Apontic directly activates hedgehog and cyclin E for proper organ growth and patterning

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Hedgehog (Hh) signaling pathway and Cyclin E are key players in cell proliferation and organ development. Hyperactivation of hh and cyclin E has been linked to several types of cancer. However, coordination of the expression of hh and cyclin E was not well understood. Here we show that an evolutionarily conserved transcription factor Apontic (Apt) directly activates hh and cyclin E through its binding site in the promoter regions of hh and cyclin E. This Apt-dependent proper expression of hh and cyclin E is required for cell proliferation and development of the Drosophila wing. Furthermore, Fibrinogen silencer-binding protein (FSBP), a mammalian homolog of Apt, also positively regulates Sonic hh (Shh), Desert hh (Dhh), Cyclin E1 (CCNE1) and Cyclin E2 (CCNE2) in cultured human cells, suggesting evolutionary conservation of the mechanism. Apt-mediated expression of hh and cyclin E can directly proliferation of Hh-expressing cells and simultaneous growth, patterning and differentiation of Hh-recipient cells. The discovery of the simultaneous expression of Hh and principal cell-cycle regulator Cyclin E by Apt implicates insight into the mechanism by which deregulated hh and cyclin E promotes tumor formation.

Animal development requires the organ growth and patterning. How these two processes are coordinated remains poorly understood. The Drosophila wing is an excellent model to study the regulation of gene expression during the organ growth and patterning. The wing disc is a sac-like structure composed of disc proper (DP) cells and peripodial epithelium (PE). During larval development, both DP and PE cells proliferate extensively and are patterned, finally give rise to the adult wing1–3. The Hh signaling and Cyclin E can contribute to growth and patterning of the wing disc during development4,5.

Hh pathway is one of the major conserved signaling pathways that control animal development from Drosophila to humans, which has been implicated in stem cell maintenance, cell migration, axon guidance and tissue regeneration6–9. Most vertebrate species have three hh: Shh, Indian hedgehog (Ihh) and Dhh, each with different expression patterns and functions. In the Drosophila wing disc, morphogen Hh expresses in posterior (P) compartment cells and spreads into approximately 12 cells-wide of anterior (A) cells along A/P boundary, where it regulates target gene expression in the A compartment to control entire wing patterning through stabilizing full-length Cubitus interruptus (CiF)4,10–12. Therefore, the expression of hh is vital during wing development. Engrailed (En) induces the expression of hh in the P compartment, at the same time, represses the Hh downstream component Ci11,13. Ci can exist in two forms: CiF and a repressor form (CiR). CiR represses the expression of hh in anterior cells12,14. However, regulatory factor that directly activates hh transcription remained to be identified.

Cyclin E belongs to the cyclin family, which is required for cell division15. Dysregulation of cyclin E correlates with various tumors, including breast cancer and lung cancer16,17. Besides, deregulated Cyclin E activity causes cell lineage-specific abnormalities, such as impaired maturation due to unregulated cell proliferation18. In Drosophila, Cyclin E is essential for G1-to-S phase transition in the posterior cells of eye disc19. It has been reported that cyclin E is a potential target gene of Hh signaling in Drosophila. Hh pathway activates cyclin E expression through its unique transcription factor Ci in the posterior cells of eye disc20. In the wing disc, Hh pathway is turned on exclusively in the A cells near A/P boundary21. However, cyclin E expresses throughout

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the wing disc. This contradiction suggests that other factors are involved in regulating the expression of cyclin E. Therefore, it is fruitful to investigate the regulation of cyclin E in the wing disc and the relationship between Cyclin E and Hh pathway.

Apt has been identified as a transcription factor involved in development of tracheae, head, heart and nervous system. Apt can suppress metastasis and is required in the nervous system for normal sensitivity to ethanol sedation. Moreover, Apt participates in JAK/STAT signaling pathway to limit border cells migration. The human homolog of Apt, FSBP, is a cancer-related factor that is expressed in many tissues. However, the role of Apt in the organ growth and patterning is unknown.

