

## Coop functions as a corepressor of Pangolin and antagonizes Wingless signaling

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**Wingless (Wg) signaling regulates expression of its target genes via Pangolin and Armadillo, and their interacting cofactors. In the absence of Wg, Pangolin mediates transcriptional repression. In the presence of Wg, Pangolin, Armadillo, and a cohort of coactivators mediate transcriptional activation. Here we uncover Coop (corepressor of Pan) as a Pangolin-interacting protein. Coop and Pangolin form a complex on DNA containing a Pangolin/TCF-binding motif. Overexpression of *Coop* specifically represses Wg target genes, while loss of *Coop* function causes derepression. Finally, we show that Coop antagonizes the binding of Armadillo to Pangolin, providing a mechanism for Coop-mediated repression of Wg target gene transcription.**

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The Wnt/Wg signaling pathway controls diverse processes such as growth, patterning, tissue and energy homeostasis, and maintenance of somatic stem cells. Misregulation of the Wnt pathway contributes to human diseases including cancer and metabolic disorders (Logan and Nusse 2004; Clevers 2006; Prestwich and Macdougald 2007). Two transcriptional regulators,  $\beta$ -catenin/Armadillo (Arm) and TCF/Lef/Pangolin (Pan), play key roles in regulating the transcriptional outputs of the canonical Wnt/Wg pathway (MacDonald et al. 2009). In the absence of Wnt/Wg ligand, the function of  $\beta$ -catenin/Arm in transcription is inhibited. A "destruction complex" composed of APC, Axin, GSK3 $\beta$ , and CK1 mediates phosphorylation of  $\beta$ -catenin/Arm and targets it for degradation via the proteasome pathway (Aberle et al. 1997; Pai et al. 1997). Also, during the off state, TCF/Lef/Pan is bound by corepressors and mediates repression of the Wnt/Wg target genes. Binding of Wnt/Wg ligand to its receptor at the cell surface primes a signaling cascade that inhibits the function of the destruction complex and results in  $\beta$ -catenin/Arm stabilization. Wnt/Wg target genes are activated when stabilized  $\beta$ -catenin/Arm enters the nucleus and recruits other coactivators to target loci through TCF/Lef/Pan (Mosimann et al. 2009).

[**Keywords:** Pangolin; Coop; corepressor; signal transduction; Wingless]

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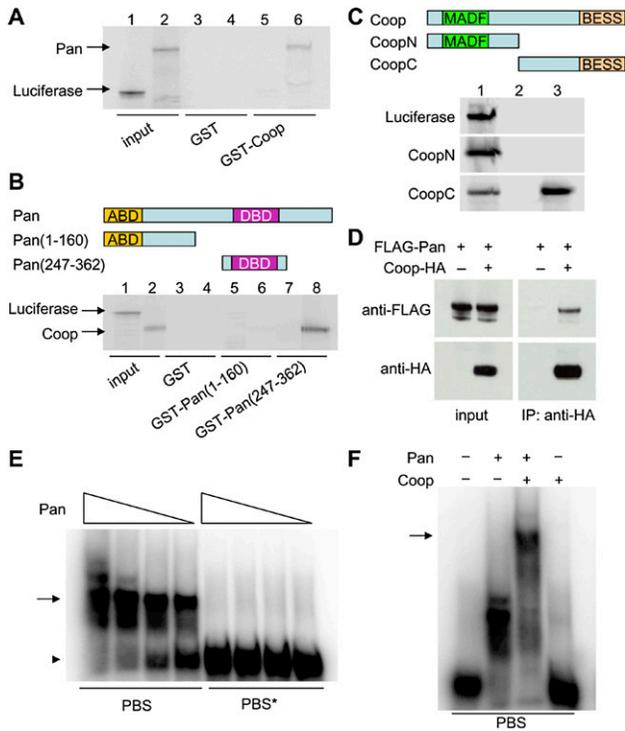
TCF/Lef/Pan can mediate either transcriptional repression or activation, depending on the context of cofactors to which it is bound. Two universal corepressors, TLE/Groucho and CtBP, are known to function in TCF/Pan-mediated repression (Cavallo et al. 1998; Brannon et al. 1999). Both proteins are capable of recruiting histone deacetylases (HDACs), and bring about chromatin modifications that promote transcriptional silencing (Chen et al. 1999; Shi et al. 2003). In addition, oligomerization of TLE/Groucho may lead to chromatin remodeling over a wider region (Song et al. 2004; Sekiya and Zaret 2007). Following the binding of  $\beta$ -catenin/Arm to TCF/Lef/Pan, many transcriptional coactivators are recruited. One such coactivator is Bcl9/Legless (Lgs), whose primary function is believed to be recruiting Pygopus (Kramps et al. 2002; Thompson et al. 2002). Pygopus can interact with the Mediator complex subunits Med12 and Med13, and the TFIID complex component TAF4 (Carrera et al. 2008; Wright and Tjian 2009). Mammalian Pygopus also binds preferentially to dimethylated Lys 4 of histone H3; the functional importance of this interaction remains to be determined (Fiedler et al. 2008; Gu et al. 2009; Kessler et al. 2009). The C-terminal region of  $\beta$ -catenin/Arm functions as a transactivation domain and interacts with many coactivators involved in chromatin remodeling, including CBP/p300, Parafibromin/Hyrax, MLL1/SET1, Brg-1/Brahma, and the NURF complex (Hecht et al. 2000; Takemaru and Moon 2000; Barker et al. 2001; Mosimann et al. 2006; Sierra et al. 2006; Li et al. 2007; Song et al. 2009).

