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The Kto-Skd Complex Can Regulate *ptc* Expression by Interacting with *Cubitus interruptus* (Ci) in the Hedgehog Signaling Pathway*

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Background: Kto-Skd plays important roles in development. But the mechanism for how this complex regulates Hedgehog pathway is unknown.
Results: Kto-Skd down-regulates *ptc* expression.
Conclusion: Kto-Skd complex can regulate *ptc* expression by interacting with Ci.
Significance: The finding that the Kto-Skd complex affects Hh pathway provides novel mechanistic insights into the regulation of A/P boundary formation in *Drosophila* wing discs.

The hedgehog (Hh) signaling pathway plays a very important role in metazoan development by controlling pattern formation. *Drosophila* imaginal discs are subdivided into anterior and posterior compartments that derive from adjacent cell populations. The anterior/posterior (A/P) boundaries, which are critical to maintaining the position of organizers, are established by a complex mechanism involving Hh signaling. Here, we uncover the regulation of *ptc* in the Hh signaling pathway by two subunits of mediator complex, Kto and Skd, which can also regulate boundary location. Collectively, we provide further evidence that Kto-Skd affects the A/P-axial development of the whole wing disc. Kto can interact with *Cubitus interruptus* (Ci), bind to the Ci-binding region on *ptc* promoter, which are both regulated by Hh signals to down-regulate *ptc* expression.

The secreted proteins of the Hedgehog (Hh)[3] family play an important role in pattern formation in both vertebrates and invertebrates (1). In *Drosophila melanogaster*, the wing imaginal disc is subdivided into A and P compartments (2), and Hh protein is synthesized and secreted by P compartment cells (3–5). Secreted Hh proteins diffuse into the A compartment to form a local concentration gradient that governs a wide variety of processes during embryonic development and adult tissue homeostasis through activation of specific target gene transcription (3, 6–9).

Activation of target gene transcription by Hh signal involves a signal transduction cascade. Specifically, secreted Hh protein can bind to the twelve-pass transmembrane receptor Patched (Ptc), thereby relieving Ptc-mediated inhibition of Smoothened (Smo), a seven-pass transmembrane protein (4, 10–12). Activated Smo in turn promotes accumulation and activation of the full-length transcription factor *Cubitus interruptus* (Ci) in the cytoplasm through a complex series of events including suppression of Ci repressor processing and post-translational modifications (9, 13, 14). Ultimately, the full-length Ci activator translocates into the nucleus and stimulates expressions of Hh target genes that function further in cell fate specification.

In anterior cells distant from the A/P boundary of the wing disc where Hh is low or absent, Ci produces a truncated form, Ci^R, which silences Hh target gene transcription, such as *decapentaplegic* (*dpp*) (15, 16). By contrast, in anterior cells at the A/P boundary where Hh is present in high concentrations, full-length Ci accumulates and stimulates gene transcription such as *patch* (*ptc*) and *engrailed (en)*. In this manner, a morphogenetic gradient of Hh controls the pattern of expressed genes that function in turn to affect the cell fate of different compartments.

A shift of the balance between repressor and activator forms of Ci is necessary and sufficient to define cell sorting behavior in the A compartment. Moreover, En, in the absence of Ci, is sufficient to specify P compartment sorting. The opposing transcriptional activities of Ci and En control cell segregation at A/P boundary by regulating a single cell adhesion molecule (17). In our studies, we confirmed a gene, named *kohtalo* (*kto*), can distort the normal A/P boundary in *Drosophila* wing discs and down-regulate *ptc* expression.

Kto is a component of Mediator, a super-molecular complex consisting of about 25 evolutionarily conserved subunits. Mediator regulates activity of the general RNA polymerase (Pol) II transcriptional machinery by transmitting information from

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[3] The abbreviations used are: Hh, Hedgehog; Kto, Kohtalo; Skd, Skuld; *ptc, patched*; Ci, *Cubitus interruptus*; A/P, anterior/posterior; Smo, Smoothened; *dpp, decapentaplegic; en, engrailed;* Pol, polymerase; Cdk8, cyclin-dependent kinase 8; CycC, Cdk8's partner C-type cyclin; CtBP, C-terminal-binding protein; Ato, Atonal; TSS, transcriptional start site; *AG4, act5c>CD2>Gal4; ap, apterous;* Co-IP, co-immunoprecipitation; ChIP, chromatin immunoprecipitation.

transcription factors bound to upstream promoter and enhancer elements to the general transcription initiation factors bound to the core promoter (18–22).

Mediator was originally described in yeast and has now been isolated from mammals and *Drosophila* (23, 24). The whole Mediator complex is composed of three core modules (25). The head and middle modules of the Mediator core complex bind to Pol II and general transcription factors, while the tail module consists largely of adaptor subunits that bind to sequence-specific transcription factors (26–28). Besides core modules, Kto (also known as Med12), Skuld (Skd, also known as Med13), cyclin-dependent kinase 8 (Cdk8), and the Cdk8 partner C-type cyclin (CycC) constitute the separable regulatory module of Mediator complex (29). Previous studies have implicated a functional role of the regulatory module mainly in transcriptional repression (21, 30), although some genes are up-regulated by this module (31–33).

Studies of Med12 and Med13 in vertebrate model organisms revealed their important functions in the development of neural crest, nervous system, cartilage, kidney, and endodermal organs (22, 34). In *Drosophila*, Kto interacts with numerous transcription factors such as Pygopus, which promotes Wnt target gene transcription by recruiting the mediator complex (35). Kto together with Skd also helps to regulate some Notch target genes by interacting with CtBP, Hairless, and another unknown cofactor (28). They are also essential for the function of transcription factor Atonal (Ato) in spatial patterning of proneural clusters in the morphogenetic furrow (36). Eye disc cell mutant of either *kto* or *skd* fail to differentiate (37), and in the wing disc, clones with loss of *kto* or *skd* results in migration of anterior cells into the posterior compartment at the A/P boundary (24, 38).