In this study, we unveiled a fundamental role of Apt in growth and patterning of the wing disc through coordinated expression of morphogen hh and cell cycle regulator cyclin E. Both loss of function and overexpression of apt resulted in defective wings. Further studies demonstrated that loss of apt function attenuated the expression of hh and cyclin E, while apt overexpression upregulated hh and cyclin E. Mutating the inherent Apt binding sites in the promoter region of hh and cyclin E compromised the expression of hh and cyclin E. Collectively, Apt directly activates the expression of hh and cyclin E to allow proper wing development. In addition, we found that Apt-dependent expression of hh and cyclin E is evolutionarily conserved in human cells.

Results

Apt is expressed in the wing disc and is required for wing development. As the first attempt to investigate the function of apt during wing development, we analyzed apt expression pattern in the wing disc by immunostaining using anti-Apt antibody. In the wing disc, Apt was detected in PE cells as revealed by co-localization with a PE marker Ubx (Fig. 1A). Apt was also detected in DP cells (Fig. 1B). These data clearly demonstrate that Apt is expressed in both the PE and DP of the wing disc, suggesting its possible role in wing development.

To analyze the role of Apt during wing development, we would examine the developing wing of homozygous apt null mutant. However, apt null homozygotes die as embryos. Therefore, we firstly examined the phenotype of apt knockdown using an MS1096-GAL4 driver. RNAi-mediated knockdown of apt resulted in a small wing, and also reduced the width between vein 3 and vein 4 (Fig. 1C–E). Overexpression of a dominant-negative form of Smoothened (SmoΔPKA) caused a “fused wing” phenotype. Knockdown of apt enhanced the “fused wing” phenotype (Fig. 1F, arrowhead indicates the “fused wing” phenotype and arrow indicates enhancement of the “fused wing” phenotype). Furthermore, we induced apt loss of function mutant clones in the wing disc using the FLP/FRT system. The formation of these clones resulted in a small wing with a blistered phenotype (Fig. 1H) compared with the control wing (Fig. 1G). To investigate the effect of apt overexpression, we employed the MS1096-Gal4 driver. Abnormal wings were induced by overexpression of apt (Fig. 1I). The wing was diminished and blistered, and the pattern of veins was disrupted and extra abnormal bristles were induced in the wing margin. In addition, when apt was overexpressed by a stronger driver (sd-Gal4), both wings and halters were lost (Fig. 1I). Taken together, the loss-of-function and overexpression analyses indicate that Apt is dispensable for wing development.

Apt activates the expression of hh in the wing disc. Given that the space between vein 3 and vein 4 is a characteristic monitor of Hh activity, the observed narrowing the space between vein 3 and vein 4 upon knockdown of apt (Fig. 1C–E) implies that Apt can modulate expression of hh in the wing disc. The enhanced dominant-negative phenotype of SmoΔPKA by knockdown of apt (Fig. 1F) supports the notion. To examine the relationship between apt and hh, we first compared the expression of apt and hh, and found that Apt and hh-lacZ were co-expressed in PE cells (Fig. 2A–C) and P compartment cells of the DP (Fig. 2D–F) in the early third instar larval disc. Furthermore, apt exhibited genetic interaction with hh. Ninety-seven percent of hhΔbar3 mutant (n = 40) showed slightly reduced area between L3 and L4 (Fig. 2H) and the remaining three percent showed wing blistering phenotype (Supplementary Fig. S5B). While heterozygotes of apt null allele showed normal wings (Fig. 2G), the same heterozygotes under the hhΔbar3 background exhibited more severe phenotypes of reduced L3-L4 area and smaller wing with blister (Fig. 2I), which reproduced the apt loss of function phenotype (Fig. 1H). Transheterozygotes of two sets of hh alleles (hhΔbar3/hh2 and hhΔm1/hh7) showed a smaller wing with an extra crossvein (Supplementary Fig. S1A–E), demonstrating that it is a loss of function phenotype of hh. While wings of animal heterozygous for hh2 or apt null mutant were normal, trans-heterozygotes of the apt null allele and hhΔm1 showed the same wing phenotype (Supplementary Fig. S1F–I). These results suggest that Apt regulates the expression of hh. To address the issue directly, we analyzed the expression of hh under loss-of-function and overexpression of Apt. The expression of hh-lacZ was significantly reduced in the apt mutant clones in the PE (Fig. 3A–C) and the DP (Fig. 3D–F). In addition, the expression of hh-lacZ also decreased in the apt-knocked down region (Fig. 3G–I). By contrast, overexpression of Apt increased the expression of hh-lacZ (Fig. 3J–L). Moreover, Apt regulates the mRNA levels of hh and its target dpp (Supplementary Fig. S2). These results demonstrate that Apt activates the expression of hh.

