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Two subunits of the *Drosophila* mediator complex act together to control cell affinity

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SUMMARY

The organizing centers for *Drosophila* imaginal disc development are created at straight boundaries between compartments; these are maintained by differences in cell affinity controlled by selector genes and intercellular signals. *skuld* and *kohtalo* encode homologs of TRAP240 and TRAP230, the two largest subunits of the *Drosophila* mediator complex; mutations in either gene cause identical phenotypes. We show here that both genes are required to establish normal cell affinity differences at the anterior-posterior and dorsal-ventral compartment boundaries of the wing disc. Mutant cells cross from the anterior to the posterior compartment, and can distort the dorsal-ventral boundary in either the dorsal or ventral direction. The Skuld and Kohtalo proteins physically interact in vivo and have synergistic effects when overexpressed, consistent with a *skuld kohtalo* double-mutant phenotype that is indistinguishable from either single mutant. We suggest that these two subunits do not participate in all of the activities of the mediator complex, but form a submodule that is required to regulate specific target genes, including those that control cell affinity.

Key words: TRAP, ARC, Transcription, Adhesion, Compartment, Boundary, *Drosophila*

INTRODUCTION

Development of many structures is controlled by organizing centers that form at the boundary between differently determined cell populations. For example, cells at the mouse mid-hindbrain boundary secrete WNT and FGF signals that pattern the surrounding region (Wassef and Joyner, 1997). In *Drosophila* imaginal discs, the integrity of these organizing centers is maintained by differences in cell affinity between the two populations that prevent cell mixing (Dahmann and Basler, 1999). Although few of the adhesion molecules that control these affinity differences have been identified, some of their upstream regulators are known.

The posterior compartment of the wing disc expresses the selector gene *engrailed* (*en*), which encodes a homeodomain transcription factor (Tabata et al., 1995). The secreted protein Hedgehog (Hh) is also limited to the posterior compartment, but can only signal to cells in the anterior compartment, where the Hh-responsive transcription factor Cubitus interruptus (Ci) is present (Schwartz et al., 1995; Tabata and Kornberg, 1994). Cells just anterior to the anterior-posterior (AP) compartment boundary respond to Hh by expressing Decapentaplegic (Dpp), a long-range organizer of pattern throughout the wing disc (Basler and Struhl, 1994; Lecuit et al., 1996; Nellen et al., 1996; Tabata and Kornberg, 1994). In addition, Hh signaling alters the affinity of these cells, preventing them from mixing with cells that do not receive the Hh signal or are unable to respond to it (Blair and Ralston, 1997; Rodriguez and Basler, 1997). En is thought to make an additional contribution to cell affinity by regulating unknown target genes in posterior cells (Dahmann and Basler, 2000).

An analogous system operates along the dorsal-ventral axis of the wing disc. The LIM domain protein Apterous (Ap) acts as a selector for the dorsal compartment (Diaz-Benjumea and Cohen, 1993). By activating dorsal-specific expression of the Notch (N) ligand Serrate and the glycosyltransferase Fringe, which makes N preferentially sensitive to the ventrally expressed ligand Delta (Dl), Ap allows N activation specifically at the dorsal-ventral (DV) boundary (Bruckner et al., 2000; Doherty et al., 1996; Kim et al., 1995; Panin et al., 1997). N then activates a stripe of Wingless (Wg), a concentration-dependent organizer of the wing pouch (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1997; Rulifson and Blair, 1995; Zecca et al., 1996). Ap also controls the expression of genes that regulate cell affinity, preventing mixing between cells of the dorsal and ventral compartments (Blair et al., 1994; Milan and Cohen, 1999). Two of its target genes that may contribute to this process are *capricious* (*caps*) and *tartan* (*trn*); both encode leucine-rich repeat proteins that are specifically expressed in the dorsal compartment at the time when affinity differences along this dimension are established (Milan et al., 2001). Misexpression of either *caps* or *trn* in the ventral compartment leads to cell death or to movement towards the DV boundary; however, dorsal cells still maintain their dorsal affinity in the absence of both genes (Milan et al., 2001). In addition to *ap* activity, N signaling is required to prevent cells from crossing the boundary in either direction (Micchelli and Blair, 1999;

Milan and Cohen, 2003; O'Keefe and Thomas, 2001; Rauskolb et al., 1999).

Sequence-specific transcription factors require the assistance of cofactors to recruit RNA polymerase II and the basal transcriptional machinery. One widely used co-activator is the mediator complex, which was first described in yeast and has now been isolated from human and mouse cells and from *Drosophila* (Malik and Roeder, 2000; Rachez and Freedman, 2001). Many transcriptional activators, as well as some repressors, require the mediator complex in order to regulate transcription in vitro, even on naked DNA templates (Boyer et al., 1999; Fondell et al., 1996; Gu et al., 1999; Ito et al., 1999; Malik et al., 2000; Naar et al., 1999; Rachez et al., 1999; Ryu et al., 1999). The largest mediator-related complexes that have been isolated contain about 20 subunits, but they seem to be divisible into functional submodules. Smaller complexes, called positive cofactor 2 (PC2) or the cofactor required for Sp1 activation (CRSP), are sufficient for co-activator activity with a number of activators in vitro (Malik et al., 2000; Ryu et al., 1999) and directly interact with the C-terminal domain of RNA polymerase II (Naar et al., 2002), suggesting that they represent a core complex containing the essential activator functions. In addition to the essential subunits of this complex, some subunits may act as adaptors for specific transcription factors. The TRAP220 subunit appears to be an adaptor for nuclear receptors; the ligand-binding domains of these receptors bind specifically to TRAP220 in vitro in a ligand-dependent manner (Ge et al., 2002; Hittelman et al., 1999; Kang et al., 2002; Yuan et al., 1998). Similarly, the *Xenopus* ARC105 subunit shows specific interactions with Smad2 and Smad3, transcription factors in the Nodal signaling pathway (Kato et al., 2002), and the mouse SUR2 (ABCC9 – Mouse Genome Informatics) subunit is specifically required for the activity of E1A-CR3, and of ELK1 that has been phosphorylated by ERK (Stevens et al., 2002).

Two of the subunits absent from the core complex to which a function has not yet been assigned by in vitro studies are the largest proteins in the complex, TRAP240 and TRAP230. The presence of these two subunits, and of Cdk8 and Cyclin C, and the absence of CRSP70, differentiate ARC, a large complex, from CRSP. ARC and CRSP have distinct activities in vitro (Taatjes et al., 2002). Distant homologs of these four proteins in yeast, Srb8-11, also form an accessory subcomplex that has been implicated in transcriptional repression (Boube et al., 2002; Lee et al., 2000; Song and Carlson, 1998; Song et al., 1996).

