POSH regulates Hippo signaling through ubiquitin-mediated expanded degradation

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The Hippo signaling pathway is a master regulator of organ growth, tissue homeostasis, and tumorigenesis. The activity of the Hippo pathway is controlled by various upstream components, including Expanded (Ex), but the precise molecular mechanism of how Ex is regulated remains poorly understood. Here we identify Plenty of SH3s (POSH), an E3 ubiquitin ligase, as a key component of Hippo signaling in *Drosophila*. *POSH* overexpression synergizes with loss of *Kibra* to induce overgrowth and up-regulation of Hippo pathway target genes. Furthermore, knockdown of *POSH* impedes dextran sulfate sodium-induced Yorkie-dependent intestinal stem cell renewal, suggesting a physiological role of POSH in modulating Hippo signaling. Mechanistically, POSH binds to the C-terminal of Ex and is essential for the Crumbs-induced ubiquitination and degradation of Ex. Our findings establish POSH as a crucial regulator that integrates the signal from the cell surface to negatively regulate Ex-mediated Hippo activation in *Drosophila*.

POSH | Hippo signaling | Expanded | *Drosophila* | intestine stem cell

During normal development, precise organ size control is critical for tissue homeostasis, and its disruption results in severe pathological conditions. The Hippo pathway is a conserved regulator of tissue growth, required to maintain proper organ size through regulating cell proliferation, apoptosis, and stem cell identity (1–3). In *Drosophila*, the core components of the Hippo pathway are Hippo (Hpo) (4–7), Warts (Wts) (8, 9), and Yorkie (Yki) (10, 11). Hpo phosphorylates Wts, which in turn phosphorylates and inactivates the transcriptional coactivator Yki. Once Yki is activated, it translocates to the nucleus and binds to transcription factors to up-regulate the transcription of growth-promoting genes (10). For the past two decades, *Drosophila* has played a leading role in elucidating the complexity of the Hippo pathway, and extensive genetic screens have identified more than 20 Hippo pathway regulators including three membrane-associated proteins, Expanded (Ex), Kibra, and Merlin (Mer) (12–17). These three proteins are known to form a complex, colocalize with each other, and act redundantly to regulate the Hippo pathway (15, 18). Strikingly, Kibra and Mer have been recently shown to regulate Hippo signaling at the medial apical cortex, independent of Ex (19), revealing a previously unrecognized mechanism in Hippo signaling control. However, despite the tremendous progress achieved toward uncovering new components of the Hippo pathway, it remains poorly understood how precisely the tumor suppressor Ex is regulated in vivo.

We performed a genetic screen using *Enhancer–promoter* (*EP*) lines in *Drosophila*, aiming to unearth oncogene(s) that can synergistically enhance the eye-overgrowth phenotype caused by loss of Kibra, an upstream component of Hippo signaling (15–17), and identified POSH (Plenty of SH3s) as a crucial regulator of Hippo signaling. POSH serves as a conserved multidomain scaffold protein for JNK pathway activation (20–23). It functions as an E3 ubiquitin ligase to degrade *Drosophila* dTAK1 (a JNK kinase kinase), mammalian Herp (homocysteine-inducible ER protein), and Hrs (hepatocyte growth factor-regulated tyrosine

kinase substrate, an endosomal protein) through its N-terminal RING finger domain (24–26). Functionally, POSH regulates several cellular activities including apoptosis, T cell differentiation, and neuronal migration (20, 27–31).

Here we found POSH is physiologically essential for Yki-mediated growth control, regeneration, and gut homeostasis. Our genetic and biochemical data show that POSH functions downstream of Crb to regulate Ex ubiquitination and degradation. These findings reveal an unrecognized function of POSH in Hippo signaling regulation and shed light on the molecular mechanism by which the Ex tumor suppressor is regulated.

Results

POSH Cooperates with Kibra to Control Tissue Growth. Based on the observation that loss of Kibra alone induces only mild growth (Fig. 1*B*) (15–17), we performed a *P* element-based gain-of-function screen utilizing the EP collection from the Berkeley *Drosophila* Genome Project (32), aiming to find modifiers that can synergize with *GMR > kibra.RNAi* to induce eye overgrowth. One candidate EP interactor was inserted in the 5′ UTR of *POSH* (Fig. S1*A*), a highly conserved RING domain containing scaffold protein, homologous to human SH3RF1, SH3RF2, and SH3RF3 (SH3 domain containing ring finger). We observed a significant synergistic increase in adult eye size (Fig. 1 *A–D* and *G*) and interommatidial cell number in the pupal retina in *GMR > kibra.RNAi + POSH* samples versus controls (Fig. 1 *H–K*). To confirm that ectopic POSH expression is responsible for the synergistic overgrowth phenotype, we examined an
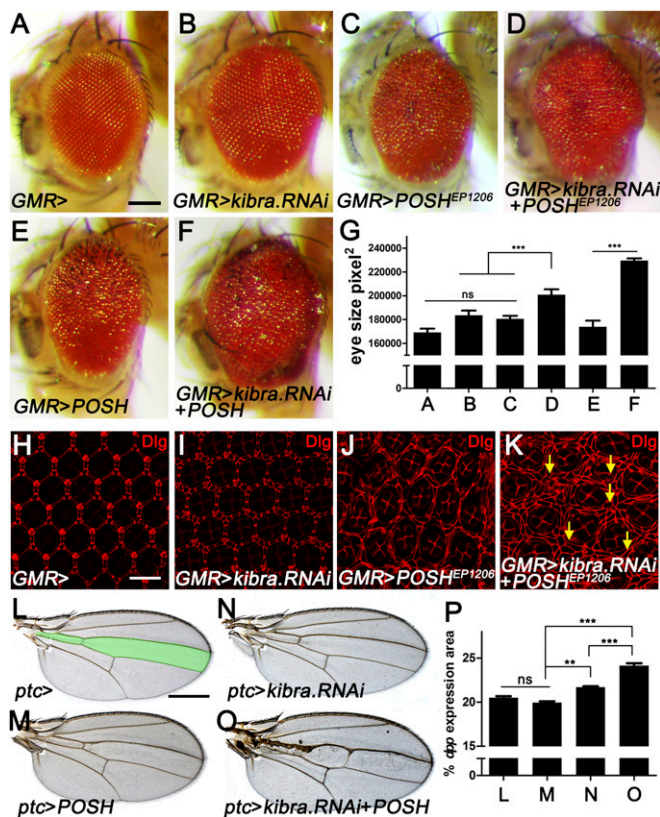
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Fig. 1. POSH synergizes with Kibra depletion to control tissue growth. (*A–F*) Light micrographs of *Drosophila* adult eyes. *kibra.RNAi* and/or *POSH* were expressed in the eye under *GMR-GAL4*. Note the synergistically increased eye size in *D* and *F*. (*G*) Quantification of eye size in *A–F* (mean + SD, *n* = 10). (*H–K*) Pupal eye discs (40 h after pupal formation) of the indicated genotypes were stained for Dlg antibody. Arrows indicate the regions where interommatidial cells are increased. (*L–O*) Light micrographs of *Drosophila* adult wings. Under the control of *dpp-GAL4* (expression pattern highlighted in green), loss of *kibra*-induced tissue growth was synergistically enhanced by coexpression of POSH. (*P*) Quantification of the ratio of the *dpp*-expressing area/total wing size in *L–O* (mean + SD, *n* = 10). (Scale bars: 100 μm in *A–F*, 20 μm in *H–K*, 500 μm in *L–O*.) **P < 0.01; ***P < 0.001; ns, not significant.