Apt directly controls hh in the wing disc. To address how Apt activates the expression of hh, we focused on a 15–kb region of the hh locus known to reproduce the normal hh expression pattern in the wing disc. We identified one potential Apt binding sequence within the region (Fig. 4A). Chromatin immunoprecipitation (ChIP) assays using early third instar wing discs detected Apt protein on the predicted wild-type Apt binding site but not in the upstream and downstream of the binding site (Fig. 4A and D). We next assessed the function of the Apt-binding site in hh using a CRISPR-Cas9 system. Since the designed gRNA contained the Apt-binding site, the four Apt-binding site deletion mutants and two insertion mutants were generated (Fig. 4B and C; Supplementary Fig. S3A). The hhΔaptD1 mutation abolished the occupancy of Apt on its binding site (Fig. 4D). Homozygotes of these mutations showed reduced expression of hh (Fig. 4E; Supplementary Fig. S3B) and exhibited the small wing and reduced vein 3–4 spacing phenotypes (Fig. 4F; Supplementary Fig. S3C and D). Effect...
of hhΔaptDB1 mutation on the hh function was also examined under the hh2 heterozygous background. While wings of animals heterozygous for hh2 or hhΔaptDB1 were normal, transheterozygotes of hhΔaptDB1 and hh2 showed the same extra vein phenotype (Fig. 4G–I) as did transheterozygotes of apt-null allele and hh2 (Supplementary Fig. S1H). Besides, we also examined the effect of hhΔaptDB1 mutation on the hh function under expression of a dominant-negative form of Smoothened (Smo−PKA). While MS > Smo−PKA alone showed reduction of the intervein space between vein 3 and vein 4 (Fig. 4J and K, arrowhead), hhΔaptDB1 exhibited more severe defects and enhanced the "fused wing" phenotype of MS > Smo−PKA (Fig. 4L). Taken together, these data demonstrate that Apt directly activates transcription of hh in the wing disc for proper wing development.

### Apt activates the cyclin E expression in the wing disc.

We have reported that Apt induces the cyclin E expression in the eye disc38. Therefore, we examined whether Apt consistently regulates cyclin E in the wing disc. To do this, we performed a double-staining experiment using Apt antibody and Cyclin E antibody. In the wild-type wing disc, Apt and Cyclin E were co-expressed (Fig. 5A–C). Furthermore, the expression of Cyclin E was significantly reduced in the apt mutant clones (Fig. 5D–F). Compared with control disc (Fig. 5G), apt knockdown decreased Cyclin E level (Fig. 5H), while apt overexpression increased Cyclin E (Fig. 5I). In addition, the
cyclin $E$ mRNA levels were decreased and increased upon RNAi-knockdown and overexpression of apt in the wing disc, respectively (Supplementary Fig. S4). These results indicate that Apt activates the expression of cyclin $E$ in the wing disc.

**Apt directly controls cyclin $E$ in the wing disc.** Since Apt directly activates the expression of cyclin $E$ in the eye disc, we anticipated a direct role of Apt in the expression of cyclin $E$ also in the wing disc. This expectation was verified by transgenic reporter assays. The reporter gene $cycE$-PlacZ with the wild type binding site recapitulated the cyclin $E$ expression in the wing disc (Fig. 5J–L). However, base substitutions in the Apt-binding site in $cycE$-PlacZ abolished the lacZ expression (Fig. 5M–O). These results indicate that Apt directly activates cyclin $E$ through its binding site in the regulatory region of cyclin $E$.

**Apt is a growth sensor to control organ growth and patterning.** Because both Hh and Cyclin $E$ are involved in cell death and cancer, we asked whether the overexpression phenotypes are caused by apoptosis. To test this, we investigated apoptosis in wing discs by staining with anti-Caspase-3 antibody. In the third instar wing disc, apt mutant clones showed few apoptotic cells (Fig. 6A). However, in the wing disc from an Apt-overexpressed larva, the number of apoptotic cells significantly increased compared with a control disc (Fig. 6B and C). This presumably explains why wing size was reduced upon strong overexpression of Apt (Fig. 1K and L). Besides, we examined the growth of wing discs upon apt knockdown in the dorsal region using an ap-GAL4 driver. Compared with a control disc (Fig. 6D), apt knockdown region exhibited growth disadvantage (Fig. 6E and H). Overexpression of Apt using the ap-GAL4 driver resulted in severely reduced dorsal region (Fig. 6F). When we inhibited cell death by simultaneous overexpression of a Caspase inhibitor P35, we observed outgrowth of cell layers from the disc in the apt and p35 overexpressed region (Fig. 6G and H).