We isolated mutations in the *skuld* [*skd*; previously named *blind spot* (Gutierrez et al., 2003)] and *kohtalo* (*kto*) genes, which encode *Drosophila* homologs of TRAP240 and TRAP230, respectively, based on their identical loss-of-function phenotypes in the eye disc (Treisman, 2001). Unlike dTrap80 and dMed6, components of the core PC2/CRSP complex, Skd and Kto are not required for cell proliferation or survival (Boube et al., 2000; Gim et al., 2001; Treisman, 2001). Here, we examine the effects of *skd* and *kto* mutations on patterning of the wing discs. We show that both genes again have identical functions, and that they regulate the differences in cell affinity that create compartment boundaries. We provide both genetic and biochemical evidence supporting the model that these two proteins act in concert, probably as a submodule of the mediator complex.

MATERIALS AND METHODS

Fly strains and genetics

The *skd* and *kto* alleles used have been previously described (Treisman, 2001). Double mutants were generated by first crossing a P(w^+) element close to *kto* (*P(w^+)Mi-2^{j3d4}*) onto the *skd^{T606}* chromosome and a P(w^+) element close to *skd* (*FRT2A*) onto the *kto^{T241}* chromosome, and then testing the complementation behavior of *w* recombinants between these two chromosomes. Other strains used were *en-lacZ* (Blair, 1992), *ap-lacZ*, *caps-lacZ* (Milan et al., 2001), *ptc-lacZ* (Johnson et al., 1995), *Dll-lacZ* (FlyBase), *hh-lacZ^{P30}* (Lee et al., 1992), *vg*-GAL4 (Simmonds et al., 1995), *da*-GAL4 (Benlali et al., 2000), *tub*-GAL4 (Lee and Luo, 1999) and UAS-*Eph* (Dearborn et al., 2002). Clones were generated by crossing to *hs*FLP122; FRT80, Ubi-GFP flies and heat-shocking larvae for 1 hour at 38.5°C in first and second instar, or in late second or early third instar to look at the behavior of late-induced clones at the DV boundary. Clones in the eye disc were generated using *ey*FLP1 rather than *hs*FLP122. Full-length *skd* and *kto* cDNAs (Treisman, 2001) were cloned into pUAST and used to generate transgenic flies. At least three independent lines were analyzed for each construct. Recombinant lines carrying both UAS-*skd* and UAS-*kto* transgenes, or two copies of each individual transgene, were generated. To make clones overexpressing *skd* and/or *kto* in the wing disc, *hs*FLP122, UAS-*GFP; FRT42*, *tub*-GAL80; *tub*-GAL4/TM6B females were crossed to *FRT42;* UAS-*skd1*, UAS-*kto6*/SM6-TM6B, or *FRT42;* UAS-*skd1*, UAS-*skd2*/SM6-TM6B or *FRT42*, UAS-*kto4;* UAS-*kto2*/SM6-TM6B males, and larvae were heat shocked for 1 hour at 38.5°C in first and second instar. Size comparisons were made on clones generated in parallel. To make *kto* clones expressing activated Ci, *hs*FLP122, UAS-*GFP; tub*-GAL4/CyO; *FRT80, tub*-GAL80 females were crossed to UAS-*HACi(m1-4); hh-lacZ, FRT80, kto^{T663}* males, and larvae were heat shocked for 1 hour at 38.5°C in first and second instar.

Antibodies and immunohistochemistry

Eye and wing discs were stained as described (Lee and Treisman, 2001). Antibodies used were rat anti-Ci (Motzny and Holmgren, 1995), rabbit anti-β-galactosidase (Cappel, 1:5000), mouse anti-Wg (Ng et al., 1996), rat anti-Elav (Robinow and White, 1991), rabbit anti-Atonal (Jarman et al., 1993), rabbit anti-Dephrin (Bossing and Brand, 2002) and rabbit anti-Eph (Dearborn et al., 2002). A peptide consisting of amino acids 361-612 of Skd was produced in *Escherichia coli* with an N-terminal His-tag, purified on Ni-NTA agarose, and used to raise rabbit polyclonal antibodies (Covance). Antiserum was used at a final concentration of 1:5000 for tissue staining. A peptide consisting of amino acids 2040-2229 of Kto was produced in *E. coli* with an N-terminal His-tag, purified on Ni-NTA agarose, and used to raise both rat and guinea pig polyclonal antibodies (Covance). Antiserum was used at a final concentration of 1:1000 for tissue staining. Fluorescence images were obtained on a Leica TCS NT confocal microscope. The NIH Image program was used to measure clone roundness: clones falling entirely within the wing pouch were outlined on an enlarged Photoshop image and the circularity was calculated as $4\pi A/L^2$, where A is the clone area and L is the perimeter (Lawrence et al., 1999). Wild-type measurements were taken from twin spots in the same set of images. Significance was calculated using a two-tailed *t*-test. To compare clone sizes, clones falling entirely within the wing pouch were outlined using NIH Image; their areas were measured in pixels and normalized to the total area on the same image of the wing pouch as outlined by Wg staining.

Protein extraction and immunoprecipitation

Proteins were extracted from 0- to 23-hour-old *white*$^-$ embryos, and from embryos expressing UAS-*skd1* and UAS-*kto4* under the control of *da*-GAL4, at 25°C. Embryos were collected with PBT (0.2% Triton in PBS), dechorionated in 50% bleach, washed with distilled

water and briefly ground in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 7.4), 1% Triton X-100, 0.1% SDS] in the presence of protease inhibitors (1 mM PMSF; 1 μg/ml each of aprotinin, pepstatin and leupeptin) and phosphatase inhibitors (1 mM NaF, 1 mM Na_2VO_4). Homogenates were rocked for 30 minutes and spun at 13,000 ***g*** for 15 minutes at 4°C. Protein amounts in the supernatant were quantified by the Bradford method to normalize quantities of extract used for immunoprecipitation between *white*$^-$-, and *skd*- and *kto*-overexpressing embryos. An aliquot of the total extract was diluted in 5×Laemmli buffer to run on the gel directly. Extracts were supplemented with lysis buffer to normalize the volume used for immunoprecipitation (IP). Guinea pig anti-Kto antibody (1:100 final dilution), or anti-Skd antibody (1:100 final dilution) or no antibody (for control IPs) was added to the samples, which were then rocked for 2 hours at 4°C. Protein-A agarose (Roche) was then added and the samples rocked for 2 hours at 4°C. Beads were spun for 30 seconds and washed four times with lysis buffer, including protease and phosphatase inhibitors, and once with the same buffer without SDS. Beads were then re-suspended in 2×Laemmli buffer.

