

Hedgehog signaling downregulates Suppressor of Fused through the HIB/SPOP-Crn axis in *Drosophila*

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Hedgehog (Hh) signaling plays vital roles in animal development and tissue homeostasis, and its misregulation causes congenital diseases and several types of cancer. Suppressor of Fused (Su(fu)) is a conserved inhibitory component of the Hh signaling pathway, but how it is regulated remains poorly understood. Here we demonstrate that in *Drosophila* Hh signaling promotes downregulation of Su(fu) through its target protein HIB (Hh-induced BTB protein). Interestingly, although HIB-mediated downregulation of Su(fu) depends on the E3 ubiquitin ligase Cul3, HIB does not directly regulate Su(fu) protein stability. Through an RNAi-based candidate gene screen, we identify the spliceosome factor Crooked neck (Crn) as a regulator of Su(fu) level. Epistasis analysis indicates that HIB downregulates Su(fu) through Crn. Furthermore, we provide evidence that HIB retains Crn in the nucleus, leading to reduced Su(fu) protein level. Finally, we show that SPOP, the mammalian homologue of HIB, can substitute HIB to downregulate Su(fu) level in *Drosophila*. Our study suggests that Hh regulates both Ci and Su(fu) levels through its target HIB, thus uncovering a novel feedback mechanism that regulates Hh signal transduction. The dual function of HIB may provide a buffering mechanism to fine-tune Hh pathway activity.

Keywords: Hh; Su(fu); HIB; Crn; SPOP; Cul3

Cell Research (2014) 24:595-609. doi:10.1038/cr.2014.29; published online 7 March 2014

Introduction

Hedgehog (Hh) signaling plays essential roles in embryonic development and adult tissue homeostasis in species ranging from insects to human [1-4]. Aberrant Hh signaling activity is associated with many human disorders including birth defects and cancers [4-7].

Hh transduces signal through binding to its receptor, a 12-transmembrane protein Patched (Ptc), which alleviates Ptc-mediated suppression of Smoothed (Smo), a

GPCR-like seven-transmembrane protein [8-14]. Activated Smo functions as the Hh signal transducer to recruit an intracellular protein complex containing the kinesin-like protein Cos2 and the kinase Fused (Fu), leading to Fu phosphorylation and activation [15-23]. Activated Fu then inhibits Ci processing to prevent the production of a Ci repressor form (CiR) and antagonizes Su(fu) to convert the latent full-length Ci into an activator form (CiA) [21, 22, 24], leading to the expression of target genes including *decapentaplegic* (*dpp*), *ptc* and *engrailed* (*en*) [1, 25, 26].

In *Drosophila* wing discs, posterior (P) compartment cells produce Hh that moves into anterior (A) compartment to form a concentration gradient and induces the A-compartment cells of the A/P boundary to express *dpp*. Dpp functions as a long-range morphogen and moves into both the A and P compartments to control the growth and patterning of cells in the whole wing [1, 27, 28]. Hh also functions as a local morphogen to specify cell pat-

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Received 19 August 2013; revised 30 November 2013; accepted 20 January 2014; published online 7 March 2014

termining near the A/P boundary with low, intermediate and peak levels of Hh to induce *dpp*, *ptc* and *en* expression, respectively [29, 30].

In A-compartment cells distant from the A/P boundary where Hh is very low or absent, Ci is processed into a truncated form Ci75 by the SCF (Skp1-Cullin-F-box protein complex) E3 ligase containing the F-box protein Slimb [31]. Ci75 (also called CiR) functions as a transcription repressor to block the expression of a subset of Hh target genes, including *dpp* [31-37], whereas in A-compartment cells near the A/P boundary Hh signaling antagonizes Ci processing and converts the accumulated full-length Ci (Ci155) into an active but unstable form (CiA), which activates Hh target gene expression [24]. The BTB protein HIB is upregulated by Hh signaling in A-compartment cells abutting the A/P boundary, where it forms a complex with Cul3 (Cul3-HIB) that functions as an ubiquitin E3 ligase. HIB recognizes the active form of Ci and targets it for ubiquitin/proteasome-mediated degradation to attenuate Hh signaling activity in a negative feedback loop [38-43].

In *Drosophila*, Su(fu) is a genetic suppressor of the Fu kinase and is antagonized by Fu function [44-50]. Su(fu) and the kinesin-like protein Cos2 retain Ci155 in the cytoplasm to oppose its nuclear import [51-56]. Studies in mammals show that SUFU can both tether Gli in the cytoplasm and enter the nucleus with Gli1 to repress transcription from Gli-dependent promoters through specific interaction with SAP18, a component of the Sin3-HDAC co-repressor complex [46, 57, 58]. In mice, abrogation of *sufu* leads to ligand-independent Hh signaling activation, and homozygous *sufu* mutant embryos die at mid-gestation with a ventralized spinal cord [59, 60]. Also, *sufu* mutations are present in 9% of medulloblastoma human patients, suggesting that *sufu* is a tumor suppressor gene [61].

Although Su(fu)/SUFU plays a conserved role in regulating Ci/Gli activity, its regulation remains poorly understood. One study has identified that Hh signaling promotes SUFU degradation through the ubiquitin-proteasome pathway in mammalian NCI-H322M lung cell line, but the E3 ligase involved remains unknown [62].

In this study, we demonstrate that Hh signaling can downregulate Su(fu) through its target protein HIB. Interestingly, regulation of Su(fu) by HIB depends on Cul3 but occurs at a level distinct from Su(fu) degradation. Through a candidate gene screen, we find that the spliceosome factor Crn can modulate Su(fu) level. Epistasis analysis suggests that HIB downregulates Su(fu) through Crn. Furthermore, we provide evidence that HIB and Crn may inhibit the formation of functional Su(fu) mRNA but not its nuclear export. Finally, we show that the mam-

malian homologue of HIB, SPOP, can substitute HIB to downregulate Su(fu) in flies.

Results

Hh signaling downregulates Su(fu)

In *Drosophila*, Su(fu) forms a complex with Ci and inhibits its transcriptional activity through tethering Ci in the cytoplasm and inhibits Ci activity even after Ci enters the nucleus [55, 63]; however, how Su(fu) is regulated by Hh signaling is still poorly understood. In wild-type wing discs either from *yw* or from *MS1096* Gal4-expressing flies, Su(fu) level is lower in A-compartment cells near the A/P boundary where Hh signaling is activated (arrows in Figure 1A-1A'', 1B-1B''). This observation prompted us to test whether Hh signaling is involved in Su(fu) downregulation. We overexpressed *UAS-Hh* through the *MS1096* Gal4 line, which drives target gene expression in the pouch region of the wing disc, and found that Hh severely reduced Su(fu) level in wing pouch (Figure 1C-1C''). Of note, overexpression of Hh could only dramatically downregulate Su(fu) in A-compartment cells but not P-compartment cells where Ci is not expressed (Figure 1C'), suggesting that Hh signaling but not Hh itself is essential for Su(fu) downregulation. To verify this hypothesis, we knocked down *smo* and *ci* by expressing *UAS-smo-RNAi* and *UAS-ci-RNAi*, respectively, with *MS1096* and found that the level of Su(fu) near A/P boundary increased to the level similar to that outside the A/P boundary (Figure 1D-1E''). Furthermore, *MS1096*-driven overexpression of *UAS-Ci^{3P}*, which is one active form of Ci with three mutated PKA kinase sites [39], downregulated Su(fu) in both A and P compartments (Figure 1F-1F''). Taken together, these results support the notion that Hh signal transduction is both necessary and sufficient for downregulating Su(fu).

Hh signaling downregulates Su(fu) through its target HIB

We next asked how Hh signaling downregulates Su(fu). *hib* is a target gene of Hh signaling pathway and its upregulation overlaps with low Su(fu) domain, as indicated by LacZ staining from the *hib-lacZ* enhancer trap line, which mimics endogenous *hib* expression pattern (Figure 2A-2A'') [39]. HIB could function as a substrate recognition component of Cul3-based E3 ubiquitin ligase to promote ubiquitination and proteasome-mediated degradation of its target proteins [39]. These observations prompted us to test whether HIB mediates the reduction of Su(fu) induced by Hh signaling. *hib^A* mutant clones were generated using the FRT/FLP-mediated mitotic recombination in wing discs and were marked by the lack of GFP expression. As shown in Figure 2B-2B'')