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**Figure 2.** Expression and genetic interaction of apt and hh. (A–F) The expression of Apt and hh-lacZ in PE cells (A–C) and DP cells (D–F). (G–I) Genetic interaction between apt and hh. Adult wings of indicated genotypes. (I) Fifty percent of apt$^{+/+}; hh$bar3/hh$bar3$ wings exhibited patterning defects and blistered wing. Total numbers of analyzed wings were G, 37; H, 40; I, 113.
Homozygotes of hh mutations for the Apt-binding site exhibited the small wing but not the blistered phenotype. However, hh and cyclin E double mutant recapitulates the smaller and blistered wing. While CycE^{+/+} flies showed normal wings, three percent of hh^{bar3}/hh^{bar3} and eighteen percent of CycE^{+/+}; hh^{bar3}/hh^{bar3} flies showed the smaller and blistered phenotypes (Supplementary Fig. S5A–C). We also observed genetic interaction between

Figure 3. Apt regulates the expression of hh. (A–F) The decreased expression of hh-lacZ in the apt^{Δ4} clones of the PE (A–C) and DP (D–F). Clones are marked by white-dotted lines. The A/P boundaries are marked by yellow-dotted lines in D–F. (G–I) Knockdown of Apt using ap-GAL4 driver resulting in the decreased expression of hh-lacZ in the dorsal region. The D/V boundaries are marked by yellow-dotted lines. (J–L) Overexpressed Apt increased the expression of hh (K).
Figure 4. Apt directly regulates the expression of hh through its binding site in the hh promoter region. (A) Schematic representation of the Apt-binding site in the genomic sequence of hh. The arrow represents transcription start site and the numbers in base pairs are distance from the start site. Up and Down mean the region relative to the Apt binding site in the hh promoter region. (B) Sequences of a wild-type allele and a heterozygous mutant of hh aptDB1. The sequence of the mutant allele was inferred by subtracting a wild-type sequence from the mixed sequence. The deleted sequence is highlighted in yellow. (C) Cas9-induced mutagenesis at the hh locus. The hh locus in Cas9-induced mutants was PCR-amplified and sequenced. The wild-type sequence is shown at the top as a reference. The Cas9-gRNA target sequence is underlined with the protospacer-adjacent motifs (PAM) indicated in green. Deleted nucleotides in hh aptDB1 are shown as dashes. The deletion size is shown next to the sequence. (D) ChIP analyses of Apt at the hh promoter. Occupancies of Apt in binding site, upstream or downstream of wild type and binding site deletion mutant were analyzed by ChIP with Apt antibody. Data are normalized to Mock. (E) RT-qPCR analyses of hh mRNA in the wing disc of
third instar larvae from yw or hhΔaptDB1. Error bars, SEM from three independent experiments. Student’s t tests, **p < 0.001. (F–L) Deletion of the Apt-binding site in the hh promoter affects wing development. The wing size and the intervein region between L3 and L4 (control value was set as 100%) were decreased in hhΔaptDB1. Error bars, SEM. Student’s t tests, **p < 0.001. hhΔ/+(G) or hhΔaptDB1/+(H) adult wings show normal phenotype. All adult wings of hhΔaptDB1/hhΔ transheterozygotes exhibited abnormal morphologies in ACV (I). An arrowhead indicates the extra ACV. (J–L) The binding site deletion mutant enhanced the fused wing phenotype of MS > SmoPKA. Total numbers of analyzed wings were G, 157; H, 132; I, 74; J, 67; K, 80; L, 97. Scale bars, 200 μm.

hh and cyclin E in the extra crossvein phenotype. While CycEΔ/+ and hhΔ/+ flies showed normal wings, fifty-four percent of CycEΔ/+; hhΔ/+ flies (n = 102) exhibited wings with the extra crossvein (Supplementary Fig. S5D–F). Collectively, these data suggest that Apt controls wing development by inducing appropriate amounts of Hh and Cyclin E.