Western blotting

Samples were boiled for 5 minutes at 95°C before running on SDS-PAGE gels [7% to detect Skd and Kto, or 12% to detect dSOH1 (Trap18 – FlyBase)]. Each immunoprecipitation was divided into equal quantities for detection of Skd, Kto or dSOH1. Wet electrotransfers of gels to nitrocellulose membranes (Bio-Rad) were performed at 50 mA overnight at 4°C. Blots were blocked for 2 hours at room temperature in TBT (0.2% Tween 20 in TBS) containing 10% low-fat milk, and then for 2 hours at room temperature in TBT containing 10% low-fat milk supplemented with rat anti-Kto (1/5000), anti-Skd (1/100,000) or anti-dSOH1 (1/10,000) (Park et al., 2001). Blots were washed four times in TBT and incubated for 1 hour in TBT containing 10% low-fat milk supplemented with anti-rat, anti-rabbit or anti-mouse HRP (1/5000 dilution, Jackson Immunoresearch). After four washes in TBT, blots were developed using the ECL photoluminescence procedure (Pierce).

RESULTS

Cells lacking *skd* or *kto* move into the posterior compartment of the wing disc

In the eye disc, loss of either *skd* or *kto* function results in inappropriately prolonged expression of the Hh target genes *decapentaplegic* (*dpp)* and *atonal* (*ato*) (Treisman, 2001), as well as loss of expression of another Hh target gene, *rough* (J.E.T., unpublished data). We wanted to determine whether Hh signaling was also altered in *skd*- or *kto*-mutant cells in the wing disc, which has a stable domain of Hh-responsive cells. As in the eye disc, we found that the two genes had indistinguishable mutant phenotypes; thus, the results shown here for each individual gene are representative of both. In clones of cells mutant for either *skd* or *kto* lying just anterior to the compartment boundary, expression of the Hh target genes *patched* (*ptc*) and *dpp* occurred normally and was accompanied by normal upregulation of the full-length, activated form of the Hh-responsive transcription factor Ci (Fig. 1 and data not shown). However, clones arising in the anterior compartment adjacent to the AP boundary frequently crossed into the posterior compartment (Fig. 1, arrows; Table 1A). These clones were clearly of anterior origin, as they expressed the anterior markers *Ci* (Fig. 1A,E) and *ptc* (Fig. 1F), failed to express the posterior marker *en-lacZ* (Fig. 1B), and had twin spots in the anterior compartment (Fig. 1C,G). Separation of anterior and posterior compartment cells has been shown to depend on the activities of both En and Ci (Dahmann and Basler, 2000). As anterior *skd* or *kto* clones did not misexpress *en* (Fig. 1B), it is probable that they affect compartmentalization by altering Ci activity. However, their effect must be selective for those target genes that control cell affinity, as Ci is still able to activate *ptc* and *dpp* in the absence of *skd* or *kto*.

If *skd* and *kto* are directly required for Ci to activate the expression of genes encoding adhesion molecules, even a constitutively active form of Ci might fail to activate such genes in the absence of *skd* or *kto*. In accordance with this model, misexpression of a form of Ci in which all the PKA sites have been mutated, preventing its cleavage to the repressor form (Chen et al., 1999b), did not prevent *kto*-mutant cells from crossing into the posterior compartment (Fig. 1I,J), although it was able to ectopically activate *ptc* expression in *kto*-mutant clones (data not shown).

Table 1. Quantification of boundary crossing behavior of *skd*^{T606}-mutant clones

A. Anterior clones touching the AP boundary

Boundary crossing behavior	Number of anterior clones
Cross from A to P	22 (58%)
Don't cross	16 (42%)

B. Clones touching the DV boundary

Boundary crossing behavior	Number of clones
Push boundary ventrally	48 (41%)
Push boundary dorsally	21 (18%)
D, straight boundary	14 (12%)
V, straight boundary	12 (10%)
D and V, straight boundary	23 (19%)

Anterior clones touching the AP boundary and all clones touching the DV boundary in discs stained for Ci and *ap-lacZ* expression were scored for crossing or distortions of the boundary. An example of a clone pushing the DV boundary ventrally is shown in Fig. 2A-D and a clone pushing it dorsally is shown in Fig. 2E-H.

Cells lacking *skd* or *kto* distort the dorsal-ventral compartment boundary

The effects of *skd* and *kto* on the AP axis appeared to be limited to alterations of cell affinity. We therefore tested the effects of loss of these genes on cell affinity at the DV boundary. Because the DV boundary does not form until the second instar, unlike the AP boundary, which forms during embryogenesis (Garcia-Bellido et al., 1973), we could examine the effects of *skd* and *kto* clones generated both before and after boundary formation. When *skd* or *kto* mutant clones that spanned the DV boundary were generated in first or early second instar larvae before boundary formation, the entire clone often moved into one of the two compartments, distorting the compartment boundary (Fig. 2; Table 1B). For example, Fig. 2A-D shows a clone located entirely in the ventral compartment that includes cells expressing the dorsal selector gene *apterous* (*ap*) in its dorsalmost part. These dorsal cells appear to have moved into the ventral compartment. When the larger part of the clone was dorsally derived, non-*ap*-expressing cells were found in the

