Pygopus activates Wingless target gene transcription through the mediator complex subunits Med12 and Med13

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Edited by Matthew P. Scott, Stanford University School of Medicine, Stanford, CA, and approved March 10, 2008 (received for review October 12, 2007)

Wnt target gene transcription is mediated by nuclear translocation of stabilized β-catenin, which binds to TCF and recruits Pygopus, a cofactor with an unknown mechanism of action. The mediator complex is essential for the transcription of RNA polymerase II-dependent genes; it associates with an accessory subcomplex consisting of the Med12, Med13, Cdk8, and Cyclin C subunits. We show here that the Med12 and Med13 subunits of the *Drosophila* mediator complex, encoded by *kohtalo* and *skuld*, are essential for the transcription of Wingless target genes. *kohtalo* and *skuld* act downstream of β-catenin stabilization both *in vivo* and in cell culture. They are required for transcriptional activation by the N-terminal domain of Pygopus, and their physical interaction with Pygopus depends on this domain. We propose that Pygopus promotes Wnt target gene transcription by recruiting the mediator complex through interactions with Med12 and Med13.

Drosophila | kinase module | *kohtalo* | *skuld* | Wnt

The mediator complex was first defined in yeast as a large multisubunit complex required for transcription of RNA polymerase II (PolII)-dependent genes. Since then, its composition and function have been shown to be conserved in *Drosophila*, mouse, and human cells (1–3). The mediator complex can directly bind to Pol II and recruit it to target promoters (4–6), but it also appears to function at a step subsequent to Pol II assembly into the preinitiation complex (7, 8). Several mediator subunits have been shown to act as adaptors for specific transcription factors, linking them to the mediator complex and allowing them to activate transcription (9–14).

Four subunits, Med12, Med13, Cdk8, and Cyclin C (CycC), form an accessory subcomplex known as the kinase module (15–17). Genetic and microarray analyses in yeast implicate the kinase module primarily in transcriptional repression (18, 19), but it also contributes to activation by GAL4 and p53 (20, 21). Many of its effects have been attributed to the Cdk8 kinase, which phosphorylates the C-terminal domain of Pol II (22, 23), the Cyclin H component of the TFIIH general transcription factor (24), and other subunits of the mediator complex (19), as well as specific transcription factors (25–29). The large Med12 and Med13 proteins are required for specific developmental processes in *Drosophila*, zebrafish, and *Caenorhabditis elegans* (30–38), but their biochemical functions are not understood.

Secreted proteins of the Wnt family play important roles in both development and oncogenesis. Transcription of Wnt target genes is mediated by nuclear translocation of stabilized Armadillo (Arm)/β-catenin and its binding to the HMG box transcription factor TCF (reviewed in ref. 39). The adaptor protein Legless (Lgl)/Bcl-9 links Arm/β-catenin to Pygopus (Pygo); the N-terminal homology domain (NHD) of Pygo is essential for Wnt-regulated transcriptional activation and is thought to interact with unknown general transcriptional regulators (40–45). We show here that the Med12 and Med13 subunits of the *Drosophila* mediator complex, encoded by *kohtalo* (*kto*) and *skuld* (*skd*) (30), are essential for the transcription of Wingless (Wg) target genes *in vivo* and a Wg-responsive reporter in cultured cells. *skd* and *kto* act downstream of Arm stabilization and are required for the function of the NHD of Pygo when fused to an exogenous DNA-binding domain. Skd and Kto interact with Pygo *in vivo* through the NHD. We suggest that this interaction recruits the mediator complex to allow for the transcription of Wg target genes.

Results

***skd* and *kto* Regulate Wingless Target Genes.** We have previously shown that the *Drosophila kto* and *skd* genes encode Med12 and Med13, two subunits of an accessory submodule of the mediator complex, and that they have identical effects on photoreceptor differentiation in the eye imaginal disc and on compartmental cell affinities in the wing disc (30, 31). In addition, we observed a consistent requirement for both *skd* and *kto* for the expression of genes that are positively regulated by the Wnt family member Wg. In the third instar larval wing disc, Wg is expressed in a narrow stripe at the dorsal–ventral (DV) boundary, the primordium of the adult wing margin (46). From this position, it activates the expression of genes that include the long-range targets *vestigial* (*vg*) and *Distal-less* (*Dll*) and the short-range target *senseless* (*sens*) (42, 47, 48). Although Wg was still present at the DV boundary in clones of *skd* or *kto* mutant cells (Fig. 1 *A* and *B*), the expression of *Dll-lacZ* (48), *lacZ* driven by the *vg* quadrant enhancer (49), and Sens was autonomously lost in these clones (Fig. 1 *C–H*). In addition, *wg-lacZ* expression was expanded in *skd* and *kto* clones on the wing margin [supporting information (SI) Fig. S1 *A* and *B*], suggesting that *skd* and *kto* are required for Wg to prevent its own transcription in neighboring cells (50). Repression of *teashirt* (*tsh*) in the wing pouch similarly requires Wg (51) and *skd* and *kto* (Fig. S1 *C* and *D*).