additional POSH-overexpression strain (33). Similarly, a strong synergistic growth effect was seen with *POSH* and *kibra.RNAi* in both adult eyes and wings (Fig. 1 *E–G* and *L–P* and Fig. S1 *B–H*). Furthermore, *POSH* expression strongly enhanced the overgrowth caused by a *kibra*-null mutant in the eye (Fig. S1 *I–L*). Taken together, these findings suggest that POSH synergizes with Kibra depletion to promote tissue growth.

POSH Promotes Hippo Target Genes Expression. The above genetic interaction between Kibra and POSH in regulating tissue growth suggests POSH is a potential component of the Hippo signaling pathway. To test this, we examined the expression of commonly used Hippo pathway target genes, including *diap1*, *expanded* (*ex*), *bantam* (*ban*), and *wingless* (*wg*) (34–36). In the wing discs, we found that coexpression of *POSH* and *kibra.RNAi* synergistically elevated *diap1* expression (Fig. 2 *A–D* and Fig. S2*A*). Furthermore, ectopic *POSH* expression is sufficient to induce strong up-regulation of *ex*, *ban*, and Wg (Fig. 2 *E–J*). Consistent with the up-regulation of Hippo target genes, POSH expression caused increased nuclear accumulation of Yki (Fig. 2 *Q* and *R*) as well as up-regulation of Yki transcriptional activity (Fig. S2*F*). Together, these data indicate that POSH is a negative regulator of Hippo signaling.

Since POSH has been previously reported as a scaffold protein promoting JNK-mediated cell death (20, 22, 23, 33), we

want to know whether POSH regulates the JNK and Hippo pathways independently. We found POSH-induced JNK activation indicated by *puc*-LacZ expression (37) and apoptosis labeled by active caspase 3 staining were suppressed by expressing a dominant negative form of Basket (fly JNK, Bsk[DN]) but not by depletion of *yki* or *sd* (Fig. S2 *B* and *C*). Conversely, POSH-induced *ex* up-regulation was impeded by depletion of *sd* but not by blocking JNK activity (Fig. S2*D*). Therefore, we conclude that POSH regulates JNK and Hippo signaling independently.

POSH Acts Through Ex to Regulate Hippo Signaling-Dependent Growth. To further dissect the mechanism by which POSH modulates Hippo signaling, we performed genetic epistasis analysis between POSH and Hippo pathway components. POSH overexpression under the *nubbin* (*nub*) promoter is sufficient to induce overgrowth and proliferation (Fig. 2 *K* and *L* and Fig. S2*E*), which are not caused by a secondary effect of JNK activation or apoptosis, as blocking JNK or caspase activation did not suppress the overgrowth phenotype (Fig. S2*G*). We found the *nub > POSH* growth phenotype was dramatically suppressed by knockdown of *yki* or *sd* or by coexpression of *wts* or *ex* (Fig. 2 *M–P*), suggesting that POSH likely acts upstream of Ex. In agreement with this, we found that *POSH* expression in the *wts*^{−/−} background had no effect on eye size or morphology (Fig. S2 *P* and *Q*) and that ectopic *POSH* synergizes with *kibra.RNAi* (Fig. 1) and loss of *mer* (Fig. S2 *L* and *M*) but not with *ex.RNAi* (Fig. S2 *H–K*). To study the physiological role of POSH in development, we knocked down POSH by RNAi in the wing. Although knockdown of POSH alone had no significant effect on growth, it synergistically enhanced the *nub > hpo*–induced small wing and resulted in a wing-loss phenotype (Fig. 2 *S–V*).