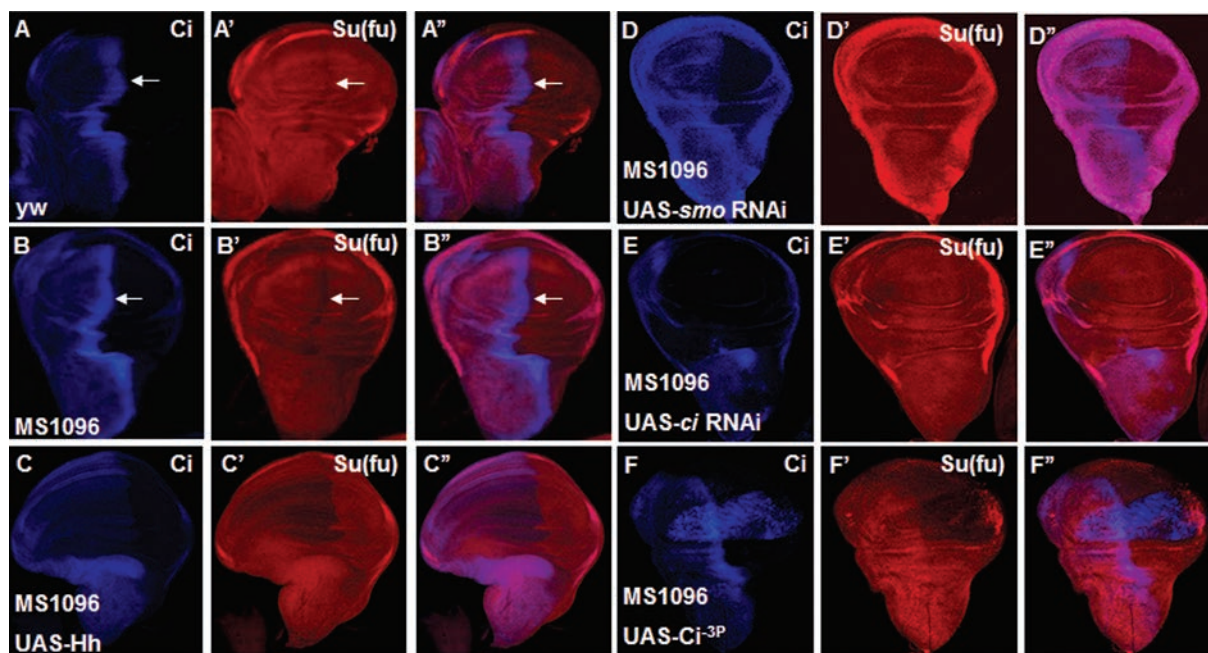


Figure 1 Hh signaling is sufficient for downregulating Su(fu). All the wing discs shown in this study are oriented with anterior to the left and ventral to the top. *UAS* transgene expression with *MS1096* gal4 are usually stronger in the dorsal region than in the ventral of wing pouch. **(A-A'')** A wing disc from *yw* flies was immunostained to show full-length Ci (Ci^{FL}) (blue) and Su(fu) (red) expression by immunostaining with corresponding antibodies. Of note, Su(fu) level is lower in A-compartment cells abutting the A/P boundary. **(B-B'')** A wing disc of *MS1096* gal4 line was immunostained to show full-length Ci (Ci^{FL}) (blue) and Su(fu) (red) expression. Su(fu) level is also lower in A/P boundary. **(C-C'')** Overexpression of *UAS-Hh* with *MS1096* decreased Su(fu) level in A-compartment cells but not in P-compartment cells where Ci is not expressed. **(D-E'')** *MS1096*-driven knockdown of *smo* **(D-D'')** or *ci* **(E-E'')** resulted in Ci reduction in the pouch of wing discs and Su(fu) (red) level at the A/P boundary was upregulated to an equal level outside the A/P boundary. **(F-F'')** *UAS-Ci^{3P}*, which is an active form of Ci with three PKA kinase sites mutated, was expressed with *MS1096* in wing discs. Su(fu) (red) level was decreased in both anterior and posterior compartments of the wing pouch.

Su(fu) level was upregulated in *hib^A* mutant clones in A-compartment cells near the A/P border. In addition, overexpression of *Hh* by *MS1096* failed to downregulate Su(fu) in anteriorly situated *hib^A* mutant clones (arrows in Figure 2C-2C''). Co-expression of *UAS-HIB* with *UAS-Hh* restored the ability of Hh to downregulate Su(fu) in *hib^A* mutant clones (arrows in Figure 2D-2D''). These results suggest that HIB is responsible for downregulating Su(fu) in response to Hh. Furthermore, overexpression of HIB in the dorsal (D) compartment of wing discs using the *Ap-Gal4* driver downregulated Su(fu) in both A- and P-compartment cells of the dorsal regions (arrows in Figure 2E-2E''). Taken together, these results support the notion that Hh signaling induces HIB, which in turns downregulates Su(fu).

Regulation of Su(fu) level by HIB depends on E3 ligase Cul3

HIB contains an N-terminal MATH domain and a C-

terminal BTB domain [39]. Usually the BTB domain interacts with the Cul family member Cul3 and the MATH domain recognizes the substrates for the 26S proteasome degradation [64]. Our previous study showed that HIB interacts with Cul3 to form an E3 ligase complex that mediates Ci degradation [39, 43]. We therefore tested whether HIB acts in conjunction with Cul3 to regulate Su(fu).

We made *hib^A* mutant clones or mutant clones for a strong loss-of-function allele of *cul3*, *cul3^{sf12}*, in eye discs since *hib* is expressed specifically in the posterior region of eye discs. We found that both *hib^A* and *cul3^{sf12}* clones in the posterior region of eye discs accumulated Su(fu) (Figure 3A-3B''), suggesting that both HIB and Cul3 are necessary for the downregulation of Su(fu).

To explore whether Cul3-HIB downregulates Su(fu) through the ubiquitin/proteasome pathway, we firstly employed a proteasome inhibitor MG132 to block 26S proteasome-mediated degradation in eye discs and

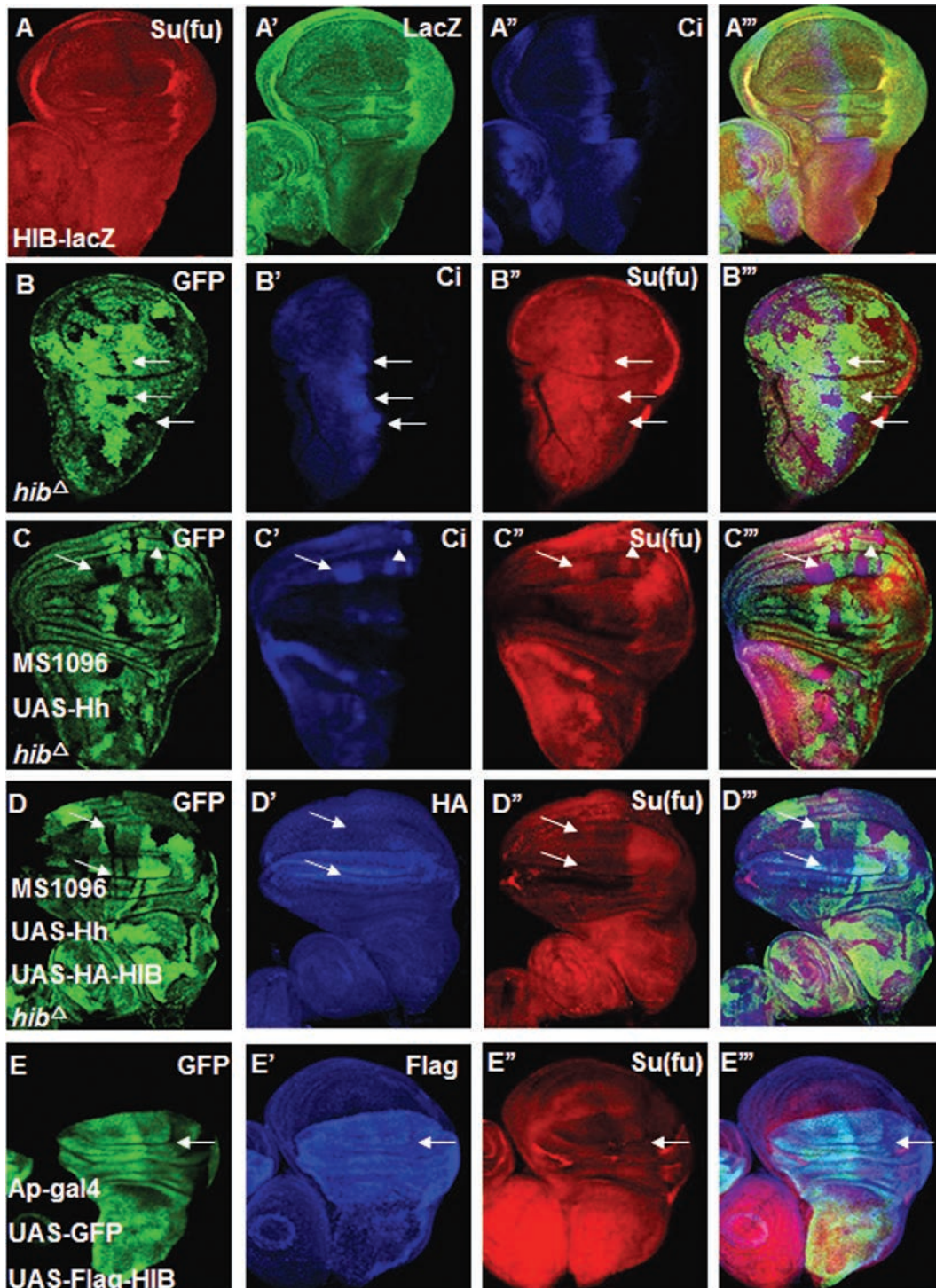


Figure 2 Hh signaling downregulates Su(fu) through HIB. (**A-A'''**) LacZ expression in wing discs from a *hib* enhancer trap line, *l(3)03477 (hib-Z)*. High LacZ staining (green) overlapped with low Su(fu) (red) region along the A/P border. (**B-B'''**) Su(fu) (red) was upregulated in the *hib*^Δ clones near the A/P boundary. *hib*^Δ clones were marked by the lack of GFP expression and were indicated by arrows. (**C-C'''**) A wing disc carrying *hib*^Δ clones and expressing *UAS-Hh* with *MS1096* was immunostained with GFP (green), Ci^{FL} (blue) and Su(fu) (red) antibodies. Su(fu) and Ci^{FL} accumulated in *hib*^Δ clones when Hh was overexpressed. (**D-D'''**) Co-expression of *UAS-Hh* and *UAS-HA-HIB* (blue) with *MS1096* could erase the accumulated Su(fu) (red) inside *hib*^Δ clones of wing discs. (**E-E'''**) Wing discs expressing *UAS-Flag-HIB* plus *UAS-GFP* with *Ap-Gal4* were immunostained with Flag (blue), GFP (green) and Su(fu) (red) antibodies. GFP marks the cells that express *UAS-Flag-HIB*. HIB overexpression led to diminishing Su(fu) level in both A- and P-compartment cells in the dorsal region of wing pouch (indicated by arrows).

