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ORIGINAL ARTICLE

SCF^{Slmb} E3 ligase-mediated degradation of Expanded is inhibited by the Hippo pathway in *Drosophila*

Hongtao Zhang[1, 2, *], Changqing Li[1, *], Hanqing Chen[1, 2], Chuanxian Wei[1, 2], Fei Dai[1, 2], Honggang Wu[1, 2], Wen Dui[1, 2], Wu-Min Deng[3], Renjie Jiao[1, 4]

[1]State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, the Chinese Academy of Sciences, Datun Road 15, Beijing 100101, China; [2]University of Chinese Academy of Sciences, Beijing 100080, China; [3]Department of Biological Science, Florida State University, Tallahassee, Florida 32304-4295, USA; [4]Guangzhou Hoffmann Institute of Immunology, School of Basic Sciences, Guangzhou Medical University, Dongfengxi Road 195, Guangzhou, Guangdong 510182, China

Deregulation of the evolutionarily conserved Hippo pathway has been implicated in abnormal development of animals and in several types of cancer. One mechanism of Hippo pathway regulation is achieved by controlling the stability of its regulatory components. However, the executive E3 ligases that are involved in this process, and how the process is regulated, remain poorly defined. In this study, we identify, through a genetic candidate screen, the SCF^{Slmb} E3 ligase as a novel negative regulator of the Hippo pathway in *Drosophila* imaginal tissues via mediation of the degradation of Expanded (Ex). Mechanistic study shows that Slmb-mediated degradation of Ex is inhibited by the Hippo signaling. Considering the fact that Hippo signaling suppresses the transcription of *ex*, we propose that the Hippo pathway employs a double security mechanism to ensure fine-tuned homeostasis during development.

***Keywords*:** *Drosophila*; Hippo pathway; SCF^{Slmb}; Expanded

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Introduction

Initially discovered in *Drosophila*, the Hippo pathway has been shown to be an evolutionarily conserved pathway that functions in the control of tissue/organ size during development, through inhibition of cell proliferation and promotion of apoptosis [1-6]. Dysregulation of the Hippo pathway correlates with the occurrence of a broad range of human cancers [7-9]. Comprehensive and in-depth study of the regulatory network of Hippo signaling holds promise for developing anticancer strategies through targeting the Hippo pathway components. An essential regulatory step of the Hippo pathway lies in the inhibitory effect of the core kinase cassette, Hippo (Hpo) and Warts (Wts), which phosphorylates the transcriptional cofactor Yorkie (Yki) to secure its cytoplasmic localization. In the absence of Hippo signaling activity, Yki enters into the nucleus where, together with transcription factors such as Scalloped, it activates the expression of genes, such as *cycE*, *Diap1* and the microRNA *bantam,* which are essential for stimulation of cell cycle progression and inhibition of apoptosis [1-3, 6, 10]. The target genes of the Hippo pathway include some of its own components, e.g., the upstream Merlin (Mer)/Expanded (Ex)/Kibra complex, which inhibit Yki activity, indicating a feedback mechanism to fine-tune the pathway activity [11-14]. This negative feedback mechanism provides a strategy for the cells to balance Hippo signaling to maintain proper homeostasis during organism development: e.g., more Hippo signaling results in less production of Ex so that the cells proliferate sufficiently. However, it is less clear whether the stability of existing Ex/Mer/Kibra needs to be controlled to ensure that no over-proliferation occurs.

Increasing evidence demonstrates that posttranslation-

*These two authors contributed equally to this work.
Correspondence: Renjie Jiao[a], Wu-Min Deng[b]
[a]Tel: +86-10-6486-7568
E-mail: rjiao@sun5.ibp.ac.cn
[b]Tel: +1-850-6451501
E-mail: wumin@bio.fsu.edu
Abbreviations: Hippo (Hpo); Warts (Wts); Yorkie (Yki); Fat (Ft); Merlin (Mer); Expanded (Ex); Dachs (D); Four-jointed (Fj); Dachsous (Ds)
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al modulations of Hippo pathway components contribute significantly to the proper Hippo pathway functions [9, 15]. One of such modulations controls the protein stability of the Hippo signaling components. To date, three members of the Hippo pathway regulatory network have been reported to be unstable in *Drosophila*. The first evidence for the role of protein degradation in Hippo pathway regulation comes from the study of Dr Irvine's lab, describing the control of Wts protein level by the function of Fat (Ft) [16]. More recently, Zyxin has been found to regulate Wts protein stability together with Dachs (D) [17]. Ft can also modulate Hippo signaling through Ex, since in *ft* mutant clones Ex becomes unstable and mislocalized [18-21], and the effect of Ft on Ex seems to be also D-dependent [21]. In contrast, Ex is found to be stabilized but mislocalized upon *crb* depletion, whereas it is destabilized upon the overexpression of Crb^{intra} [22-25]. Very recently, the adhesion protein Echinoid has been shown to modulate Hippo signaling through maintenance of the proper localization and stability of Salvador [26]. However, the underlying mechanism(s) for controlling such protein stability during Hippo signaling remains poorly understood.

Ubiquitination-coupled proteolysis implements the turnover of many proteins in eukaryotic cells [27]. Among a variety of well-characterized E3 ligases, the SCF E3 ligases, the largest E3 family, have been found to play key roles in targeted proteolysis of cell cycle regulatory proteins [27]. SCF E3 ligases contain four subunits, SkpA, Cul1, Rbx1 and the F-box protein, with the F-box protein dictating the specificity of the substrates [27, 28]. A prerequisite for substrate recognition by most SCF E3 ligases is that the substrate protein has to be phosphorylated before the degradation process is spatio-temporally coordinated [29]. Although protein degradation has been implicated in the regulation of the Hippo pathway, little is known about whether the SCF E3 ligases mediate and execute protein degradation during Hippo signaling in *Drosophila*.

In this study, we performed a genetic candidate screen of *F-box* genes in *Drosophila* aimed at identifying SCF E3s that are involved in the regulation of the Hippo pathway. We first show that the SCF^{Slmb} E3 ligase functions as an antagonist of the Hippo pathway in the developing imaginal discs. Further experiments identify Ex as a substrate of SCF^{Slmb}. Finally, we show that the Hippo pathway activity directly inhibits the Ex degradation, revealing a double security mechanism that involves inhibition of Ex degradation and suppression of *ex* transcription in the presence of Hippo signaling, to fine-tune and secure the outcomes of Hippo signaling during *Drosophila* development.

Results

Dysfunction of the SCF E3 ligases leads to Hippo pathway activation

To investigate whether SCF E3 ligases are involved in the regulation of the Hippo pathway during *Drosophila* development, two core subunits of the SCF E3 ligase complexes, *dCul1* and *dSkpA*, were selectively knocked down by RNAi in the posterior region of the imaginal wing disc, after which two reporter transgenes, *ex-lacZ* and *Diap-lacZ,* of the Hippo pathway [11] were monitored. As shown in Figure 1, knockdown of either *dCul1* (Figure 1B-1B′ and 1F-1F′) or *dSkpA* (Figure 1C-1C′ and 1G-1G′) led to a significant increase in Hippo pathway activity, as judged by the reduced expression of both *ex-lacZ* and *Diap-lacZ* (Figure 1D and 1H). These observations suggest that one or more positive regulators of the Hippo pathway are upregulated upon disruption of the SCF E3 ligase function.

Identification of slmb as a negative regulator of the Hippo pathway

We next set off to search for a specific F-box protein(s) that is responsible for the SCF-mediated regulation of the Hippo pathway. To achieve this purpose, a candidate genetic screen was carried out with flies that ectopically express Yki under the control of *GMR-Gal4*, which mimics inactivation of the Hippo pathway, giving rise to an overgrowth-like phenotype in the eye [30] (Figure 1K). The *GMR > yki* flies were crossed singly to a collection of RNAi lines designed to specifically target the predicted 45 *F-box* genes in *Drosophila*, which have been systemically described in a phenotypic screen of F-box genes [27]. The expectation was that RNAi of some of the *F-box* genes would genetically suppress the overgrowth-like eye phenotype of *GMR > yki* flies. Indeed, out of 101 RNAi lines (targeting 44 *F-box* genes) used in our screen, two independent RNAi lines (*slmb-IR*) targeting non-overlapping regions of *slmb* mRNA sequence apparently suppressed the *GMR > yki* overgrowth-like phenotype (Figure 1L), whereas flies expressing *slmb-IR* alone exhibited eye size indistinguishable from that of the *GMR-Gal4* control (Figure 1I and 1J). These results indicate that *slmb* plays a role in modulating the Hippo pathway activity, probably by mediating the degradation of one or more of the Hippo pathway components.

Loss of slmb leads to activation of Hippo signaling activity

To further substantiate the idea that Hippo pathway activity is affected by the function of *slmb* during *Drosophila* development at the molecular level, the reporter genes were again used to monitor the alteration of the