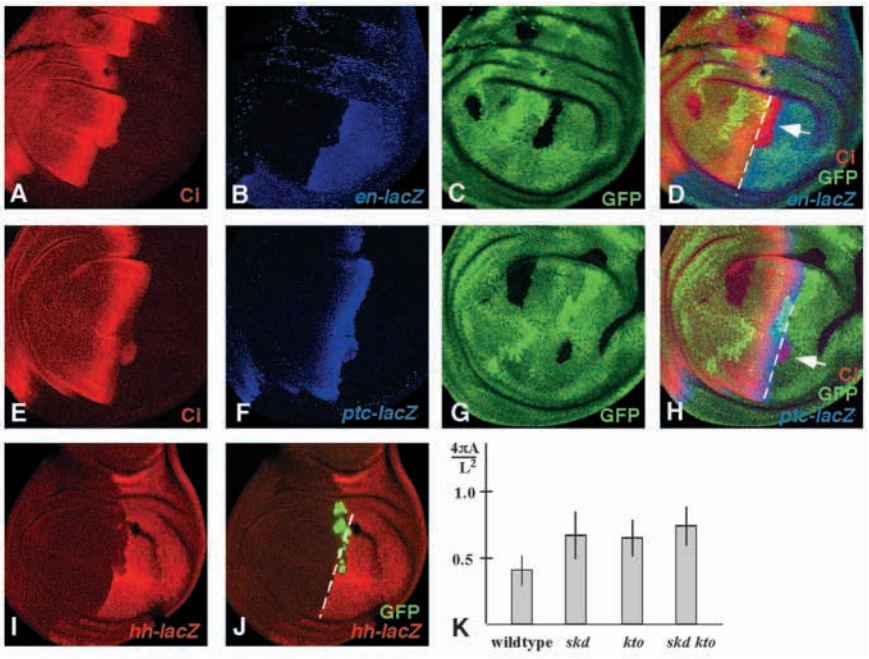

Fig. 1. *skd*- or *kto*-mutant clones cross the AP compartment boundary. (A-H) Third instar wing discs containing mutant clones, with wild-type tissue labeled with GFP (green; C,D,G,H). Ci, marking anterior cells, is stained red (A,D,E,H). (A-D) skd^{T606} clone. *en-lacZ* expression, detected by anti-β-gal staining (blue), marks the posterior compartment. (E-H) kto^{T241} clone. Anti-β-gal staining (blue) revealing *ptc-lacZ* expression (F,H). Arrows indicate anterior clones that have crossed into the posterior compartment. (I,J) kto^{T663} clone expressing HACi(m1-4), a form of Ci with all the PKA sites mutated. Anti-β-gal staining (red) reflecting *hh-lacZ* expression; the clone is positively marked with GFP (J). Ci activation does not rescue the boundary crossing behavior. The compartment boundary is indicated by a white dashed line (D,H,J). (K) *skd*- and *kto*-mutant clones are rounder than their wild-type twin spots, and *skd kto* double-mutant clones are equally round. Circularity is measured as $4\pi A/L^2$, where A is the area of the clone and L is the perimeter, and would be 1.0 for a perfect circle. The mean for wild-type twin spots is 0.41, for *skd* clones is 0.67, for *kto* clones is 0.65 and for *skd kto* clones is 0.74. Lines within the bars indicate±1 s.d. $P<0.001$ for a comparison of *skd*, *kto* or *skd kto* to wild type. The differences between *skd*, *kto* and *skd kto* are not significant.

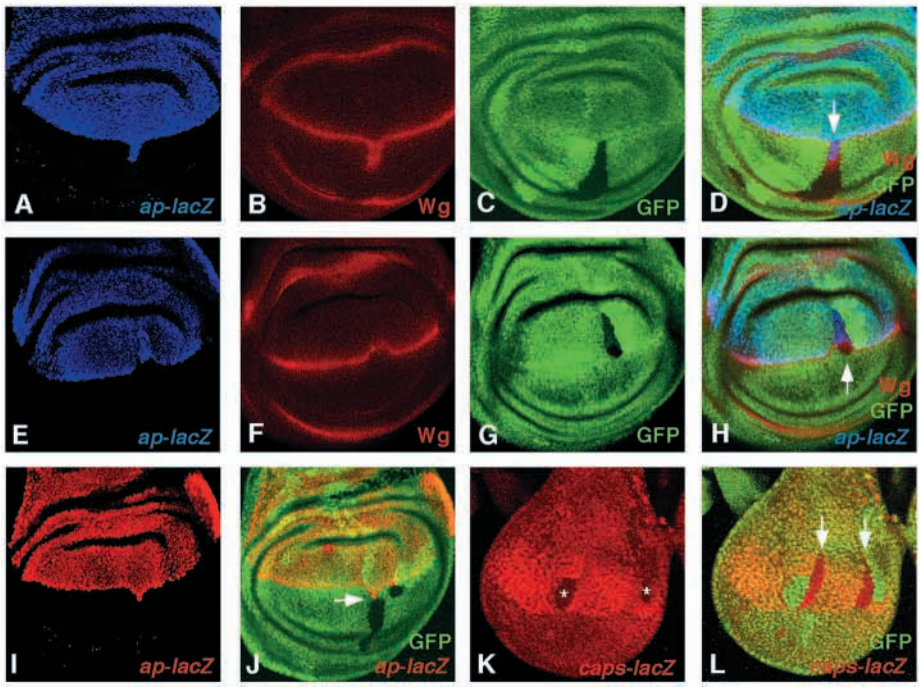

Fig. 2. *skd*- or *kto*-mutant clones distort the DV compartment boundary. (A-H) Third instar wing discs containing skd^{T606}-mutant clones. Wild-type tissue is labeled with GFP (green; C,D,G,H). *ap-lacZ* expression, marking the dorsal compartment, is stained with anti-β-gal (blue; A,D,E,H). Wg protein is stained red (B,D,F,H). Arrows indicate clones that distort the DV boundary, producing a bulge in the Wg stripe and a distortion of the *ap* expression boundary. (A-D) A clone with dorsal cells in the ventral compartment. (E-H) A clone with ventral cells in the dorsal compartment. (I-J) A kto^{T631}-clone entirely within the ventral compartment that has distorted the DV boundary (arrow). Wild-type tissue is labeled with GFP (green; J) and stained with anti-β-gal (red) to show *ap-lacZ* expression (I,J). (K,L) Second instar wing discs containing skd^{T413}-mutant clones. Wild-type tissue is labeled with GFP (green; L). Anti-β-gal (red) staining reflecting *caps-lacZ* expression (K,L). Arrows indicate dorsal clones that continue to express *caps-lacZ*. Note that the *caps-lacZ* transgene is on the same chromosome arm as *skd* and thus is not present in the wild-type twin spots (asterisks in K and brighter green staining in L).

dorsal compartment (Fig. 2E-H). The result in each case was to create a straight boundary between dorsal cells and mutant cells, or between ventral cells and mutant cells, rather than between dorsal and ventral cells. Interestingly, *wg* was still activated at the border between the *ap*-expressing and non-expressing cells, so that *wg* expression no longer formed a straight line (Fig. 2B,F). This suggests that *ap* is still able to activate *fng* and *Ser* expression in the absence of *skd* and *kto*, allowing N activation and *wg* expression at the border of the *ap* expression domain.

Because *skd*- or *kto*-mutant clones can cross the DV boundary in either direction, it is unlikely that loss of *skd* or *kto* transforms dorsal cell affinity to ventral or vice versa. *ap* promotes dorsal cell affinity in part by controlling the expression of the LRR proteins Capricious (Caps) and Tartan