In the eye disc, Wg is expressed at the lateral margins independently of *skd* and *kto* (30), and it activates the target gene *dachsous* (*ds*) (52). *ds-lacZ* expression was strongly reduced in *skd* or *kto* mutant clones (Fig. 1 *I* and *J*). A ventral wedge of Wg expression in the leg disc activates the target gene *H15*, and Wg combines with dorsally expressed Decapentaplegic (Dpp) to

Author contributions: I.C., F.J., and N.L. contributed equally to this work; I.C., F.J., N.L., F.D., and J.E.T. designed research; I.C., F.J., N.L., F.D., and J.E.T. performed research; I.C., F.J., N.L., F.D., and J.E.T. analyzed data; and I.C., F.J., and J.E.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0709749105/DCSupplemental.

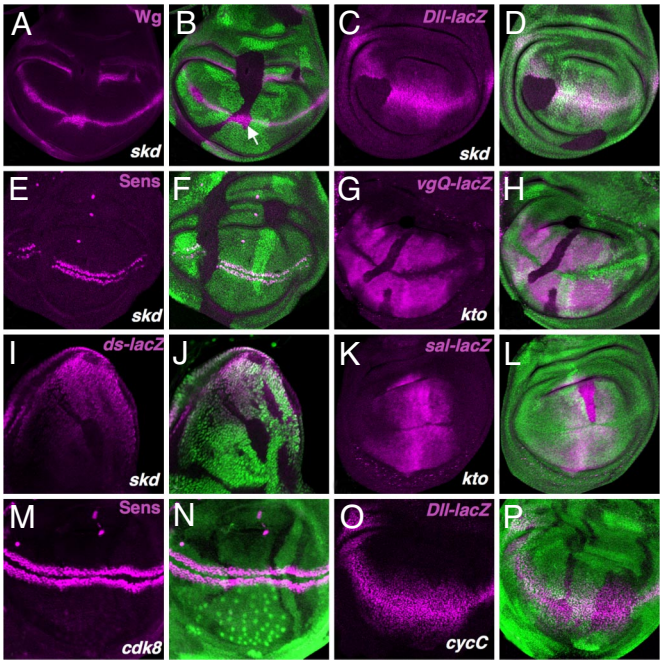

Fig. 1. *skd* and *kto* are required for the expression of Wg target genes. Third instar wing discs (*A–H* and *K–P*) and third instar eye disc (*I* and *J*) are shown. Anterior is to the left and dorsal is up in this and subsequent figures. The phenotypes of *skd* and *kto* mutations are indistinguishable (30, 31), so only one genotype is shown in each experiment. Clones homozygous for skd^{T413} (*A–F*), kto^{T241} (*G* and *H*), skd^{T606} (*I* and *J*), kto^{T555} (*K* and *L*), $cdk8^{K185}$ (*M* and *N*), or $cycC^{Y5}$ (*O* and *P*) are marked by the absence of GFP (green in *B*, *D*, *F*, *H*, *J*, *L*, *N*, and *P*). (*A* and *B*) Wg staining in magenta; the arrow in *B* indicates a clone at the wing margin. (*C*, *D*, *O*, and *P*) β-gal staining reflects *Dll-lacZ* expression in magenta. (*E*, *F*, *M*, and *N*) Sens staining is in magenta. (*G* and *H*) β-gal staining reflects *vgQ-lacZ* expression in magenta. (*I* and *J*) β-gal staining reflects *ds-lacZ* expression in magenta. (*K* and *L*) β-gal staining reflects *sal-lacZ* expression in magenta. Although Wg is still expressed at the wing margin in *skd* or *kto* mutant clones, expression of Wg target genes is lost. However, Wg target genes are unaffected in *cdk8* or *cycC* mutant clones.

induce a circular domain of *Dll* (53, 54). *skd* and *kto* clones also showed a reduction of both *H15* and *Dll* expression (Fig. S1 *I–L*). Thus, Wg fails to activate its target genes in the absence of either *skd* or *kto*. However, *skd* and *kto* were not required for the expression of the Dpp target gene *spalt* (*sal*) (Fig. 1 *K* and *L*) (55) and had no apparent effect on cell proliferation or survival, as judged by clone size and staining for mitotic and apoptotic cells (data not shown). Interestingly, Wg target gene expression does not require the entire kinase module; loss of *cdk8* or *cycC* had no effect on *sens* or *Dll* (Fig. 1 *M–P*).

