Apontic regulates cell proliferation and development by activating the expression of *hedgehog* and *cyclin E*.

**Running title: Apt regulates hh and cyclin E**

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Summary statement

We identified a novel role for Apontic as an important common regulator of the transcription of *hedgehog* and *cyclin E*. Our study provides important insights into the mechanism of organ development.

ABSTRACT

Hedgehog (Hh) signaling pathway and Cyclin E are key players in cell proliferation and development. Hyperactivation of *hh* and *cyclin E* has been linked to several types of cancer. However, transcriptional regulation of *hh* and *cyclin E* are not well understood. Here we show that an evolutionarily conserved transcription factor Apontic (Apt) is an activator of *hh* and *cyclin E* in *Drosophila*. Apt directly promotes the expression of *hh* and *cyclin E* through its binding site in the promoter regions of *hh* and *cyclin E* during wing development. This Apt-dependent proper expression of *hh* and *cyclin E* is required for cell proliferation and development of the wing. Apt-mediated expression of *hh* and *cyclin E* can direct proliferation of Hh-expressing cells and simultaneous growth, patterning and differentiation of Hh-recipient cells. The discovery of the coordinated 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.
INTRODUCTION

Animal development requires the organ patterning and growth. How these two processes are coordinated remains unclear. The *Drosophila* wing is an excellent model to study the regulation of gene expression during the organ patterning and cell growth. The wing disc is a sac-like structure composed of disc proper (DP) cells and peripodial epithelium (PE). During larval development, wing disc DP and PE cells proliferate extensively and are patterned, finally give rise to the adult wing (Milner et al., 1984). The Hh and Cyclin E can contribute to patterning and growth of the wing disc during development (Neufeld et al., 1998; Tabata and Kornberg, 1994).

Hh pathway is one of the major signaling pathways that control animal development from *Drosophila* to humans, which has been implicated in stem cell maintenance, cell migration, axon guidance and tissue regeneration (Beachy et al., 2004; Charron et al., 2003; Clement et al., 2007; Hochman et al., 2006). In the *Drosophila* wing disc, Hh expresses in posterior (P) compartment cells and spreads into the anterior compartment where it activates target genes such as *engrailed* (*en*), *patched* (*ptc*), *collier* (*col*), *decapentaplegic* (*dpp*) and *iroquois* (*iro*) (Matusek et al., 2014; Nahmad and Stathopoulos, 2009; Tabata and Kornberg, 1994) to control wing patterning. Moreover, Hh is required for transient fusion between the PE and the DP sides during regeneration of wing discs (McClure and Schubiger, 2005). Therefore, the expression of *hh* is vital in the wing disc. In the anterior (A) compartment cells, the truncated transcription repressor Ci$^{\text{R}}$ inhibits the transcription of *hh*. However, the underlying mechanism by which the posterior cells activate *hh* transcription is still to be determined.

Cyclin E belongs to the cyclin family, which is required for cell division (Knoblich et al., 1994). Dysregulation of *cyclin E* correlates with various tumors, including breast cancer and lung cancer (Donnellan and Chetty, 1999; Keyomarsi et al., 1994; Moroy and Geisen, 2004). Besides, deregulated Cyclin E activity causes cell lineage-specific abnormalities, such as impaired maturation due to unregulated cell proliferation (Minella et al., 2008). In *Drosophila*, Cyclin E is essential for G1-to-S phase transition in the posterior cells of eye disc (Richardson et al., 1995). It has been reported that *cyclin E* is a potential target gene of Hh signaling in *Drosophila*. Hh pathway activates *cyclin E* transcription through its unique transcription...
factor Ci in the posterior cells of eye disc (Duman-Scheel et al., 2002). It is known that Hh pathway is turned on exclusively in the A cells near A/P boundary (Strigini and Cohen, 1997; Tabata and Kornberg, 1994). However, cyclin E expresses throughout the wing disc (Neufeld et al., 1998). 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 wing disc and the relationship between Cyclin E and Hh pathway.