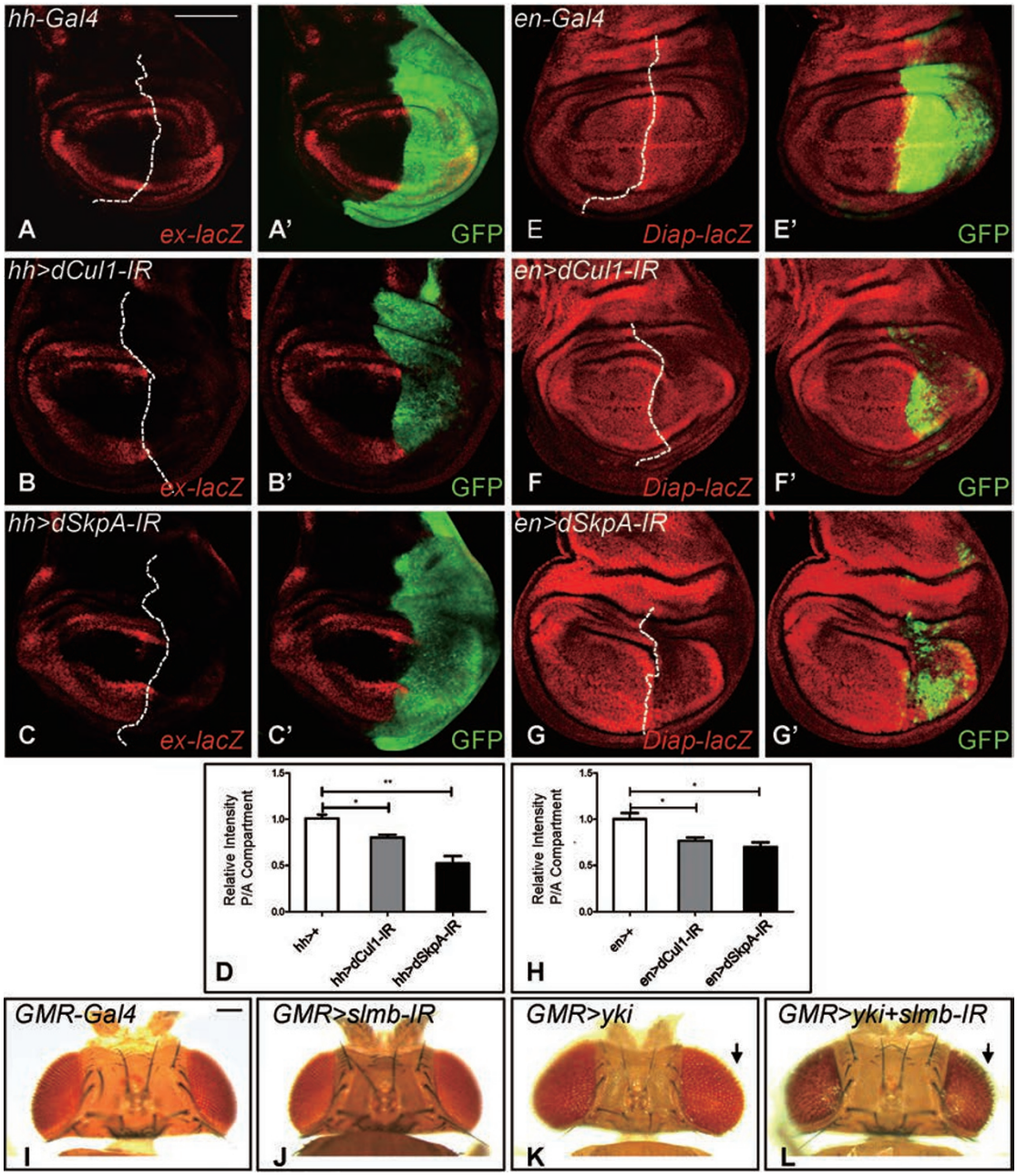

Figure 1 Disruption of the essential components of the SCF E3 ligase family led to Hippo pathway activation. **(A-C)** Wing discs from the control larva **(A)** or larvae that expressed *dCul1-IR* **(B)** or *dSkpA-IR* **(C)** under the control of *hh-Gal4 UAS-GFP* (green) were stained for *ex-lacZ* (red). **(E-G)** Wing discs from the control larva **(E)** or larvae that expressed *dCul1-IR* **(F)** or *dSkpA-IR* **(G)** under the control of *en-Gal4 UAS-GFP* (green) were stained for *Diap-lacZ* (red). Posterior is on the right for all wing discs. **(D** and **H)** Quantification of *ex-lacZ* **(D)** or *Diap-lacZ* **(H)** expression in wing discs of the indicated genotypes in **A-C** or **E-G**. **(I-L)** *slmb-IR* suppressed the overgrowth phenotype in the eye caused by overexpression of *UAS-yki* under the control of *GMR-Gal4* (arrows). Scale bar, 100 μm.

Hippo signaling. Knockdown of *slmb* in the posterior region of the wing disc led to a downregulation of both *ex-lacZ* (Figure 2A-2A′, 2D and Supplementary information, Figure S1A-S1A′) and *Diap-lacZ* (Figure 2B-2B′, 2E and Supplementary information, Figure S1B-S1B′). In line with this observation, *ex-lacZ* was also downregulated in the *slmb* mutant clones in the wing pouch (Supplementary information, Figure S1C-S1C′). It is worth to

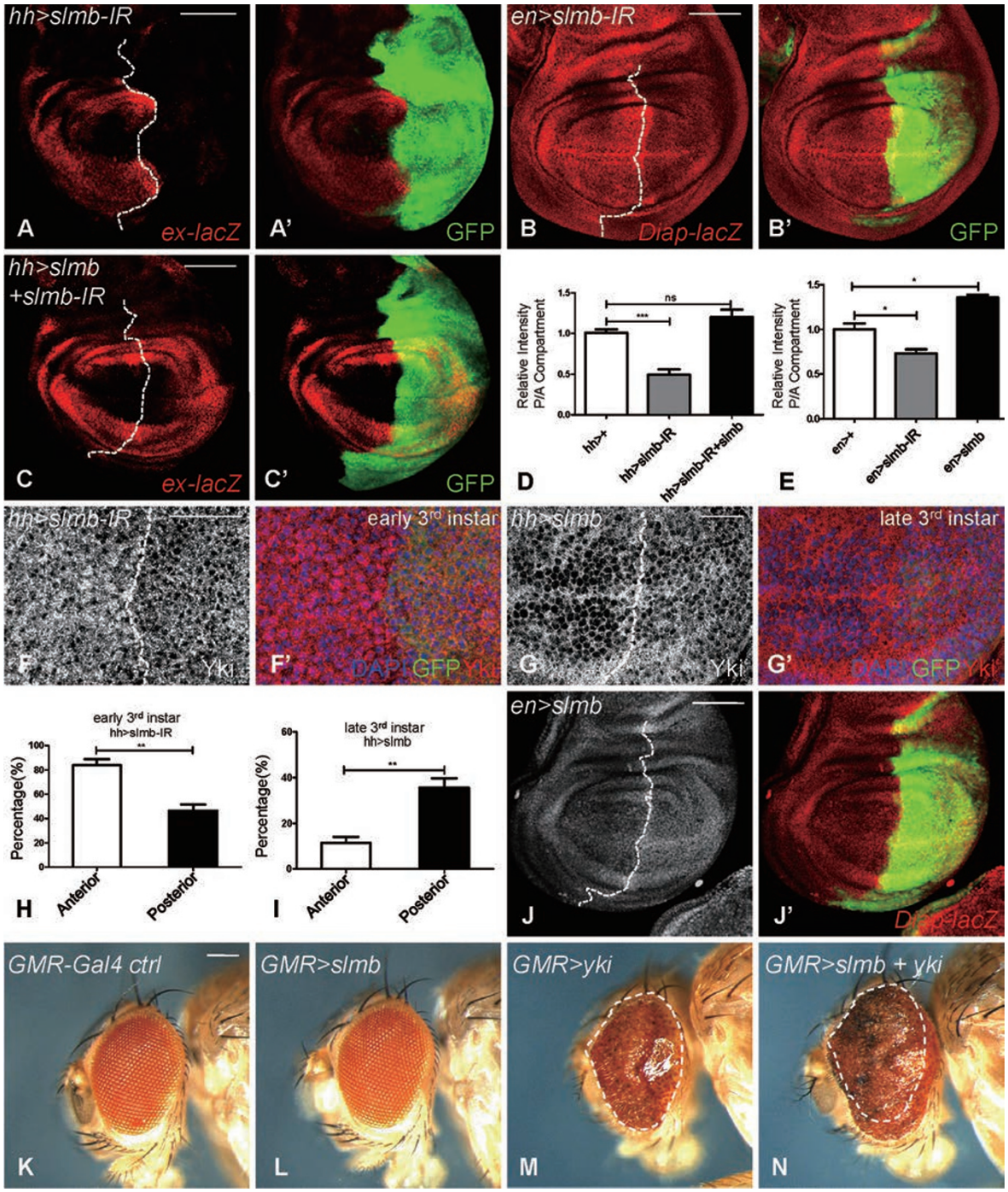

Figure 2 *slmb* functions as a negative regulator of the Hippo pathway during *Drosophila* development. **(A**-**B′)** Wing discs expressing *slmb-IR* under the control of *hh-Gal4 UAS-GFP* (green, **A′**) or *en-Gal4 UAS-GFP* (green, **B′**) were stained with anti-β-Gal antibody to show the expression of *ex-lacZ* (red, **A**-**A′**) or *Diap-lacZ* (red, **B**-**B′**). **(C**-**C′)** A wing disc expressing *hh-Gal4 UAS-GFP* (green) and *slmb-IR* + *UAS-slmb* was stained for *ex-lacZ* (red). **(D**-**E)** Quantification of *ex-lacZ* **(D)** or *Diap-lacZ* **(E)** expression in wing discs of the indicated genotypes in **A**, **C** and in Figure 1A, or in **B**, **J** and Figure 1E. **(F**-**G′)** An early third instar larval wing disc expressing *slmb-IR* **(F**-**F′)** and a late third instar larval wing disc expressing *UAS-slmb* **(G**-**G′)** under the control of *hh-Gal4 UAS-GFP* (green) were stained with anti-Yki to show the localization of Yki (red in **F′** and **G′** and white in **F** and **G**; blue is DAPI to visualize the nuclei). **(H**-**I)** Quantification of Yki nuclear localization in wing discs of the indicated genotypes in **F** and **G**. **(J**-**J′)** A wing disc from the larva expressing *en-Gal4 UAS-GFP* (green) and *UAS-slmb* were stained for *Diap-lacZ* (red in **J′** and white in **J**). Posterior is on the right for all wing discs. **(K**-**N)** Overexpression of Slmb synergistically enhances the overgrowth phenotype in the *GMR-yki* eye. Scale bar, 25 μm for **F** and **G**, 100 μm for other images.

note that, ectopic *ex-lacZ* expression was induced in wild-type cells abutting *slmb* mutant cells, which was most evident when the mutant clones were located in the notum region (Supplementary information, Figure S1C-S1C′). This non-autonomous effect of *slmb* mutant cells might have arisen from *slmb* mutant clone-induced morphogen production, such as Wg and Dpp [31]. Furthermore, the upregulation of Hippo pathway activity caused by *slmb* knockdown was suppressed by the overexpression of Slmb (Figure 2C-2C′, 2D). Another Hippo pathway reporter, *bantam-lacZ*, which has been reported to faithfully exhibit the expression of the microRNA gene *bantam* [32], was used to further confirm our observations. As expected, the expression of *slmb-IR* in the posterior compartment of the wing disc led to an evident decrease in the expression level of *bantam-lacZ* (Supplementary information, Figure S1D-S1E′). The subcellular localization of Yki has been shown to be correlated with the Hippo pathway activity in *Drosophila* imaginal wing discs [33], with a low level of nuclear Yki at late third instar larval stage and a high level of nuclear Yki at earlier third instar larval stage [17]. Consistent with the reduction in the expression of Hippo pathway reporters, we detected a decrease of the nuclear Yki upon *slmb* knockdown in the posterior region of the early third-instar larval wing disc, as compared to the wild-type anterior region (Figure 2F-2F').