***skd* and *kto* Act Downstream of Arm Stabilization.** The effect of *skd* and *kto* mutations on Wg target gene expression could be due to either a requirement of Skd- and Kto-containing mediator complexes for transcriptional activation by the TCF–Arm–Lgs–Pygo complex or an indirect effect on the expression or activity of a component of the Wg-signaling pathway. In the former case, *skd* and *kto* should be required downstream of Arm stabilization by Wg. Consistent with this site of action, we detected no reduction in the levels of Arm protein in *skd* or *kto* mutant clones (Fig. S1 *E–G*). We next tested whether Skd and Kto were required for the function of an activated form of Arm (ArmΔN) (48), which lacks the N-terminal region that is a target for destabilizing phosphorylation by Shaggy/Glycogen synthase kinase 3. When expressed in wild-type clones within the wing pouch, ArmΔN ectopically activated *Dll* expression (Fig. 2 *A* and *B*). However, ArmΔN was unable to induce *Dll* either ectopically

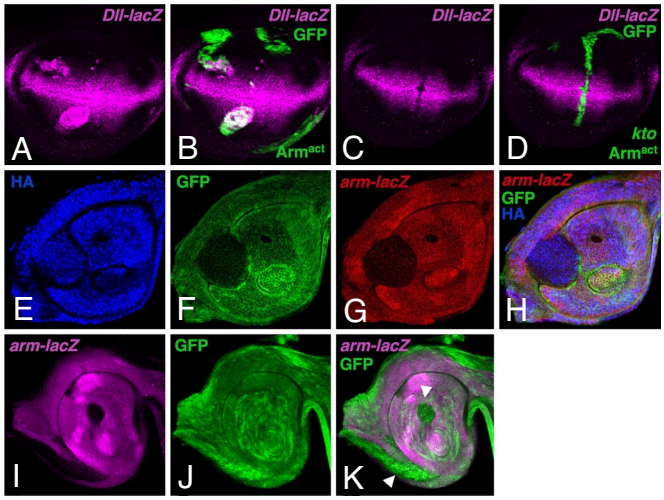

Fig. 2. *skd* and *kto* act downstream of Arm and Pygo. Third instar wing discs (*A–D*) and third instar antennal discs (*E–K*) are shown. (*A* and *B*) Clones expressing ArmΔN are marked by coexpression of GFP (green in *B*). (*C* and *D*) Clones expressing ArmΔN and homozygous for kto^{T631} are marked by coexpression of GFP (green in *D*). β-gal staining reflecting *Dll-lacZ* expression is shown in magenta in *A–D*. ArmΔN can activate *Dll* expression in wild-type, but not *kto* mutant, cells. (*E–H*) HA staining (blue in *E* and *H*) shows expression of *hs*-HAPygoGAL4 after a 2-h heat shock and a 2-h recovery. UAS-GFP expression is shown in green in *F* and *H*. Clones homozygous for skd^{T413} are marked by a lack of *arm-lacZ* expression (β-gal staining in red in *G* and *H*). Loss of *skd* does not affect the expression of *hs*-HAPygoGAL4, but abolishes its ability to activate UAS-GFP. (*I* and *K*) Clones homozygous for kto^{T555} (arrowheads) are marked by lack of *arm-lacZ* expression (β-gal staining in magenta in *I* and *K*). UAS-GFP expression driven by *tub*-GAL4 is shown in green in *J* and *K*. GAL4 can activate UAS-GFP in the absence of *kto*.

or within its normal expression domain when expressed in *skd* or *kto* mutant clones (Fig. 2 *C* and *D*). Thus, *skd* and *kto* are required for the activity of a form of Arm that is independent of upstream components of the Wg pathway. They must therefore affect transcription of target genes either directly or through a component that acts downstream of Arm.