Apontic (Apt) has been identified as a transcription factor involved in development of tracheae, head, heart and nervous system (Eulenberg and Schuh, 1997; Gellon et al., 1997; Liu et al., 2003; Su et al., 1999). Apt can suppress metastasis (Woodhouse et al., 2003) and is required in the nervous system for normal sensitivity to ethanol sedation (McClure and Heberlein, 2013). Moreover, Apt participates in JAK/STAT signaling pathway to limit border cells migration (Starz-Gaiano et al., 2009; Starz-Gaiano et al., 2008; Yoon et al., 2011). However, the role of Apt in wing development is unknown.

In this study, we found that both loss of and overexpression of apt resulted in defect wings. Further studies demonstrated that loss of apt attenuated the expression of hh and cyclin E, while apt overexpression upregulated hh and cyclin E. In addition, we found inherent Apt binding sites in the promoter region of hh and cyclin E. Mutating the sites inhibited the expression of hh and cyclin E. Collectively, Apt activates the expression of hh and cyclin E to allow proper wing development.

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 induced *apt* loss of function mutant clones in the wing disc using the *FLP/FRT* system (Theodosiou and Xu, 1998). The formation of these clones resulted in a small wing with a blistered phenotype (Fig. 1D) compared with the control wing (Fig. 1C). Furthermore, RNAi-mediated knockdown of *apt* in DP cells of the wing disc resulted in a small wing, and also reduced the width between vein 3 and vein 4 (Fig. 1E,F). Given that the space between vein 3 and vein 4 is a characteristic monitor of Hh pathway activity in adult wings, knockdown of *apt*-mediated narrowing the space indicates that Apt possibly regulates Hh pathway. RT-PCR analyses showed effective knockdown of *apt* mRNA level upon *apt*-RNAi (Fig. S1). To investigate the effect when *apt* is overexpressed, we employed *MS1096-Gal4* driver to express UAS-*apt* in both the PE and DP of the wing disc. Abnormal wings were induced by overexpression of *apt* (Fig. 1G). The wing was diminished and blistered, the pattern of veins was disrupted and extra abnormal bristles were induced in the wing margin. In addition, when UAS-*apt* was expressed by a stronger gal4 (*sd-Gal4*) in DP cells, both wings and halters were lost (Fig. 1H). Taken together, the loss-of-function and gain-of-function analyses indicate that Apt is indispensable for wing development.