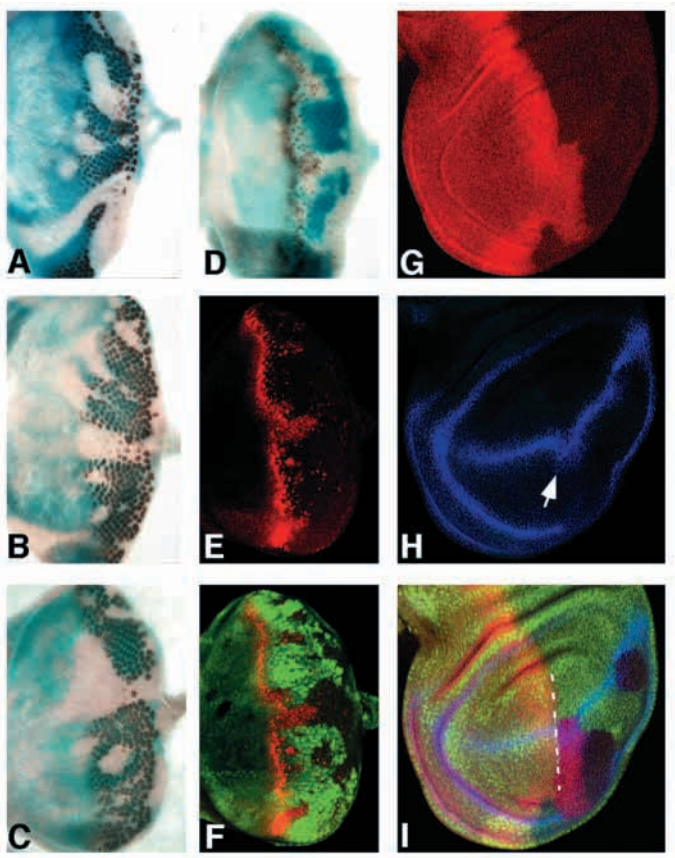

Fig. 3. *skd kto* double mutants have the same phenotype as either single mutant. (A-F) Third instar eye discs. Wild-type tissue is labeled with X-gal staining, revealing *arm-lacZ* expression (A-D), or with GFP (green; F). (A-C) Elav-stained photoreceptors (brown). (A) skd^{T616} clones, (B) kto^{T241} clones, and (C) skd^{T606}, kto^{T241} clones. Few Elav-stained photoreceptors form at the posterior of all clones, although cell growth and survival are unaffected. (D-F) Atonal staining of kto^{T314} clones (brown; D) or skd^{T606}, kto^{T241} clones (red; E,F). Ato is inappropriately maintained posterior to its normal domain in single- or double-mutant clones. (G-I) Third instar wing discs with skd^{T606}, kto^{T241} clones. Wild-type tissue is labeled with GFP (green; I). Ci is stained red (G,I) and Wg blue (H,I). Double-mutant clones cross the AP compartment boundary (white dashed line in I) and distort the DV boundary (arrow; H). Ci and Wg are expressed at normal levels within the mutant tissue.

(Trn), which are restricted to the dorsal compartment of second instar discs (Milan et al., 2001). As expected, we found that *caps-lacZ* was still expressed in dorsal *skd*-mutant clones (Fig. 2K,L). There are two other possible explanations for the phenotype. *skd*- and *kto*-mutant cells might have reduced affinity for both dorsal and ventral cells, so that the clone is pushed out of the compartment in which it has fewer cells. In support of this model, the boundaries of *skd* or *kto* clones appeared abnormally straight (Figs 1, 2), and the clones were also rounder than their wild-type twin spots (Fig. 1K) (Lawrence et al., 1999), with no significant difference between clones in the dorsal and ventral compartments. Although clones generated later in development, in late second or early third instar, did not cross the compartment boundary (data not shown), such late clones confined entirely to one compartment did sometimes distort the compartment boundary (Fig. 2I,J), perhaps as a result of cell movements minimizing contact of the mutant cells with surrounding wild-type tissue. Alternatively, *skd* and *kto* could be required for N to prevent boundary crossing; *N* clones can induce similar distortions (Rauskolb et al., 1999). Although the N target gene *wg* does not require *skd* and *kto* for its expression, a subset of other genes regulated by N are dependent on *skd* and *kto* (F.J. and J.E.T., unpublished).

The *skd* and *kto* genes act together

It is striking that the phenotypes of *skd* and *kto* mutations are identical in every respect examined. We wondered whether double mutants would show a stronger phenotype, revealing redundancy between the two proteins. In fact, the phenotype of double mutants was indistinguishable from either single mutant, as judged by the effects of clones on Elav and Ato expression in the eye disc, and Ci and Wg expression in the wing disc (Fig. 3) (Treisman, 2001). Double-mutant clones failed to respect compartment boundaries, had a rounded shape and, like single mutant clones, did not affect cell growth or survival (Fig. 1K; Fig. 3). This strongly suggests that the two proteins act as a unit and that neither can function in the absence of the other.

Although *skd* and *kto* are expressed ubiquitously (Treisman, 2001), we wanted to test the effect of overexpressing them. Overexpression might lead to hyperactivity phenotypes if Skd and Kto are limiting components of the mediator complex, or if they can act independently of the complex. Conversely, overexpression could cause dominant-negative phenotypes by sequestering other components of the complex or interacting transcription factors. We generated flies carrying each full-length cDNA under the control of UAS sites. When ubiquitously expressed, these constructs could rescue the early lethality of the respective mutants (Table 2). However, ubiquitous overexpression of either gene was itself lethal, and development was thus rescued only as far as the pupal stage.

Driving either *skd* or *kto* in more specific expression patterns caused defects in adult flies. When both genes were expressed together, these defects were stronger and more penetrant, even compared with expression of two copies of a single transgene. For example, expression of both UAS-*skd* and UAS-*kto* with the wing-specific driver *vestigial* (*vg*)-GAL4 caused a strong loss of the wing margin and reduction of the AP axis of the wing, whereas two copies of either UAS-*skd* or UAS-*kto* had a much milder effect (Fig. 4A-C). This supports the idea that Skd and Kto function in combination, producing a weaker phenotype when only endogenous levels of the other protein are available for them to interact with.

Clones overexpressing *skd* and *kto* in the wing disc did not appear to cross the AP compartment boundary, but some clones overexpressing *skd*, *kto* or both did induce distortions of the DV boundary (arrows in Fig. 4D-F). Like loss-of-function clones, *kto*- or *kto*- and *skd*-overexpression clones had smooth borders and a rounded shape (Fig. 4J), which indicates that increased levels of *skd* and *kto* cause misregulation of cell affinity molecules. Overexpression of *skd* and *kto* in combination also caused a decrease in clone size compared with expression of either gene alone (Fig. 4K), suggesting that Skd and Kto may act together to sequester components of the mediator complex and disrupt global transcription.

Table 2. UAS-*skd* and UAS-*kto* transgenes can rescue the lethality of the corresponding mutations

	% rescue to pupal stage	Number
skd rescue		
skd^{T13}, *da*-GAL4/TM6B×skd^{T606}/TM6B	0	262
skd^{T13}/TM6B×skd^{T606}, UAS*skd1*/TM6B	11	203
skd^{T13}; *tub*-GAL4/SM6.TM6B×skd^{T606}, UAS*skd1*/TM6B	63	246
skd^{T13}, *da*-GAL4/TM6B×skd^{T606}, UAS*skd1*/TM6B	58	291
skd^{T13}/TM6B×skd^{T413}; UAS*skd3*/SM6.TM6B	0	156
skd^{T13}; *tub*-GAL4/SM6.TM6B×skd^{T413}; UAS*skd3*/SM6.TM6B	54	128
skd^{T13}, *da*-GAL4/TM6B×skd^{T413}; UAS*skd3*/SM6.TM6B	109	252
kto rescue		
kto^{T555}, *da*-GAL4/TM6B×kto^{T241}/TM6B	0	535
kto^{T555}/TM6B×kto^{T241}; UAS*kto1*/SM6.TM6B	0	413
kto^{T555}, *da*-GAL4/TM6B×kto^{T241}; UAS*kto1*/SM6.TM6B	80	381

For each cross, the percentage rescue to the pupal stage is given, based on comparing the number of non-balancer pupae with the number of balancer adults that eclosed. The TM6B and SM6-TM6B balancers carried the *Humeral* and *Tubby* markers. UAS-*skd1* showed some leaky activity in the absence of a GAL4 driver, and all UAS lines caused some early lethality when expressed with the strong driver *da*-GAL4, preventing complete rescue.