POSH Is Required in Precursor Cells for Dextran Sulfate Sodium-Induced Intestinal Stem Cell Renewal. Next, to test whether POSH also represents an essential regulator of Hippo signaling beyond the imaginal discs, we examined the *Drosophila* intestine, where Hippo signaling is essential for intestinal stem cell (ISC) renewal to ensure the replenishment of damaged cells (38–42). The *POSH*[74]-null mutants are viable and have no obvious phenotype or food intake defect (Fig. 3*N*), and the gut epithelium maintains homeostasis under normal culture conditions (Fig. 3 *A*, *B*, *H*, and *I*). However, we found that dextran sulfate sodium (DSS) treatment-induced intestine cell proliferation (Fig. 3 *D* and *G*) (38) and *ban* up-regulation (Fig. 3*K*) (39) were completely blocked in *POSH* mutants (Fig. 3 *E*, *G*, and *L*) but were reverted by *yki* expression driven by the ubiquitous α-tubulin promoter (Fig. 3 *F*, *G*, and *M*), suggesting that *POSH* is physiologically required for DSS-induced Yki-mediated intestine epithelial cell renewal. The *Drosophila* midgut epithelium is mainly composed of four cell types, namely ISCs, enteroblast (EB) cells, absorptive enterocyte (EC) cells, and secretory enteroendocrine (ee) cells, while ISCs are the only mitotic cells in adult gut to maintain tissue homeostasis. We found that knockdown of *POSH* by two RNAi lines in ISCs/EBs (*esg*[ts] > GFP) suppressed DSS-induced ISC proliferation, as shown by the reduction in the number of GFP[+] (Esg) and PH3^{+} cells (Fig. 3 *O–S* and Fig. S3 *N–P*), as well as a reduction of EB cells, as indicated by *Su(H)-lacZ* staining (Fig. 3 *T–W*). Conversely, reducing POSH levels in the EC cells (*Myo1A*[ts] > GFP) had no effect on DSS-induced proliferation (Fig. S3 *J–M*). The knockdown effect of *POSH RNAi* lines was verified by qRT-PCR (Fig. S3*G*) and its ability to suppress the *GMR > POSH* eye phenotype (Fig. S3 *A–F*). Together, these data suggest that POSH functions in the ISCs to promote cell-autonomous proliferation.

POSH Physically Interacts and Ubiquitinates Ex. Given that POSH may act upstream of Ex, and that POSH encodes a RING domain containing E3 ubiquitin ligase (24), we hypothesized that POSH may regulate Ex stability. To test this, we first examined the Ex
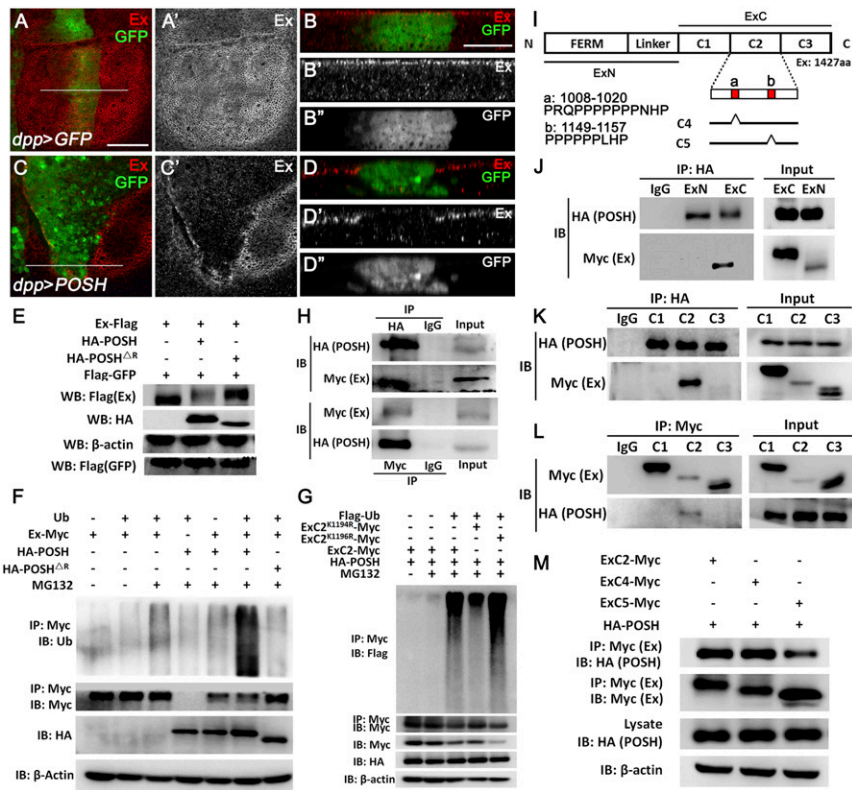
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Fig. 2. POSH negatively regulates Hippo signaling. (*A–R*) Fluorescence micrographs of wing discs. (*A–D*) *ptc-GAL4*–driven *POSH* expression synergizes with *kibra.RNAi* to up-regulate *diap1* transcription. (*E–J*) Ectopic *POSH* expression in the posterior region of wing discs is sufficient to induce *ex-lacZ*, *ban-lacZ*, and Wg up-regulation. (*K–P*) *nub > POSH*-induced tissue growth was suppressed by the depletion of *sd* or *yki* or the overexpression of *wts* or *ex*. (*Q* and *R*) Fluorescence micrographs of wing discs stained for Yki protein. Note the increased nuclear Yki signal in most POSH-expressing cells (*R* and *R'*). (*S–V*) Light micrographs of *Drosophila* adult wings. Note the complete wing loss phenotype in *nub > hpo + POSH. RNAi* flies. The penetrance and sample number are indicated on the top right of each panel. (Scale bars: 50 μm in *A–D'* and *Q–R'*, 100 μm in *E–J* and *K–P*, 500 μm in *S–V*.)

protein level in vivo. Strikingly, Ex was dramatically depleted from the apical membrane of POSH-expressing cells (Fig. 4 *A–D*), whereas no significant alterations were noted in the levels and localization of Dlg (Fig. S4 *A* and *B*). We further confirmed this in cultured S2 cells by immunoblot analysis and found that POSH decreases Ex protein levels in a RING domain-dependent manner (Fig. 4*E*). To understand the molecular mechanism by which POSH degrades Ex, we performed a ubiquitination assay and found that overexpression of *POSH*, but not of *POSH*^ΔRING (*POSH*^ΔR), increased ubiquitination on Ex (Fig. 4*F*). In accordance

with the notion that POSH negatively regulates Ex, the *GMR > ex*-induced small-eye phenotype was significantly suppressed by coexpression of *POSH* (Fig. S4 *I–K*).