***skd* and *kto* Act on a Wg Reporter in Kc Cells.** To further test whether *skd* and *kto* are directly required for transcription of Wg target genes, we examined the expression of a luciferase reporter driven by multiple TCF-binding sites (56) in cultured *Drosophila* Kc cells. This reporter was strongly activated (≈2,000-fold) by RNAi directed against *axin*, a negative regulator of Arm stability (Fig. 3*A*) (57). Expression of the TCF firefly luciferase reporter was normalized to the expression of Renilla luciferase driven by a Pol III promoter to correct for transfection efficiency (56). When we also included dsRNA homologous to *skd* or *kto*, the expression of the TCF luciferase reporter was reduced 80- to 100-fold (Fig. 3*A*). Depletion of *skd* or *kto* had much less effect on the expression of a UAS luciferase reporter that was activated (≈150-fold) by GAL4, reducing expression of this reporter ≈3-fold (Fig. 3*A*). This finding confirms that *skd* and *kto* are required downstream of Arm stabilization and shows that they act on a direct target of TCF. Interestingly, we found that the removal of Skd by RNAi also partially destabilized the Kto protein (Fig. 3*B*), suggesting that Skd may be required for Kto incorporation into the mediator complex or a stable subcomplex. We observed a similar effect *in vivo*; clones mutant for *skd* in the wing disc had reduced levels of Kto protein, whereas Skd protein was unaffected in *kto* mutant clones (Fig. 3 *C–H*).

Skd and Kto Are Recruited by Pygo. Pygo is one of the most downstream components of the Wg transcriptional complex and

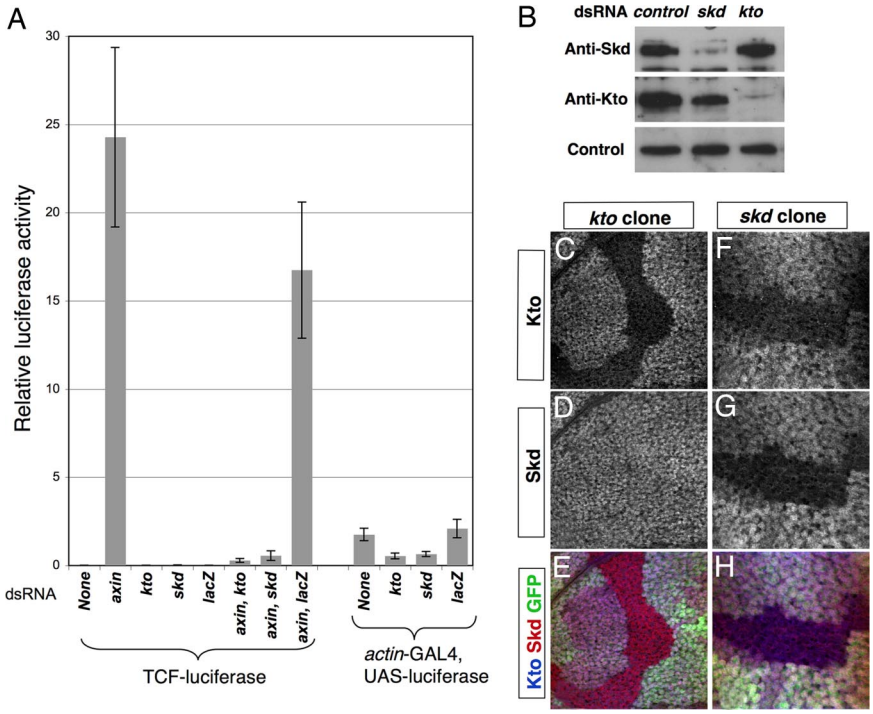

Fig. 3. *skd* and *kto* are required for the expression of a Wg reporter in cultured cells. (*A*) Ratio of TCF firefly luciferase to the transfection control Pol III-RL in Kc cells treated with the indicated dsRNAs. The TCF luciferase reporter is strongly activated when *axin* is knocked down by RNAi, but this activation is reduced 80- to 100-fold by knocking down *skd* or *kto* in addition to *axin*. In Kc cells transfected with *actin*-GAL4 and UAS-luciferase, knocking down *skd* or *kto* reduces activation of the reporter by ≈3-fold. Error bars indicate the standard deviation between the triplicate samples tested for each dsRNA. This figure is a representative example of three independent experiments. (*B*) Western blot showing the levels of Skd and Kto protein in Kc cells treated with *cdk8* (control), *skd*, or *kto* dsRNA. *skd* knockdown also partially reduces the level of Kto protein. The bottom blot shows a band that cross-reacts with the Kto antibody and serves as a loading control. (*C–H*) Wing imaginal discs with clones homozygous for kto^{T241} (*C–E*) or skd^{T606} (*F–H*) marked by the absence of GFP (green in *E* and *H*) and stained with anti-Kto (*C* and *F*; blue in *E* and *H*) and anti-Skd (*D* and *G*; red in *E* and *H*). Kto protein is reduced in *skd* mutant cells, but Skd protein is unaffected in *kto* mutant cells.