To conclude that *slmb* functions as a negative modulator of the Hippo pathway, two other possibilities which could lead to altered Hippo activation in the absence of *slmb* must be excluded. One is a possible indirect induction by Arm, which is shown to be a substrate for Slmb and autonomously accumulated in *slmb* mutant cells in the wing discs [34]. Our results did not support this possibility because overexpression of Arm did not cause Hippo activation, but Hippo inactivation, as judged by the ectopic expression of *ex-lacZ* (arrow in Supplementary information, Figure S1H-S1H′) and the enlarged posterior compartment. The second possibility is that altered Hippo activiation in the absence of *slmb* may occur through elevated Ci, which has also been shown to be a substrate for Slmb. Our results did not support this possibility either, because Ci accumulation occurs only in the anterior compartments of the wing discs upon *slmb* depletion [34], whereas our data showed that the effects of *slmb* depletion on the Hippo pathway were not restricted in the anterior region of the wing disc, but were also seen in the posterior region (Supplementary information, Figure S1C-S1C′).

Gain of function of slmb gives rise to decreased Hippo signaling activity

Our data above show that Slmb is necessary for the proper Hippo signaling activity in the imaginal discs. To further strengthen the relation between Slmb and Hippo signaling, we turned to gain-of-function analyses. We used the Gal4-UAS system to drive *UAS-slmb* expression under the control of Gal4 lines expressed in the posterior region of the imaginal discs. Contrary to the effects of loss of function of Slmb on Hippo signaling activity (i.e., decreased expression of lacZ reporters and reduced Yki nuclear localization), Slmb overexpression led to slightly but evidently increased Yki nuclear localization in the late third instar imaginal disc (Figure 2G-2G′ and 2I) and enhanced expression of the two lacZ reporters, *Diap-lacZ* (Figure 2J-2J′ and 2E) and *bantam-lacZ* (Supplementary information, Figure S1F-S1F′ and S1G), indicative of inactivated Hippo signaling activity. Although overexpression of Slmb under the control of GMR-Gal4 did not induce discernible overgrowth in the eye (Figure 2K and 2L), co-expression of Slmb with Yki synergistically enhanced the enlarged and folded eye phenotype caused by Yki overexpression alone (compare Figure 2M with 2N). Taken together, these results demonstrate that Slmb is necessary and sufficient to negatively regulate the Hippo pathway during *Drosophila* development.

Slmb functions downstream of crb and in parallel with ft and ex

To precisely determine where *slmb* functions in the Hippo pathway, genetic epistasis experiments were designed based on the decreased expression of *ex-lacZ* in the wing discs of *hh* > *slmb-IR*. RNAi lines designed to target several Hippo pathway positive regulators were tested for their effects on the expression of *ex-lacZ* after knockdown of *slmb* (*slmb*-IR). Expression of *hpo-IR* resulted in an enlarged tissue size and increased *ex-lacZ* expression as expected (Figure 3A-3A′). The increased *ex-lacZ* expression could not be suppressed by co-expression of *slmb-IR* (Figure 3B-3B′ and 3I), suggesting that *hpo* is epistatic to *slmb*. Furthermore, co-expression of either *ft-IR* or *ex-IR*, two upstream regulators acting in parallel during Hippo signaling, reverted the decreased level of *ex-lacZ* caused by *slmb-IR* to a level similar to that of *ft-IR* or *ex-IR* expression alone (Figure 3C-3D′, 3E-3F′, 3J and 3K), implying that *slmb* functions upstream of or in parallel with *ex* and *ft*. To further pinpoint where *slmb* executes its function, we examined the epistatic relationship between *slmb* and *crb*. The apical polarity determinant protein Crb acts upstream of Hippo signaling to maintain a proper protein level and the apical localization of Ex [22, 24, 25]. Overexpression of Crb^{Intra} leads to Hippo signaling inactivation due to a decreased Ex protein level, which can be rescued by complement with Ex, indicating that *crb* is upstream of or in parallel

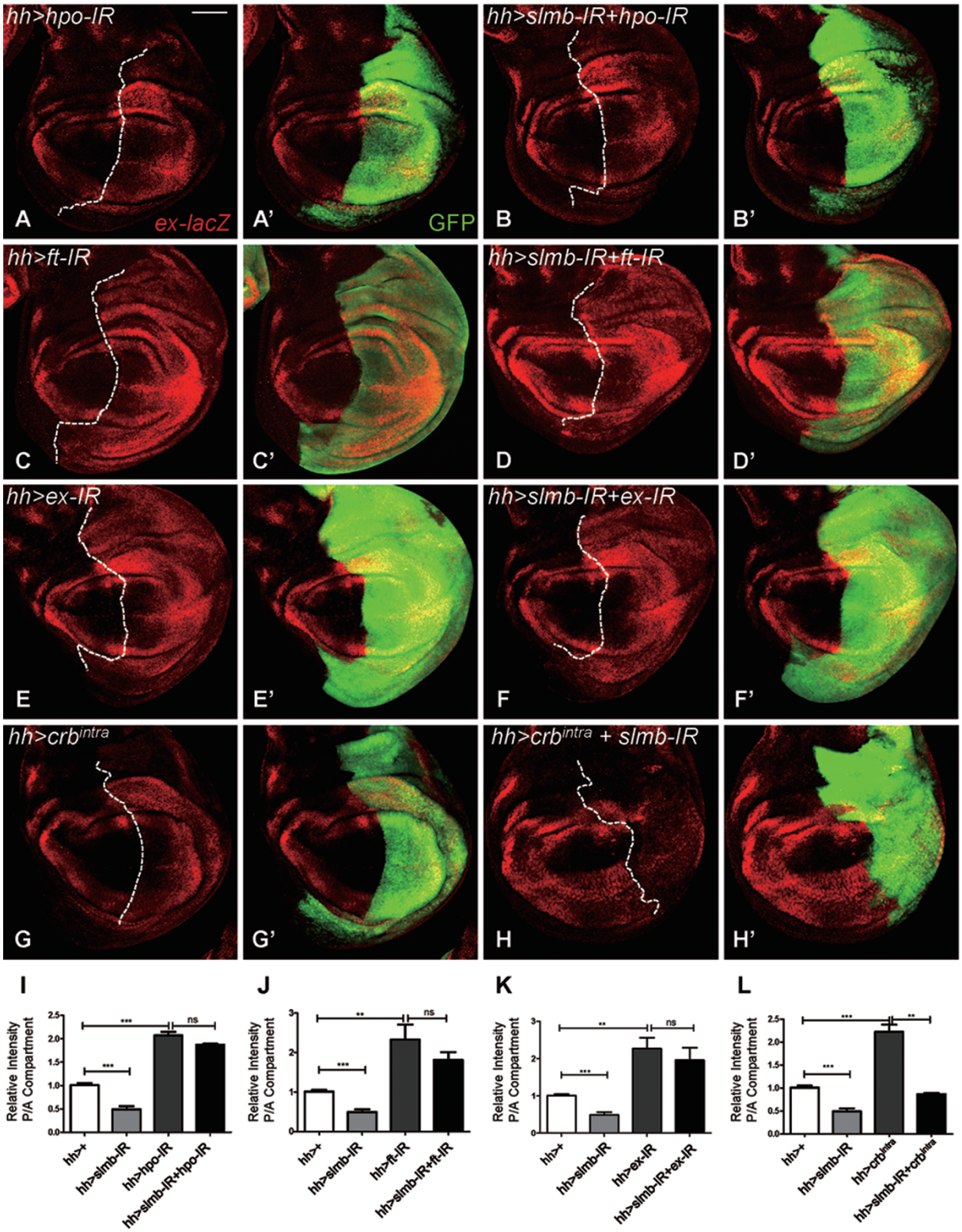

Figure 3 *slmb* functions downstream of *crb* and in parallel with *ft* and *ex* in the Hippo pathway. **(A-F′)** Wing discs expressing *hpo-IR* **(A-A′)**, *hpo-IR* + *slmb-IR* **(B-B′)**, *ft-IR* **(C-C′)**, *ft-IR* + *slmb-IR* **(D-D′)**, *ex-IR* **(E-E′)**, *ex-IR* + *slmb-IR* **(F-F′)**, *uas-crb* **(G-G′)**, *uas-crb+slmb-IR* **(H-H′)** under the control of *hh-Gal4 UAS-GFP* (green) were stained for *ex-lacZ* (red). The downregulation of *ex-lacZ* caused by *slmb* knockdown was suppressed by co-expression of either *hpo-IR* **(A-B′)**, *ft-IR* **(C-D′)**, or *ex-IR* **(E-F′)**, but not *UAS-crb*^{intra} **(G-H′)**. Posterior is on the right for all wing discs. **(I-L)** Quantification of *ex-lacZ* expression in wing discs of the indicated genotypes in **A-H**, and in Figures 1A and 2A. Scale bar, 50 μm.

with *ex*. Interestingly, the upregulation of *ex-lacZ* associated with Crb^{Intra} overexpression (Figure 3G-3G′) can be suppressed by *slmb-IR* expression (Figure 3H-3H′ and 3L), indicating that *slmb* functions downstream of *crb*.

The epistasis between *slmb* and the known components of the Hippo pathway were further verified by examining the genetic interactions between *slmb* and those genes in the adult wing. When *slmb* was knocked down under the control of *dpp-Gal4*, the area of the *dpp* expression domain between the veins L3 and L4 was reduced, which led to a deformation of the entire wing (Supplementary information, Figure S2B). Expression of a *UAS-slmb* transgene completely rescued the growth defect caused by *dpp* > *slmb-IR* (Supplementary information, Figure S2C), arguing against the possibility of any off-target effects. The wing defects associated with *slmb* knockdown between veins L3 and L4 were significantly suppressed by co-expression of *ex-IR* or *wts-IR* (Supplementary information, Figures S2D-S2G). Together with the parallel role of *ex* and *ft* during Hippo signaling [21], these results suggest that *slmb* functions downstream of *crb* and in parallel with *ex* and *ft* during Hippo signaling.