E3 ligases are known to bind directly to substrate to facilitate ubiquitination. To test whether and how POSH interacts with Ex, we divided Ex into the N-terminal half (ExN) and the C-terminal half (ExC) and further divided ExC into C1, C2, and C3 (Fig. 4*I*). We performed coimmunoprecipitation assays in S2 cells and found that ectopically expressed POSH physically interacts with Ex, and vice versa (Fig. 4*H*). Furthermore, POSH was found to

Fig. 3. POSH is required for DSS-induced ISC proliferation. (*A–F* and *H–M*) Fluorescence micrographs of adult midgut. DSS treatment-induced mitosis (*D'*) and *ban* up-regulation (*K*) were suppressed in *POSH* mutant midguts (*E'* and *L*), which were reverted by coexpression of *tub-yki* (*F'* and *M*). (*G*) Quantification of PH3^+ mitotic cells per gut (mean + SD, n = 10). (*N*) Capillary Feeder (CAFE) assay to monitor fly daily food consumption of the indicated genotypes. (*O*) Quantification of PH3^+ mitotic cells per gut in *P–S* (mean + SD, n = 10). (*P–W*) Fluorescence micrographs of adult midgut stained with PH3 or β-Gal; GFP labels precursor cells driven by *esg-GAL4*. Note the decrease in the number of PH3^+ and *su(H)-lacZ*–positive cells in *esg^ts > POSH.RNAi* after DSS treatment. (Scale bars: 100 μm in *A–F'*, *H–M*, and *P–W'*.)

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Fig. 4. POSH promotes ubiquitin-mediated protein degradation of Ex. (*A–D*) Fluorescence micrographs of wing pouch regions. *B* and *D* are vertical-section images of the white lines in *A* and *C*, respectively. Note the dramatic depletion of apical Ex in POSH-expressing cells (*D*). (*E*) The Ring domain is required by POSH to degrade Ex in S2 cells. (*F*) The Ring domain is required by POSH to promote Ex ubiquitination in S2 cells. (*G*) Mutation of Lys-1194 dramatically abrogates POSH-induced Ex ubiquitination. (*H*) Physical association between POSH and Ex in S2 cells. Lysates expressing the indicated constructs were immunoprecipitated (IP) and probed with the indicated antibodies. Myc-Ex was detected in HA-POSH immunoprecipitation. Conversely, HA-POSH was detected in Myc-Ex immunoprecipitation. (*I*) Schematic of the domain organization of Ex protein. "a" and "b" represent two potential SH3-binding motifs in the C2 region. (*J*) POSH binds to the C-terminal but not to the N-terminal half of Ex. (*K–M*) POSH binds specifically to the C2 region of Ex. Myc-C2, but not Myc-C1 or Myc-C3, was detected in HA-POSH immunoprecipitation (*K*). Conversely, HA-POSH was detected only in Myc-C2, but not in Myc-C1 or Myc-C3, immunoprecipitation (*L*). Deletion of the second SH3-binding motif in C2 impedes the POSH–Ex interaction (*M*).

coimmunoprecipitate specifically with the C2 region of the ExC fragment but not with other C-terminal regions or the ExN part (Fig. 4 *J–L*). We identified two putative SH3-binding motifs [(*i*) amino acids 1,008–1,020, and (*ii*) amino acids 1,149–1,157] in the C2 region by UniProt (www.uniprot.org) and deleted them individually from C2 to make C4 and C5 (Fig. 4*I*). We found that deletion of the second, but not the first, SH3-binding motif significantly decreased the binding between C2 and POSH (Fig. 4*M*), suggesting the second SH3-binding motif is essential for Ex to interact with POSH. Together, these data demonstrate that POSH binds to and ubiquitinates Ex, leading to its degradation. Additionally, using BDM-PUB, an online web server for protein ubiquitination site prediction (bdmpub.biocuckoo.org), we identified two lysine residues in the C2 region (K1194 and K1196) as potential ubiquitination sites. We found that mutation of K1194, but not K1196, significantly blocked POSH-induced Ex ubiquitination (Fig. 4*G*), suggesting K1194 of Ex is a target site of POSH-mediated ubiquitination.

POSH Is Required for Crumbs Intracellular Domain-Induced Growth and Ex Degradation. The apical localized transmembrane protein Crumbs (Crb) has been recently identified as a crucial regulator of Hippo signaling in *Drosophila* (43–46). The function of Crb largely depends on its intracellular domain (Crb^{intra}), and expression of Crb^{intra} also promotes growth, at least in part, by ubiquitin-dependent degradation of Ex (43, 45–48). To test whether POSH is required for Crb^{intra}-induced growth, we silenced *POSH* in Crb^{intra}-expressing cells. Interestingly, while POSH's expression and subcellular distribution remain unaffected upon Crb^{intra} overexpression (Fig. S5 *A–G*), knockdown of *POSH* significantly suppresses Crb^{intra}-induced growth (Fig. 5 *A–E* and Fig. S3 *Q–S*). We also found that *POSH* depletion significantly impedes Crb^{intra}-triggered Ex degradation (Fig. 5 *F*, *G*, and *J–N* and Fig. S5 *I–K*), suggesting that Crb functions partially through POSH to regulate Hippo signaling activity. It has been reported that Supernumerary limbs (Slmb), a SCF (Skp/Cullin/F-box) E3 ubiquitin ligase, also ubiquitinates Ex downstream of Crb (49). Consistently, we found knockdown of Slmb significantly impedes Crb^{intra}-induced Ex reduction (Fig. 5 *H*, *L*, and *N*). To examine whether Slmb and POSH act redundantly to regulate Ex levels, we knocked down Slmb and POSH simultaneously and observed full suppression of Crb^{intra}-induced Ex degradation (Fig. 5 *I*, *M*, and *N*), indicating that POSH and Slmb act in parallel to regulate Crb^{intra}-induced Ex degradation.