The Skd and Kto proteins physically interact

The synergy observed when *skd* and *kto* are co-expressed, and the identical phenotypes of single and double mutants, could result from a physical interaction between the two proteins. To test this possibility, we generated polyclonal antibodies to Skd and Kto; both were specific, as no staining was observed within clones mutant for the corresponding gene (Fig. 5A-F). As expected, both proteins were present in the nucleus and showed the ubiquitous distribution previously observed for their transcripts (Fig. 5 and data not shown). Skd and Kto appear to associate in vivo, as the two proteins could be co-immunoprecipitated both from wild-type embryos, and from embryos overexpressing UAS-*skd* and UAS-*kto* from the ubiquitous driver *daughterless* (*da*)-GAL4 (Fig. 5G,H). This may simply reflect the presence of both proteins in the mediator complex. However, when Skd and Kto were overexpressed, the amount of each protein co-immunoprecipitated by the other increased without a corresponding increase in precipitation of the core

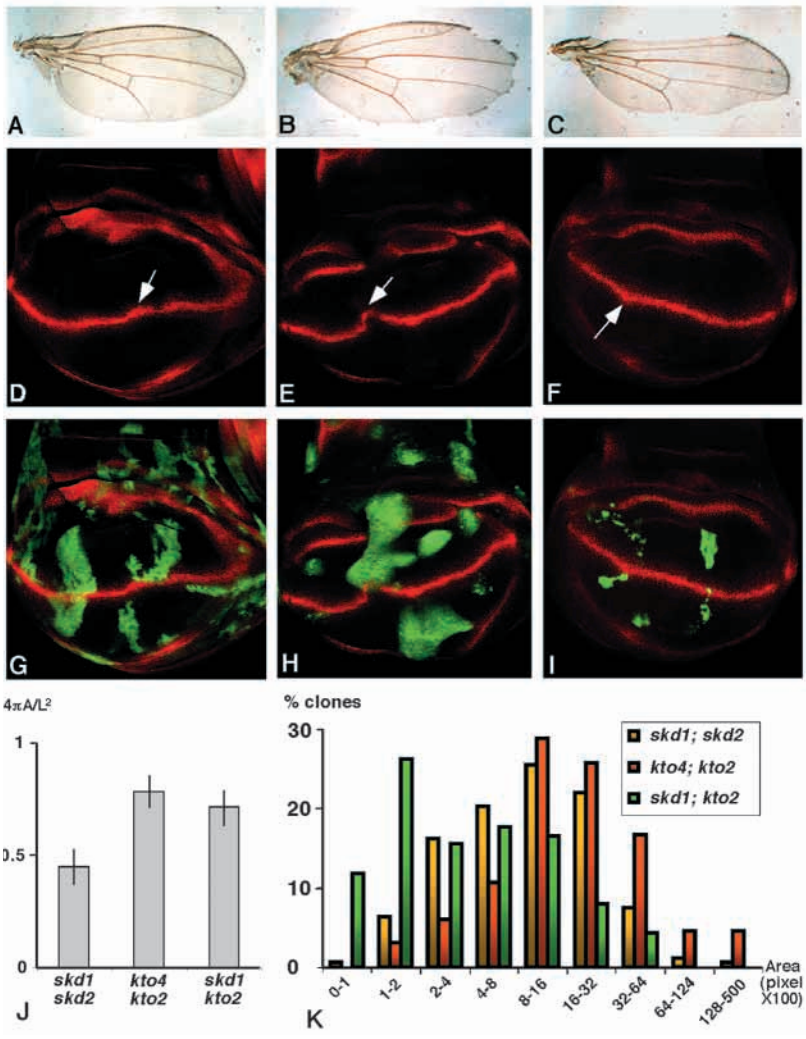

Fig. 4. *skd* and *kto* act synergistically and influence cell affinity when overexpressed. (A-C) Adult wings from flies overexpressing two copies of *skd* (A), two copies of *kto* (B), or one copy of *skd* and one copy of *kto* (C) with *vg*-GAL4. The wing margin is reduced by overexpression of either gene alone, but the effect is much stronger when both are co-expressed. (D-I) Wing discs, stained with Wg (red), in which clones expressing two copies of *skd* (D,G) two copies of *kto* (E,H), or one copy of *skd* and one copy of *kto* (F,I) have been induced. All clones are positively labeled by GFP expression (green; G-I). Arrows indicate clones that distort the DV boundary, producing a bulge in the Wg stripe. (J) Clones overexpressing two copies of *kto*, or one copy each of *skd* and *kto*, are rounder than clones overexpressing two copies of *skd*. Mean circularity for two copies of *skd* is 0.44, for two copies of *kto* 0.78 and for one copy of *skd* and one copy of *kto* 0.71. Lines within the bars indicate ±1 s.d. $P<0.001$ for a comparison of two copies of *skd* to *skd* and *kto*. The difference between two copies of *kto*, and *skd* and *kto* is not significant. (K) Size distribution of clones overexpressing two copies of *skd* (yellow), two copies of *kto* (orange), or one copy of *skd* and one copy of *kto* (green). Clones overexpressing both *skd* and *kto* are smaller in size. A total of 173 clones overexpressing two copies of *skd*, 66 clones overexpressing two copies of *kto* and 186 clones overexpressing both genes were analyzed.