FSBP positively regulates Shh and cyclin E in human cells. FSBP, the mammalian homologue of Drosophila Apt, is a cancer related factor. To examine whether FSBP regulates Shh and cyclin E, we used human 293T cells to knockdown or overexpress FSBP and analysed the mRNA levels of Shh, its signaling pathway genes and cyclin E. After transfection of FSBP siRNA, the mRNA level of FSBP decreased nearly 60 percent compared with mock. Under the condition, we observed marked decrease in the mRNA levels of Shh and Shh signaling pathway genes such as Pthc, Gli and Hhip (Fig. 7A). The levels of cyclin E (CCNE1 and CCNE2) mRNA showed less prominent but statistically significant decrease. When we overexpressed FSBP, mRNA level of FSBP increased nearly 7.5 folds, and that of Shh increased dramatically 9 folds. The mRNA levels of Shh targets and cyclin E were also increased upon overexpression of FSBP (Fig. 7B). Interestingly, FSBP also regulates the expression of Dhh, but not hh. Taking together, these data suggest that the regulation of hh/Shh and cyclin E by Apt/FSBP is conserved from Drosophila to humans.

Discussion

Morphogen Hh and cell cycle regulator Cyclin E control growth and patterning in vertebrate and invertebrate. Here, we unravel a fundamental role of transcription factor Apt/FSBP as a conserved regulator of hh/Shh and cyclin E/CCNE. During Drosophila wing development, Apt directly activates the expression of hh and cyclin E to control wing growth and patterning. Both loss-of-function and overexpression assays clearly demonstrated that Apt is vital for wing development. Further studies showed that loss of apt function attenuates, while overexpression of apt activates the expression of hh and cyclin E. Moreover, we found that the homolog of Apt, FSBP, can positively regulate Shh and its pathway genes, and CCNE in human cells.

Hyperactivation of Hh pathway and cyclin E has been implicated in many tumors.16,39. In contrast, during development, cell proliferation must be precisely regulated and coordinated with the processes of cell patterning and differentiation, which are also regulated by Hh and Cyclin E.31,42. This delicate balance is probably maintained by Apt-mediated proper expression of Hh and Cyclin E. Indeed, overexpression of Apt in the presence of apoptosis inhibitor P35 generated tumor-like outgrowth of cell layers in the wing disc. Apt-dependent expression of hh and cyclin E can direct proliferation of Hh-expressing cells and simultaneous growth, patterning and fate specification of Hh-recipient cells. Although mechanisms are quite different, this provides similar effects as an asymmetric division of a stem cell.

To assess the importance of the Apt-binding site in the promoter region of hh, we first tried a transgenic reporter assay. However, the regulatory region of hh encompassing the upstream region and the 1st intron (−15 kb) is too large to make a reporter construct for conventional P-element mediated transgenesis. Therefore, we employed the CRISPR-Cas9 system to mutagenize the endogenous Apt-binding site in the hh promoter. All 6 independent mutants exhibited the same phenotypes (reduced expression of hh, reduced wing size and the space between L3 and L4), suggesting that the observed phenotypes are not due to off-target effect of Cas9. Nevertheless, we inspected the possibility of off-target effect. Since our gRNA carries the binding sequence for Apt, a binding site of Apt in other than the hh promoter could be the most likely candidate for off-target. However, all the 6 mutants showed the wild type sequence around the Apt-binding site in the cyclin E promoter (Supplementary Fig. S6). Furthermore, we observed clear genetic interactions between hhΔaptDB1 and other hh mutants. Taken together, these data strongly suggest that the observed phenotypes are not due to off-target effect.