is thought to link the complex to the general transcriptional machinery. Pygo interacts with Lgs through its PHD domain, and its NHD can activate transcription in cultured cells through an unknown mechanism (40, 41, 44, 45). To test whether *skd* and *kto* act downstream of Pygo, we used a chimeric protein in which the PHD domain of Pygo is replaced by the DNA-binding domain of GAL4, preventing it from binding to Lgs and allowing it to recognize UAS sequences (43). When expressed under the control of *hsp70* promoter elements, PygoΔPHD-GAL4 was capable of activating UAS-GFP expression *in vivo* (Fig. 2*F*). This artificial reporter was activated in all cells independently of Wg signaling, reflecting only the activity of the Pygo NHD. Expression of UAS-GFP was lost in clones mutant for either *skd* or *kto*, although the expression of the HA-tagged PygoΔPHD-GAL4 protein was unaffected (Fig. 2 *E–H*). In contrast, the full-length GAL4 protein containing its own activation domain was able to activate UAS-GFP expression even in the absence of *skd* or *kto* (Fig. 2 *I–K*). This finding strongly suggests that transcriptional activation by Pygo specifically requires mediator complexes that contain the Skd and Kto subunits.

Several mediator complex subunits have been shown to act as adaptors for specific transcription factors, linking them to the mediator complex and allowing them to activate transcription (9–14). Similarly, Skd and Kto might promote activation by the Pygo NHD by physically interacting with Pygo and allowing it to recruit the mediator complex. Consistent with this model, we found that endogenous Skd coimmunoprecipitated HA-tagged Pygo from embryos (Fig. 4*A*) and Kc cells (Fig. 4*B*). As expected, binding was not detected when Skd was knocked down by RNAi (Fig. 4*B*). The extent of coimmunoprecipitation was strongly reduced (Fig. 4*A* and data not shown) if the NHD of Pygo was deleted or contained point mutations known to abolish its transcriptional activity (45, 58, 59). In three separate experiments, wild-type Pygo was precipitated 5.1 ± 1.1-fold more efficiently than PygoΔNHD. This result indicates that Pygo requires its NHD for strong interaction with mediator complexes containing Skd. This interaction is independent of Pygo binding to Lgs and Arm because PygoΔPHD-GAL4 also coimmunoprecipitated with endogenous Skd from embryos (Fig. 4*C*). However, we were not able to demonstrate a direct interaction of Pygo with Skd or Kto by using either yeast two-hybrid assays or GST pulldowns. A GST fusion protein containing the first 181 amino acids of Pygo interacted with *in vitro*-translated ^{35}S-labeled Skd and Kto proteins more strongly than with Luciferase, but this interaction did not map to a specific region of one of the two proteins and was not abolished by point mutations in the NHD (Fig. S2 and *SI Materials and Methods*). The failure of these experiments to reveal a direct interaction leaves open the possibility that an intermediary protein links Pygo to Skd and Kto.

Discussion

Two domains of Arm/β-catenin are important for the activation of Wnt target genes: (*i*) Arm repeats 1–4, which act by binding Lgs and thus recruiting Pygo, and (*ii*) a C-terminal transcriptional activation domain (39). The C-terminal domain has been shown to bind to the histone acetyltransferases p300 and CBP (60, 61), Hyrax/Parafibromin, which recruits histone modification complexes (62), and directly to the Med12 mediator complex subunit (63). However, this domain is insufficient for target gene activation *in vivo*, which requires Lgs, Pygo, and an amino acid in Arm that is critical for Lgs binding (44, 64). In addition,