In our study, we further detected the novel role of Kto and Skd in the regulation of A/P boundary formation in *Drosophila* wing discs. At the same time, our studies demonstrated that the regulatory module subunits Kto and Skd act together to down-regulate Hh signaling pathway indicated by lower *ptc* transcription activity. Specifically, we provided evidences that Kto and the key transcription factor of *ptc*, Ci, can interact with each other physically, and this interaction is regulated by Hh signals. Also, there is a great enrichment of Kto to the Ci binding region of *ptc* promoter (600~800 bp ahead of the transcriptional start site (TSS)) (39, 40) in the presence of Hh. Thus, our data support the conclusion that Kto together with Skd down-regulates *ptc* expression by interacting with Ci in the Hh signaling pathway.

EXPERIMENTAL PROCEDURES

Constructs—All the constructs described in our study were made from *Drosophila* genes and generated using the pUAST vector. Plasmids of *pUAST-kto* and *pUAST-skd* are kind gifts from Dr. Jessica E. Treisman (24). The constructs *pUAST-Myc-kto*, *pUAST-Myc-skd*, *pUAST-HA-kto*, and the fragments of *kto* were generated by subcloning each full-length coding region or fragments into the vectors. The generation of wild-type Myc-tagged Smo, SmoSA, and Smo*SD* were described previously (41).

Drosophila Mutants and Transgenes—*Drosophila* strains used in this study were maintained under standard conditions. The yw strain was used as host for all the P-element-mediated transformations. *act5c>CD2>Gal4 (AG4), apterous (ap)-Gal4, ptc-Gal4, ptc-lacZ, UAS-GFP* have been described (Flybase) (41–43). *kto* RNAi (NIG, #8491R-1), *skd* RNAi (NIG, #9936R-3), *Cdk8* RNAi (NIG, #10572R-1), and *CycC* RNAi (VDRC, #V27937) were obtained from NIG or VDRC.

Cell Culture, Transfection, Immunoprecipitation, and Western Blot Analysis—S2 cells were cultured in the Schneider's *Drosophila* Medium (Invitrogen) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfection was carried out using the Calcium Phosphate Transfection Kit (Specialty Media) according to the manufacturer's instructions. An *ub-Gal4* plasmid was co-transfected with *pUAST* expression vectors for all the transfection experiments. 5 mg DNA for *ub-Gal4* and 5 mg of DNA for each *pUAST* expression vector were used in a typical transfection experiment for the 10-cm dish. Cells were harvested 48 h after transfection with indicated buffers for different assays. The Hh conditional medium was obtained from the Hh stable cell line of S2 cells after 24 h induced by 0.7 mM $CuSO_4$, and was added to cells at 50% medium for 24 h stimulation before cells were harvested. For regular immunoprecipitation, cells were lysed in Nonidet P-40 buffer (50 mM Tris-Cl pH 8.0, 0.1 M NaCl, 10 mM sodium fluoride, 1 mM sodium vanadate, 1% Nonidet P-40, 10% glycerol, 1.5 mM EDTA, protease inhibitor mixture (Sigma)) for 30 min at 4 °C. After centrifugation, lysates were incubated with 2 μg indicated antibodies for 2 h at 4 °C. Samples were combined with 20 μl of protein A/G PLUS agarose (Santa Cruz Biotechnology) and incubated for 1 h on a rotator at 4 °C. Beads were washed three times with 1 ml of Nonidet P-40 buffer and then boiled in 30 μl of SDS loading buffer. For Western blot, samples were resolved by SDS-PAGE electrophoresis, transferred to PVDF membranes (Millipore), incubated with the primary antibody for 1 h and then the secondary antibody for 1 h, and visualized by chemiluminescent substrate (Thermo). Primary antibodies used in this study are mouse anti-Myc (Sigma), anti-FLAG (Sigma), anti-HA (Sigma), and rabbit anti-FLAG (Sigma).

For two-step co-immunoprecipitation (Co-IP), three dishes of S2 cells were transfected with mixture of the plasmids to express HA-Kto, FLAG-Ci, and Myc-Skd. For the control of the first immunoprecipitation, three dishes of S2 cells were transfected only with the plasmids of HA-Kto and Myc-Skd. 48 h after transfection, the cells were lysed with LSLD buffer (50 mM HEPES pH 7.4, 50 mM NaCl, 0.1% Tween 20, 20% glycerol) (44, 45), sonicated briefly, and centrifuged. The supernatant was then incubated with anti-FLAG M2-agarose (Sigma) (100 μl) for 2 h at 4 °C. The beads were washed with Lysis Buffer (50 mM HEPES pH 7.5, 0.2 mM EDTA, 10 μM NaF, 0.5% Nonidet P-40, and protease inhibitor mixture) (45) containing 150 mM NaCl three times, and the FLAG-linked protein complex was eluted with 300 μl of Lysis Buffer containing 250 mM NaCl and 3× FLAG peptide (300 μg/ml) for 2 h at 4 °C. The second immunoprecipitation was performed using 150 μl of eluated protein and 350 μl of Lysis Buffer containing 464 mM NaCl and 2 μg of mouse anti-Myc antibody or control IgG followed by addition of protein A/G PLUS agarose.

Immunostaining of Imaginal Discs—For immunostaining of wing imaginal discs, third-instar larvae were cut in half and fixed in freshly made 4% formaldehyde in PBS buffer at room temperature for 20 min, then rinsed with buffer PBT (PBS, 0.1% Triton X-100) and washed four times with buffer PBTA (PBS, 0.1% Triton X-100, 1% BSA). Larvae were incubated overnight with primary antibody diluted in PBTA at 4 °C, then washed with PBT and incubated with secondary antibody diluted in PBTA for 2 h at room temperature. After washing, wing imaginal discs were dissected and mounted in 40% glycerol. A Leica LAS SP5 confocal microscope was used to record immunostaining images. Primary antibodies used in this study are rat anti-Ci (2A1) (DSHB), rabbit anti-lacZ (MP Biomedicals), mouse anti-Ptc (DSHB).