Although the expression of Apt and Hh overlapped in the P compartment of wing disc, how Apt specifically induces hh in the P compartment is still not clear. Since Gli5 has been known to repress the expression of hh in anterior cells,54,55 Gli5 may interrupt the activation of hh by Apt in anterior cells. While our data strongly support that Apt is a transcription factor of hh, mutating the Apt-binding site on hh promoter alone induced the weak phenotype. However, the binding site mutation showed strongly enhanced “fused wing” phenotype in the background of overexpression of Smo domain-negative form (SmoPKA). These observations suggest that besides Apt, other factor(s) might also regulate hh transcription during development. Therefore, both knockdown and overexpression of Apt only moderately affected the expression of hh. Hh, as an important morphogen, plays multifaceted roles in segmentation and wing patterning. Previous findings paid more attention on the protein modification of Hh but the regulatory mechanism underlying hh transcription was not well understood. Here we identified Apt as the first regulatory factor that directly activates hh transcription.
Figure 5. Apt controls the expression of cyclin E. (A–C) Apt and Cyclin E expression visualized by immunostaining with Apt antibody and Cyclin E antibody in a control wing disc. (D–F) Decreased Cyclin E expression (E) in the apt mutant clones (D). (G–I) Cyclin E expression in the wing discs of indicated genotypes. The A/P boundaries are marked by yellow-dotted lines. (H) Knockdown of Apt with en-GAL4 decreased the expression of Cyclin E in the P compartment. (I) The expression of Cyclin E was increased in the P compartment from en > apt fly. (J–L) The reporter cycEPlacZ (K) was co-expressed with the endogenous Apt (J) in the wing disc. (M–O) Base substitutions in the Apt-binding site in cycEMPlacZ abolished the lacZ expression (N).
Figure 6. Apt as a growth sensor during development. (A–C) Wing discs from larvae of indicated genotypes labelled to visualize apoptotic cells with Caspase-3 antibody. Apoptosis was barely detectable in the apt mutant clones and their wild-type background (A) and in the disc from MS1096-GAL4 (B). (C) Overexpressed Apt in the wing disc increased the number of apoptotic cells. (D–H) wing discs of indicated genotypes labelled to visualize GFP (Green) and DAPI (White). (D) Control disc. (E) Dorsal region of disc was reduced upon knockdown of Apt with ap-GAL4 driver. (F–G) Overexpression of Apt reduced the disc (F), and simultaneous overexpression of apt and Caspase inhibitor P35 resulted in overgrowth of the wing disc (G). Scale bars, 50 um. (H) The ratio of GFP-positive wing pouch area to total wing pouch area decreased in the apt-RNAi disc, while it increased in the apt and p35-overexpressed disc.
**Materials and Methods**

**Fly stocks.** All the adult phenotypes were obtained from females. Strains used were as follows. aptPΔ422, aptP25, apt167 (gift from M. Starz-Gaiano), cycEPlacZ and cycEMPlacZ38, UAS-apt (gift of D. Montell), UAS-GFP (gift of Y. Hiromi), MS>Smo gastrulin31,32. hhMir was obtained from Drosophila Genetic Resource Center. hh2, hhMrt, hhbar3, CycE2, CycEP, hh-LacZ, MS1096-GAL4, sd-GAL4 (8609) and ap-Gal4 were obtained from Bloomington Drosophila Stock Center. UAS-apt RNAi lines were obtained from Tsinghua Fly Center and Vienna Drosophila Resource Center. CAS-0001, TBX-000, TBX-0004, TBX-0010 were obtained from NIG-FLY Stock Center.

**Clonal analysis.** Homozygous apt loss-of-function clones were generated by hs-FLP/FRT recombination34. FRT42D and aptPΔ4/CyO were recombined to generate FRT42D, aptPΔ4. Six pairs of FRT42D, aptPΔ4 cross to Glo/CyO were allowed to lay eggs in G418-containing medium, and then test each line with aptP2/CyO. hs-FLP; FRT42D, Ubi-GFP/CyO crossed with FRT42D, aptPΔ4/CyO were performed at 25 °C. Heat shocks were performed 32-56 hours after egg-laying for 1.5 hours at 37 °C.

**Generation of CRISPR constructs.** To induce mutations in the Apt-binding site in the hh promoter region, we used a Cas9–gRNA system. We designed gRNA in the hh promoter region carrying the binding sequence of Apt (Fig. 3A). The corresponding sequence was introduced into the pBFv-U6.2 vector and the gRNA transgenic flies were generated as described37. gRNA females were crossed to Cas9 males to obtain the founder animals. Male founders were crossed to female balancer. Offspring male flies were balanced and stocked. Genomic DNA was extracted from each offspring male and used for molecular characterization. PCR primers were designed to construct gRNA expression vectors and to amplify the promoter region of cyclin E (Supplementary Table S1).

