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 Kirb 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 Cumbs-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.

Result

POSH Cooperates with Kirb to Control Tissue Growth. Based on the observation that loss of Kirb alone induces only mild growth (Fig. 1B) (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 > kirb.RNAi to induce eye overgrowth. One candidate EP interactor was inserted in the 5’ UTR of POSH (Fig. S1A), 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 > kirb.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

Significance

Here we performed a genetic screen in Drosophila and identified POSH (Plenty of SH3s), an E3 ubiquitin ligase, as a regulator of Hippo pathway. We found POSH ubiquitinates and degrades Ex (Expanded) to inactivate Hippo signaling. Intriguingly, POSH is particularly crucial for damage-induced intestinal stem cell renewal in a Yorki-dependent manner, highlighting the essential physiological role of POSH as a stress sensor in gut epithelia. Given the conservation of the Hippo signaling pathway between Drosophila and human, our findings here suggest that POSH might play a similar role in mammalian growth control.

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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, Bsk46) 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. S2D). 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 nubbun (nub) promoter is sufficient to induce overgrowth and proliferation (Fig. 2 K and L and Fig. S2E), 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. S2G). We found that 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. S2P 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 POSH74-null mutants are viable and have no obvious phenotype or food intake defect (Fig. 3N), 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. 3K) (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 promotor (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 (esg2 > GFP) suppressed DSS-induced ISC proliferation, as shown by the reduction in the number of GFP+ (Esq) and PHS+ 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 (MyoA < 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. S3G) 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. 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.
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 POSHΔRING (POSHΔR), 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
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\textsuperscript{intr}), and expression of Crb\textsuperscript{intr} also promotes growth, at least in part, by ubiquitin-dependent degradation of Ex (43, 45–48). To test whether POSH is required for Crb\textsuperscript{intr}-induced growth, we silenced POSH in Crb\textsuperscript{intr}-expressing cells. Interestingly, while POSH’s expression and subcellular distribution remain unaffected upon Crb\textsuperscript{intr} overexpression (Fig. S5 A–G), knockdown of POSH significantly suppresses Crb\textsuperscript{intr}-induced growth (Fig. 5 A–E and Fig. S3 Q–S). We also found that POSH depletion significantly impedes Crb\textsuperscript{intr}-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 Supermumery limbs (Slmb), a SCF (Skp/Cullin/F-box) E3 ubiquitin ligase, also ubiquinates Ex downstream of Crb (49). Consistently, we found knockdown of Slmb significantly impedes Crb\textsuperscript{intr}-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\textsuperscript{intr}-induced Ex degradation (Fig. 5 I, M, and N), indicating that POSH and Slmb act in parallel to regulate Crb\textsuperscript{intr}-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\textsuperscript{intr} 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.

Ubiquitation 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, 61–66). 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\textsuperscript{intr}-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 stimuli during normal feeding. To maintain gut homeostasis, timely ISC proliferation is essential to
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. S3F). 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 (Fig. S2). Consistent with 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 exist 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 GMRC stock center were used for this study: *nub-GAL4, nub-GAL4, hh-GAL4, en-GAL4, UAS-GFP, UAS-p35, POSH(POSH74), ban-lacZ, ex-lacZ, diap1-lacZ, and UAS-slimb.RNAI (33898). The following wing lines were collected from the Vienna Drosophila Resource Center: UAS-POSH.RNAI (v2, v2655), UAS-Kiara.RNAI (v106507), UAS-yki.RNAI (v40497), UAS-ex.RNAI (v22994), UAS-flgl.RNAI (v51249), UAS-dlg.RNAI (v41136), and UAS-fruit.RNAI (v3939). The strains kibraRNAi, UAS-Kibra (15), puc-lacZ (69), UAS-bac(1C) (70), mef (71), wts (9) were previously described. UAS-crmRNAi (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 (33) was 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, Myo1A-GAL4 UAS-GFP tub-GAL80, and suh-lacZ were gifts from Jin Jiang, University of Texas Southwestern, Dallas; UAS-ex.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 1x PBST containing 5% normal goat serum overnight at 4 °C. They were incubated 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–j-Gal (1:100) (DSHB), mouse anti-Wg (1:100) (DSHB), rabbit anti-Ph (1:300) (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-DIAPI (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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**Fig. 5.** POSH is required for Crb<sup>min</sup>-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-<sup>CRM</sup>-induced Ex degradation. (N) Quantification of Ex signal intensity ratio of GFP<sup>+</sup> region/GFP<sup>−</sup> 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.