RNAi, Real-time PCR, and Luciferase Reporter Assay—The double-strand RNA was synthesized using the *in vitro* Transcription T7 Kit from TakaRa. After cells were transfected for 24 h, the culture medium was changed to Serum Free Medium with 20–50 μg of dsRNA/10^6 cells for 1–6 h starvation. Then fresh medium with serum was added, and cells were cultured for 24–36 h. The primers used for the generation of DNA fragments of *kto* are designed according to a previous study (35) with minor modifications. The sequences are shown below: *Renilla*-T7-F: 5′-GATCACTAATACGACTCACTATAGGG-ATGACTTCGAAAGTTTATGATCCAG-3′, *Renilla*-T7-R: 5′-GATCACTAATACGACTCACTATAGGGTTATCTTG-ATGCTCATAGCTATAATG-3′; *kto*-T7-F: 5′-GATCACTA-ATACGACTCACTATAGGGAATGTGGACGACGATTTG-GTT-3′, *kto*-T7-R: 5′-GATCACTAATACGACTCACTATA-GGGCTCTTGCGAGTGAAGCGGCCGGTT-3′.

kto RNAi efficiency was tested by real-time PCR with *GAPDH* as an internal control. Primer sequences are as follows: *GAPDH*-realtime-F, 5′-TGCTGGAGCCGAGTATGTGG-3′, *GAPDH*-realtime-R, 5′-GCCGAGATGATGACCTTCTTGG-3′; *kto*-realtime-F, 5′-ATAAAGGATGAAATGAAGGCGG-3′, *kto*-realtime-R, 5′-CTGTTGCTGAGTATTGACCAC-3′.

The *ptc-luciferase* reporter was described previously (41). In all luciferase assays, 1×10^6 S2 cells were transfected with 300 ng of *ptc-luc* reporter, 6 ng of *Renilla*, 300 ng of *ub-Gal4*, 200 ng of *ci155*, 100 ng of *su(fu)*, and 300 ng of the indicated constructs in each well of a 24-well plate. The luciferase activity was tested after 48 h using the dual-reporter luciferase system on a 96 luminometer (Promega). The normalized data are expressed as mean of at least three replicates obtained from at least three independent experiments.

Chromatin Immunoprecipitation—S2 cells were used for ChIP assay. Crosslinking was performed in 1% formaldehyde and sonication was carried out at 675 W for 3 s and paused for 7 s up to 35 cycles to shear DNA to an average fragment size of 200–400 bp (46). Sonication buffer or FA lysis buffer was used for Myc or FLAG IP, respectively. After de-crosslinking and protein digestion, DNA was precipitated and real-time PCR was performed. The primers used for real-time PCR are: *ptc*-1-F: 5′-TGGCGGTTTCAGTTTAATGAAGG-3′, *ptc*-1-R: 5′-TCTATTGTTATTCGCATGCCGT-3′; *ptc*-2-F: 5′-CAACAAA-CCAGCAGCCAAGG-3′, *ptc*-2-R: 5′-AATATACCCAAATAG-CTCCGCCAC-3′; *ptc*-3-F: 5′-ACAATAGAAATTAGCGCTC-TCGTG-3′, *ptc*-3-R: 5′-GCATACGGACTTAATGCTTGGA-3′.

RESULTS

Loss of Function of Kto and Skd Causes a Shift of A/P Boundary to Posterior Compartment and Increases the Width of Ptc Domain—*kto* and *skd*, the *Drosophila* homologues of Med12 and Med13, respectively, play a very important role in development (24, 28, 35–37). Like their homologues in mammals, Kto and Skd form a complex and play a negative role in the regulation of some gene expression. Furthermore, Kto and Skd have indistinguishable loss-of-function phenotypes in wing morphogenesis, as well as identical effects on gene expression (24, 28, 35–37), suggesting that Kto and Skd function in a manner dependent on each other. To investigate whether Kto and Skd are also involved in the regulation of Hh signaling downstream gene expression, we overexpressed *kto* RNAi or *skd* RNAi with *apterous (ap)-Gal4-GFP-ptc-lacZ*, which can drive target gene overexpression in the dorsal region of *Drosophila* wing imaginal disc. Our results showed that knockdown of either *kto* or *skd* resulted in Ci expanding to P compartment compared with an internal control (Fig. 1, compare *A*′, *a* with *B*′, *b* and *C*′, *c*). Meanwhile, Ptc also expanded from the A/P boundary to P compartment with a much wider stripe and slightly up-regulated expression level (Fig. 1, compare *A*″, *a* with *B*″, *b* and *C*″, *c*). Consistent with these *in vivo* observations, further *ptc*-luciferase reporter assays also suggested that loss of *kto* up-regulated *ptc* expression level (Fig. 2*D*). Moreover, double knockdown of *kto* and *skd* could further increase the width of *ptc* expression domain, and enhance the expansion of Ci and Ptc to the P compartment (Fig. 1, *D-D*‴, *d*). On the other hand, we noticed that knockdown of *kto* or *skd*, or both, had no dramatic effect on the expression pattern of *ci* or *ptc* in the A compartment away from the A/P boundary.

Previous studies suggested that Cdk8 and CycC interact with each other and furthermore form a large complex with Med12 (Kto)/Med13 (Skd) for gene regulation during multiple cellular processes such as adaptation to environmental stresses including nutrient deprivation and heat shock (18, 29, 47). On the other hand, Med12 (Kto)/Med13 (Skd) sub-complex appears to have biological functions independent of the Cdk8-CycC sub-complex (48, 49). To investigate whether both Cdk8-CycC and Kto-Skd sub-complexes are involved in the phenotype of shifted A/P boundary and wider *ptc* expression stripe, we overexpressed *Cdk8* RNAi and *CycC* RNAi with *ap-Gal4-GFP-ptc-lacZ* (the RNAi efficiency has been detected and data not shown). Our results showed that knockdown of both *Cdk8* and *CycC* did not affect the *ci* or *ptc* expression pattern, and A/P boundary formation (Fig. 1, *E–F*‴, *e–f*).