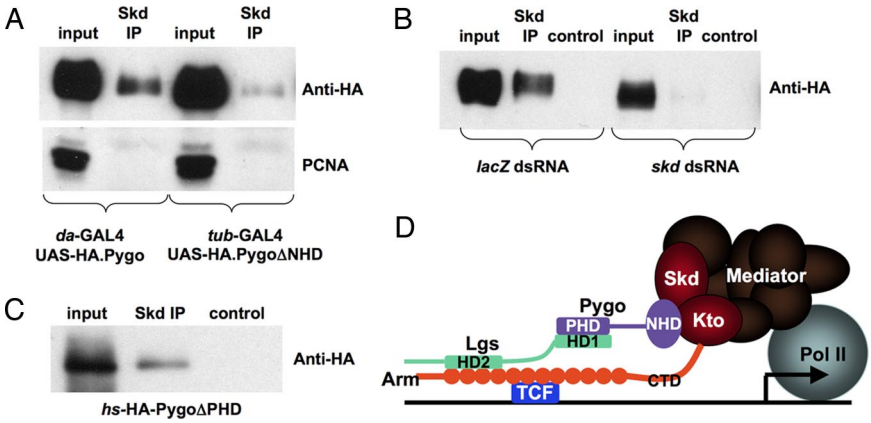

Fig. 4. Pygo physically interacts with Skd. (*A*) Anti-Skd immunoprecipitations (IPs) of extracts from embryos expressing UAS-HAPygo or UAS-HAPygoΔNHD (58) with the ubiquitous drivers *daughterless* (*da*)-GAL4 or *tubulin* (*tub*)-GAL4. Input lanes show 1% of the input for the IP. HAPygoΔNHD is less efficiently coimmunoprecipitated with anti-Skd than full-length Pygo. The lower blot shows that the unrelated nuclear protein PCNA does not coimmunoprecipitate with anti-Skd. (*B*) Coimmunoprecipitation of HAPygo with anti-Skd from Kc cells treated with *lacZ* or *skd* dsRNA. Removing Skd protein greatly reduces Pygo coimmunoprecipitation, demonstrating the specificity of the Skd antibody. Input lanes show 0.5% of the input, and control lanes show IPs with Protein A beads but no primary antibody. (*C*) Coimmunoprecipitation of a Pygo construct that lacks the PHD domain, HA-PygoΔPHDGAL4, with anti-Skd. Input lane shows 1% of the input, and the control lane shows an IP with no primary antibody. This figure shows that the interaction with Skd is independent of Pygo binding to the Lgs/Arm/TCF complex. (*D*) Model consistent with our results. Pygo, one of the most downstream components of the Wg-responsive transcriptional complex, may recruit the mediator complex through interactions of its NHD with Skd/Med13 and Kto/Med12, leading to transcriptional activation of Wg target genes. The C-terminal domain of Arm also directly interacts with Med12 (63), enhancing binding to the mediator complex; this interaction may explain why *skd* and *kto* have a stronger effect than *pygo* on Wg target genes.

although the C-terminal domain is a strong activator in cell culture, it is not sufficient to replace the function of Arm *in vivo* when fused to dTCF, whereas the activation domain of Pygo is (43, 65). It has been proposed that Pygo interacts with unidentified general transcriptional regulators through its NHD (41). Our results suggest that the Pygo NHD recruits the mediator complex through the Kto/Med12 and Skd/Med13 subunits and that these subunits are essential for its activation function (Fig. 4*D*).

An alternative view of the role of Pygo is that it acts as a nuclear anchor for Lgs and Arm (66). This model has been further refined by recent data showing that Pygo is constitutively localized to Wg target genes in a manner dependent on its NHD and on TCF, and it might function there to capture Arm (58). However, our finding that PygoΔPHD-GAL4 is sufficient to activate UAS-GFP expression in all cells *in vivo* strongly supports an additional activation function for Pygo. We suggest that this function reflects its ability to recruit the mediator complex. Interestingly, the *C. elegans* Med12 and Med13 homologues have been implicated in the transcriptional repression of Wnt target genes although these effects have not been shown to be direct (34, 36). Their dispensability for Wnt target gene activation may reflect the absence of *pygo* homologues in the worm genome.