found that MG132 blocked Su(fu) downregulation in the posterior region of eye discs (Figure 3D-3D''), suggesting that 26S proteasome is involved in Su(fu) regulation. Furthermore, we applied the Ub-K0, which blocks polyubiquitination. To test whether polyubiquitination is necessary for Su(fu) regulation, we co-expressed *Ub-K0* with *UAS-Hh* (Figure 3E-3E'') or *UAS-HIB* (Figure 3F-3F'') in wing discs, and found that Ub-K0 attenuated both Hh- and HIB-mediated Su(fu) reduction in wing discs, suggesting that polyubiquitination is indispensable for Su(fu) reduction. Moreover, we overexpressed HIB- Δ 3box by *AP-Gal4* in wing discs to investigate the change of Su(fu) level. Compared with wild-type HIB, HIB- Δ 3box lacks amino acids from 299 aa to 330 aa that are essential for conjugating with Cul3 and forming a functional Cul3-HIB E3 ligase (our unpublished data). We found that HIB- Δ 3box no longer downregulated Su(fu) level (Figure 3G-3G''). We also applied a dominant-negative form of Cul3, designated Cul3^{KR}, to address the effect of Cul3 on Su(fu) level in wing discs.

Cul3^{KR} impairs its Cul3 E3 ligase activity due to the lysine (K) to arginine (R) replacement on the neddylation site [39, 65]. We found that co-expression of *Cul3^{KR}* with *HIB* blocked Su(fu) downregulation mediated by HIB (Figure 3H-3I). Taken together, these results suggest that Cul3-HIB E3 ligase activity and the ubiquitin/proteasome pathway are required for Su(fu) downregulation.

HIB does not directly regulate *Su(fu)*

In the Cul3-HIB E3 ligase complex, HIB specifically recognizes substrates for ubiquitination through direct binding to them [39, 64]. Therefore, we test whether HIB forms a complex with Su(fu) by immunoprecipitation (IP) experiments. Intriguingly, the result showed that HIB did not bind Su(fu) in S2 cells (Supplementary information, Figure S1A). Previous studies indicate that HIB/SPOP binds S/T-rich degrons that are found in multiple copies in a large number of HIB-interacting proteins [43, 66]. Su(fu) does not contain such motifs. Taken together, these observations suggest that Su(fu) is not a direct sub-

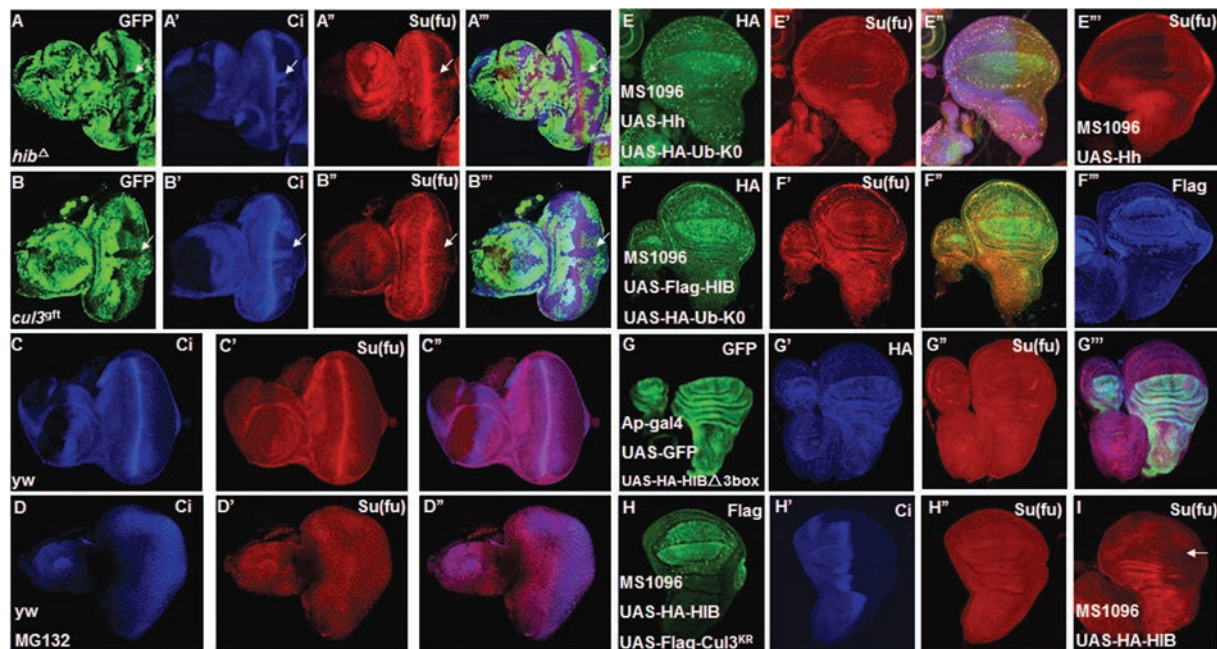


Figure 3 HIB downregulates Su(fu) depending on Cul3 and the ubiquitin/proteasome pathway. **(A-A''')** *hib*^Δ clones were generated in eye discs, followed by immunostaining with GFP (green), Ci^{FL} (blue) and Su(fu) (red) antibodies. Ci^{FL} and Su(fu) were accumulated in posterior *hib*^Δ clones. Clones were marked by the lack of GFP expression and indicated by arrows. **(B-B''')** An eye disc carrying *cul3^{gft}* clones was immunostained to show the accumulation of Ci^{FL} (blue) and Su(fu) (red) in the posterior compartments of eye discs. **(C-C''')** Endogenous Su(fu) expression pattern was shown in eye discs. **(D-D''')** Proteasome inhibitor MG132 blocked Su(fu) reduction in the posterior region of eye discs. **(E-E''')** Co-expression of *Ub-K0* and *UAS-Hh* with *MS1096* attenuated the Su(fu) reduction in wing discs. **(F-F''')** Co-expression of *Ub-K0* and *UAS-HIB* with *MS1096* blocked the Su(fu) reduction in wing discs. **(G-G''')** HIB- Δ 3box did not downregulate Su(fu) in wing discs. HIB- Δ 3box has lost Cul3-HIB E3 ligase activity due to lack of its C-terminal 299 aa-330 aa that mediates formation of a functional Cul3-HIB complex. **(H-I)** *UAS-HIB* was expressed alone **(I)** or together with *UAS-Cul3^{KR}* **(H-H''')**, a dominant-negative form of Cul3, in the wing discs using *MS1096*. Co-expression of *UAS-Cul3^{KR}* with *UAS-HIB* prevented HIB-mediated reduction of Su(fu).

