was significantly lower in tissues expressing *pix* RNAi. Data represents mean +/- SEM. * indicates a *p*-value <0.05, n=4.
(D-E') *D. melanogaster* third instar larval wing imaginal discs expressing the indicated RNAi lines under the control of *en-Gal4.* Transcriptional activity of the *ex* gene (D and D') or the *ban* gene (E and E') were reported by β-galactosidase expression (grayscale). In the merged images GFP (green) demarcates the posterior compartment (D'), whereas in (E') Cubitus interruptus staining demarcates the anterior compartment.

Supplemental Figure 2, related to Figure 2. Greater ptilinum overgrowth occurs when mutant for *fat* and *git*, compared to *fat* alone.
Adult female *D. melanogaster* heads from git^{ex21c} mutant animals (A), containing homozygous fat^{fd} clones generated with *eyeless-Flp* (B), or homozygous fat^{fd} clones in a git^{ex21c} mutant background (C).

Supplemental Figure 3, related to Figure 3. Pix and Git overexpression limits wing size.
Adult female *D. melanogaster* wings expressing the following transgenes under the control of *nub-Gal4*: (A) *UAS-Dicer*; (B) *UAS-pix, UAS-git* (two copies of each transgene and Gal4 driver). (C) Quantification of wing area of the indicated genotypes. n=15 in (A), n=16 in (B). Data represents mean +/- SEM. *** indicates a *p*-value <0.001.

Supplemental Figure 4, related to Figure 4. Pak1 and Pak3 depletion do not affect the ability of Pix and Git to enhance Hippo's ability to retard wing size.
Adult female *D. melanogaster* wings expressing the following transgenes under the control of *nub-Gal4*: (A) *UAS-lacZ;* (B) *UAS-hpo;* (C) *UAS-hpo, UAS-pix, UAS-git*; *UAS-lacZ RNAi;* (D) *UAS-hpo, UAS-pix, UAS-git*; *UAS-Pak1 RNAi;* (E) *UAS-hpo, UAS-pix, UAS-git*; *UAS-Pak3 RNAi*. (F) Quantification of Hpo phosphorylation in (Figure 4D), n=3, error bars represent mean SEM. * indicates a *p*-value <0.05.

Supplemental Table 1, related to Figure 1. **Binding partners of Hippo identified by mass spectrometry.** Data from experimental and control purifications were analyzed by SAINT. For each protein identified in the Hippo purification, the number of unique peptides is shown in column E (Spec). This number was compared to data from six biological replicates of control samples using extracts from untransfected S2 cells (column J, ctrlCounts). AvgP indicates the probability of a protein being a *bona fide* interactor, with values greater than 0.8 considered significant. Hippo itself is

highlighted green (hpo), and Git and Pix (RtGEF) are highlighted yellow. Both Git and Pix were identified with a highly significant SAINT probability and were not detected in control purifications.