Taken together, these results indicated that loss of *kto-skd* in *Drosophila* wing imaginal disc causes distorted A/P boundary; and that Kto-Skd regulates *ptc* expression pattern suggested by a wider Ptc stripe domain in a manner independent of Cdk8-CycC.

Kto-Skd Complex Can Down-regulate the Expression of ptc Mainly in High Levels of Hh—Since the formation of the A/P boundary is quite complicated, needing multiple signaling pathways and protein factors, we were more interested in whether *kto* and *skd* can directly regulate the expression of *ptc* in Hh signaling pathway. Hh signaling is important for meta-

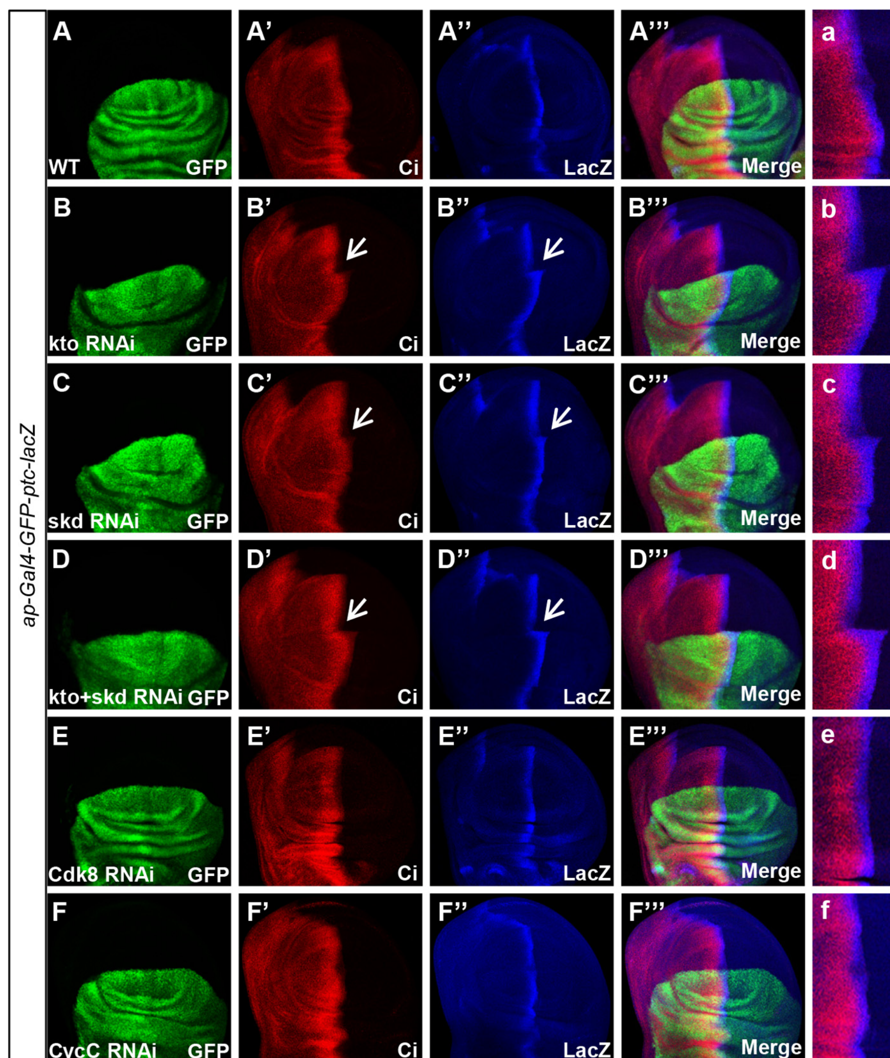

FIGURE 1. **Loss of function of both *kto* and *skd* enlarges the A compartment size and increases the width of the Ptc domain.** Wing imaginal discs expressing *UAS-GFP-ptc-lacZ* alone (*A–A‴*) or combined with *UAS-kto* RNAi (*B–B‴*), *UAS-skd* RNAi (*C–C‴*), both *UAS-kto* RNAi and *UAS-skd* RNAi (*D–D‴*), *UAS-Cdk8* RNAi (*E–E‴*), *UAS-CycC* RNAi (*F–F‴*) driven by the dorsal compartment-specific driver *ap-Gal4* were stained with anti-Ci or LacZ antibody to show Ci (*red*) and LacZ signal (*blue*). The GFP signal marks gene expression regions. *White arrows* mark the boundary of dorsal and ventral compartments of the wing imaginal disc. *a–d*, magnified images show the signals of both Ci and LacZ. *B–B‴*, *b*, knockdown of *kto* resulted in Ci and Ptc expanding to the P compartment and Ptc expression region at the A/P boundary becoming wider with a slight up-regulated expression level compared with the wild type control (*A–A‴*, *a*). *C–C‴*, *c*, knockdown of *skd* induced the similar phenotype with knockdown of *kto*. *D–D‴*, *d*, wing disc with both *kto* RNAi and *skd* RNAi showed more obvious phenotypes than with either of the two gene RNAi. *E–F‴* and *e–f*, wing imaginal discs with *Cdk8* or *CycC* RNAi showed close to the wild-type phenotype.

zoan development by forming long range signal to control cell fate in a manner dependent on the Hh protein concentration gradient (1, 9, 42, 50, 51). In *Drosophila* wing imaginal disc, Hh produced by P compartment cells acts as a local morphogen. Secreted Hh proteins diffuse into the neighboring A compartment cells and induce different gene expression. Low levels of Hh are sufficient to activate *dpp* expression, while higher levels of Hh are required to activate *ptc* expression, and peak levels of Hh are required to activate *en* expression. Since the effect of the Kto-Skd complex on Hh signaling we have detected was mainly observed in the dorsal region of the wing disc, we employed *AG4-Dicer2-GFP* driver to overexpress *kto* RNAi or *skd* RNAi in disc randomly, including both dorsal and ventral regions. Our result showed that loss of *kto* or *skd* clones arising in the A compartment adjacent to A/P boundary both in dorsal and ventral regions displayed an increased expression level of Ci and Ptc (Fig. 2, *A–C‴*, *a–c′*). Notably, Ci and Ptc were up-regulated only near the A/P boundary where there is a high level of Hh, but is not increased in the A compartment far from the boundary location. These phenotypes are consistent with the former results we have observed in the dorsal part of the wing disc and indicate that the regulation of *ptc* expression by Kto-Skd complex is mainly controlled by high Hh levels.