Apart from Crb, knockdown of other cell polarity regulators, including Lethal(2) giant larvae (Lgl), Discs large 1 (Dlg), and Fat (Ft), are also known to promote tissue growth via Hippo signaling inactivation (34, 44, 50–55), which, however, were not suppressed by knockdown of *POSH* (Fig. S4 *L–Q*), highlighting the specificity of Crb as the upstream regulator of POSH. Together, our data support a model in which Crb^{intra} regulates tissue growth via POSH-mediated Ex degradation.

Discussion

Drosophila has been widely considered an excellent model organism to uncover novel cancer-regulating genes of various signaling pathways for the past two decades (56–58). Here, we have conducted an *EP*-based overexpression genetic screen and identified POSH as an important upstream regulator of Hippo signaling in *Drosophila*.

Ubiquitination is a crucial process for protein degradation and affects almost all cellular processes, including cell death, cell cycle, and tumorigenesis (59, 60). Interestingly, several key members of the Hippo pathway were found to be negatively regulated by ubiquitination (49, 61–63). Here we found that POSH specifically binds to the C-terminal region of Ex and promotes its ubiquitination and degradation. We further show that Crb^{intra}-induced Ex degradation is partially suppressed by depletion of *POSH*. Given that the human homolog of POSH is highly overexpressed in colon cancers (64), it would be interesting to further explore whether a conserved mechanism exists in human POSH-related cancer progression.

The *Drosophila* gut is under continuous attack due to exposure to pathogens and chemical stimulus during normal feeding. To maintain gut homeostasis, timely ISC proliferation is essential to
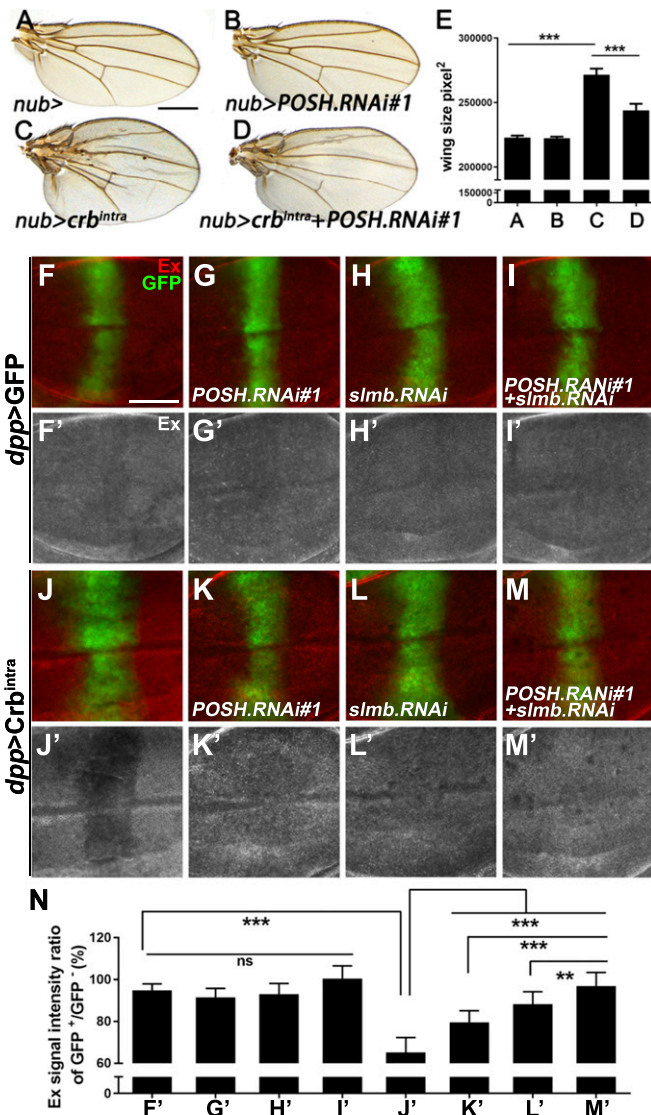
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Fig. 5. POSH is required for Crb^{intra}-induced Ex degradation. (*A–D*) Light micrographs of *Drosophila* adult wings. (*E*) Quantification of wing size in *A–D* (mean + SD; *n* = 10). (*F–M*) Widefield fluorescence micrographs of wing pouch regions. Coexpression of *POSH* and *slmb* RNAi fully suppresses *dpp* > *crb^{intra}*-induced Ex degradation. (*N*) Quantification of Ex signal intensity ratio of GFP$^+$ region/GFP$^-$ region in *F–M* (mean + SD; *n* ≥ 10). (Scale bars: 500 μm in *A–D*, 50 μm in *F–M*.) **P < 0.01; ***P < 0.001; ns, not significant.

ensure the replenishment of damaged cells (38). We show that *POSH* is not required for gut homeostasis under normal conditions but is essential for ISC renewal and proliferation following DSS treatment (Fig. 3). Intriguingly, it has been shown that Yki is also dispensable for normal gut homeostasis (39, 42), highlighting the essential physiological role of the POSH–Yki axis as stress sensors in gut epithelia. However, it is worth noting that unlike YAP (the mammalian Yki homolog) inhibition, which showed a dramatic increase in the mortality rate against DSS treatment (65), *POSH* mutants survived better than wild-type flies (Fig. S3*H*). A possible explanation is that in *POSH* mutants JNK activation is compromised (28, 33), which leads to reduced caspase activation, while, conversely, loss of Yki/YAP has been shown to induce apoptosis (10, 65).