In this study, we report the identification, via a proteomic approach, of a novel corepressor acting in the Wg signaling pathway. This protein, encoded by *CG1621*, interacted with Pan in a series of independent assays. In addition, overexpression of *CG1621* specifically repressed Wg target genes, while the knockdown of *CG1621* derepressed them. Thus, we refer to it as *Coop* (corepressor of Pan). Similar to the function of TLE/Groucho in the Wnt/Wg pathway, we showed that the interaction between Coop and Pan obstructed the recruitment of Arm, suggesting that the competition between the corepressors and  $\beta$ -catenin/Arm could be a common mechanism to regulate the transcriptional outputs of the Wnt/Wg signaling pathway.

### Results and Discussion

#### *Coop interacts directly with Pan*

Coop (CG1621) was found in an attempt to identify novel Pan-interacting proteins by a mass spectrometry-based proteomic approach (Supplemental Fig. S1A). It contains an N-terminal MADF domain and a C-terminal BESS domain (Bhaskar and Courey 2002). This architecture is conserved in 16 *Drosophila* proteins, one *C. elegans* protein, and one zebrafish protein (Supplemental Fig. S1B). The putative dimeric BESS domain does not exist in mammals, and there is one uncharacterized MADF-containing protein in humans (ZSCAN29/ZNF690). Earlier studies on other members of this family indicated that they might be involved in transcriptional regulation (Cutler et al. 1998; Bhaskar and Courey 2002). We first confirmed the interaction between Coop and Pan in a series of pull-down experiments. In lysates of *Drosophila* Kc cells, Pan was coimmunoprecipitated by Coop (Fig. 1D). Consistent with



**Figure 1.** Coop and Pan interact with each other and form a complex on DNA. (A–C) In vitro translated samples were used for GST pull-down. (A) Control Luciferase and full-length Pan (lanes 1,2, 15% input) were pulled down by GST (lanes 3,4) or GST-Coop (lanes 5,6). (B) Control Luciferase and Coop (lanes 1,2, 5% input) were pulled down by GST (lanes 3, 4), GST-Pan (1–160) (lanes 5,6), or GST-Pan (247–362) (lanes 7,8). (C) Control Luciferase, CoopN (1–160), and CoopC (161–358) (lane 1, 5% input) were pulled down by GST (lane 2) or GST-Pan (247–362) (lane 3). (D) Kc cells were transfected with Flag-tagged Pan, or cotransfected with Flag-tagged Pan and HA-tagged Coop. Coop was immunoprecipitated by anti-HA antibody-conjugated beads and was analyzed by Western blotting. (E) Pan (247–362) caused a mobility shift (arrow) of a DNA probe harboring a Pan-binding site (PBS), but did not affect the mobility of a DNA probe harboring a mutated Pan-binding site (PBS\*). Free probes are indicated by the arrowhead. (F) Coop alone did not bind to a DNA probe harboring a PBS, but caused a supershift in the presence of Pan (arrow).