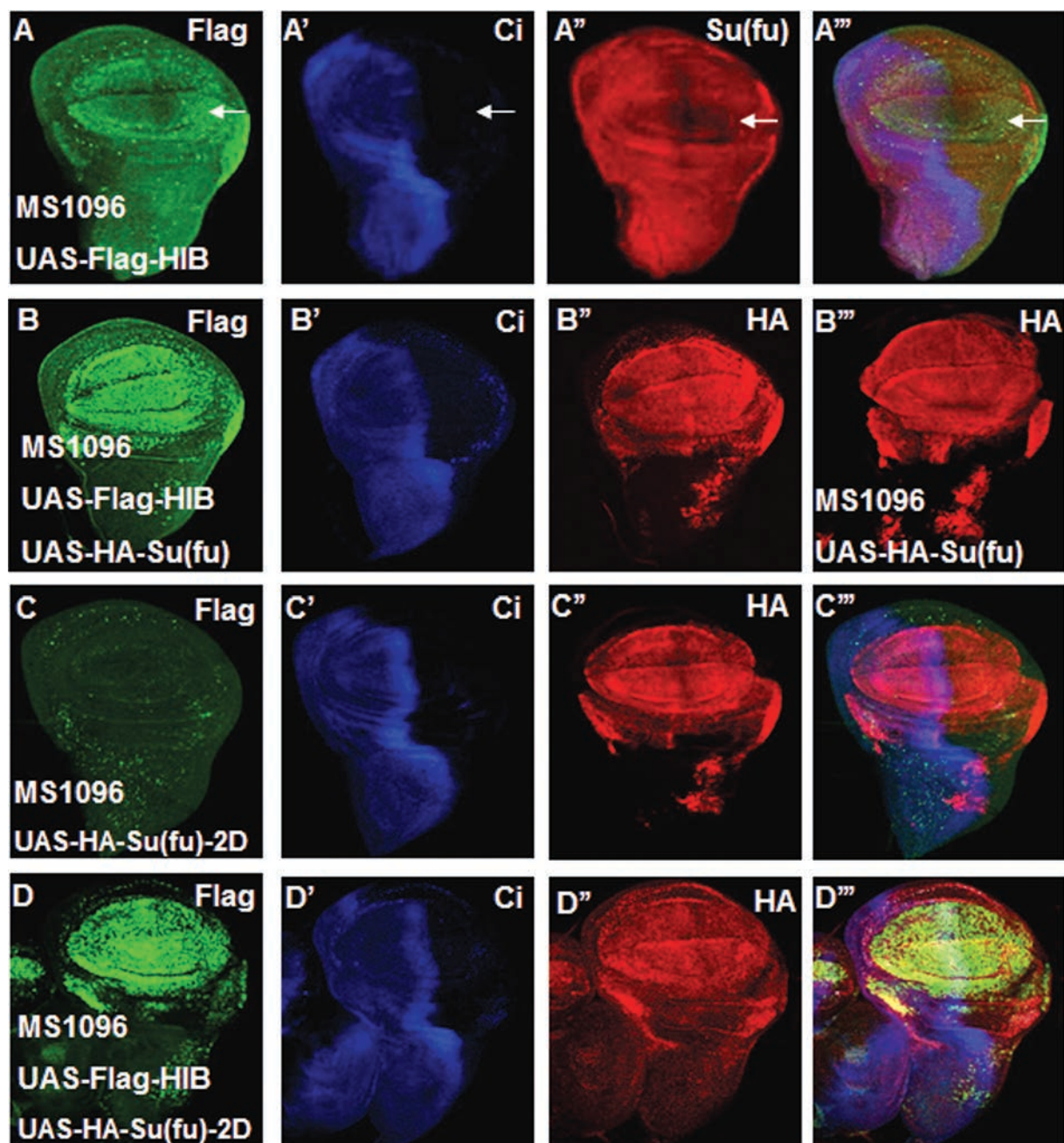


Figure 4 HIB does not affect Su(fu) stability. **(A-A''')** A wing disc overexpressing *UAS-Flag-HIB* with *MS1096* was immunostained with Flag (green), Ci^{FL} (blue) and Su(fu) (red) antibodies. Overexpression of HIB downregulated the endogenous Su(fu) level (arrow in **A''**) in the dorsal region of wing pouch. **(B-B''')** HA-Su(fu) was expressed alone (**B''**) or together with Flag-HIB (**B-B''**) with *MS1096*. Overexpression of *HIB* did not change the exogenous HA-Su(fu) (red) level shown by HA antibody staining (compare **B''** with **B'''**). **(C-D''')** Wing discs overexpressing HA-Su(fu)-2D alone (**C-C''**) or together with Flag-HIB (**D-D''**) with *MS1096* were immunostained to show the expression of HA-Su(fu)-2D, Flag-HIB and Ci. Co-expression of *Flag-HIB* with *HA-Su(fu)-2D* did not change HA-Su(fu)-2D level (compare **D''** with **C''**).

strate of the Cul3-HIB E3 ligase and its level is indirectly affected by Cul3-HIB.

In *Drosophila* wing discs, overexpression of Flag-HIB downregulated endogenous Su(fu), as indicated by anti-

Su(fu) antibody staining (Figure 4A-4A'''); however, co-expression of Flag-HIB with HA-Su(fu) did not affect the exogenously expressed Su(fu) recognized by anti-HA antibody staining (Figure 4B-4B'''). To further determine

whether HIB regulates Su(fu) protein stability, we transfected S2 cells with *Flag-HIB* alone or together with *HA-Su(fu)* and treated the cells with cycloheximide (CHX) to block protein synthesis. We found that HIB did not affect protein stability of the endogenous Su(fu) or exogenous HA-Su(fu) (Supplementary information, Figure S1B and S1C); similarly, Hh did not affect the stability of endogenous Su(fu) in S2 cells (Supplementary information, Figure S4B), suggesting that Su(fu) is unlikely to be affected by Cul3-HIB at post-translational stage.

CKI and Fu kinases trigger Su(fu) phosphorylation at

S321 and S324 upon Hh stimulation [21]. To test whether phosphorylated Su(fu) at S321 and S324 stimulated by Hh facilitates its reduction by Cul3-HIB E3 ligase, we made a construct *HA-Su(fu)-2D* in which amino acids S321 and S324 were changed to aspartic acids (D) to mimic phosphorylation. We overexpressed *HA-Su(fu)-2D* together with *HIB* in wing discs and found that HA-Su(fu)-2D level did not change in the presence of HIB overexpression (Figure 4D-4D’’’). In addition, we co-transfected *HA-Su(fu)-2D* and *Flag-HIB* into S2 cells and treated them with CHX for different periods of time,

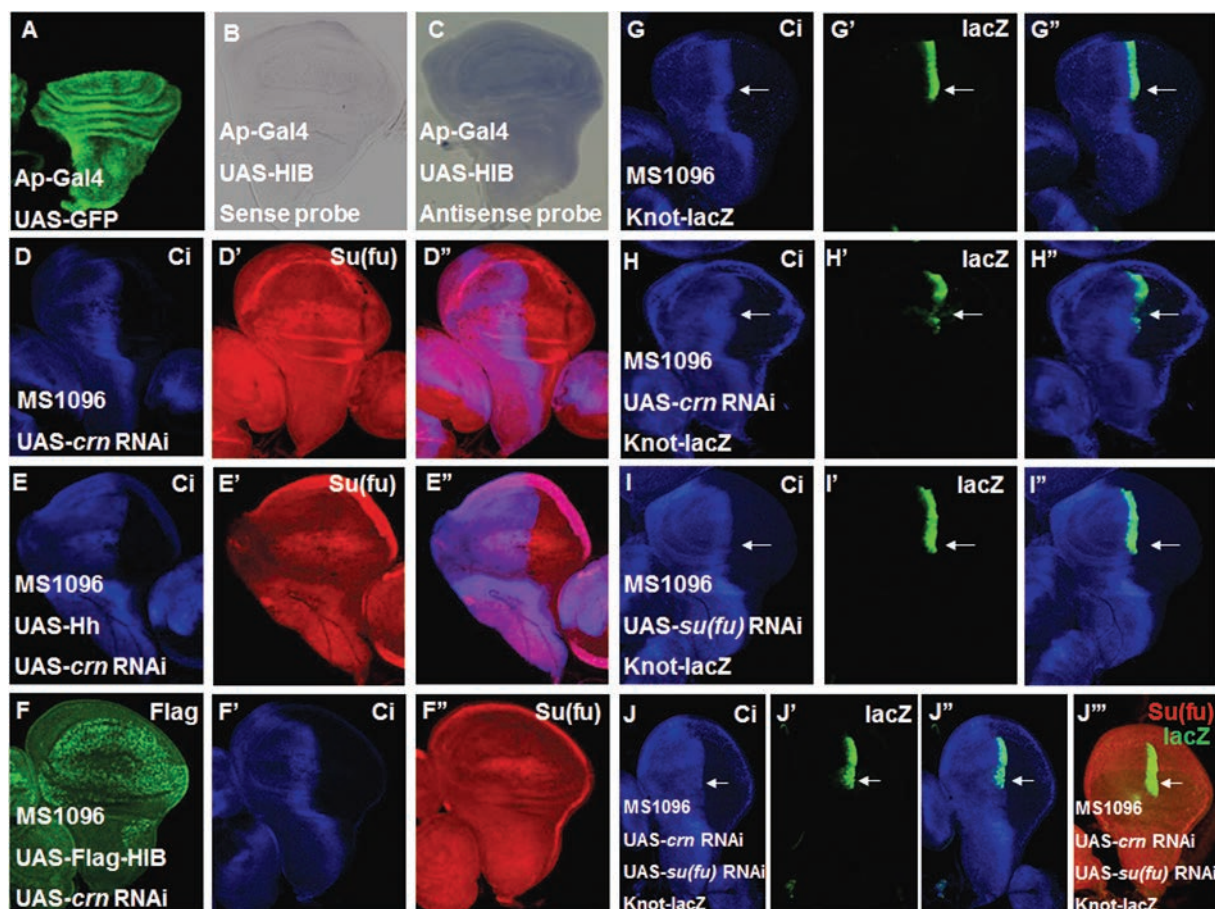


Figure 5 HIB inhibits Su(fu) level through spliceosome factor Crooked neck (Crm). **(A)** *UAS-GFP* (green) marked the *Ap-Gal4*-mediated gene expression pattern. *AP-Gal4* drives *UAS* transgenes to be specifically expressed in the dorsal region of wing discs. **(B, C)** Overexpression of *HIB* with *AP-Gal4* did not change *su(fu)* mRNA level in the dorsal region of wing discs as determined by *in situ* hybridization assay. Su(fu) sense probe was used as control **(B)**, and Su(fu) antisense probe detected *su(fu)* mRNA level **(C)**. **(D-D’’)** Knockdown of *crm* with *MS1096* upregulated Su(fu) protein level in the wing pouch region. **(E-F’)** Wing discs expressing *UAS-crm-RNAi* together with *UAS-Hh* **(E-E’)** or *UAS-Flag-HIB* **(F-F’)** were immunostained with *Ci^{FL}* (blue) and Su(fu) (red) antibodies. Simultaneous overexpression of *UAS-Hh* or *UAS-Flag-HIB* and *UAS-crm-RNAi* phenocopied *crm* knockdown. **(G-J’)** *crm* knockdown through *MS1096* significantly reduced the expression of *Knot-lacZ* (indicated by arrows) near the A/P border (compare **H’** with **G’**), and co-overexpression of *su(fu)* RNAi prevented the reduction in *Knot-lacZ* expression (indicated by arrows) in wing discs **(I-J’)**. **(J’’’)** Co-overexpression of *su(fu)* RNAi downregulated endogenous Su(fu) level (red) revealed by immunostaining with an anti-Su(fu) antibody. Of note, *MS1096* exhibited higher expression in the dorsal region than in the ventral region of wing discs.

followed by western blot analysis. We found that HA-Su(fu)-2D stability was not affected by HIB (Supplementary information, Figure S1D). These results rule out the possibility that phosphorylation of Su(fu) at S321 and S324 might render its regulation by Cul3-HIB.