**Apt regulates the expression of hh in the wing disc**

The observation that knockdown of *apt* narrowed the space between vein 3 and vein 4 implied that Apt might modulate Hh pathway in wings. As an important initiator of Hh pathway, *hh* gene expresses in the wing disc (Cho et al., 2000; Tabata and Kornberg, 1994). We first compared the expression of *apt* and *hh* in the wing disc, and found that Apt and *hh-lacZ* were co-expressed in PE cells (Fig. 2D-F) and posterior compartment cells of the DP (Fig. 2G-I) in the second instar larval disc. Furthermore, *apt* exhibited genetic interaction with *hh*. Transheterozygotes of two sets of *hh* alleles (*hh*bar3/*hh*2 and *hh*Mir/*hh*2) exhibited smaller wing with an extra crossvein (Fig. S2), demonstrating that it is a phenotype of *hh* mutant. While wings of animals heterozygous for *hh*2 or *apt*-null allele were normal, transheterozygotes of *apt*-null allele and *hh*2 showed the same wing phenotype (Fig. 2A-C). These results raised the possibility that Apt regulates transcription of *hh*. To test the possibility, we analyzed the expression of *hh* under loss-of-function and overexpression of Apt. The expression of *hh-lacZ*
and Hh was significantly reduced in the apt mutant clones in the PE (Fig. 2J-L; Fig. S3A-C) and the DP (Fig. 2M-O; Fig S3D-F). Moreover, the expression of hh was significantly reduced in the wing disc of larvae upon RNAi-knockdown of apt (Fig. S4). By contrast, overexpression of Apt increased the expression of hh-lacZ (Fig. 2P-R) and hh (Fig. S4, S5G-I). The expression of Hh also decreased in the apt mutant clones of the eye disc and the salivary gland (Fig. S5A-F). These results indicate 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 (Lee et al., 1992). We identified one potential Apt binding sequence (Liu et al., 2003) within the region (Fig. 3A). We next assessed the function of the Apt-binding site in hh using a CRISPR-Cas9 system (Kondo and Ueda, 2013). Since the designed gRNA contained the Apt-binding site, four Apt-binding site deletion mutants and two insertion mutants were generated (Fig. 3B-C; Fig. S6A). Homozygotes of these mutations showed reduced expression of hh mRNA and Hh protein (Fig. 3D-E; Fig. S6B) and exhibited the small wing and reduced vein 3-4 spacing phenotypes (Fig. 3F; Fig. S6C-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. 3G-I) as did transheterozygotes of apt-null allele and hh2. Taken together, these data suggest that Apt directly regulates the expression 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 disc (Liu et al., 2014). Therefore, we examined whether Apt regulates cyclin E also in the wing disc. To do this, we first performed a double-staining experiment. In the wild-type wing disc, Apt and Cyclin E were co-expressed (Fig. 4A-C). Furthermore, the expression of Cyclin E was significantly reduced in the apt mutant clones (Fig. 4D-F; Fig. S7A). The expression of cyclin E mRNA was also reduced upon RNAi-knockdown of apt in the wing disc (Fig. S7B). By
contrast, the expression of Cyclin E and its mRNA was increased by overexpression of Apt in
the wing disc using MS1096-Gal4 and UAS-apt (Fig. 4G-I; Fig. S7B). These results indicate
that Apt activates the expression of cyclin E in the wing disc.

We then asked whether the regulation of cyclin E by Apt is mediated through Hh. To test
this idea, we compared the expression of hh and cyclin E upon overexpression of Apt in the
wing disc. Both hh and Cyclin E were induced by overexpression of Apt (Fig. 2Q,4H). However, their expression patterns were different. Cyclin E was induced in all region of the
wing disc, whereas the expression of hh was restricted in the posterior compartment.
Moreover, the expression of Cyclin E was not changed by RNAi–knockdown of hh using
MS1096-Gal4 and UAS-hhRNAi (Fig. 4J) and in an hh gain of function mutant hhMrt that
exhibits ectopic expression of hh in the anterior compartment (Tabata and Kornberg, 1994)
(Fig. 4K). These data suggest that the activation of cyclin E by Apt is independent of Hh in
the wing disc (Fig. 4L).

**Apt directly controls cyclin E in the wing disc**
Since Apt directly activates the expression of cyclin E in the eye disc (Liu et al., 2014), 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 (Liu et al., 2014)
carries the endogenous promoter and the cyclin E regulatory element containing a wild-type
Apt-binding site (cycEPlacZ) (Fig. 5A) or a mutated site (cycEMPacZ) (Fig. 5E). Although
cycEPlacZ with the wild type binding site recapitulated the cyclin E expression in the wing
disc (Fig. 5B-D), base substitutions in the Apt-binding site in cycEMPacZ abolished the lacZ
expression (Fig. 5F-H). These results indicate that Apt directly activates cyclin E through its
binding site in the regulatory region of cyclin E.