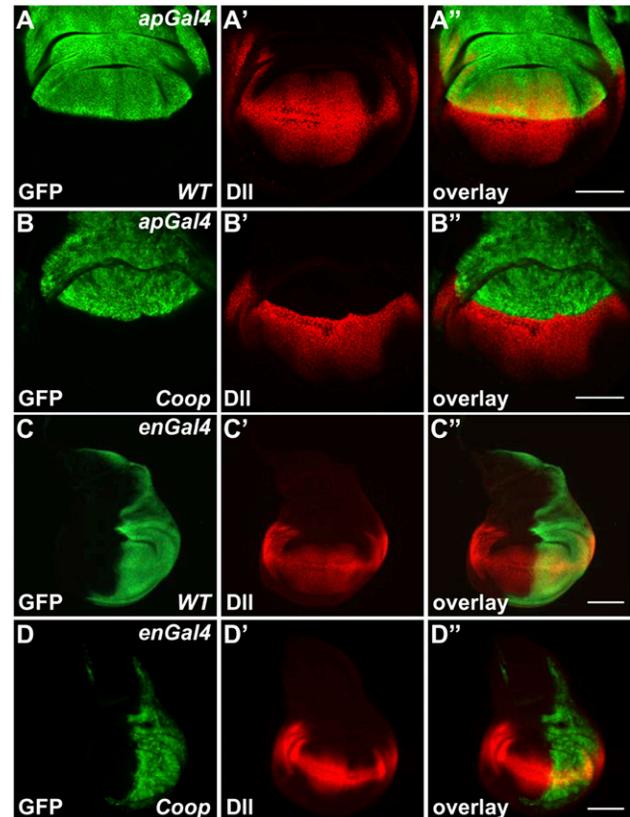
a role in transcription, Coop was localized in the nucleus in these cells (Supplemental Fig. S1C). To determine if this interaction was direct, we performed GST pull-down experiments. A GST fusion of Coop could pull down in vitro translated Pan (Fig. 1A). In the reverse experiment, Coop was pulled down by a Pan central fragment (247–362) harboring its DNA-binding domain, but not by a Pan N-terminal fragment (1–160) (Fig. 1B). We further mapped this interaction to the C-terminal part of Coop (161–358), which contains its BESS domain (Fig. 1C).

Since the DNA-binding domain of Pan may be involved in the interaction with Coop, we asked whether Coop affected the DNA affinity of Pan. We addressed this question in a gel shift assay. Pan (247–362) induced the mobility shift of a DNA oligonucleotide containing a consensus Pan/TCF-binding site, but did not affect that of a control oligonucleotide in which the binding site was mutated (Fig. 1E). Coop alone did not bind to this DNA probe. However, in the presence of Pan, Coop induced a supershift of this oligonucleotide (Fig. 1F). This suggests that the interaction between Coop and Pan does not

interfere with Pan binding to DNA. Instead, it suggests that Coop and Pan form a complex on Pan targets.

*Overexpression of Coop represses Wg targets*

To test whether the interaction between Coop and Pan affects Wg signaling in vivo, we overexpressed *Coop* in wing imaginal discs and monitored expression of Wg targets, including *Distal-less (Dll)*, *senseless (sens)*, and *Notum/wingful (wf)* (Nolo et al. 2000; Gerlitz and Basler 2002; Giraldez et al. 2002). The expression of *Coop* in the dorsal compartment of wing discs by *apterous-Gal4 (apGal4)* resulted in the loss of Dll protein in this domain (Fig. 2A,B). This repression occurred at the transcriptional level, as the expression of a *Dll-lacZ (DllZ)* reporter transgene was similarly repressed in the same assay (Supplemental Fig. S2A–C). The expression of *Coop* in the posterior compartment of wing discs by *engrailed-Gal4 (enGal4)* also repressed *Dll* expression, but in a different pattern: Dll staining was lost in the domain away from the dorsal–ventral (D/V) boundary, and was only weakened in the domain close to the D/V boundary (Fig. 2C,D). The Wg morphogen is expressed at the D/V boundary and forms a concentration gradient in the wing disc. It is possible that our observations reflected a dosage-dependent effect of Coop on different levels of Wg