HIB affects Su(fu) level through Crn

According to the above results, Su(fu) level is not affected by HIB at post-translation stage, raising the possibility that Su(fu) could be regulated at the level of mRNA. To determine whether HIB regulates Su(fu) mRNA expression, we overexpressed HIB with *AP-Gal4* in the dorsal region of the wing disc and used the ventral region of the same disc as internal control. We checked the *su(fu)* mRNA level by *in situ* hybridization and found that overexpression of *HIB* driven by *AP-Gal4* did not affect *su(fu)* mRNA level in dorsal region of the wing discs (Figure 5A-5C). In addition, knocking down *hib* in S2 cells did not change endogenous *su(fu)* mRNA level (Supplementary information, Figure S6B). However, it is still possible that HIB affects Su(fu) mRNA nuclear export or inhibits the formation of functional *su(fu)* mRNA to finally result in downregulating Su(fu).

Considering that HIB E3 ligase activity is necessary for Su(fu) downregulation, we reasoned that HIB inhibits Su(fu) through an intermediate substrate. To identify the HIB substrate that regulates Su(fu), we knocked down over 30 genes that encode putative HIB-interacting proteins annotated by the flyBase through their transgenic RNAi expression in wing discs and examined Su(fu) level via immunostaining with the anti-Su(fu) antibody (Supplementary information, Table S1). We found that knockdown of the spliceosome factor Crn using *MS1096* resulted in an upregulation of Su(fu) level (Figure 5D-5D’). Of note, the more dramatic effect on Su(fu) expression in the dorsal region is because *MS1096* drives higher levels of *UAS-crn-RNAi* in this region. Furthermore, we found that knockdown of *crn* blocked the downregulation of Su(fu) induced by overexpression of either Hh or HIB (Figure 5E-5F’). Similarly, we found that knockdown of *crn* in S2 cells upregulated the level of endogenous Su(fu) in the absence or presence of HIB or Hh overexpression (Supplementary information, Figure S6A, S6C and S6E). Taken together, these results suggest that Crn may act downstream of Hh and HIB to downregulate Su(fu). Multiple *crn* RNAi lines showed the same effect on Su(fu) level and a *UAS-Crn* transgene could rescue the phenotype of *crn* RNAi, suggesting that Su(fu) upregulation is caused by *crn* inactivation but not by an off-target effect (data not shown). Finally, we found that the read-out of high levels of Hh signaling activity, *knot-lacZ*, was significantly downregulated

by *crn* RNAi (Figure 5G-5H’), and this reduction was alleviated when Su(fu) level was downregulated by co-overexpression of *su(fu)* RNAi in wing discs (Figure 5I-5J’), suggesting that inactivation of Crn attenuates Hh signaling activity through Su(fu).

HIB reduces Su(fu) level by modulating Crn subcellular localization

Based on the information shown in flyBase, HIB binds Crn in a yeast two-hybrid assay. However, HIB failed to bind Crn through IP assay when they were co-transfected in S2 cells (Supplementary information, Figure S2), indicating that Crn might not be a direct substrate of HIB-Cul3 E3 ligase. Strikingly, when transfected into S2 cells the majority of HA-tagged Crn (HA-Crn) was localized in the cytoplasm (Figure 6A-6A’), whereas when co-transfected with *HIB*, HA-Crn was largely retained in the nucleus and co-localized with HIB (Figure 6C-6C’). Similarly, when expressed in wing discs, HA-Crn was localized mainly in the cytoplasm in A-compartment cells away from the A/P boundary (Figure 6E-6E’); however, when co-expressed with either HIB (Figure 6F-6F’) or Hh (Figure 6G-6G’), and Supplementary information, Figure S5E-S5E’), Crn was enriched in the nucleus, suggesting that Hh signaling and HIB can modulate Crn cellular localization. Furthermore, upon treatment with Leptomycin B (LMB), which is a specific nuclear export inhibitor, we found that the majority of Crn was localized in the nucleus both in S2 cells and in the wing discs in the absence of HIB co-overexpression (Figure 6D-6D’, 6H-6H’). As shown in Figure 6F’, nuclear localization of Crn correlated with lower Su(fu) level in the cells of the wing disc. Meanwhile, in the wing discs treated with LMB, the endogenous Su(fu) level was reduced as shown by western blot analysis (Figure 6I-6J, lane 2 compared with lane 1). Knockdown of *crn* blocked LMB-induced downregulation of Su(fu) and accumulated Su(fu) to a level higher than that of control discs (Figure 6I-6J, compare lane 3 with lanes 1 and 2). In addition, when co-transfected with *HIB* in S2 cells, HA-Crn could downregulate endogenous Su(fu) level (Figure 6K). Taken together, these results suggest that HIB may reduce Su(fu) level by modulating Crn cellular localization.

We further checked whether HIB regulates *su(fu)* mRNA nuclear export through Crn and found that it did not. Cytoplasmic and nuclear mRNA for the Su(fu) coding region were similar among S2 cells transfected with either *HIB* or *Crn* alone, or their combination (Supplementary information, Figure S8), suggesting that HIB and Crn downregulate Su(fu), most possibly by inhibiting the formation of functional *su(fu)* mRNA.