**Apt controls cell proliferation by inducing hh and cyclin E**
Because both Hh and Cyclin E are involved in cell proliferation (Jiang and Hui, 2008;
Knoblich et al., 1994), defects in apt would affect the cell number in the wing disc. As
expected, we observed significant decrease in the cell number in an apt mutant clone using
DAPI staining (Fig. 6B). Moreover, phalloidin labeling revealed disruption of the linear
arrangement of cells in the clone (Fig. 6C). When Apt was overexpressed in the wing disc, the
number of DAPI-stained cells was not significantly changed from that in the control disc
(compare Fig. 6F with 6D). However, the linear arrangement of cells was disrupted (compare
Fig. 6G with 6E). Since Hh and Cyclin E are required for the regulation of apoptosis
(Guerrero and Ruiz i Altaba, 2003; Hwang and Clurman, 2005; Ruiz i Altaba, 1999), 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 from apt mutant clones and wild type, few apoptotic cells were observed (Fig.
6H-J). However, in the wing disc from an Apt-overexpressed larva, the number of apoptotic
cells was significantly increased (Fig. 6K). This presumably explains why wing size was
reduced upon overexpression of Apt (Fig. 1G).

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 CycE2/+ flies showed normal wings, three percent of hhbar3/hhbar3 and
eighteen percent of CycE2+; hhbar3/hhbar3 flies showed the smaller and blistered phenotypes
(Fig. S8A-C). We also observed genetic interaction between hh and cyclin E in the extra
crossvein phenotype. While CycEJP/+ and hh2/+ flies showed normal wings, fifty-four percent
of CycEJP/+; hh2/+ flies exhibited wings with the extra crossvein (Fig. S8D-F). Collectively,
these data suggest that Apt controls wing development by inducing appropriate amounts of
Hh and Cyclin E.

DISCUSSION

Here, we revealed that the transcription factor Apt regulates Drosophila wing development, at
least in part, through directly activating the expression of hh and cyclin E to control wing
patterning and growth. Both loss-of-function and gain-of function assays clearly demonstrated
that Apt is vital for wing development. Further studies showed that knockdown of apt
attenuated, while overexpression of apt activated the expression of hh and cyclin E.

In the wing disc, Hh exclusively expresses in the P compartment. After many modifications,
the mature Hh ligands are secreted from the P compartment and reach ~12cell rows near A/P
boundary of the A compartment (Basler and Struhl, 1994; Tabata et al., 1992; Tabata and
Ci expresses solely in the A compartment (Slusarski et al., 1995). Without
the Hh, full-length Ci is ubiquitinated by SCF$^{\text{Slimb}}$ to partial degradation, culminating in
formation a truncated transcriptional repressor termed Ci$^{R}$. Ci$^{R}$ enters into the nucleus to
repress the expression of $hh$ in the A compartment (Aza-Blanc et al., 1997; Jiang and Struhl,
1998; Smelkinson and Kalderon, 2006). In this study, we found the ubiquitous expression of
Apt in the wing disc (Fig. 1A,B). However, the expression of $hh$ is restricted in the P
compartment of DP cells. Overexpression of apt in the wing disc with the MS1096-GAL4
driver emerges the ectopic expression of $hh$ in the A compartment, suggesting that Apt is
sufficient to turn on $hh$ expression. We speculate that during the normal development progress,
Apt might cooperates with others factors (such as Ci$^{R}$) to restrict the expression region of $hh$.
It is interesting to investigate the relationship between Ci$^{R}$ and Apt.

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) (Lee et al., 1992) is too large to make a reporter construct
for conventional P-element mediated transgenesis. Therefore, we employed the CRISPR-Cas9
system (Kondo and Ueda, 2013) 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 (Fig. S9). Furthermore, transheterozygotes between $hh^{2}$ and $hh^{\text{aptDB1}}$ exhibited the
$hh$ mutant phenotype, smaller wing with extra crossvein. Taken together, these data strongly
suggest that the observed phenotypes are not due to off-target effect.