According to the results above, we proposed that *kto* and *skd* can regulate the Ptc pattern and subtly affect this gene expression level in the wing disc. To further investigate the effects of Kto and Skd in S2 cells, we employed the *ptc*-luciferase assay (Fig. 2*D*). Our results suggested that increased levels of Kto or Skd suppressed *ptc*-luciferase activity, especially in the presence of Hh. Consistently, knockdown of *kto* in S2 cells enhanced the activity of Hh signaling pathway as shown by the *ptc* reporter (Fig. 2*D*). Taken together, *kto* and *skd* can regulate *ptc* expression level mainly in the presence of high levels of Hh.

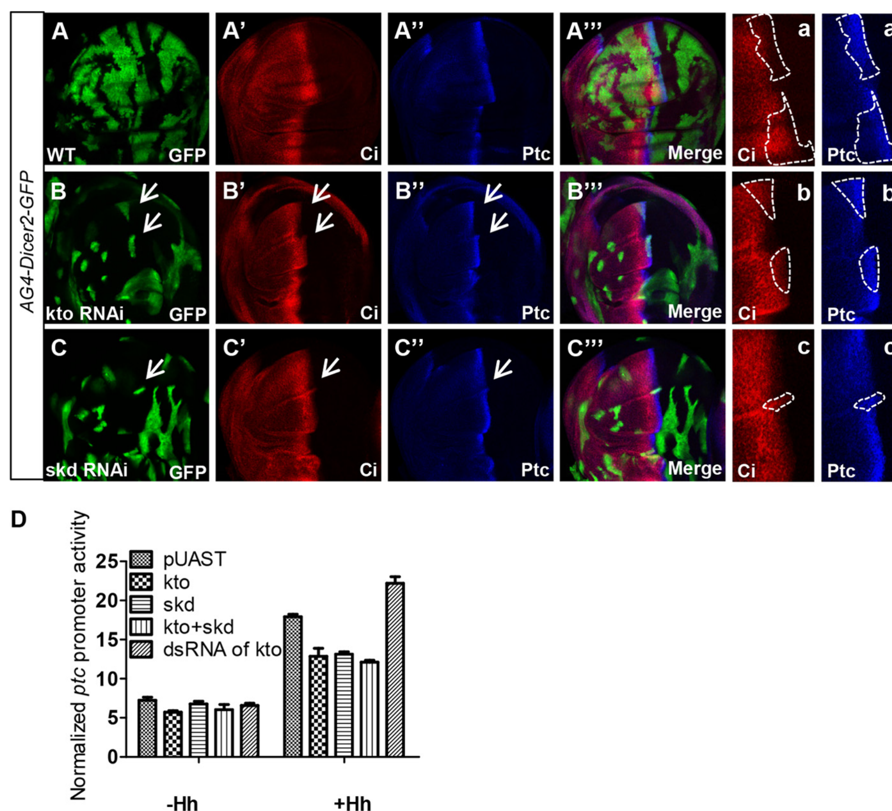

FIGURE 2. **Kto-Skd complex can down-regulate the expression of *ptc* mainly in high levels of Hh.** *A–C'''*, wing imaginal discs expressing UAS-GFP alone (*A–A'''*) or together with *UAS-kto* RNAi (*B–B'''*), *UAS-skd* RNAi (*C–C'''*) driven by *AG4-Dicer2* were stained with anti-Ci (*red*) and anti-Ptc antibody (*blue*). GFP signals (*green*) label the clones in which target genes were knocked down. *Arrows* indicate clones along the A/P boundary both dorsal and ventral in which Ci and Ptc are up-regulated. *a–c'*, magnified images show the protein levels of Ci and Ptc. *D*, S2 cells were transfected with plasmids of *UAS-kto, UAS-skd,* or *dsRNA of kto*, respectively, on the basis of *ptc*-luciferase assay system. Overexpression of Kto or Skd suppressed *ptc*-promoter activity, especially in the presence of Hh, and knockdown of *kto* in S2 cells up-regulated the activity of *ptc*-luciferase reporter.

Kto-Skd Interacts with Ci in a Hh Signal-dependent Manner—Previous studies have shown that *Drosophila* wing disc compartments are maintained by different adhesive properties of cells on opposite sides of the A/P compartment boundary in the wing disc, which are controlled by Ci in anterior cells close to the boundary and by En in posterior cells (24, 52). According to these studies and our results described above, we hypothesize that loss of *kto-skd* may affect the transcription activity of Ci, and then lead to the A/P boundary change and altered *ptc* expression activity. Previous research demonstrates that Gli3, the homologue of Ci in vertebrates, can bind to the Med12 subunit and intact Mediator complex both *in vitro* and *in vivo* (38). In this context, our observation that loss of *kto* and *skd* resulted in wider Ptc stripe and subtle up-regulation of *ptc* expression (Figs. 1 and 2) suggested that Kto and Skd may regulate Hh pathway downstream genes by interacting with Ci. To test this possibility, we investigated the interaction between Ci and Kto-Skd. We first co-expressed 3× FLAG-tagged Ci (FLAG-Ci) with Myc-tagged Kto (Myc-Kto) or Skd (Myc-Skd) in S2 cells, respectively, and treated with or without Hh. The Co-IP results suggested that Kto and Skd do interact with Ci; and that the interaction can be greatly enhanced by Hh treatment (Fig. 3, *A* and *B*). This result is consistent with our *in vitro* and *in vivo* findings that suppression of *ptc* expression by Kto and Skd is more obvious with Hh treatment in *ptc*-luciferase assay (Fig. 2*D*), and expression of *ci* and *ptc* can only be up-regulated near the A/P boundary, in which there is a high level Hh signal (Fig. 2, *A–C'''*, *a–c'*). To further confirm that the interaction between Ci and Kto-Skd is regulated by Hh activity, we employed Smo mutants. Smo C-tail phosphorylation and activation can be mimicked by SmoSD mutant, in which three PKA sites (Ser-667, Ser-687, and Ser-740) and adjacent CKI sites are mutated to aspartic acid. Another Smo mutant, SmoSA, which has three PKA sites mutated to alanine, was used to mimic a situation in which Smo C-tail fails to be phosphorylated and does not response to Hh signal (41–43). We then co-expressed Myc-Kto or Myc-Skd and FLAG-Ci with Myc-SmoWT, Myc-tagged SmoSA (Myc-SmoSA), or Myc-tagged SmoSD (Myc-SmoSD) in S2 cells. Our Co-IP results showed that Kto and Ci have the stronger interaction when Myc-SmoSD was co-expressed. In contrast, co-expressing Myc-Kto, FLAG-Ci, with Myc-SmoSA leads to a much weaker interaction between Kto and Ci; while Myc-SmoWT causes a medial level of interaction between the Kto and Ci protein (Fig. 3*C*). Similar results were observed for Myc-Skd and FLAG-Ci (Fig. 3*D*).

