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 determined cell populations. For example, cells at the mouse centers that form at the boundary between differently developed structures is controlled by organizing centers. These centers 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 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;
Sequence-specific transcription factors require the recruitment of co-activators 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 Friedman, 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 nuclear receptors; the ligand-binding domains of these proteins may act as adaptors for specific transcription functions. In addition to the essential subunits of this complex, some subunits are required by in vitro studies 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 from CRSP. ARC and CRSP have distinct activities in vitro (Taatjes et al., 2002). Distant homologs of these four proteins from Xenopus, which encode 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+]M2-1504) onto the skd1-166 chromosome and a P(w+) element close to skd (FR212A) onto the kto4-41 chromosome, and then testing the complementation behavior of v recombinants between these two chromosomes. Other strains used were en-lacZ (Bliar, 1992), ap-lacZ, caps-lacZ (Milan et al., 2001), ptc-lacZ (Johnson et al., 1995), DI-lacZ (FlyBase), hh-lacZ (Lee et al., 1992), vg-GAL4 (Simmonds et al., 1995), da-GAL4 (Benali et al., 2000), tub-GAL4 (Lee and Luo, 1999) and UAS-Eph (Dearborn et al., 2002). Clones were generated by crossing to hsFLP122; 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 eyFLP1 rather than hsFLP122. 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, hsFLP122, UAS-GAL4; 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 Ca2+, hsFLP122, UAS-GFP; tub-GAL4/Cyo; FRT80, tub-GAL80 females were crossed to UAS-HAC(m1-4); hh-lacZ, FRT80, kto7053 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 were used rat anti-β-C (Mozny 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 (Bosson 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 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, Srh18-11, 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 skald [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.

**Protein extraction and immunoprecipitation**

Proteins were extracted from 0- to 23-hour-old white-eyed 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 homogenized in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5% Nonidet P-40, 5 mM EDTA, and 10 mM MgCl2, containing a cocktail of protease inhibitors (Brockman et al., 1997). Precipitation of the core mediator complex was performed as described (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, Srh18-11, 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).

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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 Na2VO4). 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 5xLaemmli 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 2xLaemmli 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

| Table 1. Quantification of boundary crossing cell affinity of skd166A-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-F.

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

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. 1L), although it was able to ectopically activate ptc expression in kto-mutant clones (data not shown).

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

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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. 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) skdT606 clone. en-lacZ expression, detected by anti-β-gal staining (blue), marks the posterior compartment. (E-H) ktoT241 clone. Anti-β-gal staining (blue) revealing ptc-lacZ expression (F,H). Arrows indicate anterior clones that have crossed into the posterior compartment. (LJ) ktoT663 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 4πA/L², 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 skdT606-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 ktoT663 clone entirely within the dorsal 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 (IJ). (K,L) Second instar wing discs containing skdT413-mutant clones. Wild-type tissue is labeled with GFP (green; L). Anti-β-gal (red) staining reflecting caps-lacZ expression (KL). 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).
skd-mutant cells might have reduced 2K,L. There are two other possible explanations for the was still expressed in dorsal caps-lacZ instar discs (Milan et al., 2001). As expected, we found that are expressed at normal levels within the mutant tissue. dashed line in I) and distort the DV boundary (arrow; H). Ci and Wg Double-mutant clones cross the AP compartment boundary (white clones, although cell growth and survival are unaffected. (D-F) Atonal staining of ktoT314 clones (brown; D) or skdT606, ktoT241 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 skdT606, ktoT241 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. (Trm), 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 with 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 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.
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

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

<table>
<thead>
<tr>
<th>skd rescue</th>
<th>% rescue to pupal stage</th>
<th>Number</th>
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<tr>
<td>skdT11; da-GAL4/TM6B×skdT060/TM6B</td>
<td>0</td>
<td>262</td>
</tr>
<tr>
<td>skdT11/TM6B×skdT060, UASskd1/TM6B</td>
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<td>203</td>
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<tr>
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<tr>
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<td>54</td>
<td>128</td>
</tr>
<tr>
<td>skdT11; UAS-GAL4/TM6B×skdT411; UASskd3/S66.TM6B</td>
<td>109</td>
<td>252</td>
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<table>
<thead>
<tr>
<th>kto rescue</th>
<th>% rescue to pupal stage</th>
<th>Number</th>
</tr>
</thead>
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<tr>
<td>ktoT355; da-GAL4/TM6B×ktoT241/TM6B</td>
<td>0</td>
<td>535</td>
</tr>
<tr>
<td>ktoT355/TM6B×ktoT241, UASKto1/S66.TM6B</td>
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<td>413</td>
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<tr>
<td>ktoT355, da-GAL4/TM6B×ktoT241, UASKto1/S66.TM6B</td>
<td>80</td>
<td>381</td>
</tr>
</tbody>
</table>

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 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

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, 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.
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 DEC-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 bidirectional 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 (Domínguez, 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 (Raczez 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; Borggreve 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 dMed60 are both cell-lethal, suggesting that these subunits are essential for crucial functions of the mediator complex (Bouje 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; Yen 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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A mediator submodule controls cell affinity.