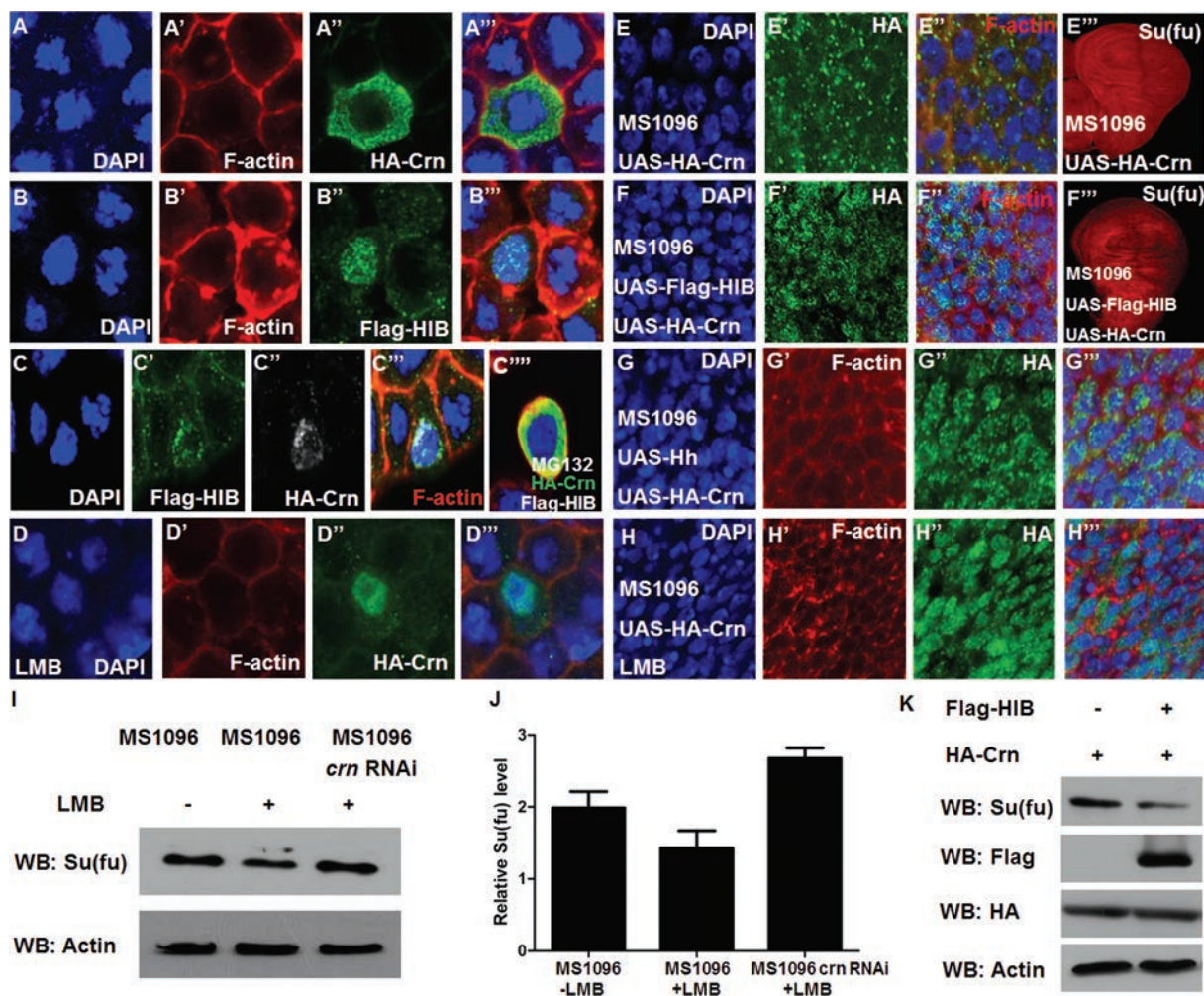


Figure 6 HIB affects Su(fu) level by modulating Crn cellular localization. **(A-C''')** S2 cells transfected with *UAS-HA-Crn*, *UAS-Flag-HIB*, or both were immunostained to show the subcellular localization of HA-Crn and Flag-HIB. The nuclei were stained with DAPI (blue) and the membrane was stained with TRITC-labeled phalloidin that preferentially labels filamentous actin (F-actin) (red). When expressed alone, HA-Crn was located mainly in the cytoplasm and barely in the nucleus **(A-A''')**, whereas Flag-HIB was located in the nucleus **(B-B''')**. When co-expressed with Flag-HIB, HA-Crn was co-localized with Flag-HIB at speckle-like sites in the nucleus **(C-C''')**; however, treating cells with MG132 resulted in cytoplasmic localization of HA-Crn **(C''')**. **(D-D''')** LMB treatment resulted in nuclear localization of HA-Crn in the absence of Flag-HIB co-expression in S2 cells. **(E-G''')** Wing discs expressing *UAS-HA-Crn* alone **(E-E''')** or together with *UAS-Flag-HIB* **(F-F''')** or *UAS-Hh* **(G-G''')** with *MS1096* were immunostained to show HA (green), Su(fu) (red in **E'''**, **F'''**), DAPI (blue) and F-actin (red in **E'''**, **F'''**). High-magnification views of anterior compartment cells were shown in **(E-E''')**, **(F-F''')**, and **(G-G''')**. When expressed alone, HA-Crn was located mainly in cytoplasm **(E-E''')** and did not affect endogenous Su(fu) level **(E''')**; however, when co-overexpressed with Flag-HIB, Crn was localized in the nucleus **(F-F''')** and downregulated endogenous Su(fu) level **(F''')**. Similarly, when co-overexpressed with *UAS-Hh*, Crn was also localized in the nucleus **(G-G''')**. **(H-H''')** LMB treatment led to nuclear localization of HA-Crn in the wing discs in the absence of HIB or Hh co-expression. **(I)** Endogenous Su(fu) level was reduced as shown by western blot in LMB-treated wing discs; however, the endogenous Su(fu) level was restored or even upregulated when *crn* was knocked down at the same time. **(J)** Quantification of the western blot results of endogenous Su(fu) level in **(I)** analyzed by ImageJ. **(K)** Co-expression of *Flag-HIB* with *HA-Crn* decreased Su(fu) protein level in S2 cells as determined by western blot analysis. Actin was used as a loading control.

SPOP can regulate Su(fu) in *Drosophila*

SPOP, the mammalian homologue of HIB, plays a conserved role in regulation of Ci/Gli degradation [39,

47, 67]. To investigate whether SPOP can substitute HIB to suppress Su(fu) in flies, first we checked whether SPOP has the ability to regulate Su(fu). The results

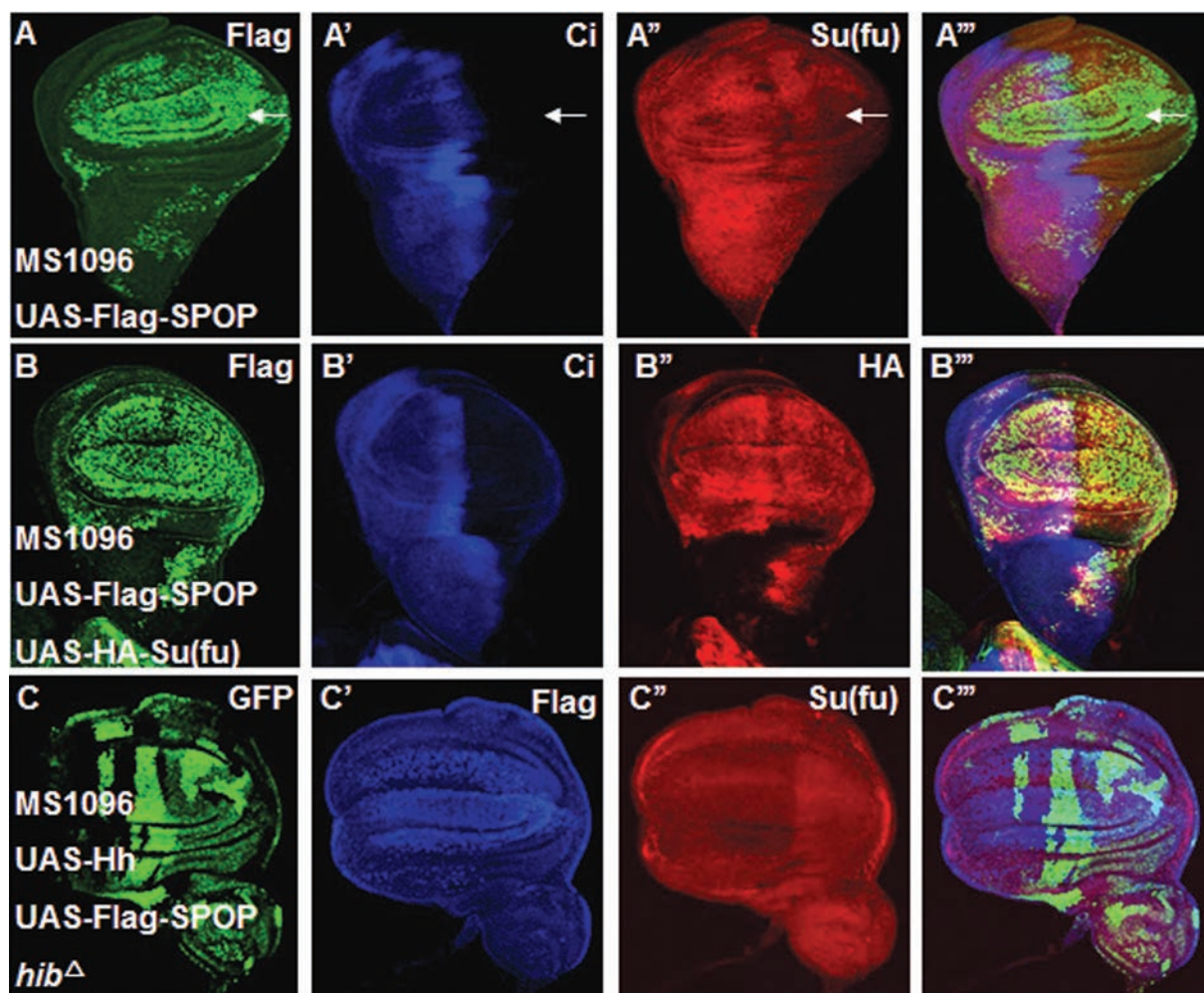


Figure 7 SPOP can substitute HIB to regulate Su(fu). **(A-A''')** Overexpression of *UAS-Flag-SPOP* with *MS1096* affected endogenous Su(fu) as revealed by staining with anti-Su(fu) antibody. **(B-B''')** Overexpression of *Flag-SPOP* together with *HA-Su(fu)* did not decrease exogenous HA-Su(fu) level as revealed by staining with anti-HA antibody (red). **(C-C''')** Su(fu) (red) was not accumulated inside *hib^Δ* clones co-expressing *UAS-Hh* and *Flag-SPOP* (blue) with *MS1096*.

showed that overexpression of SPOP in wing discs driven by *MS1096* or *AP-gal4* could downregulate endogenous Su(fu) (Figure 7A-7A''', and Supplementary information, Figure S3A-S3B'''); however, like HIB, overexpression of SPOP together with HA-Su(fu) did not affect exogenous HA-tagged Su(fu) level (Figure 7B-7B'''). Similarly, in S2 cells, exogenous HA-Su(fu) stability was not affected by SPOP (Supplementary information, Figure S3C). Second, when generating *hib^Δ* mutant clones and overexpressing *Hh* and *SPOP* with *MS1096*, we found that SPOP could erase the accumulated Su(fu) inside *hib^Δ* clones (Figure 7C-7C'''). All these results suggest that SPOP can substitute HIB to affect Su(fu) level in the same manner in *Drosophila*.

Discussion

Su(fu) is a conserved negative regulator of the Hh pathway, but until now how Su(fu) is regulated is largely unknown. Our work demonstrates that in *Drosophila* Hh signaling can regulate Su(fu) level and that this regulation is mediated by the Hh target HIB that functions in conjunction with Cul3 to downregulate Su(fu). Furthermore, we identify the splicing factor Crn as a mediator of HIB function and put HIB and Crn in the same pathway to achieve the suppression on Su(fu). Finally, we show that SPOP, like HIB, can downregulate Su(fu) in flies.

As a major signaling pathway involved in many key aspects of animal development, the Hh pathway activity needs to be precisely regulated *in vivo*, which is likely

to rely on multiple layers of regulatory mechanisms. Our previous studies demonstrated that the BTB protein HIB is induced by Hh and forms a negative feedback to attenuate Hh signaling activity by targeting the active form of Ci for degradation [39, 43]. Intriguingly, here we found that HIB also downregulates Su(fu) level *in vivo*, suggesting that HIB plays a dual role in Hh signaling.

In *Drosophila* wing imaginal discs we found that Su(fu) level is downregulated by overexpression of Hh, and this regulation relies on Hh signal transduction. We further demonstrate that the Hh pathway target gene *hib* mediates the regulation of Su(fu) through the HIB/SPOP-Crn axis in *Drosophila*. Of note, since overexpression of *Hh* is somehow a little bit more efficient to downregulate Su(fu) than overexpression of *HIB*, we cannot rule out the possibility that Hh acts through additional factors together to downregulate Su(fu).