To further determine whether Kto-Skd and Ci function together in complex, we performed an additional two-step Co-IP, which showed that Kto-Skd indeed could form a bigger complex with Ci (Fig. 3*E*). Meanwhile, knockdown of *skd* decreased the interaction between Kto and Ci; knockdown of *kto* also impaired interactions between Skd and Ci (data not shown).

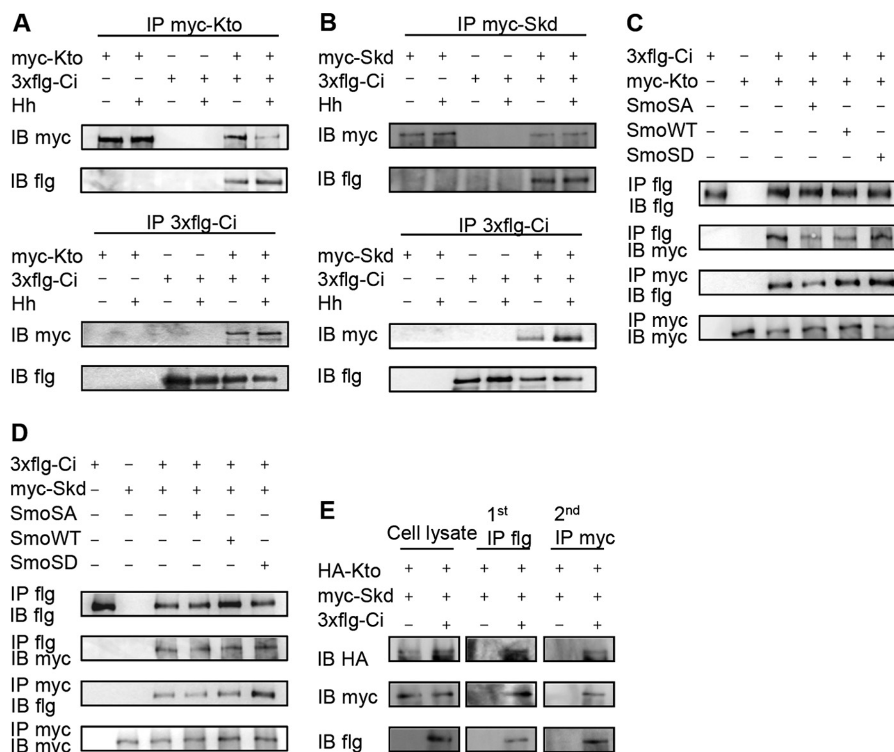

FIGURE 3. **Hh signal regulates the interaction between Ci and Kto or Skd.** *A–E*, S2 cells were transfected with the indicated constructs and analyzed by Co-IP and Western blot. *A* and *B*, Myc-Kto and Myc-Skd interact with 3×FLAG-Ci (*flg-Ci*), and the interaction may be greatly enhanced by the treatment of Hh. *C* and *D*, S2 cells were co-transfected with Smo mutants, SmoSA, SmoWT, or SmoSD, to mimic differential Hh pathway activity. SmoSD, which mimics high level activity of the Hh pathway, dramatically enhances the interaction between 3×FLAG-Ci and Myc-Kto (*C*) or Myc-Skd (*D*). In contrast, SmoSA and SmoWT, which mimic none or low levels of Hh, cannot promote interaction of the proteins. *E*, HA-Kto, Myc-Skd, and 3×FLAG-Ci were together overexpressed in S2 cells for two-step Co-IP. HA-Kto and Myc-Skd may be precipitated in the first IP of 3×FLAG-Ci. HA-Kto and 3×FLAG-Ci may be precipitated in the second IP of Myc-Skd. These suggested Kto, Skd, and Ci could form a large complex.

Taken together, these results suggested that Ci interacts with Kto-Skd complex in a way dependent on Hh signaling activity. Higher activity of Hh signaling could enhance this interaction.

Kto and Ci Interact with Each Other through Their N-terminal Parts—To map the specific region in Kto that interacts with Ci, we prepared several FLAG-tagged Kto fragments corresponding to amino acids 1–289, 290–1309, 1310–1868, 1869–2531, respectively (Fig. 4*A*). We then tried to precipitate Myc-Ci from extracts of S2 cells transfected by Kto fragments. Our result showed that the N-terminal half of Kto interacted with Ci, while the C-terminal half of Kto, including amino acids 1310–1868 and 1869–2531, failed to interact with Ci (Fig. 4*B*). Consistently, full-length of Ci could precipitate the Kto fragments corresponding to amino acids 1–289, 290–1309 (Fig. 4*B*). We then further dissected the large fragment of Kto (amino acids 290–1309) by generating two small fragments corresponding to amino acids 290–740 and 741–1309, respectively (Fig. 4*A*). Co-IP results showed that both of these two Kto fragments interacted with Ci (Fig. 4*C*). Together, these results indicated that the whole N-terminal-half of Kto is critical for interaction with Ci.