Although our data strongly support that Apt is a transcription factor of $hh$, mutating the Apt
binding site on $hh$ promoter does not induce severe phenotypes. Beside Apt, other factors
might also regulate $hh$ transcription. Therefore, both knockdown and overexpression of apt
only moderately affect the expression of $hh$. Hh, an important morphogen, plays multifaceted
roles in segmentation and wing patterning. Previous findings paid more attention on the
protein modification of Hh. The mechanism underlying hh transcription is not clear. Here our
studies unveil that Apt acts as a transcription factor of hh.

While Hh has been implicated in induction of Cyclin E through Ci (Duman-Scheel et al.,
2002), subsequent researches have shown that Cyclin E accumulates in the Mad\textsuperscript{1-2}Su(H)ci
mutant cells (Firth and Baker, 2005). So whether Hh activates cyclin E is controversial. In this
study we showed that RNAi-mediated knockdown of hh or ectopic expression of hh in the
anterior compartment did not change the expression of cyclin E. Taken together, these
observations argue against the notion that hh regulates cyclin E in the wing disc.

Hyperactivation of Hh pathway has been complicated in many tumors (Clement et al., 2007;
Jiang and Hui, 2008). It will be fruitful to investigate whether Apt is upregulated in
Hh-related tumors. The previous work indicates that Apt involves in tumorigenesis
(Woodhouse et al., 2003). It is also interesting to explore whether Apt regulates tumorigenesis
through activating Hh signaling. Our finding that Apt regulates wing development through
activating hh raises a possibility that Apt acts as a potential clinical target for Hh-related
tumors.

MATERIALS AND METHODS

Drosophila Strains

Strains used were as follows. apt\textsuperscript{P14} (Eulenberg and Schuh, 1997), apt\textsuperscript{P2} (Liu et al., 2003),
cycE\textsuperscript{PlacZ} and cycEM\textsuperscript{PlacZ} (Liu et al., 2014), UAS-apt (gift of D. Montell), UAS-GFP (gift
of Y. Hiromi). hh\textsuperscript{Mtr} was obtained from Drosophila Genetic Resource Center. hh\textsuperscript{2}, hh\textsuperscript{Mtr}, hh\textsuperscript{bar3},
CycE\textsuperscript{2}, CycE\textsuperscript{op}, hh-LacZ, MS1096-GAL4, sd-GAL4, dpp-Gal4, ptc-GAL4 and UAS-hhRNAi
were obtained from Bloomington Drosophila Stock Center. UAS-aptRNAi was obtained from
Tsinghua Fly Center. y\textsuperscript{2}cho\textsuperscript{v1}; attP40[nos-Cas9/CyO, y\textsuperscript{1} v\textsuperscript{1} P{nos-phiC3\int.NLS}X; attP40,
y\textsuperscript{2} cho\textsuperscript{v1}, y\textsuperscript{2} cho\textsuperscript{v1}/Y{hs-ld}; Sp/CyO, y\textsuperscript{2} cho\textsuperscript{v1}; PrDr/TM6C were obtained from NIG-Fly.

Clonal analysis

Homozygous apt loss-of-function clones were generated by hs-FLP/FRT recombination
(Theodosiou and Xu, 1998). FRT42D and apt\textsuperscript{P14}/CyO were recombined to generate FRT42D,
apt\textsuperscript{P14}. Six pairs of FRT42D, apt\textsuperscript{P14} cross to Gla/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, aptP14/CyO were performed at 25°C. Heat shocks were performed 32-56 hours after egg-laying for 1.5 hours at 37.5°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 described (Kondo and Ueda, 2013). 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 (Table S1).

**RT-qPCR analysis**

Wing discs were dissected from 40 third instar larvae. Total RNA was prepared from the dissected tissues using an RNAprep Pure Tissue kit TIANGEN #DP431). cDNAs were synthesized using a Prime Script™1st strand cDNA synthesis kit (TaKaRa #6210A). qPCR was conducted with Bio-Rad CFX96 real-time system using a SuperReal PreMix 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 were designed to amplify the hh region (Table S1).