**Figure 2.** Overexpression of *Coop* represses Wg target *Dll* in a dosage-dependent manner. (A–A'') The expression of *apGal4* alone did not affect *Dll* expression. (B–B'') The expression of *Coop* by *apGal4* abolished *Dll* expression. (C–C'') The expression of *enGal4* did not affect *Dll* expression. (D–D'') The expression of *Coop* by *enGal4* strongly reduced *Dll* expression. Bar, 100  $\mu$ m.

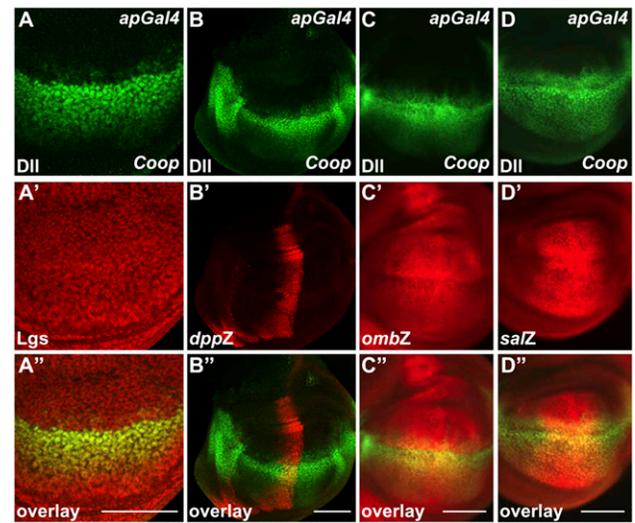
signaling. To test this idea, we also expressed *Coop* at lower levels, either in clones or in the center of wing discs by *spalt enhancer-Gal4* (*salEGal4*). Indeed, we observed the repression of *Dll* expression preferentially in the domain with lower levels of Wg signaling (Supplemental Fig. S2D–G). These results implied that *Coop* functions to antagonize incoming Wg signaling.

*sens* is a high-threshold target of Wg in wing discs and is expressed in two to three rows of cells flanking the D/V boundary. The expression of *sens* starts at the center of the wing disc and extends to the periphery at late larval stage (Fang et al. 2006). The expression of *Coop* by *enGal4* resulted in the repression of *sens* in the posterior compartment of wing discs, while the expression of *Coop* by *apGal4* prevented *Sens* from extending to the periphery in the dorsal compartment (Supplemental Fig. S3A–D). Consistent with our observations with *Dll*, lower levels of *Coop* expression by *act>CD2>Gal4* or *salEGal4* did not affect *sens* expression (data not shown).

*wf* is another known Wg target gene. We previously isolated a 4-kb upstream element from the *wf* locus, which responded to Wg signaling in cell culture. A *lacZ* transgene under its control (*wfZ*) mimicked *wf* expression in wing discs (Supplemental Fig. S3E,F). The expression of *Coop* repressed this *wfZ* in wing discs and *wf* reporter in cultured *Drosophila* cells (Supplemental Fig. S3G,H). In addition to Wg targets in the wing disc, the expression of *Coop* also repressed *H15*, a Wg target gene in the leg disc (Supplemental Fig. S4; Brook and Cohen 1996). Thus, in the case of the four Wg target genes we analyzed, *Coop* functioned consistently as a negative transcriptional regulator.