As a target of Hh signaling, *hib* is upregulated by high levels of Ci activity in A-compartment cells abutting the A/P boundary. HIB-mediated downregulation of Su(fu) in this region may boost the levels of active Ci to counterbalance HIB-mediated degradation of CiA, allowing Ci activity to stay above critical thresholds required for appropriate expression of Hh target genes (Figure 8A). Hence, the dual role of HIB may serve to buffer the steep drop of Ci activity by modulating both Ci and Su(fu), and finally accomplish fine-tuning of Ci activity. The dual role of HIB may explain an earlier observation that Ci accumulated inside *hib* clones only mildly upregulated *dpp-lacZ* but not *ptc-lacZ* level because upregulated Su(fu) might prevent a surge in Ci activity [39]. Therefore, our study unveils a novel mechanism for fine-tuning

Hh pathway activity.

Although in wild-type flies loss of *su(fu)* does not show overt phenotype due to the presence of another negative regulator Cos2, loss of Su(fu) can impact Hh signaling outcome in a genetically sensitized background, i.e., when the pathway is compromised by expressing a dominant-negative Smo, Smo^{-PKA}. As shown in Supplementary information, Figure S7, expression of Smo^{-PKA} resulted in fusion between veins 3 and 4 in the proximal region of *Drosophila* adult wings, indicative of reduced Hh signaling. This phenotype was suppressed by removing Su(fu), indicating that Su(fu) plays a negative role in Hh signaling. On the other hand, excessive Su(fu) as shown either by previous overexpression studies or by Crn loss of function can attenuate Hh signaling activity. Hence, altering Su(fu) levels can influence Hh signaling output. Overall, our study demonstrates that Hh regulates both Ci and Su(fu) levels through its target HIB, thus uncovering a novel feedback mechanism that regulates Hh signal transduction. We think that the dual function of HIB may provide a buffer to fine-tune Hh pathway activity to ensure that the appropriate level of pathway activity is achieved for a given level of Hh.

It is interesting that HIB regulates Su(fu) at the mRNA layer instead of protein turnover. We find that HIB does not affect Su(fu) stability, *su(fu)* mRNA level, or *su(fu)* mRNA nuclear export (Supplementary information, Figure S8), implying that HIB downregulates Su(fu) most likely by inhibiting the formation of functional *su(fu)* mRNA. Interestingly, we find that HIB reduces Su(fu) level by modulating Crn subcellular localization. Based on our results that downregulation of Su(fu) by

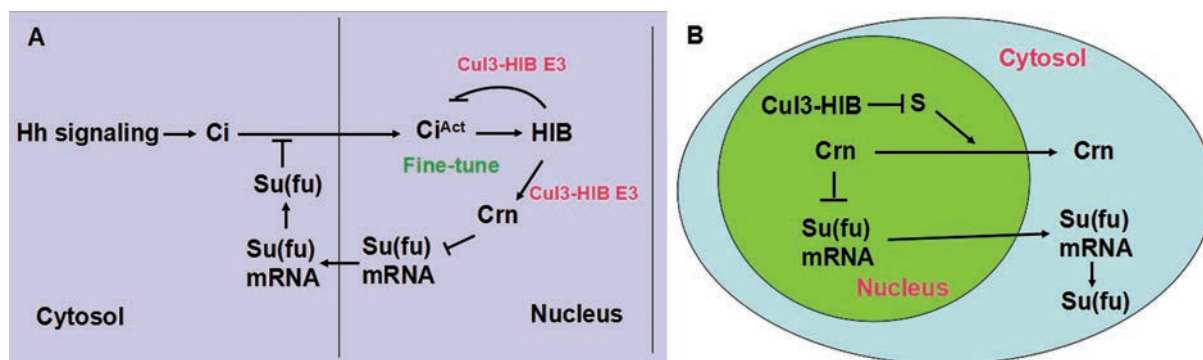


Figure 8 A model of Su(fu) regulation by HIB. **(A)** Hh induces *hib* expression through the transcription factor Ci. HIB degrades activated Ci (CiA) by forming Cul3-HIB E3 ligase complex. Cul3-HIB also downregulates Su(fu) level through Crn that likely interferes with normal translation from *su(fu)* mRNA. Therefore, Hh modulates both Ci and Su(fu) through its target HIB to fine-tune the pathway activity. **(B)** Cul3-HIB degrades an unknown substrate “S”, which is essential for Crn nuclear export. In the absence of HIB, “S” promotes cytoplasmic localization of Crn; in the presence of HIB, Cul3-HIB degrades “S” to promote nuclear accumulation of Crn, which inhibits the formation of functional *su(fu)* mRNA, leading to reduced Su(fu) protein synthesis.

HIB depends on Cul3 and Crn, we propose a working model as shown in Figure 8B. HIB together with Cul3 functions as an E3 ligase to degrade an unknown substrate “S” that is necessary for exporting Crn from nucleus to cytoplasm. Without HIB, Crn is mainly located in the cytoplasm because “S” can promote Crn export, whereas in the presence of HIB, Cul3-HIB E3 ligase degrades “S” to block Crn export. This model is further supported by the observation that MG132 blocked Crn nuclear localization induced by HIB (Figure 6C’’’’).

Since HIB did not affect *su(fu)* mRNA nuclear export, *Su(fu)* gene has no introns and the 5’ or 3’UTR structure of *su(fu)* mRNA plays a very important role in *su(fu)* translation (Supplementary information, Figure S9A), we speculate that Crn accumulated in the nucleus may affect the 5’ or 3’UTR structure of *su(fu)* mRNA or mediate interaction between 5’ and 3’UTR of *su(fu)* mRNA with some unidentified factors. These changes do not seem to affect *su(fu)* mRNA export from the nucleus to the cytoplasm, but may affect *su(fu)* mRNA translation. This idea was supported by the *su(fu)* mRNA translation assay as shown in Supplementary information, Figure S9B. Compared with control S2 cells, we found more Su(fu) proteins translated from the same amount of *su(fu)* mRNA upon knockdown of *hib* during 4 h after blocking the *su(fu)* transcription with Act D (Supplementary information, Figure S9B and S9C). The exact mechanism by which Crn regulates Su(fu) expression awaits further investigation.

Su(fu)/SUFU plays a conserved role in regulating Ci/Gli activity; however, the regulation of mammalian SUFU remains poorly understood. We found that Hh signaling downregulates Su(fu) through its target HIB/SPOP, which forms a complex with Cul3 to function as an E3 ubiquitin ligase, and that HIB-Cul3 downregulates Su(fu) via regulating the nuclear/cytoplasmic shuttling of a putative spliceosome factor Crn, thus uncovering a novel mechanism for regulating Hh signal transduction. We demonstrated that the mammalian homologue of HIB, SPOP, also downregulates Su(fu) in the same fashion as HIB does in flies. Moreover, the mouse homologue of *crn*, *crnk11* siRNAs reduced ShhN signaling by 50% [68], indicating that both SPOP and Crnk11 may have a conserved role in vertebrate Hh signaling. Therefore, it will be interesting to investigate whether SPOP and Crnk11 can regulate SUFU through a similar mechanism in mammalian systems.

Materials and Methods

Mutations and transgenes

yw, *hib-lacZ*, *Ptc-lacZ*, *Knot-lacZ*, *MS1096*, *UAS-ci-RNAi*, *hib^Δ*, *Ap-Gal4*, *UAS-Hh*, *UAS-HA-Su(fu)*, *UAS-Flag-Su(fu)*, *UAS-*

Myc-Ci, *UAS-HA-Ci^{3P}*, *UAS-Flag-HIB*, *UAS-HA-HIB*, *UAS-HA-CiN*, *UAS-hib-RNAi*, *UAS-Flag-SPOP*, *UAS-Flag-Cul3^{KR}*, *UAS-Smo^{-PKA}* and *UAS-GFP* have been described [39, 42, 55, 63, 68, 69]. The *crn* RNAi lines, *su(fu)* RNAi lines and other RNAi lines used in small-scale genetic screen were obtained from the National Institute of Genetics Stock Center, Japan and the Vienna Drosophila RNAi Center, Austria. *eyflp*, *hsflp*, *cul3⁹¹²* and FRT82 flies were obtained from the Bloomington Stock Center. To make *UAS-HA-Ub-K0*, ubiquitin (*ub*) cDNA was amplified by PCR with substitution of amino acids at all seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) to Arg, then inserted into *NotI* and *XhoI* sites of the pUAST-HA vector. For *UAS-HA-HIB-Δ3box*, the sequence encoding *hib* lack of amino acids from 299 aa to 330 aa was cloned into pUAST-HA digested with *BglII* and *XhoI*. To construct *UAS-HA-Su(fu) 2D*, *su(fu)* cDNA was amplified by PCR with substitution of amino acids at both S321 and S324 to Asp, then cloned into *BglII* and *XhoI* sites of pUAST-HA vectors. The coding sequence for *crn* was amplified and inserted into *BglII* and *XhoI* sites of the pUAST-HA vector to make construct *UAS-HA-Crn*. The dsDNA sequences for 5’UTR and 3’UTR of *su(fu)* were synthesized in Genscript, then cloned into *BglII* or *KpnI* or between these two sites of the *pUAST-Flag-Su(fu)* vector to make the constructs *UAS-5’UTR-Flag-Su(fu)*, *UAS-Flag-Su(fu)-3’UTR* and *UAS-5’UTR-Flag-Su(fu)-3’UTR*, respectively.

