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

Feifei Mao¹, Xiaofeng Yang¹, Lin Fu, Xiangdong Lv, Zhao Zhang, Wenzong Wu, Siqi Yang, Zhaocai Zhou, Lei Zhang, and Yun Zhao²

*From the State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, China*

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, CiΔ, which silences Hh target gene transcription, such as *deca*pentaplegic (*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

---

*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.
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 progenitor 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 SmoSD 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. actSc>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 μg DNA for ub-Gal4 and 5 μg 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 CuSO4, 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 mM NaCl, 1 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 Lysin Buffer containing 250 mM NaCl and 3X 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 Lysin 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.
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 ptc up-regulated ptc expression level (Fig. 2D). 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 Kto 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-
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. 2D). 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. 2D). Taken together, kto and skd can regulate ptc expression level mainly in the presence of high levels of Hh.
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 and 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 3xFLAG-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. 2D), 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’). 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 respond 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. 3C). Similar results were observed for Myc-Skd and FLAG-Ci (Fig. 3D).

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. 3E). 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 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-skdRNAi (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.
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. 4A). 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. 4B).

Consistently, full-length of Ci could precipitate the Kto fragments corresponding to amino acids 1–289, 290–1309 (Fig. 4B). 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. 4A). Co-IP results showed that both of these two Kto fragments interacted with Ci (Fig. 4C). 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 large fragment of Kto (amino acids 290–1309). As shown in Fig. 4D, 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. 4D). In contrast, the C terminus of Myc-Ci (amino acids 1160–1397) failed to interact with Kto (Fig. 4D). 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. 2D). 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. 5A), 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. 5B). 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...
Numerous genes are involved in morphogenesis, in which Hh signaling plays a critical role. In wing disc, posterior cells secrete Hh to induce a stripe of neighboring anterior cells across the compartment boundary to secrete Dpp, which can exert a long-range organizing influence on surrounding wing tissue. 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/H11630). 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 2D). 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. 2D) (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

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 secrete Hh to induce a stripe of neighboring anterior cells across the compartment boundary to secrete 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/H11630). 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 2D). 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. 2D) (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
Kto-Skd Regulates ptc Expression by Interacting with Ci

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. 5A). 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.

Acknowledgments—We thank Dr. Jessica E. Treisman (Skirball Institute of Biomolecular Medicine and Department of Cell Biology, NYU School of Medicine, New York) for sharing reagents. We thank Dr. Gang Wang (Institute of Biochemistry and Cell Biology, CAS, China) for critical comments on the study. We apologize to colleagues whose work is not cited because of space limitation. We also thank DSHB, VDRC, NIG, and Bloomington Stock Center for reagents and fly stocks.

REFERENCES

1. Ingham, P. W., and McMahon, A. P. (2001) Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15, 3089–3087
2. Garcia-Bellido, A., Ripoll, P., and Morata, G. (1973) Developmental compartmentalisation of the wing disk of Drosophila. Nature: New Biology 245, 251–253
3. Nestoras, K., Lee, H., and Mohler, J. (1997) Role of knot (kn) in wing patterning in Drosophila. Genetics 147, 1203–1212
4. Basler, K., and Struhl, G. (1994) Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. Nature 368, 208–214
5. Müller, J. L., Callea, M., Capdevila, J., and Guerrero, I. (1997) Hedgehog activity, independent of decapentaplegic, participates in wing disc patterning. Development 124, 1227–1237
6. Nüsslein-Volhard, C., and Wieschaus, E. (1980) Mutations affecting segment number and polarity in Drosophila. Nature 287, 795–801
7. Jiang, J., and Hui, C. C. (2008) Hedgehog signaling in development and cancer. Dev. Cell 15, 801–812
8. Chen, Y., and Struhl, G. (1996) Dual roles for patched in sequestering and transducing Hedgehog. Cell 87, 553–563
9. Fuse, N., Maiti, T., Wang, B., Porter, I. A., Hall, T. M., Leaby, D. J., and Beachy, P. A. (1999) Sonic hedgehog protein signals not as a hydrolytic enzyme but as an apparent ligand for patched. Proc. Natl. Acad. Sci. U. S. A. 96, 10992–10999
10. Kalderon, D. (2000) Transducing the hedgehog signal. Cell 103, 371–374
11. Jiang, J. (2002) Degrading Ci: who is Cul-pale? Genes Dev. 16, 2315–2321
12. Lum, L., and Beachy, P. A. (2004) The Hedgehog response network: sensors, switches, and routers. Science 304, 1755–1759
13. Aza-Blanc, P., Ramírez-Weber, F. A., Laget, M. P., Schwartz, C., and Kornberg, T. B. (1997) Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. Cell 89, 1043–1053
14. Ménétrot, N., and Basler, K. (1999) Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. Cell 96, 819–831
15. Dalmann, C., and Basler, K. (2000) Opposing transcriptional outputs of Hedgehog signaling and engrailed control compartmental cell sorting at the Drosophila A/P boundary. Cell 100, 411–422
16. Conaway, R. C., Sato, S., Tomomori-Sato, C., Yao, T., and Conaway, J. W. (2005) The mammalian Mediator complex and its role in transcriptional regulation. Trends Biochem. Sci. 30, 250–255
17. Malik, S., and Roeder, R. G. (2010) The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nature Reviews. Genetics 11, 761–772
18. Malik, S., and Roeder, R. G. (2005) Dynamic regulation of pol II transcription by the mammalian Mediator complex. Trends Biochem. Sci. 30, 256–263
19. Björklund, S., and Gustafsson, C. M. (2005) The yeast Mediator complex and its regulation. Trends Biochem. Sci. 30, 240–244
Kto-Skd Regulates ptc Expression by Interacting with Ci