#### The specificity of *Coop* in the Wg signaling pathway

Next we tested whether *Coop* generally affected transcription, or specifically affected Wg signaling. We first examined the effect of *Coop* on the expression of Notch targets *wg* and *cut*. The expression of *Coop* by *enGal4* or other drivers did not affect expression of either gene (Supplemental Fig. S5A,B; data not shown), confirming that the effect we observed on Wg targets was not due to the interference in the upstream level of Wg signaling. We then expressed *Coop* by *apGal4*, which strongly repressed Wg targets, and monitored the expression of other non-Wg targets. *Lgs* plays a key role in transducing Wg signal. We observed no effect on *Lgs* expression by *Coop* (Fig. 3A). Similarly, the expression of *Coop* had no effect on the Hedgehog (Hh) target gene *decapentaplegic* (*dpp*), or the Dpp targets *optomotor blind* (*omb*) and *spalt* (*sal*) (Fig. 3B–D; Supplemental Fig. S6). Similarly, *hh*, *patched* (*ptc*), and *en* expression were unaffected (Supplemental Fig. S5C–E). In our study we noticed that overexpression of *Coop* had a weak negative effect on the Gal4/UAS system (Supplemental Fig. S7). It is therefore possible that *Coop* expression was reduced, leading to an underestimation of the strength of *Coop*'s activity. Whatever the case, the effect does not compromise any of our conclusions, as none of the targets we monitored in this study was driven by the Gal4/UAS system. Taken together, our results suggest that the repressive effect of *Coop* is probably mediated by its interaction with Pan, and not an indirect effect on *wg* expression or components of the Wg signaling cascade. Importantly, *Coop* does not appear to affect Hh or Dpp signaling, suggesting its effect on the Wg pathway is fairly specific.

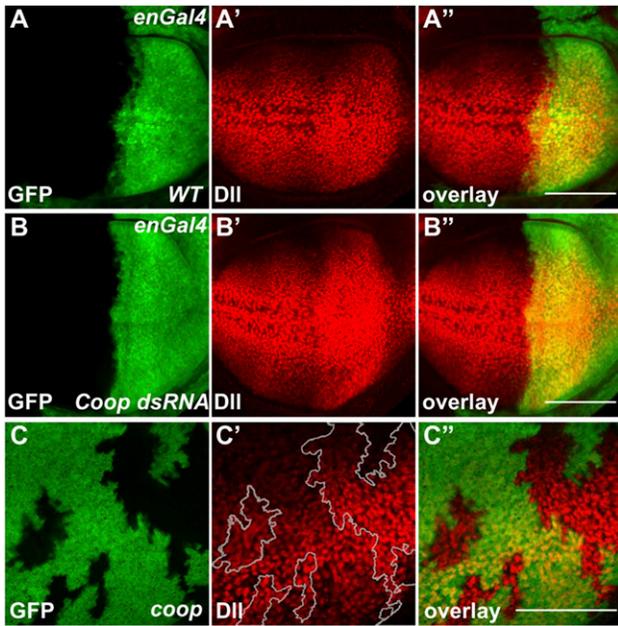


**Figure 3.** *Coop* is not a universal repressor. (A–D) The expression of *Coop* by *apGal4* strongly repressed *Dll*, but had no effect on *Lgs* (A'), *dppZ* (B'), *ombZ* (C'), or *salZ* (D') expression. *Dll* staining is in green, and staining of *Lgs* or the *lacZ* reporters is in red. Bar, 100  $\mu$ m.

#### Loss of *Coop* derepresses Wg targets

In the next step, we asked whether *Coop* was required for the proper regulation of Wg target gene expression. We first examined the effect of *Coop* RNAi on the basal transcriptional levels of Wg target genes in *Drosophila* Kc cells. For comparison, we also performed RNAi against Groucho or CtBP, two known corepressors of the Wg pathway. To our surprise, the knockdown of *Coop* mRNA in Kc cells by dsRNA treatment was very inefficient. In our hands, the levels of most mRNAs could be knocked down to 10%–15% after 4 d (data not shown); however, the levels of *Coop* mRNA remained at 60%–70% after 4 d and 40%–50% after 7 d (Supplemental Fig. S8A–C). Even so, under this condition, the knockdown of *Coop* mRNA caused a twofold to threefold increase in Arm-independent basal expression of Wg target gene *nkd* and *CG6234* (Zeng et al. 2000; Fang et al. 2006; Chang et al. 2008), similar to the effect observed with Groucho RNAi, but slightly weaker than the effect seen with CtBP RNAi. In addition, the combination of *Coop* and CtBP RNAi showed an additive effect (Supplemental Fig. S8D–F).