The kinase module of the mediator complex is commonly thought to have a repressive function; it has been shown to sterically hinder recruitment of Pol II (67), and Ras signaling promotes transcriptional elongation by inducing loss of this module from the mediator complex bound to C/EBP-regulated promoters (68). However, recent results suggest that the kinase module can play a role in transcriptional activation as well as repression (20, 21). An exclusively repressive function would be difficult to reconcile with the observation that the genome-wide occupancy profiles of Cdk8 and Med13 characterized by ChIP match that of the core mediator complex (69, 70). Our results support an essential and direct function for the Med12 and Med13 subunits in the activation of Wg target genes. The transcriptional and phenotypic profiles of mutants in the four subunits of the yeast kinase module are very similar (19, 71, 72). However, *Drosophila cdk8* and *cycC* are only required for a subset of the functions of *skd* and *kto* (73) that does not include Wg target gene activation (Fig. 1). Therefore, Med12 and Med13 may have gained additional functions during the evolution of higher eukaryotes. The identical defects of the two mutants may reflect the requirement for Skd to stabilize the Kto protein. Similarly, Med24 stabilizes Med16 and Med23 and promotes their incorporation into the mediator complex (74).

Several mediator complex subunits act as adaptors that link specific transcription factors to the mediator complex. For example, Med1 interacts with nuclear receptors (10, 75); Med23 interacts with phosphorylated Elk-1, the adenovirus E1A protein, and Heat shock factor (11, 13); Med16 interacts with differentiation-inducing factor (13); and Med15 interacts with Smad2/3 and Sterol regulatory element-binding protein (9, 14). Our results show that, despite their location in a module that is not part of the core mediator complex, Med12 and Med13 act as adaptors for Pygo. These subunits also are likely to act as adaptors for additional transcription factors because mutations in *Drosophila* and other organisms have other phenotypes that cannot be explained by loss of Wg signaling (30, 31, 33, 37). Indeed, Med12 has been shown to interact with both Sox9 and Gli3 (32, 76). The yeast Med13 homologue is a target for Ras-regulated PKA phosphorylation (77), suggesting the interesting possibility that Wg or other signals might directly regulate the activity of Med12 or Med13. Finally, because *skd* and *kto* are not essential for normal cell proliferation or survival, they may provide targets for the treatment of Wnt-driven cancers.

Materials and Methods

Fly Stocks and Genetics. All *skd* and *kto* alleles used are null alleles described in ref. 30. Other fly stocks used were *P{PZ}Dll*^{01092}, *P{PZ}ds*^{05142}, *da*-GAL4, *tub*-GAL4 (Flybase), *vgQ-lacZ*, *sal1.1-lacZ* (78), *H15-lacZ* (53), *cdk8*^{K185}, *cycC*^{Y5} (73), UAS-HAPygo, UAS-HAPygoΔNbox, UAS-HAPygoNnpf (58, 59), and UAS-*fluΔArm* (48). *hs*-HA-PygoΔPHD-GAL4 was made by cloning PygoΔPHDG4HA (43) into the pCaSpeR-hs vector (GenBank U59056).

To generate *skd*, *kto*, *cdk8*, or *cycC* mutant clones in the wing or leg disc, *FRT80*, *skd* (or *kto* or *cdk8*)/TM6B or *FRT80*, *skd* (or *kto* or *cdk8*); *Dll-lacZ* (or *vgQ-lacZ* or *sal-lacZ* or *H15-lacZ*)/SM6-TM6B males were crossed to *hs*FLP122; *FRT80*, *Ubi-GFP*/TM6B females, or *FRT82*, *cycC*; *Dll-lacZ*/SM6-TM6B males were crossed to *hs*FLP122; *FRT82*, *Ubi-GFP*/TM6B females, and larvae were heat shocked for 1 h at 38.5°C in both first and second instar. Clones in the eye disc

were generated by using *ey*FLP1 instead of *hs*FLP122. Clones misexpressing activated Arm were generated by crossing *FRT80* (or *FRT80*, *skd* or *kto*); UAS-*flu*Δ*Arm*, *Dll-lacZ*/SM6-TM6B males to *hs*FLP122, UAS-*GFP*; *tub*-GAL4; *FRT80*, *tub*-GAL80 females and heat-shocking larvae for 1 h at 38.5°C in both first and second instar. *skd* or *kto* mutant clones in an *hs*-PygoΔPHD-GAL4 or *tub*-GAL4 background were made by crossing *FRT80*, *skd* (or *kto*); *hs*-PygoΔPHD-GAL4 (or *tub*-GAL4)/SM6-TM6B males to *ey*FLP1, UAS-*GFP*; *FRT80*, *arm-lacZ* females. For *hs*-PygoΔPHD-GAL4, larvae were dissected after a 2-h heat shock at 38.5°C and a 2-h recovery period.