22. Hong, S. K., Haldin, C. E., Lawson, N. D., Weinstein, B. M., Dawid, I. B., and Hukriede, N. A. (2005) The zebrafish kothalo/trap230 gene is required for the development of the brain, neural crest, and pronephric kidney. *Proc. Natl. Acad. Sci. U. S. A.* 102, 18473–18478.

23. Malik, S., Gu, W., Wu, W., Qin, J., and Roeder, R. G. (2000) The U.S.A.-derived transcriptional coactivator PC2 is a submodule of TRAP/SMCC and acts synergistically with other PCs. *Mol. Cell* 5, 753–760.

24. Janody, F., Martirosyan, Z., Benali, A., and Treisman, J. E. (2003) Two subunits of the Drosophila mediator complex act together to control cell affinity. *Development* 130, 3691–3701.

25. Kang, J. S., Kim, S. H., Hwang, M. S., Han, S. J., Lee, Y. C., and Kim, Y. J. (2001) The structural and functional organization of the yeast mediator complex. *J. Biol. Chem.* 276, 42003–42010.

26. Chadick, J. Z., and Asturias, F. J. (2005) Structure of eukaryotic Mediator complexes. *Trends Biochem. Sci.* 30, 264–271.

27. Taatjes, D. J. (2010) The human Mediator complex: a versatile, genomewide regulator of transcription. *Trends Biochem. Sci.* 35, 315–322.

28. Janody, F., and Treisman, J. E. (2011) Requirements for mediator complex subunits distinguish three classes of notch target genes at the Drosophila wing margin. *Developmental Dynamics* 240, 2051–2059.

29. Loncle, N., Boube, M., Joulia, L., Boschiero, C., Werner, M., Cribs, D. L., and Bourbon, H. M. (2007) Distinct roles for Mediator Cdk8 module subunits in Drosophila development. *EMBO J.* 26, 1045–1054.

30. Borggrefe, T., Davis, R., Erdjument-Bromage, H., Tempst, P., and Kornberg, R. D. (2002) A complex of the Srb8-, -9, -10, and -11 transcriptional regulatory proteins from yeast. *Mol. Cell. Biol.* 22, 3265–3278.

31. Larschan, E., and Winston, F. (2005) The Saccharomyces cerevisiae Srb8–Srb11 complex functions with the SAGA complex during Gal4-activated transcription. *Mol. Cell. Biol.* 25, 114–123.

32. Meyer, K. D., Donner, A. J., Knuese, M. T., York, A. G., Espinosa, J. M., and Taatjes, D. J. (2008) Cooperative activity of cdk8 and GCN5L within Mediator directs tandem phosphoacetylation of histone H3. *EMBO J.* 27, 1447–1457.

33. Belakavadi, M., and Fondell, J. D. (2010) Cyclin-dependent kinase 8 positively cooperates with Mediator to promote thyroid hormone receptor-dependent transcriptional activation. *Mol. Cell. Biol.* 30, 2437–2448.

34. Wang, X., Yang, N., Uno, E., Roeder, R. G., and Guo, S. (2006) A subunit of the mediator complex regulates vertebrate neuronal development. *Proc. Natl. Acad. Sci. U. S. A.* 103, 17284–17289.

35. Carrera, I., Janody, F., Leeds, N., Duveau, F., and Treisman, J. E. (2008) Pygopus activates Wingless target gene transcription through the mediator complex subunits Med12 and Med13. *Proc. Natl. Acad. Sci. U. S. A.* 105, 6644–6649.