We next examined whether reducing *Coop* function affected the activation of Wg target genes in vivo. We first tested this by expressing dsRNA against *Coop* in the wing disc and monitoring expression of Wg targets. *Coop* is expressed ubiquitously in imaginal discs (Supplemental Fig. S9A–C). The knockdown of *Coop* enhanced expression of *Dll* and *wf* (Fig. 4A,B; Supplemental Fig. S10A,B), suggesting that endogenous *Coop* also affects Wg-mediated target activation. Starting from an enhancer P element (EP) line, we generated two *Coop* alleles (hereafter referred to as *coop*) that encode truncated proteins (Supplemental Fig. S11). *coop* mutant flies seemed normal, but showed enhanced Wg signaling in a sensitized background: Loss of *Coop* enhanced a rough eye phenotype caused by ectopic Wg signaling (*sev-Wg*) (Supplemental Fig. S10C–E). In *coop* mutant clones, we also observed moderately enhanced expression of *Dll* in the wing disc (Fig. 4C). This is consistent with our in vivo RNAi results, indicating that *Coop*



**Figure 4.** Loss of Coop enhances expression of *Dll* in vivo. (A) Expression of *Dll* in wild-type control. (B) Expression of dsRNA targeting *Coop* mRNA by *enGal4*-enhanced *Dll* expression. (C) Expression of *Dll* was enhanced in *coop* clones induced by *hs-flp*, *FRT42 ubi-GFP Minute/FRT42 coop<sup>R62</sup>*. Bar, 100  $\mu$ m.

is a repressor of Wg target genes. Although *coop* clones had a weaker effect than RNAi, this difference might be due to the perdurance of Coop protein. The RNAi was induced earlier than the clones of *coop*, thus probably eliminating Coop more thoroughly (cf. Supplemental Fig. S9D,E).

There are 15 sequence homologs of Coop in *Drosophila*, and it is possible that one or several of them also had similar roles in Wg signaling. We examined some *Coop* family members, and found overexpression of *CG6854* and *Adf1* also repressed Wg signaling in *Drosophila* cultured cells (data not shown). As a next step, we tested the possibility that *CG6854* or *Adf1* acts like Coop in vivo. Overexpression of *CG6854* strongly repressed expression of *Dll* and *sens* (Supplemental Fig. S12A–E). However, in contrast to Coop, it also repressed expression of *wg* and targets of other pathways (Supplemental Fig. S12F,G; data not shown), suggesting the repressive effect of *CG6854* may be less specific than that of Coop. *Adf1* behaved like *CG6854* (data not shown). Taking these results together, we propose that, unlike Coop, *Adf1* and *CG6854* are not specific repressors of the Wg pathway.

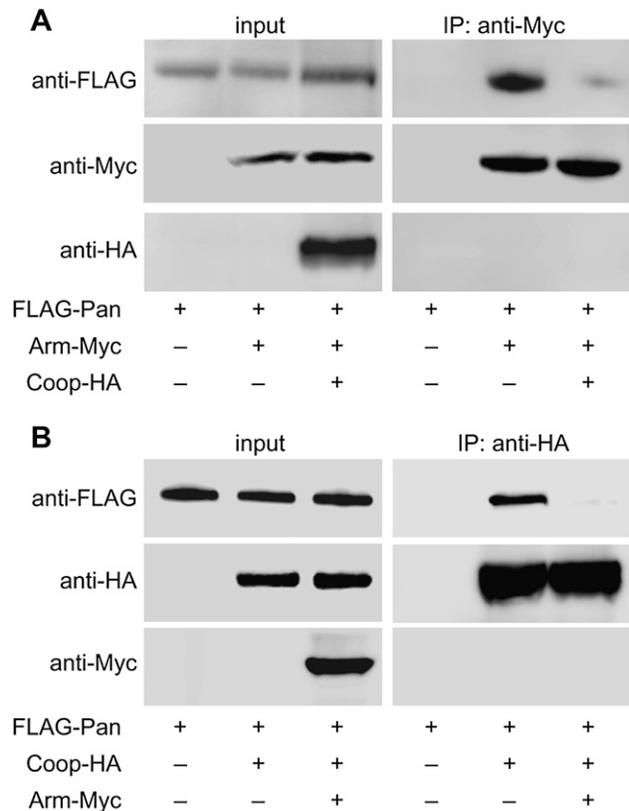
*Coop competes with Arm for binding to Pan*