To identify the Kto-binding region in Ci, we screened a panel of Myc-tagged Ci truncation derivatives for their respective abilities to bind the biggest fragment of Kto (amino acids 290–1309). As shown in Fig. 4*D*, Ci76 (amino acids 1–700) strongly interacted with Kto, and so did the N-terminal (amino acids 1–440) and middle (amino acids 440–1160) fragments (Fig. 4*D*). In contrast, the C terminus of Myc-Ci (amino acids 1160–1397) failed to interact with Kto (Fig. 4*D*). Together, these results indicated that the N-terminal region of Ci is responsible for recruiting Kto.

Kto and Ci Can Be Recruited to the Same ptc Promoter Locus—Kto can down-regulate *ptc* expression indicated by *ptc*-luciferase assay in S2 cells (Fig. 2*D*). Besides, both of the two Kto fragments (amino acids 1–289 and 290–1309) that are able to interact with Ci can also dramatically suppress *ptc*-luciferase activity especially in the presence of Hh (Fig. 5*A*), which indicates that the two Kto fragments are suppressive domains.

Since Kto and its fragments can interact with Ci to down-regulate *ptc* expression, we supposed that Kto is likely recruited to the Ci binding sites around the *ptc* promoter locus. Ci binding sites on *ptc* promoter were mapped 600∼800 bp ahead of the transcriptional start site (TSS) (39, 40) (Fig. 5*B*). To test this possibility, we performed chromatin immunoprecipitation (ChIP) assays in S2 cells. Our ChIP assays revealed that in the presence of Hh, both Ci and Kto can be recruited to the *ptc* promoter locus especially the regions corresponding to primers of ptc-1 and ptc-2 (Fig. 5, *C* and *D*). These results support that Kto can be recruited to the Ci regulatory region of the *ptc* promoter locus in the presence of Hh signal.

DISCUSSION

In *Drosophila*, controlled by morphogens, different organs of adult are subdivided into precisely defined regions, including

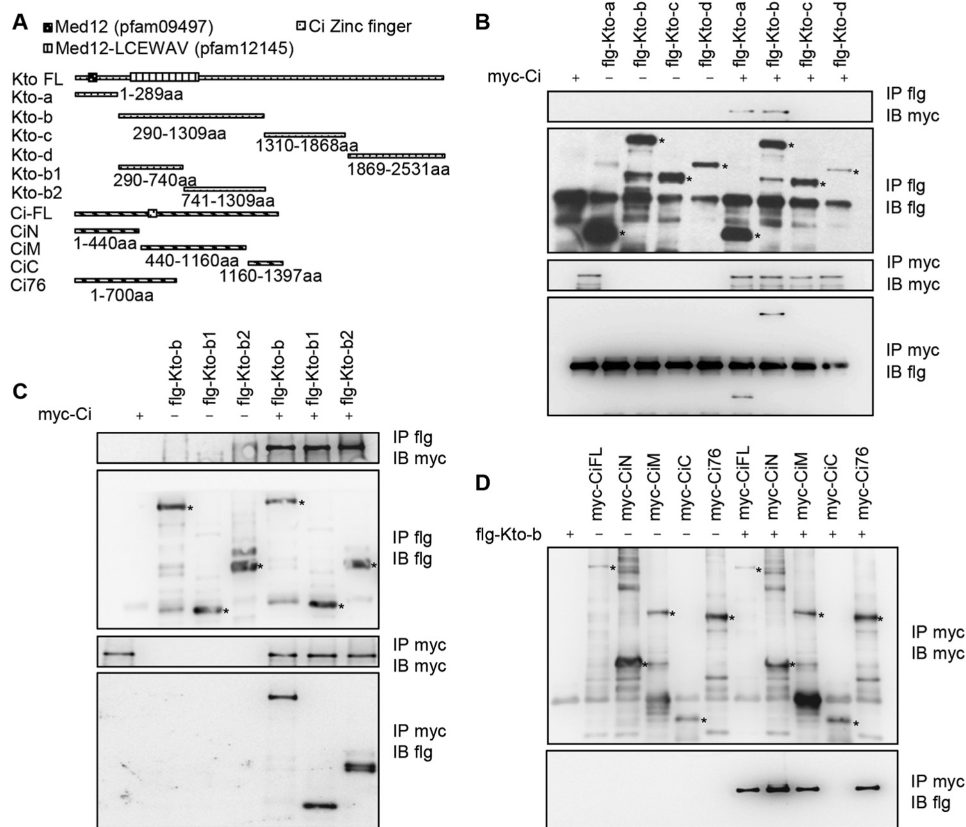

FIGURE 4. **Kto and Ci interact with each other through their N-terminal parts.** *A*, schematic drawings of Kto and Ci and their fragments. *B–D*, S2 cells were transfected with combinations of DNA constructs as indicated. After 48 h transfection, lysates from transfected S2 cells were immunoprecipitated with anti-FLAG (*flg*) or anti-Myc agarose beads. *Stars* indicate the target proteins. *B* and *C*, N-terminal part of Kto interacted with Ci. *D*, Kto fragment (amino acids 290–1309) could bind to most parts of Ci.

the A and P compartments (17, 53). Numerous genes are involved in morphogenesis, in which Hh signaling plays a critical role. In wing disc, posterior cells secret Hh to induce a stripe of neighboring anterior cells across the compartment boundary to secret Dpp, which can exert a long-range organizing influence on surrounding wing tissue (53). Genes encoding the Mediator components Med12 and Med13, known as Kto and Skd in *Drosophila*, also play essential roles in cell arrangements and morphogenesis. In this work, we showed that knockdown of both *kto* and *skd* in the wing disc resulted in Ci expanding to P compartment (Fig. 1, *A--D‴*). Consistent with previous studies (24), our data suggest that both *ci* RNAi and the constitutively active form of Ci cannot rescue the phenotype of the shifted A/P boundary (data not shown). We supposed that maybe the phenotype of A/P boundary distortion caused by loss of *kto-skd* could be due to a skewed balance between Ci in A compartment and En in P compartment with altered Hh signaling activity.