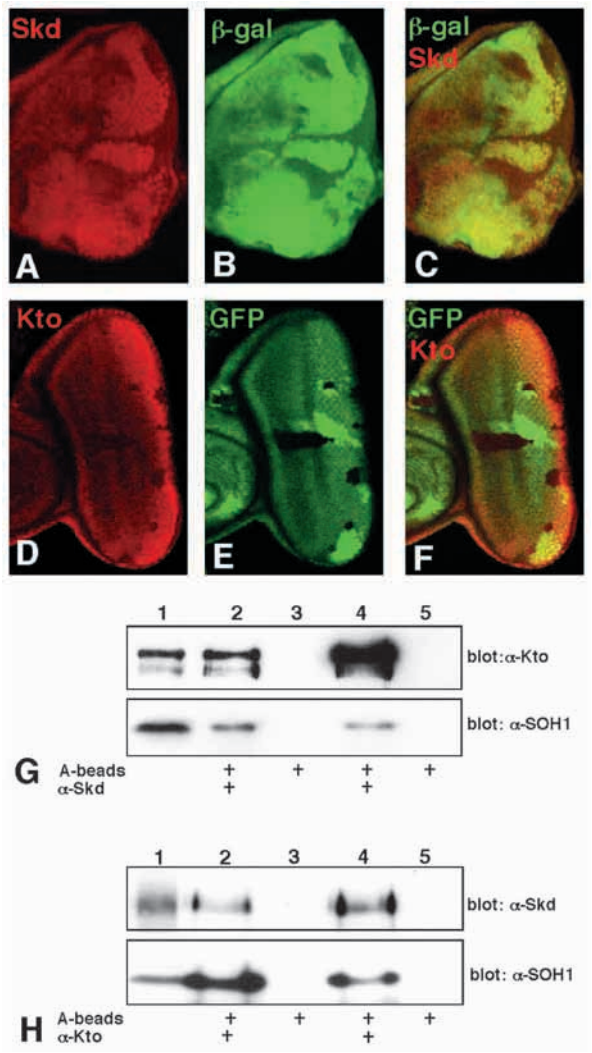

Fig. 5. The Skd and Kto proteins associate in vivo. (A-F) Third instar eye discs. (A-C) show skd^{T413}-mutant clones. Wild-type tissue is labeled by anti-β-gal staining reflecting *arm-lacZ* expression (green; B,C). Rabbit anti-Skd staining (red; A,C). (D-F) kto^{T241}-mutant clones. Wild-type tissue is labeled with GFP (green; E,F). Rat anti-Kto staining (red; D,F). In both cases, the antibodies do not stain mutant tissue, but show ubiquitous nuclear staining in wild-type tissue. The same pattern was observed for the guinea pig anti-Kto antibody (not shown). (G-H) Coimmunoprecipitation of Skd, Kto and SOH1. (G) Coimmunoprecipitation of Kto and Soh1 with an anti-Skd antibody. The upper gel is blotted with rat anti-Kto and the lower gel with anti-SOH1. Lane 1, 10% *white*$^-$ total embryonic extract; lane 2, IP with anti-Skd from *white*$^-$ extract; lane 3, control IP from *white*$^-$ extract; lane 4, IP with anti-Skd from extract overexpressing UAS-*skd* and UAS-*kto* with *da*-GAL4; lane 5, control IP from extract overexpressing UAS-*skd* and UAS-*kto* with *da*-GAL4. (H) Coimmunoprecipitation of Skd and Soh1 with the guinea pig anti-Kto antibody. The upper gel is blotted with anti-Skd and the lower gel with anti-SOH1. Lane 1, 2.5% *white*$^-$ total extract; lane 2, IP with anti-Kto from *white*$^-$ extract; lane 3, control IP from *white*$^-$ extract; lane 4, IP with anti-Kto from extract overexpressing UAS-*skd* and UAS-*kto* with *da*-GAL4; lane 5, control IP from extract overexpressing UAS-*skd* and UAS-*kto* with *da*-GAL4.

mediator component dSOH1 (Fig. 5G,H), which suggests that Skd and Kto can also associate outside the complex.

Because *skd* and *kto* are required for the expression of the Ci target genes that control cell affinity, we attempted to co-immunoprecipitate Skd and Kto with endogenous Ci, as well as with an HA-tagged activated form of Ci (Chen et al., 1999b). However, we were unable to detect any interaction above background levels (data not shown). Skd and Kto may therefore act indirectly on Ci target genes, may act directly on such genes without forming direct contacts with Ci, or may have an interaction with Ci too transient or unstable to be detected by immunoprecipitation.

DISCUSSION

Cell affinity differences require *skd* and *kto* function

To create a point or line source of a morphogen, populations of cells that signal to each other must be prevented from intermixing. Cells are thought to preferentially adhere to other cells that express the same surface adhesion molecules and to minimize their contacts with those expressing a different set of molecules (Dahmann and Basler, 1999). During segmentation of the vertebrate hindbrain, adhesion boundaries are formed by stripes of ephrin and Eph receptor expression (Xu et al., 1999). In *Drosophila*, there are few known candidates for the adhesion molecules regulating compartment boundary formation. However, some of the upstream regulators of these molecules have been identified. The different adhesive properties of cells on opposite sides of the AP compartment boundary in the wing disc are controlled by Ci activation in anterior cells close to the boundary and by En in posterior cells. The effect of Ci is more significant than that of En, as anterior cells containing only the repressor form of Ci, but lacking En, sort into the posterior compartment (Dahmann and Basler, 2000). Anterior cells mutant for *skd* or *kto* likewise sort into the posterior compartment. This phenotype suggests that *skd* and *kto* are required for the expression of adhesion molecules that are normally activated by Ci. We cannot test this model directly because no adhesion molecule has yet been found to have a distribution matching that of active Ci. An alternative possibility is that loss of *skd* or *kto* leads to the upregulation of an adhesion molecule that normally plays no role in compartment boundary formation. Upregulation of DE-cadherin has been shown to cause fusion of anterior and posterior clones, disrupting the compartment boundary (Dahmann and Basler, 2000). We do not favor this model because the crossing behavior of *skd* and *kto* clones is unidirectional along the AP axis, and upregulation of an unrelated adhesion molecule should produce bidirectional crossing.

Compartmentalization along the DV axis takes place during the second larval instar. *skd*- or *kto*-mutant clones generated after this time do not appear to cross the compartment boundary in either direction, which suggests that loss of *skd* or *kto* does not induce a switch from dorsal to ventral affinity or vice versa. Consistent with this, *skd* and *kto* are not required for the dorsal expression of Caps, an adhesion molecule that when misexpressed in ventral cells forces them toward the compartment boundary (Milan et al., 2001). When *skd*- or *kto*-mutant cells are generated before the compartment boundary

has formed, the boundary does not form a straight line within the clone; instead, an affinity boundary appears to separate mutant from either dorsal or ventral wild-type tissue. The bi-directional nature of these distortions again indicates that the mutant cells have not taken on the affinity of either compartment. *skd*- or *kto*-mutant clones also form straight boundaries with wild-type tissue in both the dorsal and ventral compartments, and round up to minimize their contact with wild-type cells. Loss of *skd* and *kto* may prevent the establishment of both dorsal and ventral affinity, or may promote the acquisition of a novel affinity. N activation is required to establish the DV boundary; however, alterations in N signaling relocate the Wg stripe to the border of the mutant clone, rather than to the border of *ap* expression (Micchelli and Blair, 1999; Rauskolb et al., 1999). In *skd*- and *kto*-mutant clones, Wg remains at the border of the *ap* expression domain. If the effects of *skd* and *kto* on affinity are mediated by changes in N signaling, they must alter transcriptional regulation by N in a way that leaves *wg* expression unaffected. This would be consistent with changes in the expression of other N-regulated genes that we have observed in *skd*- and *kto*-mutant clones (F.J. and J.E.T., unpublished).