**Chromatin immunoprecipitation (ChIP).** ChIP assays of wing discs were performed as previously described49. Briefly, 100 early third instar wing discs were dissected in PBS and fixed by 1% formaldehyde at room temperature for 20 minutes. Sonicated chromatin was immunoprecipitated using 10μl anti-Apt antibody. Quantitative PCR using 4μl of the purified DNA.
RT-qPCR analysis. Total RNAs were prepared from the dissected tissues using an RNAprep Pure Tissue kit (TIANGEN #DP431). cDNAs were synthesized using a Prime Script™ II1 st strand cDNA synthesis kit (TaKaRa #6210A). The real-time qPCR was conducted with Bio-Rad CFX96 real-time system using a SuperRealPreMix Plus (SYBR Green) Kit (TIANGEN #FP205) in a 20 ul reaction containing 2 pmol of relevant primers. The amount of mRNA was normalized to that of control tubulin mRNA. PCR primers used are shown in Supplementary Table (Supplementary Table S1).

Antibodies and immunohistochemistry. Staining of larval tissues was performed as described previously. Larvae were dissected in PBS, fixed with 4% formaldehyde for 40 minutes on ice and then permeabilized for 15 minutes at room temperature in PBS containing 0.5% NP-40. The following primary antibodies were used in overnight incubations at 4 °C in blocking solution: rabbit anti-Apt (1:1000), rabbit anti-β-galactosidase (1:2000, Cappel), rabbit Caspase3 (1:50, Cell Signaling Technology), mouse anti-β-galactosidase (1:500, Sigma), mouse anti-Ub (1:10, Developmental Studies Hybridoma Bank (DSHB)), goat anti-Cyclin E (1:200, Santa Cruz). The secondary antibodies used were as follows: Alexa 488 donkey anti-rabbit IgG conjugate (1:500, Molecular Probes), Alexa 488 donkey anti-mouse IgG (1:500, Molecular Probes), Cy3-conjugated donkey anti-mouse IgG (1:500, Sigma), Cy3-conjugated goat anti-rabbit IgG (1:500, CWBIO), bovine anti-goat IgG-CFL 555 (1:500, Santa Cruz). Mounting used VECTASHIELD Mounting Medium with DAPI (Vector Labs). The caspase-3 staining was did as described previously.

Cell culture and transfection. 293 T cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum and 100 U/ml of penicillin/streptomycin. For RNA interference experiment, FSBP siRNA was designed by ourselves, the sequences were: siFSBP-F: 5′-GCCUGUGUAAGAGACTGGAAAdTdT-3′, siFSBP-R: 5′-UCUCUGUCUCUACAGGCdTdT-3′. After cells were cultured for 24 h in 12-well plate, the culture medium was changed to serum-free medium. Mock is no siRNA treatment. siRNA duplexes were transfected at a final concentration of 20 nM using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells are harvested for real-time PCR after cultured for 48–96 h with serum containing medium. For over-expression experiment, transfection was carried out using PEI (polyethylenimine) transfection method. 293 T cells were transfected in 60 mm plates with 5 μg plasmid pcDNA3.1-FSBP which was constructed by our lab, and pcDNA3.1-HisA/V5 plasmid was transfected as control. Forty-eight to ninety-six hours after transfection, cells are harvested for real-time PCR analyses with standard protocols. The primers were used as showing in the Supplementary Table S1.

Microscopy and image treatment. Images were acquired in Olympus FV1200 confocal microscope and Olympus cellSens, treated with Adobe Photoshop CS6 image programs. Wing size and space between vein 3 and vein 4 were measured on each picture using the ImageJ computer program.

Statistical analysis. Results are given as means ± SEM; each experiment included at least three independent samples and was repeated at least three times. Group comparisons were made by two-tailed unpaired Student’s t-tests. *P < 0.05; **P < 0.01, and ***P < 0.001.

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Author Contributions
X.F. Wang, S. Hirose and Q.X. Liu designed research, X.F. Wang, Y. Shen, Q. Cheng and C.L. Fu performed experiments and X.F. Wang, Z.Z. Zhou, S. Hirose and Q.X. Liu analyzed data and wrote the manuscript.

Additional Information
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