Besides the disturbed A/P boundary, loss of *kto* and *skd* also induces wider Ptc expression stripe and slight up-regulation of *ptc* transcription level (Figs. 1 and 2*D*), while overexpression of both *kto* and *skd* suppresses *ptc* expression (Fig. 2*D*). In Hh signaling pathway, *ptc* transcription is specifically controlled by Ci activity. In the presence of high level Hh, full-length Ci accumulates and is activated to stimulate *ptc* expression. Our observation of Kto-Skd complex regulating *ptc* expression suggests that these two proteins may function as co-factors of Ci to facilitate a fine control of *ptc* transcription.

Our data demonstrate that loss of either *kto* or *skd* slightly up-regulates *ptc* expression and induces wider Ptc stripe, both of which are closely related to Ci activity. Kto and Skd physically interact with Ci in a manner dependent on Hh signaling activity (Fig. 3, *A--D*). Considering that the high activity of Hh signaling could enhance their interactions with Ci, it is likely that Kto and Skd are recruited to Ci and thereby restrict its activation to a proper extent.

Previous studies consistently show that loss of either *kto*, *skd*, or both genes may cause highly similar functional consequences, and that overexpression of these two genes in combination led to a more severe phenotype (Fig. 2*D*) (24). These suggest that the two proteins function in a form of pairing with each other. Kto-Skd together with Cdk8-CycC constitutes the regulatory submodule of the Mediator. Interestingly, loss of Cdk8 and CycC failed to induce shifted A/P boundary and wider Ptc stripe. Although all four subunits of the regulatory module have very similar mutant phenotypes in yeast (54–56), loss of function of *kto-skd* appears to cause more severe defects in *Drosophila* development when compared with loss of Cdk8-CycC (29, 35). Moreover, recent studies showed that together with Med13 (Skd), Med12 (Kto) can lead to transcriptional repression independent of the kinase activity of Cdk8 (24, 37, 48), suggesting that Med12 (Kto) and Med13 (Skd) have

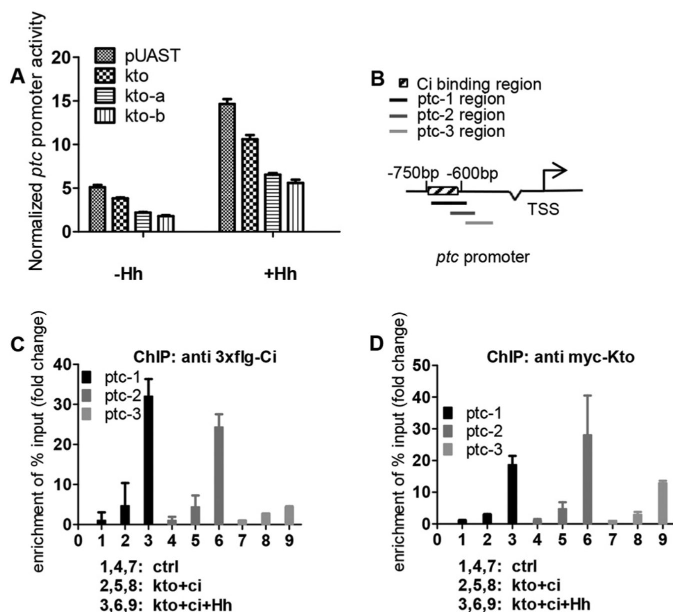

FIGURE 5. **Kto and Ci can localize on the same *ptc* promoter locus.** *A*, S2 cells were transfected with plasmids of *UAS-kto, UAS-kto-a,* or *UAS-kto-b* respectively on the basis of *ptc*-luciferase assay system. Overexpression of Kto or the mapped two fragments of Kto suppressed *ptc*-promoter activity, which indicates that the two Kto fragments are both suppressive domains. *B*, schematic drawings of Ci binding locus on the *ptc* promoter and the regions covered by the ChIP primers. *C* and *D*, S2 cells were transfected with combinations of DNA constructs as indicated. After 48 h of transfection, lysates from transfected S2 cells were prepared for the ChIP assay. Data from ChIP signals were normalized to 1/10 of input and shown as the fold change to the first group (mean ± S.D.; $n = 3$).

evolved additional functions in higher eukaryotes. Taken together, these observations suggested that Kto and Skd act independently of Cdk8-CycC in the regulation of *ptc* expression controlled by Hh signals.

Kto interacts with the transcription factor Ci through multiple domains and both the two fragments we have mapped can down-regulate the *ptc* expression activity (Fig. 5*A*). These two fragments do not play a dominant-negative role in the regulation of *ptc* since they still down-regulate the transcription activity of *ptc* as the dosage increase of their expression level (data not shown).

In summary, our studies verified novel roles of Kto-Skd in regulating A/P boundary formation in *Drosophila* wing discs and more importantly in affecting *ptc* expression. As repressors, Kto and Skd function together to down-regulate *ptc* transcription. Both Kto and Skd can physically interact with Ci, which is regulated by Hh signaling activity, and Kto can be recruited to the Ci regulatory region of the *ptc* promoter locus in the presence of Hh. But it should be noted that Hh signaling is not the only signaling pathway regulated by the Kto-Skd complex. In *Drosophila*, *kto* and *skd* are also involved in Wnt pathway and Notch pathway, both of which play essential roles in the development of wing discs. Loss of *kto* and *skd* can distort these two pathways and then affect the development process. How Kto-Skd complex differently regulates these pathways to control the development is not very clear and needs further study in the future. Collectively, our present study provides important insights into the relationship between Kto-Skd complex and the Hh signaling pathway transcription factor Ci in *ptc* transcription activity.

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