The SCF^{Slmb} complex regulates the stability of Ex in Drosophila imaginal discs

Given that SCF E3 ligases target polyubiquitinated substrates for proteasomal degradation [27], it is plausible to hypothesize that a positive regulator upstream of the Hippo pathway is subject to SCF^{Slmb}-mediated degradation. Considering the epistatic relationship that we have shown above and the fact that both Ft and Crb regulates the stability of Ex [18, 20-22, 24, 25], we hypothesized that Ex may be the target of Slmb. To test this hypothesis, we first examined the effect of *slmb* depletion on the endogenous level of Ex. Ex has been shown to co-localize with Mer at the apical membrane region in the wing disc [35]. Intriguingly, an apparent upregulation of Ex was observed when *slmb* was knocked down in the posterior region of the wing disc, as compared to the wild-type anterior region (Figure 4A-4B′ and 4G). The increased Ex was mainly localized in the apical region of the cells, where it is normally localized, as shown by the transverse section (Supplementary information, Figure S3A-S3B′). In contrast, knockdown of *slmb* did not lead to apparent upregulation but downregulation of Mer (Supplementary information, Figure S3C-S3C′), probably due to the feedback effect of Hippo pathway activation. Consistently, in the *slmb* mutant clones of the wing disc, more Ex was detected than in the surrounding wild-type cells (Figure 4C-4C′). An increase of Ex level was also observed when *slmb* was knocked down by *mirr-Gal4* in the eye disc (Supplementary information, Figure S3D-S3D′) and the increased Ex was apically localized as well (Supplementary information, Figure S3D). Importantly, the increased Ex was attributed to reduced degradation of the protein rather than increased expression of *ex* because the transcription of *ex* was downregulated (Figure 2A-2A′).

We found that knockdown of either *dCul1* or *dSkpA*, two essential components for SCF^{Slmb} function, induced an accumulation of the endogenous apically-localized Ex (Figure 4D-4E′ and 4G-4H) in the wing disc. Further corroborating the idea that Ex is degraded through an SCF^{Slmb}-mediated proteasomal process. Taken together, these results suggest that Slmb regulates Ex stability dependent of a functional SCF^{Slmb} complex.

In line with the notion that Slmb mediates Ex degradation, overexpression of *slmb* in the posterior region of the wing disc led to a decrease of apically localized Ex in the posterior compartment (Figure 4F-4F′ and 4G; image was taken from an apical focus plane). The reduction in the apical Ex was not due to an altered localization of Ex from the apical region to the basal-lateral membrane, because the transverse section image also showed an overall weaker staining of Ex than that in the wild-type anterior compartment (Supplementary information, Figure S3E-S3E′). The decrease of Ex was also reflected by the increased Yki activity (Figure 2G-2G′). These results indicate that *slmb* is both necessary and sufficient for controlling the stability of Ex *in vivo* during *Drosophila* development.

Slmb interacts with Ex and promotes its ubiquitination and degradation in S2 cells

To further substantiate the relationship between Slmb and Ex, we performed a series of experiments with cultured S2 cells. First, we showed that Ex stability in S2 cells is also associated with the function of Slmb as observed in the imaginal tissues. In line with the *in vivo* results, co-expression of HA-tagged Slmb downregulated the protein level of Flag-tagged Ex, while the expression of a dominant-negative form of Slmb (Slmb-ΔF) enhanced its stability (Figure 5A). However, co-expression of HA-tagged Slmb or Slmb-ΔF failed to induce any changes in the protein level of either Flag-tagged Hpo or V5-tagged Wts (Supplementary information, Figure S4A and S4B), indicating a specific role of Slmb in the regulation of Ex stability. Second, immunoprecipitation results showed that ubiquitinated Ex (Myc-tagged) was detected in the presence of both Ubiquitin (FLAG-tagged) and MG132 (Figure 5B). The level of Ex ubiquitination was reduced when cells were treated with dsRNA of *slmb* (Figure 5C-5D), suggesting that Slmb is specifically required for the ubiquitination of Ex. To test whether

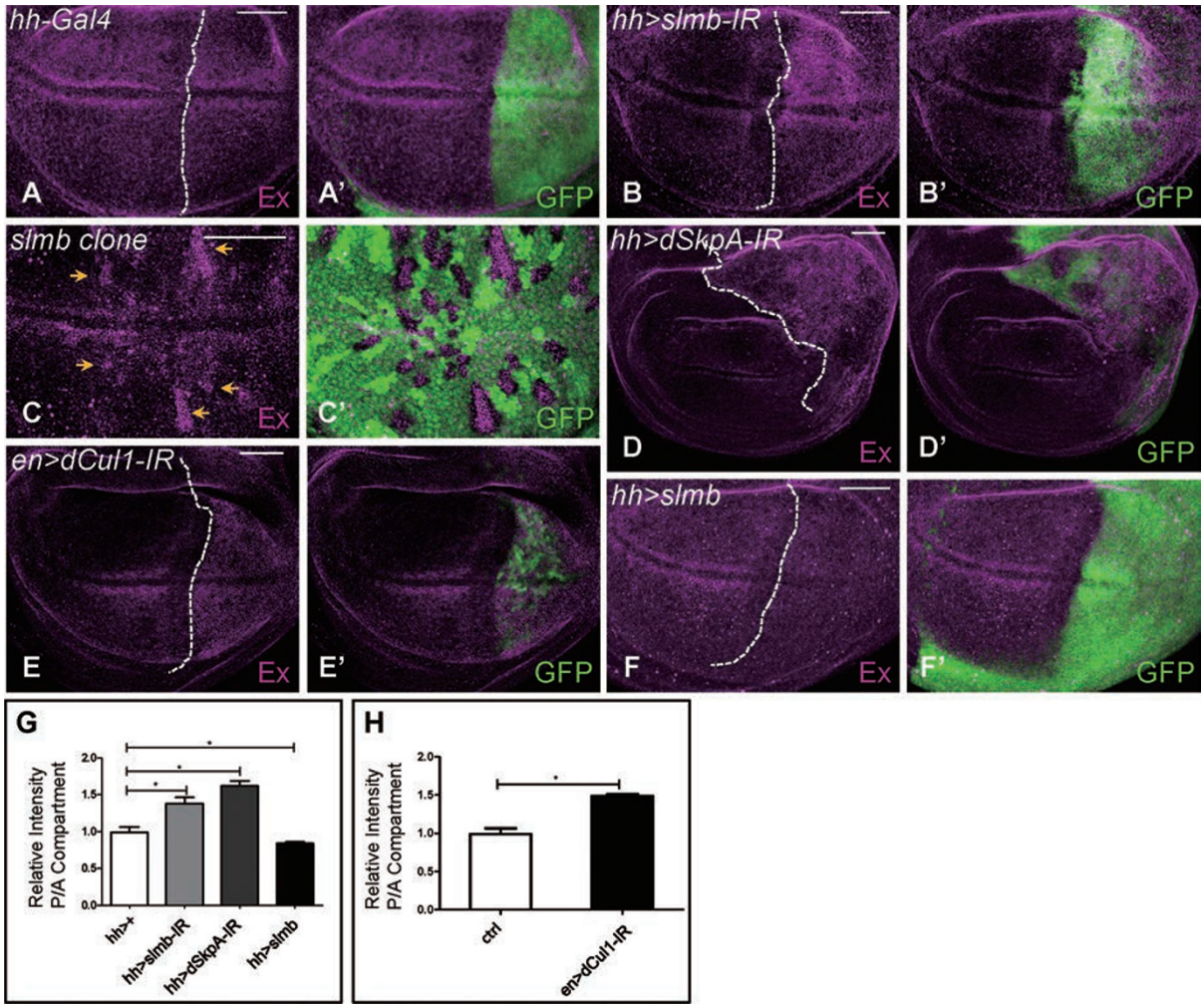

Figure 4 SCF^{Slmb} is both necessary and sufficient for maintaining Ex stability in *Drosophila* imaginal discs. **(A-E')** Loss of function of SCF^{Slmb} led to Ex accumulation. Wing discs from the control larva **(A-A')** or larvae that expressed *slmb-IR* **(B-B')**, *dSkpA-IR* **(D-D')** or *dCul1-IR* **(E-E')** were stained for Ex (magenta). A wing disc carrying *slmb* mutant clones (marked by the absence of GFP, yellow arrows) was stained for Ex (magenta) **(C-C')**. **(F-F')** Gain of function of Slmb led to a reduction of Ex. A wing disc from the larva that expressed *hh-Gal4 UAS-GFP* (green) and *UAS-slmb* was stained for Ex (magenta). Posterior is on the right for all wing discs. **(G-H)** Quantification of Ex protein level changes in wing discs of the indicated genotypes. Scale bar, 50 μm.

Ex is physically associated with Slmb at the molecular level, differentially tagged proteins of Ex and Slmb were transiently transfected into cultured S2 cells. Co-immunoprecipitation results showed that FLAG-tagged Ex and HA-tagged Slmb were detected in the same precipitation complex (Figure 5F, lanes 1-2). Intriguingly, both the N- and C-terminal fragments of Ex could independently immunoprecipitate HA-tagged Slmb (Figure 5F, lanes 3-4), and the N-terminal part has less affinity. To further dissect the region(s) in Ex responsible for its interaction with Slmb, we arbitrarily divided the C-terminal region into three parts (ExC1, from aa 654 to 911; ExC2, from aa 912 to 1164; ExC3, from aa 1 168 to 1 427, Figure 5E) with a similar size. Co-immunoprecipitation between Slmb and each of the three parts showed that Slmb interacted mainly with the middle part of Ex C-terminal region (ExC2; Figure 5G). These results suggest that Slmb and Ex may be associated with each other *in vivo*. To further address whether a direct physical association between Slmb and Ex may exist, we conducted *in vitro* GST pull-down assays with GST-Slmb and His-ExC2. The result showed that Slmb interacted directly with ExC2 (Figure 5H).