Having established that Coop has a defined role in Wg signaling, in contrast to *CG6854* and *Adf1*, we then investigated the mechanism by which Coop represses Wg target genes. As the interaction between Arm and Pan is essential for activation of Wg target genes, we tested whether Coop functions by preventing this process. In cultured *Drosophila* Kc cells, we overexpressed Pan and Arm in the absence or presence of overexpressed Coop, and coimmunoprecipitated Pan via Arm. The presence of Coop greatly reduced the amount of Pan coimmunoprecipitated (Fig. 5A). Similarly, when we coimmunopreci-

pitated Pan via Coop, the presence of overexpressed Arm also prevented this interaction (Fig. 5B). These results suggest that the binding of Coop to Pan and the binding of Arm to Coop are mutually exclusive.

As Coop can interact with a domain in Pan that is conserved in other TCFs, we were therefore interested in examining whether Coop could also affect Wnt signaling. Interestingly, activation of a Wnt signaling reporter was repressed by ectopic expression of *Coop* in HEK293T cells (Supplemental Fig. S3I). It is likely that Coop achieved this by interfering with the conserved interaction between  $\beta$ -catenin and TCF. These results indicate a way in which Wnt signaling could be additionally regulated by a functional homolog of Coop. Since BESS domain proteins apparently do not exist in mammals, we postulate that a Coop-like repressor function is carried out by a TCF-interacting protein that is not necessarily structurally related to Coop. Proteomic analysis of TCF interaction partners may help to identify functional homologs of Coop in mammals.

It has been shown in vitro that  $\beta$ -catenin and TLE1 compete for binding to Lef1 (Daniels and Weis 2005). By competing for an overlapping binding site adjacent to the DNA-binding domain of Lef1, TLE1 prevents the recruitment of  $\beta$ -catenin. Here we show that Coop and Arm also compete to bind Pan. Thus, the levels of nuclear  $\beta$ -catenin/Arm, determined by the levels of Wnt/Wg signaling, decide



**Figure 5.** Coop and Arm compete for Pan binding. (Left panels) Presence of transfected Pan, Arm, and Coop are shown by Western blotting. (A, right panels) Coop reduced the amount of Pan coimmunoprecipitated along with Arm. (B, right panels) Arm reduced the amount of Pan coimmunoprecipitated along with Coop.

the transcriptional activity of TCF/Lef/Pan. This dosage-dependent, reversible mechanism helps to shape Wnt/Wg gradient-induced expression of downstream targets.

Ectopic Wnt signaling, transduced via interaction between  $\beta$ -catenin and TCF, is often detected in human cancers. Several  $\beta$ -catenin-binding proteins, including ICAT and Chibby, can interfere with the interaction of these two proteins (Tago et al. 2000; Takemaru et al. 2003). Here we report the identification of Coop as another potential blocker of the  $\beta$ -catenin–TCF interaction. As  $\beta$ -catenin has divergent functions in more than Wnt signaling, TCF-binding proteins may help to specifically decrease the transcriptional outputs of ectopic Wnt signaling. Given the specific effect of Coop in the Wg pathway, we believe Coop may function as a specific inhibitor of Arm–Pan interaction in *Drosophila*. Further studies to map the Coop–Pan interaction may uncover novel ways to prevent the interaction between  $\beta$ -catenin and TCF.

## Materials and methods

### Antibodies and immunochemistry

The following antibodies were used: mouse anti-Dll (1:500; a gift from Ian Duncan), mouse anti-Wg (4D4, 1:500; Hybridoma Bank), guinea pig anti-Sens (1:800; a gift from Hugo Bellen), mouse anti-Cut (2B10, 1:20; Hybridoma Bank), rabbit anti-HA (1:1000 for Western blot, and 1:200 for immunostaining; ICL), mouse anti-HA (1:1000; Covance Research Products), rabbit anti-Lgs (1:500), rabbit anti- $\beta$ -galactosidase (1:2000; Cappel), mouse anti-Flag (1:5000 for Western blot, and 1:1000 for immunostaining; Sigma), mouse anti-Myc (9E10, 1:1000; Hybridoma Bank), Alexa-fluor-conjugated secondary antibodies (Molecular Probes), HRP-conjugated secondary antibodies (1:2000; Jackson Laboratories). Anti-Coop antibody was generated using purified Coop (1–160). For immunoprecipitation, mouse anti-HA-conjugated or rabbit anti-myc-conjugated agarose beads (Sigma) were used. X-Gal staining was performed as described (Song et al. 2004).