36. Lim, J., Lee, O. K., Hsu, Y. C., Singh, A., and Choi, K. W. (2007) Drosophila TRAP230/240 are essential coactivators for Atonal in retinal neurogenesis. *Dev. Biol.* 308, 322–330.

37. Treisman, J. (2001) Drosophila homologues of the transcriptional coactivation complex subunits TRAP240 and TRAP230 are required for identical processes in eye-antennal disc development. *Development* 128, 605–615.

38. Zhou, H., Kim, S., Ishii, S., and Beyer, T. G. (2006) Mediator modulates Gli3-dependent Sonic hedgehog signaling. *Mol. Cell. Biol.* 26, 8667–8682.

39. Alexandre, C., Jacinto, A., and Ingham, P. W. (1996) Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. *Genes Dev.* 10, 2003–2013.

40. Müller, B., and Basler, K. (2000) The repressor and activator forms of *Cubitus interruptus* control Hedgehog target genes through common generic gli-binding sites. *Development* 127, 2999–3007.

41. Zhao, Y., Tong, C., and Jiang, J. (2007) Hedgehog regulates smoothened activity by inducing a conformational switch. *Nature* 450, 252–258.

42. Jia, J., Tong, C., Wang, B., Luo, L., and Jiang, J. (2004) Hedgehog signalling activity of Smoothened requires phosphorylation by protein kinase A and casein kinase l. *Nature* 432, 1045–1050.

43. Zhang, Y., Mao, F., Lu, Y., Wu, W., Zhang, L., and Zhao, Y. (2011) Transduction of the Hedgehog signal through the dimerization of Fused and the nuclear translocation of Cubitus interruptus. *Cell Research* 21, 1436–1451.

44. Wotton, D., Lo, R. S., Lee, S., and Massagué, J. (1999) A Smad transcriptional corepressor. *Cell* 97, 29–39.

45. Harada, J., Kokura, K., Kane-Ishii, C., Nomura, T., Khan, M. M., Kim, Y., and Ishii, S. (2003) Requirement of the co-repressor homeodomain-interacting protein kinase 2 for ski-mediated inhibition of bone morphogenetic protein-induced transcriptional activation. *J. Biol. Chem.* 278, 38998–39005.

46. Huang, C., Xiang, Y., Wang, Y., Li, X., Xu, L., Zhu, Z., Zhang, T., Zha, Q., Zhang, K., Jing, N., and Chen, C. D. (2010) Dual-specificity histone demethylase KIAA1718 (KDM7A) regulates neural differentiation through FGF4. *Cell Research* 20, 154–165.

47. Carlson, M. (1997) Genetics of transcriptional regulation in yeast: connections to the RNA polymerase II CTD. *Annu. Review Cell Dev. Biol.* 13, 1–23.

48. Knuese, M. T., Meyer, K. D., Bernecky, C., and Taatjes, D. J. (2009) The human CDK8 subcomplex is a molecular switch that controls Mediator coactivator function. *Genes Dev.* 23, 439–451.

49. Galbraith, M. D., Donner, A. J., and Espinosa, J. M. (2010) CDK8: a positive regulator of transcription. *Transcription* 1, 4–12.

50. Smelkinson, M. G., Zhou, Q., and Kalderon, D. (2007) Regulation of CI-SCFSlimb binding, Ci proteolysis, and hedgehog pathway activity by Ci phosphorylation. *Dev. Cell* 13, 481–495.

51. Desvaud, E., Yang, L. L., Hill, K., Cox, B., Uloa, F., Ribeiro, A., Mynett, A., Novitch, B. G., and Briscoe, J. (2007) Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. *Nature* 450, 717–720.

52. Dahmann, C., and Basler, K. (1999) Compartment boundaries at the edge of development. *Trends Genetics* 15, 320–326.

53. Zecca, M., Basler, K., and Struhl, G. (1995) Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the Drosophila wing. *Development* 121, 2265–2278.

54. Song, W., and Carlson, M. (1998) Srb/mediator proteins interact functionally and physically with transcriptional repressor Sfl1. *EMBO J.* 17, 5757–5765.

55. Samuelsen, C. O., Baraznenok, V., Khorosjutina, O., Spahr, H., Kieselbach, T., Holmberg, S., and Gustafsson, C. M. (2003) TRAP230/ARC240 and ARC250 Mediator subunits are functionally conserved through evolution. *Proc. Natl. Acad. Sci. U. S. A.* 100, 6422–6427.

56. van de Peppel, J., Kettelarij, N., van Bakel, H., Kockelkorn, T. T., van Leenen, D., and Holstege, F. C. (2005) Mediator expression profiling epistasis reveals a signal transduction pathway with antagonistic submodules and highly specific downstream targets. *Mol. Cell* 19, 511–522.