**Antibodies and Immunohistochemistry**

Staining of larval tissues was performed as described previously (Liu et al., 2014). Larvae were dissected in PBS, fixed in 25 mM PIPES-KOH (pH 7.0), 0.5 mM EDTA, 0.25 mM MgSO4 and 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) (Liu et al., 2014), rabbit anti-Hh (1:800, gift of T. Tabata), rabbit anti-GFP (1:200, Molecular Probes), mouse anti-GFP (1:400, Molecular Probes), rabbit anti-β-galactosidase (1:2000, Cappel), rabbit Caspase3 (1:50, Cell Signaling Technology), mouse anti-β-galactosidase (1:500, Sigma), FITC-conjugated phalloidin (1:200, Sigma), mouse anti-Ubx (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 (Rudrapatna et al., 2013).

Microscopy and Image Treatment

Images were acquired in Leica TCS SP5 confocal microscope and Olympus cellSens, treated with Adobe Photoshop CS6 image programs. Wing size and space between vein 3 and vein 4 or that between vein 1 and vein 2 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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Competing interests
The authors declare no competing financial interests.

**Author Contributions**

X.F.W., S.H., and Q.X.L. designed research, X.F.W., Q.C., and C.L.F. performed experiments and X.F.W., Z.Z.Z., S.H., and Q.X.L. analyzed data and wrote the manuscript.

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**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/suppl

**References**


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expressed specifically in posterior compartment cells and is a target of engrailed regulation. *Genes Dev* 6, 2635-2645.


**Figure legends**

**Fig. 1.** Apt is expressed in the wing disc and required for wing development.

(A) A single optical section of the PE of the wing disc from a second instar larva and the co-localization of Apt and Ubx (PE marker). (B) A single optical section of the DP of the wing disc from a second instar larva. (C) The adult wing of *yw*. (D) The adult wing of *aptpΔ* clones. The arrow indicates the blistered wing. (E) The adult wings of *sd-GAL4; UAS-GFP* and *sd-GAL4; UAS-aptRNAi*. (F) Quantification of the intervein region between L3 and L4 relative to that between L1 and L2 (*sd>GFP* value was set as 100%) of *sd-GAL4; UAS-GFP* and *sd-GAL4; UAS-aptRNAi* by ImageJ. Error bars, SEM. Student’s t tests, ***p < 0.001. (G) The adult wing of *MS1096-GAL4; UAS-apt*. (H) The adult wing of *sd-GAL4; UAS-apt*. Scale bars, 200 um.

**Fig. 2.** Apt regulates the expression of *hh*.

(A-C) Genetic interaction between *apt* and *hh*. Adult wings of *aptpΔ/+; +/-* (A) and +/-; *hh2/+* (B) show normal pattern. (C) Forty percent of *aptpΔ/+; hh2/+* wings exhibited abnormal morphologies in anterior crossvein (ACV). An arrowhead indicates the extra ACV. Total...
The expression of Apt (D) and hh-lacZ (E) in PE cells. (G-I) The expression of Apt (G) and hh-lacZ (H) in DP cells. (J-L) The decreased expression of hh-lacZ (K) in the apt<sup>PΔ4</sup> clones of the PE (J). Clones are marked by white-dotted lines. (M-O) The decreased expression of hh-lacZ (N) in the apt<sup>PΔ4</sup> clones of the DP (M). Clones are marked by white-dotted lines. (P-R) Overexpressed Apt (P) increased the expression of hh (Q).