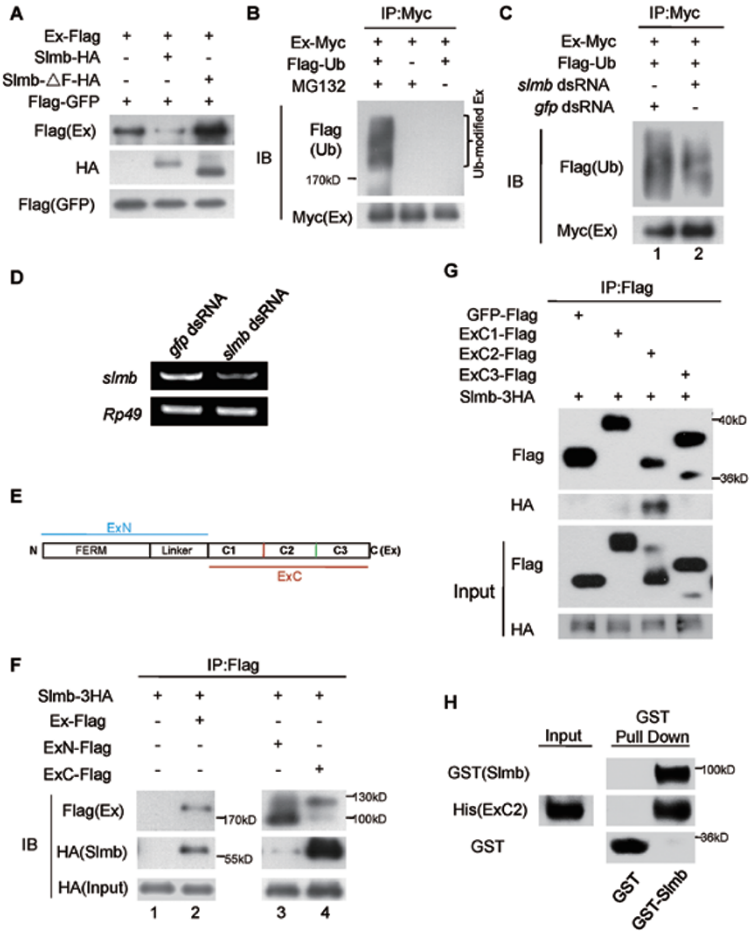

Figure 5 Slmb is physically associated with Ex and required for its ubiquitination in S2 cells. **(A)** Slmb promotes Ex degradation in S2 cells. Flag-tagged Ex were co-transfected with HA-tagged Slmb or Slmb-ΔF into S2 cells. GFP was used as a transfection control. **(B)** Ex ubiquitination in S2 cells. Signals on the upper gel indicate the ubiquitinated Ex. Note the ubiquitination of Ex can only be detected with the addition of MG132. **(C)** RNAi of *slmb* led to reduced Ex ubiquitination. S2 cells transfected with Myc-tagged Ex and Flag-tagged ubiquitin were divided into two parts and treated with dsRNAs of either *gfp* (lane 1) or *slmb* (lane 2). The efficiency of *slmb* RNA interference is assessed and shown in **D**. **(E)** Schematic of the domain organization of Ex protein. **(F)** Slmb was physically associated with Ex in S2 cells. Lanes 1-4: Slmb-HA was detected in the immunoprecipitates with anti-Flag antibody from the S2 cell extracts that co-expressed Ex-Flag (lane 2), ExN-Flag (N-terminal half of Ex, aa 1-653, lane 3), or ExC-Flag (C-terminal half of Ex, aa 654-1427, lane 4). **(G)** HA-tagged Slmb bound mainly to the middle part of Ex C-terminal region (ExC2). C-terminal region of Ex was divided into three parts (ExC1 258 aa, ExC2 253 aa, ExC3 260 aa). HA-tagged Slmb was co-immunoprecipitated by Flag-tagged ExC2, but not ExC1 or ExC3. **(H)** Direct interaction of Slmb with ExC2 *in vitro*. Bacterial cell lysates with His-ExC2 expression were incubated with Glutathione agarose beads containing purified GST (Ctrl) or GST-Slmb. Note that GST-Slmb bound to ExC2, but GST did not. Molecular weights of the protein marker used are indicated to the right of the gels. IB: immunoblot; IP: immunoprecipitation.

Degradation of Ex by Slmb is inhibited by Wts

We have shown that Slmb degrades Ex through an SCF E3-mediated proteasomal proteolysis mechanism. We next wondered how this process is controlled. We noted that, in wild-type wing discs the expression pattern of *ex-lacZ* and the pattern of endogenous Ex protein are apparently different. *ex-lacZ* expression is highest in proximal cells (Figure 1A), whereas more Ex protein is detected in the distal cells than the proximal cells (e.g., Figure 4A, Supplementary information, Figure S3A, and the anterior regions of Figure 4E and Supplementary information, Figure S5A). More interestingly, when *slmb* or *dCul1* was depleted, Ex becomes evenly distributed in cells of both proximal and distal part of the disc (Figure 4B and 4E, and Supplementary information, Figure S3B, posterior part). These observations led us to hypothesize that Ex in the proximal cells of the wing disc may be more prone to degradation by Slmb than that in the distal cells.

The "complementary" pattern of *ex-lacZ* and Ex is reminiscent of the expression patterns of Four-jointed (Fj) and Dachsous (Ds), two Ft signaling regulators expressed in opposite gradients [36-41]. Specifically, the distal wing disc cells express the highest level of Fj and the proximal wing disc cells express the lowest level, whereas Ds expression shows the opposite pattern [39, 41]. This reminiscence promoted us to wonder whether there is any connection between the Ft signaling and Slmb-Ex interaction. With high *fj* expression in the distal cells, the Ft signaling activity is assumed to be also higher than that in the proximal cells since Fj is able to promote the ability of Ft to bind to its ligand Ds [42]. Functional connections between Ft and Ex have been established by several studies [18-21] and the genetic interaction between *ex* and *ft* occurs only in the Ft-Hippo signaling. Based on these, we hypothesized that the high Ft-Hippo signaling activity in the distal wing disc cells weakens the ability of Slmb to degrade Ex. In fact, Ft is shown to maintain the stability of Ex [18, 20, 21] although the underlying mechanism(s) is poorly defined.

Interestingly, upon carefully examining the amino acid sequence of Ex, a Wts/Lats consensus ^{1111}HXRXXS^{1116} motif in the middle part of Ex C-terminal region was detected (Figure 6A), suggesting that there might be a