Immunostaining and in situ hybridization

Immunostaining and *in situ* hybridization of imaginal discs were performed with standard protocols [70]. Antibodies were used in this study: rat anti-Ci (2A) (1:50; DSHB); mouse anti-Flag (M2) (1:200; Sigma); mouse anti-HA (F7) (1:200; Santa Cruz); rabbit anti-β Gal (1:500; Cappel); mouse anti-Su(fu) (1:100; DSHB); rabbit anti-GFP (1:500; Santa Cruz); TRITC-labeled phalloidin (1:250; Sigma) and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (1:1000; Santa Cruz). For *in situ* hybridization assay, the primers for *su(fu)* and *crn* sub-cloning are as follows: *su(fu)*-F, 5’-ATAAGAATGCGGCCGCATGGCCGAGGCGAATTGGACA-3’, *su(fu)*-R, 5’-GGGGTACCTCACATTTTCA-GAGGAGC-3’; *crn*-F, 5’-GGGGTACCGCCGGCGGAGGTGCAAATTAC-3’, *crn*-R, 5’-CCCAAGCTTCGCGCCCTGTCGTGCTCCTT-3’. *su(fu)* and *crn* RNA probes were synthesized from the above corresponding regions.

Generating clones of mutant cells

Clones of mutant cells were generated by FLP/FRT-mediated mitotic recombination as described [39, 70]. Genotypes for generating clones are as follows: *hib* clones in wing discs with or without expressing UAS transgenes: *MS1096 hs-flp*; (*UAS-Hh* or/and UAS transgenes); FRT82 *hib^Δ/FRT82 hs-Myc-GFP*. *hib* clones in eye discs with or without expressing UAS transgenes: *ey-flp*; (*GMR-Gal4/UAS* transgenes); FRT82 *hib^Δ/FRT82 hs-Myc-GFP*. *cul3* clones in eye discs: *ey-flp*; *cul3⁹¹²FRT40/hs-Myc-GFP* FRT40.

Cell culture, transfection, RNA interference, immunoprecipitation, western blot assays

S2 cells were cultured in the Schneider’s *Drosophila* Medium (Invitrogen) with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Transfection was carried out using the calcium phosphate transfection method. Usually S2 cells are

transfected in 10-cm plates with no more than 20 µg of total DNA for an *ubiquitin-Gal4* construct and other co-transfected pUAST expression vectors. 36-48 h after transfection, cells are harvested for immunoprecipitation and western blot analysis with standard protocols as previously described [71, 72]. The following antibodies were used for immunoprecipitation and immunoblotting: mouse anti-Myc (9E10) (1:5 000; Santa Cruz); mouse anti-HA (1:5 000; Santa Cruz); mouse anti-Su(fu) (1:500; DSHB); mouse anti-β-actin (1:10 000; Genscript); and goat anti-mouse HRP (1:10 000; Jackson ImmunoResearch). For RNA interference experiment, dsRNA was generated through *in vitro* transcription by using the MEGAscript T7 kit (Ambion). After cells were transfected for 24 h, the culture medium was changed to serum-free medium with 15-50 µg dsRNA/10⁶ cells for 8-12 h starvation. Then fresh medium with serum was added and cells were cultured for 48-36 h. The primer sequences of the target genes are as follows: *hib* RNAi-F, 5'-GAATTAATACGACTCACTATAGGGAGAATGGC-GGTCAGCCGTGTACCAT-3', *hib* RNAi-R, 5'-GAATTAATACGACTCACTATAGGGAGACCATCTCATGCTCGAACATG-3'; *crn* RNAi-F, 5'-GAATTAATACGACTCACTATAGGGAGAATGGAGCGGCCACAGAAGATG-3', *crn* RNAi-R, 5'-GAATTAATACGACTCACTATAGGGAGACAAATACCCGCC-GAGAGCCG-3'; *hh* RNAi-F, 5'-GAATTAATACGACTCACTATAGGGAGAAGAAGTTCATCCGACGAGAC-3', *hh* RNAi-R, 5'-GAATTAATACGACTCACTATAGGGAGAAGCT-CATTTTCACGCGTTTC-3'.

MG132, LMB, CHX and Act D treatment

For MG132 (Calbiochem) treatment assay, eye discs were cultured in supplemented M3 medium containing 40 µM MG132 for 4 h, followed by immunostaining. S2 cells were treated with 20 µg/ml MG132 for 4 h before cells were harvested. For LMB (Sigma) treatment, S2 cells were treated with LMB at a final concentration of 5 nM for 2 h before cells were harvested for Crn localization assay. Wing discs from mid-third instar larvae were cultured in Cl-8 cell medium without or with 50 ng/ml LMB treatment for 4 h, endogenous Su(fu) was shown by western blotting and then the results were analyzed by ImageJ. For Cycloheximide (CHX, Calbiochem) treatment, S2 cells were plated in 10-cm dishes and transfected with the indicated plasmids after 18-24 h. After another 24 h, the cells were transferred into six-well cell culture plates at equivalent densities. Cells were treated with 20 µg/ml CHX for the indicated times before harvesting. For Actinomycin D (Act D, Beyotime) treatment, S2 cells were treated with 1 µg/ml Act D for the indicated times before harvesting.

RNA isolation from cell extracts, reverse transcription PCR (RT-PCR) and real-time quantitative PCR (RTQ-PCR)

S2 cells were transfected with *UAS-HA-Crn* or *UAS-Flag-HIB* or both of them, respectively. After two days, the cells were harvested, 3 ml of them was prepared for the total RNA isolation, and the rest 7 ml was washed with PBS and incubated in 400 µl of hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4/10 mM NaCl/3 mM MgCl₂/0.1% (vol/vol) Nonidet P-400) containing protease inhibitors (Roche) for 20 min on ice. After centrifuged at 2 500×g at 4 °C for 5 min, supernatant was removed in a new tube as cytosol fraction, and the rest (nuclei pellet) was washed for 10 min with hypotonic lysis buffer for three times. Nuclei pellet was resuspended in 100 µl RIPA buffer (50 mM Tris-HCl, pH 7.4/150

mM NaCl/1% (vol/vol) Nonidet P-400/0.5% (wt/vol) sodium desoxycholate/0.1% (wt/vol) SDS) containing protease inhibitors and frozen at -80 °C for 1 h. All of the total cells, cytosol fraction and nuclei pellet RNA were extracted and the reverse transcription was carried out using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa). RT-PCR primers for *Drosophila su(fu)* coding region (upstream: 5'-GACAAAAAACCTGAGGTGAAGCC-3' and downstream: 5'-AGTTTGGTGCCTGCGCTT-3') were synthesized by Invitrogen (Shanghai, China). For detecting the expression of *hh*, *ci*, *hib*, *crn* and *su(fu)* in S2 cells, the PCR primers are as follows: *hh* (upstream: 5'-ATGGATAACCACAGCTCAGTGCCT-3' and downstream: 5'-TCAATCGTGGCGCCAGCTCT-3'), *ci* (upstream: 5'-GCAGTATATGCTTGTGTGC-3' and downstream: 5'-TCTTTGACTGAATGAACCCC-3'), *hib* (upstream: 5'-ATG-GCGGTCAGCCGTGTACC-3' and downstream: 5'-TCAGCT-CATTTTCACGCGTT-3'), *crn* (upstream: 5'-ATGGAGCGGC-CACAGAAGAT-3' and downstream: 5'-TCAGTCACCGCTATC-CGTCG-3'), *su(fu)* (upstream: 5'-ATGGCCGAGGCCAATTTG-GA-3' and downstream: 5'-TCACATTTTCAGAGGAGCAG-3'). For Q-PCR, S2 cell and wing disc RNA were isolated by using Trizol reagent (Invitrogen) and then reverse-transcribed by PrimeScript RT reagent kit (TaKaRa). Finally, the real-time PCR was done using the SYBR Premix Ex Taq (TaKaRa) according to the instrument of StepOnePlus (Applied Biosystem). Standard Q-PCR primers for *Drosophila su(fu)* (upstream: 5'-AAGCGCAGGCAC-CAAAC-3' and downstream: 5'-CCAAAGTGAGCGCCA-GAT-3'), *hib* (upstream: 5'-GCTACACGCAGGTCAAAGTG-3' and downstream: 5'-CCGTAAACACCATTTTAGTT-3'), *ci* (upstream: 5'-CCTCTTGCCTATTCTGAATT-3' and downstream: 5'-GAATCTGATGTTCCACCCG-3') and actin (upstream: 5'-CGAAGAAGTTGCTCTGGTTGTGTCG-3' and downstream: 5'-GGACGTCCCAATCGATGGGAAG-3') were synthesized by Genscript. Actin was used as a control.

Statistical analysis

Imaging data were analyzed using Image J. Statistical tests were performed in GraphPad Prism 5. All measures are reported as mean ± SEM.

Acknowledgments

We thank Dr Yun Zhao (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China), Fly Stocks of National Institute of Genetics of Japan (NIG-Fly), Vienna *Drosophila* RNAi Center (VDRC), the Bloomington Stock Center and Developmental Studies Hybridoma Bank at the University of Iowa for providing fly stocks and reagents. This work was supported by grants from the National Natural Science Foundation of China (30971679, 31071264 and 31271531) and the National Key Scientific Program of China (2011CB943902, 2010CB945102) to Qing Zhang. Jin Jiang is supported by grants from NIH (GM061269 and GM06745), Welch foundation (I-1603) and NSFC (31328017).

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