**Fig. 3.** 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. (B) Sequences of a wild-type allele and a heterozygous mutant of hh<sup>hapdB1</sup>. 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 protoscaler-adjacent motifs (PAM) indicated in green. Deleted nucleotides in hh<sup>hapdB1</sup> are shown as dashes. The deletion size is shown next to the sequence. (D) RT-qPCR analyses of hh mRNA in the wing disc of third instar larvae from yw or hh<sup>hapdB1</sup>. Error bars, SEM from three independent experiments. Student’s t tests, *p < 0.05. (E) The wing disc of third instar larvae from yw or hh<sup>hapdB1</sup> was stained with an anti-Hh antibody. The expression levels of Hh were determined by mean fluorescence. Error bars, SEM. ***p < 0.001. (F-I) Deletion of the Apt-binding site in the hh promoter affects wing development. The wing size and the intervein region between L3 and L4 relative to that between L1 and L2 (control value was set as 100%) were decreased in hh<sup>hapdB1</sup>. Error bars, SEM. Student’s t tests, ***p < 0.001. hh<sup>+/+</sup> (G) or hh<sup>hapdB1</sup>+/+ (H) adult wing shows a normal phenotype. All adult wings of hh<sup>hapdB1</sup>/hh<sup>2</sup> transheterozygotes exhibited abnormal morphologies in ACV (I). An arrowhead indicates the extra ACV. Total numbers of analyzed wings were G, 157; H, 132; I, 74. Scale bars, 200 um.
Fig. 4. Apt controls the expression of cyclin E.

(A-C) The expression of Apt (A) and Cyclin E (B) in the PE of control wing disc. (D-F) Decreased Cyclin E (E) expression in the apt mutant clones (D). (G-I) Overexpressed Apt with MS1096-GAL4 (G) increased the expression of Cyclin E (H). (J, K) The expression of Cyclin E in the wing disc from the hhRNAi knockdown (J) and hh gain of function mutant animals (K). (L) Regulation of hh and cyclin E by Apt in the wing disc. Wing discs from third instar larvae of wild-type (A-C), hs-FLP; FRT42D, Ubi-GFP/FRT42D, aptΔ (D-F), MS1096-GAL4; UAS-apt (G-I), MS1096-GALA; UAS-hhRNAi (J) and hhMrt (K) animals were stained with the anti-Apt antibody (A, G), the anti-GFP antibody (D), the anti-Cyclin E antibody (B, E, H, J and K). (C, F, I) Merged images of A and B, D and E, G and H, respectively.

Fig. 5. Apt directly regulates the expression of cyclin E through its binding site in the cyclin E promoter region.

(A) Schematic illustration of the lacZ reporter construct driven by the cyclin E promoter carrying the wild type Apt binding site. (B-D) The reporter cycEPlacZ (C) was coexpressed with the endogenous Apt (B) in the wing disc. (E) Schematic illustration of the lacZ reporter construct driven by the cyclin E promoter carrying the mutant Apt binding site. (F-H) The expression of reporter cycEMPlacZ (G). Wing discs of third instar larvae were stained with anti-Apt antibody (B and F) and anti–β-galactosidase antibody (C and G). (D and H) Merged images of B and C and of F and G, respectively.

Fig. 6. Apt is required for production of proper cell number and arrangement of wing discs.

(A-C) Cell number (B, marked by DAPI) and array of cells (C, marked with phalloidin) were affected in an apt mutant clone (A, lack of the GFP signals and marked with a broken line). (D, E) Cell number (D) and array (E) from a wild-type wing disc were visualized with DAPI (D) and phalloidin (E). (F, G) Overexpression of Apt in the wing pouch resulted in slight decrease in the cell number (F) and irregular arrangement of cells (G). (H-J) Apoptosis was barely detectable in the apt mutant clones (H, I) and wild-type wing disc (J). (K)
Overexpressed Apt in the wing disc increased the number of apoptotic cells. The wing discs of hs-FLP; FRT42D, Ubi-GFP/FRT42D, apt$^{P14}$ (A-C, H, I), wild-type (D, E and J) and MS1096-GAL4; UAS-apt (F, G and K) animals were stained with the anti-GFP antibody (A and H), DAPI (B, D and F), phalloidin (C, E and G) and Caspase-3 antibody (H-K).
Figure 1
Figure 2
Figure 4
Figure 6