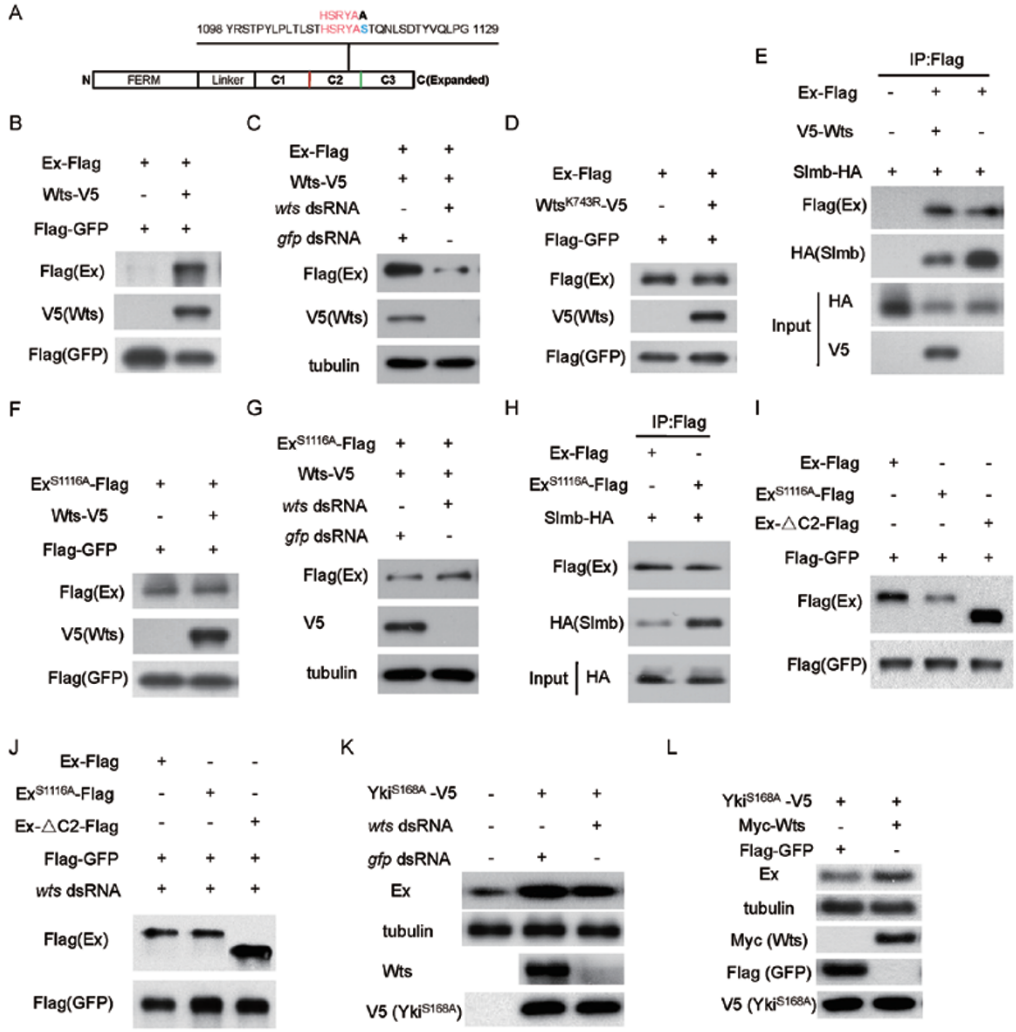

Figure 6 Wts protects Ex from Slmb-mediated degradation in S2 cells. **(A)** Schematic of the protein structure of Ex and the relative position of the putative Wts phosphorylation site (HSRYAS). **(B-C)** Wts promotes stability of Ex. Co-expression of V5-tagged Wts with Flag-tagged Ex led to increased Ex **(B)**, which can be reverted by addition of dsRNA of *wts* **(C)**. **(D)** The ability of Wts to stabilize Ex is dependent on its kinase activity. Substitution of Arginine at 743, which is essential for the kinase activity of Wts, with Lysine rendered Wts unable to stabilize Ex. **(E)** Wts antagonizes the association between Slmb and Ex. Note the co-immunoprecipitated HA-tagged Slmb by Flag-tagged Ex was decreased in the presence of V5-tagged Wts. **(F-G)** Ex stabilization by Wts is dependent on the putative Wts phosphorylation site. V5-tagged Wts was unable to stabilize Flag-tagged Ex when the Serine at 1116 of Ex was mutated to Alanine. **(H)** Ex^{S1116A} exhibits higher affinity to Slmb than Ex. When similar amounts of Ex or Ex^{S1116A} were immunoprecipitated, Ex^{S1116A} pulled down more Slmb than Ex. **(I)** Ex, Ex^{S1116A} or ExΔC2 was transiently transfected into S2 cells and their steady state protein levels were compared. GFP was employed as the transfection control. Note that ExΔC2 appeared to be the most stable, whereas Ex^{S1116A} appeared to be the least stable. **(J)** S2 cells transiently transfected with Ex, Ex^{S1116A} or ExΔC2 were treated with *wts* dsRNA. Compared with **I**, the difference in steady state protein level between Ex and Ex^{S1116A} became smaller, whereas the difference between ExΔC2 and Ex^{S1116A} was not significantly changed upon *wts* knockdown. **(K-L)** The effect of Wts on endogenous Ex stability was evaluated in the presence of Yki^{S168A}. Yki^{S168A} overexpression led to an increase in Ex protein level as expected (compare the left lane with the middle lane in **K**). When Wts was knocked down, endogenous Ex was evidently decreased (compare the right lane with the middle lane in **K**), and when Wts was overexpressed, endogenous Ex was markedly increased **(L)**.

direct interaction between Wts and Ex. We thus set out to explore the possible influence of the Wts kinase on Ex stability. Since there is negative feedback by the Hippo pathway on the expression of Ex, we are unable to directly manipulate the Hippo pathway *in vivo* to examine its effects on endogenous Ex stability. The cultured S2 cells

were used to address this issue. Tagged forms of Wts and Ex were co-transfected into the S2 cells before the tagged Ex protein level was monitored. Indeed, ectopic expression of Wts could stabilize Ex, and knockdown of Wts led to a decrease in Ex abundance (Figure 6B-6C). To evaluate more precisely the effects of Wts on Ex stability, we examined the half-life of Ex with Wts overexpression or knockdown treatment after CHX blockage of translation. In normal condition, the half-life of Flag-tagged Ex was around 2.5 h (Supplementary information, Figure S4C and S4F). Consistent with our finding that Wts stabilizes Ex, overexpression of Wts extended the half-life of Ex to about 4 h (Supplementary information, Figure S4C and S4F), whereas knockdown of endogenous *wts* led to a shortened half-life of less than 2 h (Supplementary information, Figure S4C and S4F). The effect of Wts was dependent on its kinase activity because the kinase-dead form of Wts (Wts^{K743R}) failed to stabilize Ex (Figure 6D). To investigate how Wts may promote the stability of Ex, the influence of Wts on the physical association between Ex and Slmb was examined. The hypothesis is that Wts may prevent Slmb from binding to Ex by modifying Ex, e.g., through phosphorylation. In line with our hypothesis, the association between Slmb and Ex was strongly attenuated in the presence of Wts (Figure 6E). To evaluate the functional significance of the potential Wts consensus motif, we mutated the serine residue to alanine (S1116A), which resulted in the failure of stabilization of Ex^{S1116A} by Wts as compared with wild-type Ex (Figure 6F-6G, Supplementary information, Figure S4D and S4F). In addition, Ex^{S1116A} exhibited a stronger binding affinity to Slmb than its wild-type counterpart (Figure 6H). Moreover, Ex^{S1116A} became less stable than the wild-type Ex *in vitro*, as shown by both the steady state level (Figure 6I) and the half-life of the protein (Supplementary information, Figure S4D and S4F), supporting the idea that the serine at the site of 1116 (S^{1116}) may be critical for its stability. Interestingly, Ex and Ex^{S1116A} became similar in stability when endogenous Wts was knocked down (Figure 6J and Supplementary information, Figure S4F), further strengthening the notion that Wts stabilizes Ex through S^{1116}. Notably, Slmb showed the highest affinity to the region that contains the Wts consensus motif (ExC2, Figure 5G), implying the possibility that a modification of Ex by Wts may change the protein structure of Ex, interfering with its binding to Slmb. Given that Wts phosphorylates Ex to prevent Slmb from binding to the C2 region or Ex, we hypothesized that Ex ΔC2 (Ex lacking the C2 region) should be more stable than wild-type Ex, and Ex ΔC2 should no longer respond to Wts dosage change. Supporting this hypothesis, the steady state level of Ex ΔC2 was indeed higher than that of Ex (Figure 6I), and did not decline with *wts* knockdown whereas wild-type Ex did (Figure 6J). Also, Ex ΔC2 exhibited longer half-life than Ex (Supplementary information, Figure S4E and S4F), underscoring the essential role of the C2 region in the regulation of Ex stability. In support of a possible direct association between Wts and Ex, these two proteins were reciprocally immunoprecipitated by each other from the S2 cell extracts (Supplementary information, Figure S4G).

As described thus far, the regulation of Ex by Wts was demonstrated in S2 cells using overexpressed tagged Ex protein. To further explore the physiological relevance of this regulation, we set out to probe the role of Wts in the regulation of endogenous Ex. As mentioned previously and shown in Supplementary information, Figure S4H, endogenous Ex level is affected by Wts dosage change due to the feedback regulation, as manifested by the increase of Ex with *wts* dsRNA treatment and decrease of Ex upon Wts overexpression. To bypass the feedback transcriptional regulation of *ex* by Wts, constitutively active form of Yki (Yki^{S168A}) was used. In consistency with our findings described earlier, overexpression of Wts upregulated, while knockdown of *wts* downregulated, the endogenous Ex level in the presence of Yki^{S168A} (Figure 6K-6L). Taken together, these results strongly suggest a model in which Wts regulates the interaction between Slmb and Ex, likely through phosphorylation at S^{1116} to stabilize Ex.

Discussion

Slmb is one of the well-characterized F-box proteins in *Drosophila*, which participates in a variety of cellular processes through mediating the degradation of different targets. Here we report that Slmb degrades Ex, which negatively regulates and is directly inhibited by Hippo signaling. Our results reveal for the first time an inhibitory role of Hippo signaling for Ex stability, which suggest that a double security mechanism may exist in Hippo signaling to secure the balanced outcomes during *Drosophila* development (Figure 7). This mechanism is essential for animal development because both under- and over-proliferation can cause a failure to maintain proper tissue and organ size.

During preparation of our manuscript, we noted that the biochemical interaction between Slmb and Ex was very recently reported by Ribeiro and colleagues [43]. They identified Slmb as an Ex-associated protein via an AP-MS approach and showed that Slmb is required for Crb-mediated degradation of Ex. The novel findings in our study include: (1) independent identification of the interaction of *slmb* and *ex* through a genetic candidate

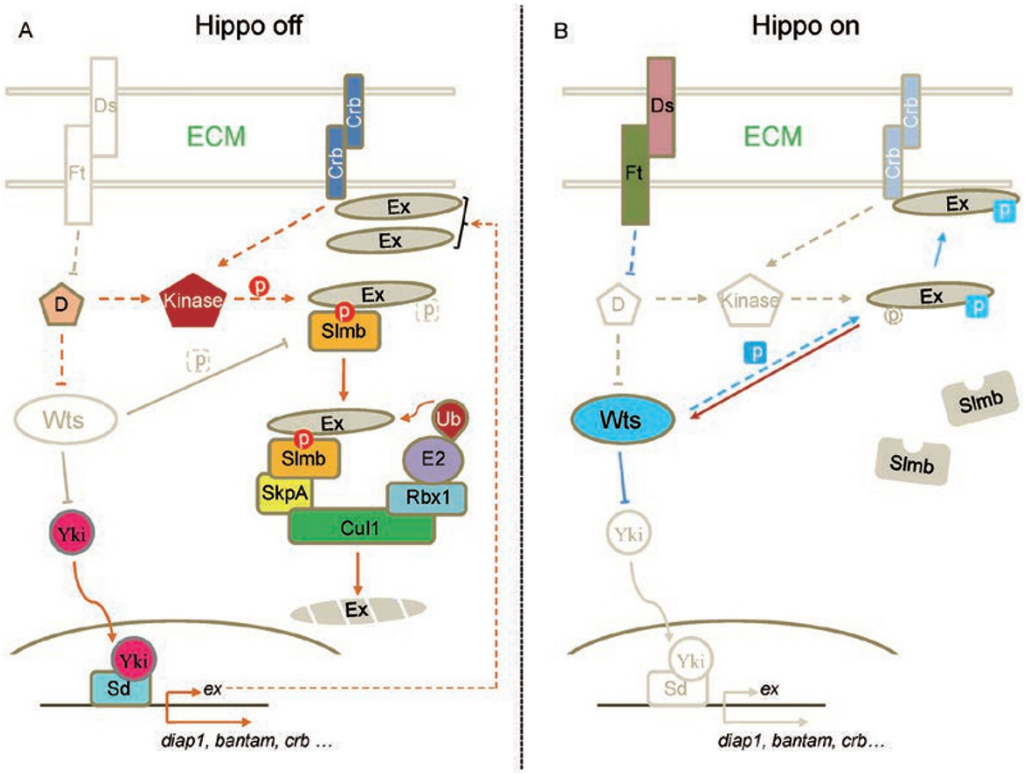

Figure 7 A double security mechanism to regulate the cellular Ex level by Hippo signaling. The cellular abundance of Ex is regulated by the Hippo signaling at both transcriptional and post-translational level. Transcription of *ex* is activated by Yki. Previous studies [18, 20-22, 24, 25] have shown that Ft maintains a proper level of both Ex and Wts through antagonizing the activity of D; Crb promotes the degradation of Ex via an undefined mechanism(s). In this figure, we propose, as described in our paper, that **(A)** when Hippo signaling is 'off', Crb and D may activate an unknown kinase(s) that is/are necessary for the phosphorylation of Ex, a prerequisite for SCF^{Slmb}-mediated degradation. Alternatively, D may promote the degradation of Ex merely through its regulation of Wts. In the meantime, transcription of *crb* is upregulated [50], which may contribute further to the degradation of Ex. In case that the cellular Ex is not sufficient, Hippo signaling employs a negative feedback mechanism, via upregulating the transcription of *ex* by Yki, to compensate for the degraded Ex; **(B)** when Hippo signaling is 'on', Wts becomes associated to and phosphorylates Ex, which prevents Slmb from binding it to mediate the degradation. The degradation of Ex may be further attenuated by the downregulated *crb* transcription. In case that the cellular Ex becomes more than needed, Hippo signaling shuts down the transcription of *ex* via inactivating Yki. The paled symbols indicate inactive components or processes that are not in function. ECM: extra-cellular matrix.

screen; (2) dissection of the role of *slmb* in Hippo signaling; (3) identification of the functional interaction between *slmb* and *ex* regulated by the Hippo pathway. The interaction of Slmb and Ex described in Ribeiro and colleagues' paper was mainly characterized through biochemical approaches, which leaves the *in vivo* relevance of this interaction as well as the role of *slmb* in Hippo signaling less addressed. For example, upregulation of Ex in *slmb* mutant cells is not sufficient to conclude that Slmb degrades Ex, because the increase of Ex could be due to either enhanced stability of Ex or increased transcription of *ex*. The former possibility may lead to activation of the Hippo pathway and downregulation of *ex-lacZ*, consistent with Slmb degradation of Ex, whereas the latter possibility may result from inactivation of the Hippo pathway. Our study clearly discriminates between these two possibilities and reveals a negative role of Slmb in Hippo signaling regulation.