### Fly stocks and plasmid constructs

The following fly lines were used for overexpression: *apGal4/SM5a<sup>+</sup>TM6b*, *enGal4/cyo*, *act>CD2>Gal4/TM6b*, *salEGal4/TM6b*, *dppGal4/cyo*, *UAS-GFP/cyo*, *UAS-Coop*, *UAS-CG6854*, *UAS-Adf1*. Coop RNAi lines were from VDRC. The *coop<sup>R62</sup>* and *coop<sup>R56</sup>* alleles contain nonsense mutations at W89 and Q182, respectively. The PCR fragments corresponding to Coop 1–358, 1–160 (CoopN), and 161–358 (CoopC); Pan 1–719, 1–160, and 247–362; and Arm 1–853 were cloned into expression vectors and used for interaction studies.

### Protein interaction studies

For GST pull-down, [<sup>35</sup>S]methionine-labeled proteins were diluted into binding buffer (20 mM HEPES at pH 7.9, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 0.2% NP-40, 0.5 mM DTT, protease inhibitor cocktail) and incubated with glutathione beads bearing GST or GST fusion proteins. For coimmunoprecipitation, transfected cells were lysed in the buffer containing 20 mM phosphate (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 0.5 mM DTT, and protease inhibitor cocktail. After extensive washing, bound proteins were eluted and resolved by Nu-PAGE, and analyzed by autoradiography or Western blotting.

### Kc cell culture

RNAi was performed as described (Worby et al. 2001). The dsRNA-treated cells were incubated for 4 d, and a second round of dsRNA treatment was performed. Total RNA was isolated after another 3 d. Quantitative RT-PCR was performed and analyzed as described previously (Mosimann et al. 2006). The mRNA levels of Actin5C,  $\alpha$ -tubulin, and TBP were used

as internal control for normalization. Two independent sets of dsRNA were used to target Coop mRNA and gave similar results.

### EMSA

We previously identified a 200-base-pair (bp) Wg-responsive element ~8 kb upstream of the *wf* transcription starting site (M Kuster and K Basler, unpubl.). We used a 61-bp oligonucleotide from this element containing a single putative Pan-binding site (PBS) and flanking sequence for our assay (forward strand, tttCTCTGCCGACGTCGCTGCC GCTGCCGCT GCTCTGCAGCTGCCGGCTGAACACTTTGATGTC; reverse strand, tttGACATCAAAGTGTTCAGCCGGCAGCTGCAGAGCAGCGGCAG CGGCAGCGACGTCGGCAGAG). The control oligonucleotide has the same sequence, with a mutated Pan-binding site (PBS<sup>\*</sup>). The oligonucleotides were end-labeled by <sup>32</sup>P, and were incubated with purified Pan (247–362) and/or GST-Coop in binding buffer (20 mM HEPES at pH 7.9, 50 mM KCl, 6 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.1 mg/mL BSA, 2 mM DTT, 50  $\mu$ g/mL d[I-C], 5% glycerol). The bound and free DNA were resolved in 6% DNA retardation gel (Invitrogen) followed by autoradiography.

### Reverse-phase LC-MS analysis

Proteins isolated via immunoprecipitation were reduced with 5 mM TCEP and treated with 10 mM iodoacetamide to modify cysteine residues. Tryptic digestion was carried out overnight. Samples were purified by reverse-phase C-18 chromatography (Sep-PacK, Waters). For mass spectrometry analysis samples were resuspended in buffer A (5% acetonitrile, 0.2% formic acid). ESI-based LC-MS/MS analyses were performed on an LTQ linear ion trap (Thermo Fisher Scientific). MS/MS spectra were searched using the Mascot software version 2.2 (Matrix Science).

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