Both Hippo and JNK signaling have well-established roles in regulating cell proliferation, growth, and survival. Despite the well-documented cross-talk between JNK and Hippo signaling in

various contexts, ranging from ISC renewal (39, 42) to cell growth (66, 67) and migration (68), the mechanisms by which JNK intersects with Hippo in growth and apoptosis control have not been well studied. Here we identified POSH as an essential linker that bridges JNK and Hippo signaling. On one hand, POSH stimulates Hippo-mediated growth by promoting Ex ubiquitination and degradation; on the other hand, POSH induces JNK-dependent apoptosis, independent of Hippo signaling (Fig. S2). Considering the important roles of both JNK and Hippo activity in tumorigenesis, our findings provide a molecular basis for further investigation of mammalian POSH homologs as potential linkers of JNK- and Hippo-mediated cancer progression.

Given that studies have overwhelmingly proven that *Drosophila* is an excellent model for gaining insight into human cancer biology (57) and the conservation of the Hippo pathway between *Drosophila* and human, our findings here bring forth the exciting prospect that similar mechanisms may exists in both normal development and cancer progression.

Experimental Procedures

***Drosophila* Stocks.** All crosses were raised on standard *Drosophila* medium at 25 °C unless otherwise indicated. The following strains from the Bloomington *Drosophila* Stock Center were used for this study: *GMR-GAL4, ptc-GAL4, nub-GAL4, hh-GAL4, en-GAL4, UAS-GFP, UAS-p35, POSH^{EP1206}, POSH^{74}, ban-lacZ, ex-lacZ, diap1-lacZ*, and *UAS-slmb.RNAi* (#33898). The following RNAi lines were collected from the Vienna *Drosophila* Resource Center: *UAS-POSH.RNAi* (#2, v26655), *UAS-kibra.RNAi* (v106507), *UAS-yki.RNAi* (v40497), *UAS-ex.RNAi* (v22994), *UAS-lgl.RNAi* (v51249), *UAS-dlg.RNAi* (v41136), and *UAS-fat.RNAi* (v9396). The strains *kibra^{del}, UAS*-Kibra (15), *puc-lacZ* (69), *UAS-bsk^{DN}* (70), *mer^4* (71), *wts^{X1}* (9) were previously described. *UAS-crb^{intra}* (43, 48) was a gift from Georg Halder, University of Leuven, Leuven, Belgium and Elisabeth Knust, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany; *UAS-POSH.RNAi* (#1) and *UAS-POSH* (33) were gifts from Zhiheng Xu, Chinese Academy of Sciences, Beijing; *UAS-hpo* and *UAS-wts* were gifts from Shian Wu, Nankai University, Tianjin, China; *esg-GAL4 UAS-GFP tub-GAL80^{ts}, Myo1A-GAL4 UAS*-GFP tub-GAL80^{ts}, and *su(H)-lacZ* were gifts from Jin Jiang, University of Texas Southwestern, Dallas; *UAS-sd.RNAi* (72) was a gift from Lei Zhang, Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China; and *UAS-ex* (73) was a gift from Peter Bryant, University of California, Irvine, CA.

Immunostaining. Eye and wing discs of third-instar larvae were dissected in PBS and fixed in PBS containing 4% formaldehyde for 15 min, and fly intestines were fixed for 30 min. The samples were then blocked in 1× PBST containing 5% normal goat serum overnight at 4 °C and were incubated first with primary antibody overnight at 4 °C or for 2 h at room temperature and then with a fluorescence-conjugated secondary antibody for 2 h at room temperature. The following antibodies were used: mouse anti-Dlg (1:200) [Developmental Studies Hybridoma Bank (DSHB)], mouse anti–β-Gal (1:100) (DSHB), mouse anti-Wg (1:100) (DSHB), rabbit anti-PH3 (1:100) [Cell Signaling Technology (CST)], rabbit anti-active Caspase 3 (1:400) (CST), rabbit anti-Yki and rabbit anti-Kibra (1:1,000) (gifts from Duojia Pan, University of Texas Southwestern, Dallas), guinea-pig anti-Ex (1:1,000) (a gift from Richard Fehon, University of Chicago, Chicago), mouse anti-DIAP1 (1:200) (a gift from Bruce Hay, California Institute of Technology, Pasadena, CA), and guinea pig anti-POSH (1:200) (a gift from Sean Sweeney, University of York, York, England). Secondary antibodies were anti-rabbit Alexa (1:1,000) (CST) and anti-guinea pig-Cy3 (1:1,000) and anti-mouse Cy3 (1:1,000) (Jackson ImmunoResearch).