β-TrCP, the human orthologue of Slmb, has been identified to mediate the degradation of YAP and TAZ at high cell density in a Lats/Wts phosphorylation-dependent manner [44, 45]. The fact that the phosphodegron in YAP does not exist in Yki raises the question of whether *slmb* plays a similar role in Yki degradation in *Drosophila*, given that the regulation of Hippo signaling during animal development is highly conserved. Our findings indicate that at least in the wing discs, Yki is not a *bona fide* substrate for Slmb because neither upregulation of

Yki upon *slmb* depletion (Figure 2F-2F′) nor downregulation of Yki upon Slmb overexpression (Figure 2G-2G′) was detected in our experiments. However, the seeming discrepancy between Slmb and β-TrCP in terms of their roles in Yki/YAP degradation may be a reflection of different cellular contexts. β-TrCP-mediated YAP degradation takes place at high cell density when contact inhibition occurs, whereas Slmb-mediated Ex degradation is shown mainly in tissues that mostly contain proliferating cells. Given the conserved role of Slmb and β-TrCP in various cellular processes, it is possible that Slmb may degrade Yki in certain cells with lesser or no proliferating potential. Thus, the possibility that Slmb may regulate the turnover of Yki is not formally excluded.

Studies in both *Drosophila* and the mammals have indicated the existence of a short conserved stretch of amino acids, known as degron, in some of the targets of Slmb/β-TrCP [46, 47]. The degron is responsible for target recognition and binding. Slmb/β-TrCP usually recognizes a consensus DSGXXS motif or its variant(s), in which the two serine residues need to be phosphorylated to facilitate the binding of the target to Slmb/-TrCP [46, 47]. By searching for such degron(s) in Ex, we identified two putative motifs that conform to the consensus sequence (^{188}DSGEETS^{194} and ^{452}TSGIVS^{457}). However, they did not appear to function as degrons because: 1) Substitution of the serine residues with alanine in either motif or both motifs did not compromise the binding ability of Ex to Slmb (data not shown); 2) The ubiquitination status of Ex was not changed with mutations in these two motifs (data not shown). To our surprise, Ribeiro and colleagues reported that the ^{452}TSGIVS^{457} motif is the degron for Slmb-mediated degradation, as both the Slmb-ExN (Ex-N terminal part) interaction and ubiquitination level of ExN decrease when the serine residues are mutated [43]. Comparing their study with ours, we noticed that all the conclusions they made regarding the degron motif were based on experiments employing only the N-terminal part of Ex, whereas we used the full-length Ex. The N-terminal function may have been "masked" or hidden in the presence of the C-terminal function in our study because our data strongly suggest a very important role of ExC in the regulation of Slmb-Ex interaction and Ex stability (Figures 5G, 5H, 6I, 6J, Supplementary information, Figure S4F).

Another important question is how Slmb-mediated degradation of Ex is regulated during development. The previous findings that the apical polarity determinant protein Crb controls both sub-cellular localization and stability of Ex promoted our hypothesis that Crb may act as a coordinator in the control of Ex stability. Indeed, our observations show that Slmb is likely the executive player of Crb-mediated Ex degradation, as the Crb-mediated degradation of Ex in the imaginal wing discs is fully suppressed upon the depletion of Slmb (Supplementary information, Figures S5A-S5C). Our *in vivo* observations here manifest the *in vitro* results from Ribeiro and colleagues [43]. Previous study by Robinson and colleagues [25] showed that Ex is mislocalized and the protein level is elevated with unchanged transcription in *crb* mutant clones, suggesting that Crb-mediated Ex apical localization may be the prerequisite for the degradation of Ex. In other words, Slmb-mediated Ex degradation seems to require a proper apical localization of Ex. Crb has previously been reported to physically interact with Ex and maintain its proper localization through Crb^{intra} [22, 24, 25]. When Crb^{intra} is co-expressed with Ex in cultured S2 cells, Ex becomes hyper-phosphorylated and unstable [24]. As phosphorylation is known to prime substrate recognition and ubiquitination by SCF E3 ligases, we propose that the recruitment of Ex to the apical membrane by Crb probably facilitates phosphorylation by an unknown kinase and the subsequent recognition of Ex by SCF^{Slmb} for degradation (Figure 7), which is also reported by Ribeiro and colleagues [43]. Here a critical question arises: Where would the phosphorylation signal(s) come from? One possibility is that the signal that directs Ex for degradation may come from Hippo pathway activity, because (1) there exists a feedback regulation system for the abundance of Ex protein: more Ex activates Hippo signaling, leading less production of Ex and less Ex downregulates Hippo signaling, leading to more production of Ex; (2) it is known that β-TrCP-mediated YAP/TAZ degradation depends on Hippo pathway activation [44, 45], which makes it possible that the cells use a similar strategy to control Slmb-mediated turnover of Ex; (3) it seems that the Slmb-mediated proteolysis of key components of different cellular signaling processes is often coordinated by the relevant signaling activity. For example, it has been shown that signaling by Hh and Wg prevents Slmb-mediated proteolysis of Ci and Arm, respectively [34, 48, 49]. Opposing our hypothesis that the Hippo pathway may direct Ex for degradation, the Hippo pathway itself is able to prevent Ex from degradation by Slmb instead of providing the phosphorylation signal required for Ex degradation. This is demonstrated by the stabilization of Ex when Wts is co-expressed in S2 cells. Wts most probably stabilizes Ex through a direct phosphorylation at the Wts consensus motif on Ex, which may interfere with the Slmb-mediated degradation of Ex by (1) antagonizing the phosphorylation of Ex that is required for Ex degradation, and/or (2) preventing Slmb from recognizing Ex (Figure 7). Interestingly, Ribeiro and colleagues reported a prediction of a non-canonical

degron of ^{1116}STQNIS^{1121} in the C-terminal region of Ex. Given that this putative degron is essentially overlapping with the Wts phosphorylation site (S^{1116}), we asked whether it plays a role in the regulation of Ex stability. To this end, we mutated both T^{1117} and S^{1121}, two amino acids in the putative degron which could be potentially phosphorylated. The resultant $Ex^{T1117AS1121A}$ showed less stability than its wild-type counterpart (Supplementary information, Figure S4I and S4J). To exclude the possibility that the T^{1117} may have a structural role in maintaining a proper Wts function at S^{1116}, we made a single mutation of Ex^{S1121A}. Ex^{S1121A} was more stable than the wild-type Ex (Supplementary information, Figures S4I and S4J), suggesting a role of S^{1121} as a key residue in the putative degron for Slmb recognition. Future work will be needed to demonstrate the phosphorylation at S^{1121} and identify the responsible kinase so that its role in antagonizing Wts-mediated Ex stabilization can be finally established.

Given that Crb can promote the Slmb-mediated degradation of Ex through its N terminal part [43], and Wts can protect Ex from degradation by Slmb through its C terminal part, we wondered whether there is crosstalk between these two processes. For this purpose, we transfected S2 cells with either Wts or Crb alone or both, respectively, in the presence of tagged Ex to monitor Ex phosphorylation status and its stability. As shown in Supplementary information, Figure S4K, in the presence of Wts, transfected Crb^{Intra} could still induce the phosphorylation (slower migrating band) and degradation of Ex (less stable), suggesting that at least in S2 cells Crb-promoted degradation of Ex may be dominant over Wts-mediated protection. However, more experiments are needed before a definite conclusion can be made. Therefore, we currently do not know precisely whether and how Wts-mediated protection of Ex degradation and Crb-mediated degradation of Ex influence each other in the physiological conditions. Nevertheless, to the best of our knowledge, there is at least one way *in vivo* that Wts may antagonize Crb-mediated regulation of Ex, i.e., Wts or Hippo pathway activation is able to downregulate the endogenous expression of Crb, as *crb* is also regulated at the transcription level by Hippo signaling [50], which may in turn negatively regulate Crb-mediated Ex degradation.

Notably, the stabilization of Ex by Hippo signaling is consistent with the *in vivo* correlation of Ft-Hippo signaling activity and Ex protein expression pattern. In fact, Ft seems to promote Ex stability through antagonizing the function of Slmb because the decrease in the apical Ex protein resulting from *ft* knockdown is reverted by *slmb* knockdown, leading to the apical Ex accumulation at a similar level to that caused by *slmb* knockdown alone (Supplementary information, Figure S5D-S5F). Given that Ft affects stability of both Ex and Wts, it is logical that Ft stabilizes Ex, at least partially, through its ability to stabilize Wts. In addition, since both Ft and Crb are apically localized transmembrane proteins that play important roles in controlling the apical localization of Ex and thus its abundance (Figure 7), it will be interesting to investigate how these proteins function cooperatively in the regulation of Ex and Hippo signaling.