The adhesion molecules underlying compartment boundary formation have been notoriously elusive, even in screens specifically designed to identify them (Vegh and Basler, 2003), perhaps because multiple molecules each make a small contribution to cell affinity. Caps and Trn appear to confer some aspects of dorsal affinity on ventral cells, but loss of both molecules does not cause dorsal cells to cross into the ventral compartment (Milan et al., 2001); thus, if these proteins are involved in compartmentalization they must be redundant with other signals. We considered the possibility that the *Drosophila* Ephrin and Eph receptor might act downstream of *skd* and *kto* to control cell affinity differences. However, neither Dephrin (Ephrin – FlyBase) nor Eph (Bossing and Brand, 2002; Dearborn et al., 2002; Scully et al., 1999) showed compartment-specific expression in the wing disc, and their expression levels were unaltered in *skd*- and *kto*-mutant clones (data not shown). In addition, overexpression of wild-type Eph failed to rescue the boundary crossing behavior of *skd*-mutant clones (data not shown). Although we have not been able to identify the crucial adhesion molecules for boundary formation, our demonstration that they are likely to be among the target genes of Skd and Kto may aid in their discovery.

Skd and Kto regulate a subset of Ci target genes

Our results imply that Skd and Kto assist Ci to regulate the genes that confer anterior cell affinity, yet *skd* and *kto* are clearly not required for Ci to activate *dpp* or *ptc*. This represents the first situation in which the effects of Hh on cell affinity are specifically disrupted without a global effect on Hh signaling. Hh regulates Ci both by blocking its cleavage to a repressor form, and by converting the full-length protein to a transcriptional activator and transporting it to the nucleus (Aza-Blanc et al., 1997; Chen et al., 1999a; Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998; Wang and Holmgren, 2000). Some Ci target genes are thought to be controlled primarily by the repressor or the activator form, whereas others respond to both (Methot and Basler, 1999). However, both the repressor and activator forms have been shown to act through common DNA binding sites in a minimal *dpp* enhancer (Muller and Basler, 2000). The effects of Skd and Kto cannot be specific for the repressor form of Ci, as *hh*, which is a target of the repressor form (Methot and Basler, 1999), is not de-repressed in *skd*- or *kto*-mutant cells in the anterior wing disc (data not shown).

skd and *kto* likewise affect only a subset of Hh target genes in the eye disc. In *skd*- or *kto*-mutant clones in the eye disc, Hh is still able to activate *ato* and *dpp* expression, although at slightly reduced levels (Treisman, 2001). However, expression of another Hh target gene, *rough* (Dominguez, 1999), is lost in these clones (J.E.T., unpublished). The enhancer sequences mediating Hh regulation of these genes have not yet been analyzed in detail; it will be interesting to determine what features of an enhancer make it dependent on Skd and Kto to recruit the mediator complex. We also do not know whether Skd and Kto interact directly with Ci, or with other factors binding to the same enhancer element. Although we cannot detect a stable interaction between Skd and Ci by immunoprecipitation, it is still possible that a transient interaction or an interaction with a small proportion of the total Ci protein occurs in vivo. It is also possible that Skd and Kto do not affect the activity of Ci directly, but assist a transcription factor downstream of Ci that activates a subset of its target genes.

TRAP230 and TRAP240 may constitute a submodule of the mediator complex

Our current and previously published data (Treisman, 2001) demonstrate that loss of either *skd, kto* or both genes has exactly the same effect, and that the two genes produce a more severe phenotype when overexpressed in combination. We have also shown here that the Skd and Kto proteins interact with each other, and it seems that this interaction can occur outside the mediator complex. These observations strongly suggest that Skd and Kto function as a unit. Both proteins might interact simultaneously with transcription factors such as Ci; alternatively, one of the two proteins might be required to attach the other to the mediator complex. The SUR2, TRAP100 and TRAP95 subunits of the mouse complex appear to associate as a submodule, with both SUR2 and TRAP100 required for its incorporation (Ito et al., 2002; Stevens et al., 2002). TRAP240 and TRAP230 may form another such submodule. Together with Cdk8 and Cyclin C, they are present only in larger forms of the mediator complex, such as ARC, TRAP, DRIP or NAT, but not in smaller forms, such as PC2 and CRSP (Rachez and Freedman, 2001). Interestingly, the larger ARC complex fails to promote transcription from an Sp1 and SREBP-dependent enhancer that is strongly activated by CRSP (Taatjes et al., 2002). Although the roles of individual subunits in promoting this selectivity have not been defined, it is possible that TRAP230 and TRAP240 could prevent the mediator complex from acting on certain enhancers while promoting its activity on others. However, it is also possible that the repressive effect is caused by phosphorylation of TFIIH by Cdk8 (Akoulitchev et al., 2000), or to the absence of Crsp70. Distant homologs of TRAP240, TRAP230, Cdk8 and Cyclin C, the yeast Srb8-11 proteins, also form a separable submodule of the mediator complex that can repress transcription by phosphorylating RNA polymerase II, but that can also phosphorylate activators (Ansari et al., 2002; Borggrefe et al., 2002; Boube et al., 2002; Hengartner et al., 1998; Lee et al., 2000; Nelson et al., 2003; Song and Carlson, 1998).

These observations suggest that the mediator complex consists of a core complex, perhaps PC2/CRSP or a smaller subset of these subunits, and accessory subcomplexes that interact with specific sets of transcription factors (Malik and Roeder, 2000). In *Drosophila*, mutations in *dTrap80* and *dMed6* are both cell-lethal, suggesting that these subunits are essential for crucial functions of the mediator complex (Boube et al., 2000; Gim et al., 2001). Loss of any of several core components of the *C. elegans* mediator complex causes embryonic lethality (Kwon et al., 2001; Kwon and Lee, 2001; Kwon et al., 1999), whereas mutations in *sur-2*, *sop-1*, encoding a TRAP230 homolog, and *sop-3*, encoding a TRAP220 homolog, cause milder defects (Singh and Han, 1995; Zhang and Emmons, 2000; Zhang and Emmons, 2001). Human TRAP220 has been shown to interact specifically with nuclear receptors (Hittelman et al., 1999; Yuan et al., 1998), and knocking out the mouse gene prevents cells from responding to thyroid hormone and estrogen (Ito et al., 2000; Kang et al., 2002). It is not known with which transcription factors TRAP230 and TRAP240 interact. In addition to their effects on cell affinity, we have evidence that *skd* and *kto* are required for the expression of some Wg and N target genes (F.J. and J.E.T., unpublished). These very large and highly conserved proteins are likely to present a large number of interaction surfaces, or perhaps even exhibit enzymatic activities. Their further study will shed light on the functions of the mediator complex and its interactions with specific developmental signaling pathways.

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