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1. Pan D (2007) Hippo signaling in organ size control. *Genes Dev* 21:886–897.
2. Hayashi S, Yokoyama H, Tamura K (2015) Roles of hippo signaling pathway in size control of organ regeneration. *Dev Growth Differ* 57:341–351.
3. Mo JS, Park HW, Guan KL (2014) The hippo signaling pathway in stem cell biology and cancer. *EMBO Rep* 15:642–656.
4. Harvey KF, Pfleger CM, Hariharan IK (2003) The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* 114:457–467.
5. 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.
6. Udan RS, 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.
7. 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.
8. Justice RW, Zilian O, Woods DF, Noll M, Bryant PJ (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.
9. Xu T, Wang W, Zhang S, Stewart RA, Yu W (1995) Identifying tumor suppressors in genetic mosaics: The Drosophila lats gene encodes a putative protein kinase. *Development* 121:1053–1063.
10. Huang J, Wu S, Barrera J, Matthews K, Pan D (2005) The hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila homolog of YAP. *Cell* 122:421–434.
11. Oh H, Irvine KD (2008) In vivo regulation of Yorkie phosphorylation and localization. *Development* 135:1081–1088.
12. Staley BK, Irvine KD (2012) Hippo signaling in Drosophila: Recent advances and insights. *Dev Dyn* 241:3–15.
13. Meng Z, Moroishi T, Guan KL (2016) Mechanisms of hippo pathway regulation. *Genes Dev* 30:1–17.
14. Hamaratoglu F, et al. (2006) The tumour-suppressor genes NF2/Merlin and expanded act through hippo signalling to regulate cell proliferation and apoptosis. *Nat Cell Biol* 8:27–36.
15. Yu J, et al. (2010) Kibra functions as a tumor suppressor protein that regulates hippo signaling in conjunction with Merlin and expanded. *Dev Cell* 18:288–299.
16. Baumgartner R, Poernbacher I, Buser N, Hafen E, Stocker H (2010) The WW domain protein kibra acts upstream of hippo in Drosophila. *Dev Cell* 18:309–316.
17. Genevet A, Wehr MC, Brain R, Thompson BJ, Tapon N (2010) Kibra is a regulator of the salvador/warts/hippo signaling network. *Dev Cell* 18:300–308.
18. Enderle L, McNeill H (2013) Hippo gains weight: Added insights and complexity to pathway control. *Sci Signal* 6:re7.
19. Su T, Ludwig MZ, Xu J, Fehon RG (2017) Kibra and merlin activate the hippo pathway spatially distinct from and independent of expanded. *Dev Cell* 40:478–490.e473.
20. Xu Z, Kukekov NV, Greene LA (2003) POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis. *EMBO J* 22:252–261.
21. Xu Z, Kukekov NV, Greene LA (2005) Regulation of apoptotic c-Jun N-terminal kinase signaling by a stabilization-based feed-forward loop. *Mol Cell Biol* 25:9949–9959.
22. Tsuda M, Seong KH, Aigaki T (2006) POSH, a scaffold protein for JNK signaling, binds to ALG-2 and ALIX in Drosophila. *FEBS Lett* 580:3296–3300.
23. Kukekov NV, Xu Z, Greene LA (2006) Direct interaction of the molecular scaffolds POSH and JIP is required for apoptotic activation of JNKs. *J Biol Chem* 281:15517–15524.
24. Tsuda M, Langmann C, Harden N, Aigaki T (2005) The RING-finger scaffold protein plenty of SH3s targets TAK1 to control immunity signalling in Drosophila. *EMBO Rep* 6:1082–1087.
25. Kim GH, Park E, Kong YY, Han JK (2006) Novel function of POSH, a JNK scaffold, as an E3 ubiquitin ligase for the Hrs stability on early endosomes. *Cell Signal* 18:553–563.
26. Tuvia S, et al. (2007) The ubiquitin E3 ligase POSH regulates calcium homeostasis through spatial control of herp. *J Cell Biol* 177:51–61.
27. Yang T, et al. (2012) POSH localizes activated Rac1 to control the formation of cytoplasmic dilation of the leading process and neuronal migration. *Cell Reports* 2:640–651.
28. West RJ, Lu Y, Marie B, Gao FB, Sweeney ST (2015) Rab8, POSH, and TAK1 regulate synaptic growth in a Drosophila model of frontotemporal dementia. *J Cell Biol* 208:931–947.
29. Cunningham CA, Knudson KM, Peng BJ, Teixeiro E, Daniels MA (2013) The POSH/JIP-1 scaffold network regulates TCR-mediated JNK1 signals and effector function in CD8(+) T cells. *Eur J Immunol* 43:3361–3371.
30. Cunningham CA, Cardwell LN, Guan Y, Teixeiro E, Daniels MA (2016) POSH regulates CD4+ T cell differentiation and survival. *J Immunol* 196:4003–4013.
31. Lyons TR, et al. (2007) Regulation of the Pro-apoptotic scaffolding protein POSH by Akt. *J Biol Chem* 282:21987–21997.
32. Rørth P, et al. (1998) Systematic gain-of-function genetics in Drosophila. *Development* 125:1049–1057.
33. Zhang M, Zhang Y, Xu Z (2010) POSH is involved in Eiger-Basket (TNF-JNK) signaling and embryogenesis in Drosophila. *J Genet Genomics* 37:605–619.
34. Cho E, et al. (2006) Delineation of a fat tumor suppressor pathway. *Nat Genet* 38:1142–1150.
35. 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.
36. Thompson BJ, Cohen SM (2006) The hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in Drosophila. *Cell* 126:767–774.
37. Martín-Blanco E, et al. (1998) Puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. *Genes Dev* 12:557–570.
38. Amcheslavsky A, Jiang J, Ip YT (2009) Tissue damage-induced intestinal stem cell division in Drosophila. *Cell Stem Cell* 4:49–61.