Immunohistochemistry. Imaginal discs were stained as described previously (30). Antibodies used were mouse anti-Wg (1:5; Developmental Studies Hybridoma Bank), rabbit anti-β-gal (1:5,000; Cappel), mouse anti-β-gal (1:200; Promega), guinea pig anti-Sens (1:1,000) (79), rabbit anti-Tsh (1:2,000) (80), rabbit anti-CM1 (1:500; BD PharMingen), rabbit anti-phosphohistone H3 (1:200; Upstate Biotechnology), rabbit anti-Skd (1:5,000), rat anti-Kto (1:1,000) (31), rabbit anti-GFP (1:5,000; Molecular Probes), and rat anti-HA (1:100; Roche). Images were collected on a Leica TCS NT or Zeiss LSM 510 confocal microscope.

Cell Culture, RNAi, and Luciferase Assays. Twelve-well plates of Kc cells were incubated with 1–2 μg of dsRNA for 3 days as described previously (81). See *SI Materials and Methods* for sequences of primers used to make dsRNA. Cells were then transfected by using Qiagen Effectene Transfection Reagent with 200 ng of TOP-Flash and 200 ng of Pol III-Renilla luciferase (Pol III-RL) (56) and incubated for 2 days. Knockdown of *skd* or *kto* did not visibly affect the growth or survival of the cells, and the Pol III-RL values were not significantly altered (*skd* RNAi/*lacZ* or no RNAi = 1.56 ± 0.63; *kto* RNAi/*lacZ* or no RNAi = 1.47 ± 0.39 in four experiments). Cell lysis and luciferase assays were performed with the dual luciferase reporter assay system (Promega) according to the manufacturer's protocol.

Immunoprecipitation and Western Blotting. First, 3- to 20-h embryos were collected in PBS/0.1% Triton, dechorionated, and ground in lysis buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 2 mM EDTA, 0.5% Nonidet P-40, 0.01% NaDeoxycholate, and 10% glycerol] containing protease inhibitors (Roche). To induce expression of *hs*-PygoΔPHDG4HA (43), embryos were heat shocked for 75 min at 40°C and allowed to recover for 1 h at room temperature before collection. Extract was passed three times through a syringe, nutated 25 min at 4°C, and spun for 15 min at 16,000 × *g*. Then, 2–3 mg of total protein was preabsorbed for 2 h with 30 μl of Protein A beads and immunoprecipitated overnight with 10 μl of anti-Skd. After a 3-h incubation with 50 μl of Protein A beads, the beads were washed five times with lysis buffer with 0.1% Nonidet P-40 and no NaDeoxycholate. Kc cells were transfected with actin-GAL4 and UAS-HAPygo (58) by using Effectene and treated with 11 μg of dsRNA for either *skd* or *lacZ*. After 5 days, cells were harvested and lysed in lysis buffer; ≈700 μg of extract was used for each immunoprecipitation.

Finally, 5–10 μg of protein per lane was run on 10% SDS/PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C in 5% milk/TBT (0.3% Tween 20 in TBS). Blots were incubated in 5% milk/TBT with guinea pig anti-Kto (1:5,000), rabbit anti-Skd (1:5,000) (31), or rat anti-HA (1:1,000) (Roche) for 2 h at room temperature; washed in TBT; incubated with HRP-conjugated secondary antibodies (1:12,500) in 5% milk/TBT for 2 h; and washed in TBT. Blots were developed by using the ECL photoluminescence procedure (Pierce).

ACKNOWLEDGMENTS. We thank H. Bellen (Baylor College of Medicine, Houston), M. Bienz (Medical Research Council, Cambridge, U.K.), M. Boube (Université Paul Sabatier, Toulouse, France), S. Carroll (University of Wisconsin, Madison), R. Dasgupta (New York University), C. Estella (Columbia College of Physicians and Surgeons, New York), M. de la Roche (Medical Research Council), A. Salzberg (Technion, Haifa, Israel), G. Struhl (Columbia College of Physicians and Surgeons), N. Tanese (New York University), B. Thompson (EMBL, Heidelberg), the Bloomington Drosophila Stock Center, and the Developmental Studies Hybridoma Bank for fly stocks and reagents; E. Bach, R. Dasgupta, and N. Tanese for technical advice; Y. Zorina for technical assistance; and S. Astigarraga, K. Hofmeyer, K. Legent, J.-Y. Roignant, and J. Steinhauer for critical comments. This work was supported by National Institutes of Health Grant GM56131 and the Irma T. Hirschl/Monique Weill-Caulier Trust.

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