As a membrane-associated protein, Ex transduces signals from outside of the cell (by binding to the transmembrane protein Crb) to inside of the cell (by promoting activity of Hpo and Wts, or directly binding to Yki, leading to inhibition of Yki). Expression of Ex is regulated in a negative-feedback manner by the Hippo pathway. Together with our findings in this study that Wts regulates the protein level of Ex, it is plausible to propose that a fine-tuned Ex level, maintained by the double security mechanism, is essential for Hippo pathway regulation and thus critical for the homeostatic cell proliferation during development. It would be interesting if future studies were to focus on illustrating the developmental abnormalities when the Hippo pathway is unable to stabilize Ex. The Hippo signaling may act through Ex to respond to various external/environmental and internal cues to maintain precise homeostasis not only during development but also throughout the whole process of life.

Materials and Methods

Fly strains and genetics

RNAi lines used in this study include *ex-IR* (BL28703), *wts-IR* (BL33064), *hpo-IR* (BL33614), *ft-IR* (from Dr J Ni); all of these four RNAi lines phenocopy their corresponding mutants in terms of Hippo pathway deregulation. Also included are *dCul1-IR* (BL29520 and VDRC v108558), *dSkpA-IR* (BL28974 and VDRC v107815), *slmb-IR* (BL33898 and VDRC v107825; defects caused by both lines can be reverted by co-expression of *UAS-slmb*). UAS lines used in this study include *UAS-yki* (from Dr Z Wang, Institute of Genetics and Developmental Biology, CAS, Beijing, China), *UAS-yki-V5* (BL28819), *UAS-arm* (BL8369), *UAS-Crb^{intra}* (from Dr E Knust, Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany), *UAS-slmb* (from Dr E Verheyen, Simon Fraser University, BC, Canada) [51], *UAS-slmb-flag* (from Dr Y Zhao, Institute of Biochemistry and Cell Biology, CAS, Shanghai, China). Gal4 lines used in this study include *GMR-Gal4*, *mirr-Gal4* (BL29650), *dpp-Gal4, en-Gal4 UAS-GFP* and *hh-Gal4 UAS-GFP* (from Dr Z Wang). Other lines employed in this study include *ex^{e1}*, *Diap-lacZ* (BL12093), *bantam-lacZ* (BL10154), *slmb^{P1493} FRT82* (from Dr E Verheyen) [51].

Crosses with *UAS-Crb^{intra}* were kept at 18 °C. For assaying the phenotypes of the adult eyes and wings, and for clonal analysis, crosses were kept at 25 °C. Clonal analysis of *slmb^{P1493}* was performed as previously described [34]. Other crosses were per-

formed at 28 °C to obtain stronger phenotypes than those obtainable at 25 °C. In assays to examine the interaction between two UAS transgenes, control crosses were carried out with a test UAS line and a *UAS-GFP* transgene to avoid potential effects resulting from titration of the Gal4 proteins.

Immunohistochemistry and antibodies

Wing and eye discs were dissected from *Drosophila* third instar larvae and immunostaining was performed as previously described [52]. Primary antibodies used in this study were as follows: rabbit anti-β-galactosidase (1:2 000), rabbit anti-Yki (1:50), guinea pig anti-Ex (from Dr R Fehon, The University of Chicago, IL, USA; 1:2 500) [53], guinea pig anti-Mer (from Dr R Fehon, 1:5 000), mouse anti-DE-Cadherin (Developmental studies Hybridoma Bank, 1:100). The secondary antibodies were conjugated either to FITC (Jackson Immunoresearch, 1:200) or Cy3 (Jackson Immunoresearch, 1:200), or DyLight (Jackson Immunoresearch, 1:100). The images were taken with Leica TSC SP5 confocal laser scanning microscope.

Cell culture, transfection, protein stability assay and RNA interference

Drosophila S2 cells were maintained at 25 °C in Schneider's *Drosophila* Medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin and 50 mg/ml streptomycin. The pAc5.1/V5-His or the pMT/V5-His vector was used for generating protein expression constructs in S2 cells. Plasmids of pAc-Ex-Flag, pAc-ExN-Flag (aa 1-653), pAc-ExC-Flag (aa 654-1 427), pAc-ExC1-Flag (aa 654-911), pAc-ExC2-Flag (aa 912-1 164), pAc-ExC3-Flag (aa 1 168-1 427), pAc-$Ex^{S1117AS1121A}$-Flag, pAc-Ex^{S1121A}-Flag, pAc-Ex^{S1116A}-Flag, pAc-ExΔC2-Flag, pMT-Ex-Myc, pAc-Flag-Ub, pAc-6Myc-Wts were constructed by a ligation-independent cloning strategy. For Wts^{K743R}-V5 and Wts^{T1077A}-V5, point mutation was induced via the megaprimer method of site-directed mutagenesis. Other plasmids used in this study were as follows: Ex-Flag (from Dr H McNeill, University of Toronto, ON, Canada), pAc-Slmb-3HA and pAc-SlmbΔF-3HA (from Dr I Edery, Rutgers University, New Jersey, USA), pAc-$Crb^{Myc\text{-}Intra}$ (from Dr D Pan, Johns Hopkins University, MD, USA). S2 cells were transfected with relevant plasmids using X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's instructions.

For Ex half-life measurement, cells expressing Ex, Ex^{S1116A} or ExΔC2 were treated with CHX 48 h after transfection and harvested at the indicated time points to measure the total amount of Ex, Ex^{S1116A} or ExΔC2 by western blot. The half-life of the proteins was estimated in normal condition, Wts overexpression condition and Wts knockdown condition, respectively. All the related quantifications were performed with ImageJ (NIH). Briefly, the intensity of each band was calculated and divided by the co-transfected GFP intensity. The ratio of such calculation at each time point was then normalized to the ratio of the starting point (0 h) to make the starting value '1'. All of the quantification results were plotted in GraphPad.

For RNA interference, dsRNA was prepared with the RiboMAXTM large scale RNA Production System-T7 kit (Promega). The primers for GFP control were 5′-ttaatacgactcactataggggagaATGGTGAGCAAGGGCGAGGAGCTG-3′ (forward) and 5′-ttaatacgactcactataggggagaCTTGTACAGCTCGTCCATGCCGAGAG-3′ (reverse). The primers for *slmb* RNAi and *wts* RNAi were designed as previously described [28, 54]. For a 6 cm plate, 5 ml culture media in each well was treated with 75 μg dsRNA for 48 h after transfection or $CuSO_4$ addition. The efficiency of dsRNA treatment was evaluated by RT-PCR using the SuperScript III First-Strand Synthesis System (Invitrogen) or by western blot.

In vivo ubiquitination, co-immunoprecipitation and western blot

For *in vivo* ubiquitination assay (Figure 5B and 5C), S2 cells were transiently transfected with Myc-tagged Ex and FLAG-tagged ubiquitin or FLAG-GFP as a control. At 48 h posttransfection, MG132 (final concentration of 75 μM) was added into the medium. Cells were harvested 7 h later in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) supplemented with 1 mM PMSF and protease inhibitor cocktail (Calbiochem) to make total cell extracts. After incubation with the extracts, the anti-Myc-conjugated beads were then washed and subjected to immunoblot analysis. For co-immunoprecipitation assays, S2 cells were co-transfected with plasmids expressing relevant tagged proteins. Cells were lysed with the immunoprecipitation lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA) supplemented with 1 mM PMSF and protease inhibitor cocktail, and the lysates were then incubated with rabbit anti-Flag antibody (Sigma) and protein A/G agarose beads, or anti-Flag M2 affinity gel (Sigma) or mouse anti-Myc agarose (Abmart) followed by western blot using relevant antibodies. Western blot was performed as previously described [55]. The following antibodies were used in western blot analyses: guinea pig anti-Ex (from Dr R Fehon, 1:5 000), mouse anti-Flag (Sigma, 1:20 000), mouse anti-HA (Abmart, 1:1 000), mouse anti-Myc (Cwbiotech, Beijing, 1:5 000) , mouse anti-tubulin (Cwbiotech, Beijing, 1:200 000), mouse anti-V5 (Invitrogen, 1:10 000), rabbit anti-Wts (from Dr K Irvine, Rutgers University, NJ, USA, 1:5 000). All western blot signals were visualized using the Immobilon Western Chemiluminescent HRP Substrate (Millipore).

GST pull-down assay

pGEX-4T1-Slmb and pET28a-ExC2 were constructed by a ligation-independent cloning strategy and were transformed into the *E.coli* strain Rossetta2 (DE3) (Cwbiotech, Beijing). Bacterial culture and induction of fusion proteins were described previously [56]. Bacterial cells expressing GST or GST-Slmb were lysed in PBS containing protease inhibitors (Roche) before sonication. The cell lysates were then incubated with Glutathione agarose beads (Cwbiotech, Beijing) at 4 °C overnight and the beads were washed for three times with PBS. Bacterial cell lysates with His-ExC2 expression were pre-cleared by incubation with GST-glutathione beads at 4 °C for 2 h, and the lysates were then split into two parts with equal volume and incubated with immobilized GST or GST-Slmb fusion proteins at 4 °C overnight. Beads were washed four times with PBS and the binding proteins were eluted with sample loading buffer for SDS-PAGE followed by western blot. Antibodies used were as follows: mouse anti-GST (Cwbiotech, Beijing, 1:20 000 for GST and 1:5 000 for GST-Slmb), mouse anti-His tag (Cwbiotech, Beijing, 1:1 000).

Statistical analysis

Quantification of the fluorescence signals was performed as

previously described [55]. Briefly, for each image, we first performed manual cropping to remove the unselected area outside the wing pouch of the wing disc. Then the average fluorescence intensities were calculated for anterior and posterior part of the wing pouch, respectively. All calculations were performed on MATLAB (MathWorks, Natick, MA). For data plotting, the average fluorescence intensity ratio of posterior and anterior area of wing pouch in each disc was calculated, shown as relative intensity P/A compartment (except for Yki nuclear localization analysis). For Yki nuclear localization analysis, the same square areas in both the anterior and posterior compartments of one wing pouch were selected and the percentage of nuclei with nuclear Yki accumulation in each square was calculated. Each area contained about 30 nuclei. Three independent squares were randomly picked for each analysis. For all the quantification, statistical significance of difference between two groups was determined using two tailed Student's *t*-test in Prism 5 (GraphPad), and $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. $n \geq 2$ for each genotype. All indicated experimental errors represent SEM.

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