39. Karpowicz P, Perez J, Perrimon N (2010) The hippo tumor suppressor pathway regulates intestinal stem cell regeneration. *Development* 137:4135–4145.
40. Ren F, et al. (2010) Hippo signaling regulates Drosophila intestine stem cell proliferation through multiple pathways. *Proc Natl Acad Sci USA* 107:21064–21069.
41. Shaw RL, et al. (2010) The hippo pathway regulates intestinal stem cell proliferation during Drosophila adult midgut regeneration. *Development* 137:4147–4158.
42. Staley BK, Irvine KD (2010) Warts and Yorkie mediate intestinal regeneration by influencing stem cell proliferation. *Curr Biol* 20:1580–1587.
43. Chen CL, et al. (2010) The apical-basal cell polarity determinant crumbs regulates hippo signaling in Drosophila. *Proc Natl Acad Sci USA* 107:15810–15815.
44. Grzeschik NA, Parsons LM, Allott ML, Harvey KF, Richardson HE (2010) Lgl, aPKC, and crumbs regulate the salvador/warts/hippo pathway through two distinct mechanisms. *Curr Biol* 20:573–581.
45. Ling C, et al. (2010) The apical transmembrane protein crumbs functions as a tumor suppressor that regulates hippo signaling by binding to expanded. *Proc Natl Acad Sci USA* 107:10532–10537.
46. Robinson BS, Huang J, Hong Y, Moberg KH (2010) Crumbs regulates salvador/warts/hippo signaling in Drosophila via the FERM-domain protein expanded. *Curr Biol* 20:582–590.
47. Klebes A, Knust E (2000) A conserved motif in crumbs is required for E-cadherin localisation and zonula adherens formation in Drosophila. *Curr Biol* 10:76–85.
48. Wodarz A, Hinz U, Engelbert M, Knust E (1995) Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. *Cell* 82:67–76.
49. Zhang H, et al. (2015) SCF(Slmb) E3 ligase-mediated degradation of expanded is inhibited by the hippo pathway in Drosophila. *Cell Res* 25:93–109.
50. Bennett FC, Harvey KF (2006) Fat cadherin modulates organ size in Drosophila via the salvador/warts/hippo signaling pathway. *Curr Biol* 16:2101–2110.
51. 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.
52. Willecke M, et al. (2006) The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. *Curr Biol* 16:2090–2100.
53. Doggett K, Grusche FA, Richardson HE, Brumby AM (2011) Loss of the Drosophila cell polarity regulator scribbled promotes epithelial tissue overgrowth and cooperation with oncogenic Ras-Raf through impaired hippo pathway signaling. *BMC Dev Biol* 11:57.
54. Sun G, Irvine KD (2011) Regulation of hippo signaling by Jun kinase signaling during compensatory cell proliferation and regeneration, and in neoplastic tumors. *Dev Biol* 350:139–151.
55. Humbert PO, et al. (2008) Control of tumourigenesis by the scribble/Dlg/Lgl polarity module. *Oncogene* 27:6888–6907.
56. Gonzalez C (2013) Drosophila melanogaster: A model and a tool to investigate malignancy and identify new therapeutics. *Nat Rev Cancer* 13:172–183.
57. Pastor-Pareja JC, Xu T (2013) Dissecting social cell biology and tumors using Drosophila genetics. *Annu Rev Genet* 47:51–74.
58. Sonoshita M, Cagan RL (2017) Modeling human cancers in Drosophila. *Curr Top Dev Biol* 121:287–309.
59. Hoeller D, Hecker CM, Dikic I (2006) Ubiquitin and ubiquitin-like proteins in cancer pathogenesis. *Nat Rev Cancer* 6:776–788.
60. Nakayama KI, Nakayama K (2006) Ubiquitin ligases: Cell-cycle control and cancer. *Nat Rev Cancer* 6:369–381.
61. Ho KC, et al. (2011) Itch E3 ubiquitin ligase regulates large tumor suppressor 1 stability [corrected]. *Proc Natl Acad Sci USA* 108:4870–4875.
62. Ma B, et al. (2015) Hypoxia regulates hippo signalling through the SIAH2 ubiquitin E3 ligase. *Nat Cell Biol* 17:95–103.
63. Bae SJ, et al. (2015) NEDD4 controls intestinal stem cell homeostasis by regulating the hippo signalling pathway. *Nat Commun* 6:6314.
64. Kim TW, et al. (2014) SH3RF2 functions as an oncogene by mediating PAK4 protein stability. *Carcinogenesis* 35:624–634.
65. Cai J, et al. (2010) The hippo signaling pathway restricts the oncogenic potential of an intestinal regeneration program. *Genes Dev* 24:2383–2388.
66. Ma X, et al. (2015) Impaired hippo signaling promotes Rho1-JNK-dependent growth. *Proc Natl Acad Sci USA* 112:1065–1070.
67. Dunn B, Ma X (2017) The dark side of hippo signaling: A cancer promoter role. *Fly (Austin)* 11:271–276.
68. Ma X, et al. (2017) Hippo signaling promotes JNK-dependent cell migration. *Proc Natl Acad Sci USA* 114:1934–1939.
69. Ma X, et al. (2014) Bendless modulates JNK-mediated cell death and migration in Drosophila. *Cell Death Differ* 21:407–415.
70. Ma X, et al. (2015) Wallenda regulates JNK-mediated cell death in Drosophila. *Cell Death Dis* 6:e1737.
71. Fehon RG, Oren T, LaJeunesse DR, Melby TE, McCartney BM (1997) Isolation of mutations in the Drosophila homologues of the human neurofibromatosis 2 and yeast CDC42 genes using a simple and efficient reverse-genetic method. *Genetics* 146:245–252.
72. Zhang L, et al. (2008) The TEAD/TEF family of transcription factor scalloped mediates hippo signaling in organ size control. *Dev Cell* 14:377–387.
73. Boedigheimer MJ, Nguyen KP, Bryant PJ (1997) Expanded functions in the apical cell domain to regulate the growth rate of imaginal discs. *Dev Genet* 20:103–110.

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