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Abstract

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Wnt/Wingless Signaling Requires BCL9/Legless-Mediated Recruitment of Pygopus to the Nuclear β-Catenin-TCF Complex

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Summary

Wnt/Wingless signaling controls many fundamental processes during animal development. Wnt transduction is mediated by the association of β-catenin with nuclear TCF DNA binding factors. Here we report the identification of two segment polarity genes in *Drosophila*, *legless* (*lgs*), and *pygopus* (*pygo*), and we show that their products are required for Wnt signal transduction at the level of nuclear β-catenin. Lgs encodes the homolog of human BCL9, and we provide genetic and molecular evidence that these proteins exert their function by physically linking Pygo to β-catenin. Our results suggest that the recruitment of Pygo permits β-catenin to transcriptionally activate Wnt target genes and raise the possibility that a deregulation of these events may play a causal role in the development of B cell malignancies.

Introduction

Wnt proteins are secreted signaling molecules that have pivotal roles in animal development. They direct growth and cell fates in processes as diverse as embryonic segmentation, limb development, and CNS patterning (reviewed by Wodarz and Nusse, 1998). Recent evidence also implicates Wnt signaling in the postembryonic regulation of stem cell number and stem cell differentiation in epithelia, such as those of the skin and colon (Taipale and Beachy, 2001; Hülsken et al., 2001).

Wnt/Wg proteins exert many of their effects on animal development by activating the expression of specific target genes in responding cells. Many of the conserved components of the cellular machinery responsible for transducing Wnt signals from the cell surface to the nucleus have been identified: receptors of the Frizzled and LRP families, the cytosolic proteins Dishevelled (Dsh), Glycogen Synthase Kinase 3 (GSK3/Zeste-white3/Shaggy), Axin, APC, and β-catenin/Armadillo, as well as the transcription factors TCF (LEF/Pangolin; reviewed by Wodarz and Nusse, 1998). The underlying principle of this transduction pathway is to prevent the accumulation of β-catenin in the cytoplasm when Wnt signals are absent, but to allow the build-up of high β-catenin levels when they are present. An intricate machinery has been identified which normally marks β-catenin for proteasome-mediated destruction by an N-terminal phosphorylation event (reviewed by Peifer and Polakis, 2000). Axin and APC are important components of this β-catenin destruction complex, as they appear to allow GSK3 to access and phosphorylate β-catenin. Upon activation of the Wnt pathway, β-catenin escapes this phosphorylation reaction, accumulates in the cytoplasm, and enters the nucleus, where it associates with TCF proteins to function as a transcriptional coactivator of target genes (reviewed by Peifer and Polakis, 2000).

This set-up, in which the key transducer is continuously held in check, is highly susceptible to mutations in its inhibitory components. The loss of any of the three elements of the β-catenin destruction complex leads to an increase in β-catenin levels and, hence, to the constitutive activation of the pathway. While this may reduce cellular viability, as upon loss of GSK-3 function, it can also lead to cell fate changes, uncontrolled proliferation, and tumorigenic behavior, as in the cases of APC and Axin (Polakis, 2000). Attempts to counter these harmful situations must aim at curbing the nuclear activities of β-catenin, either by preventing the formation of the β-catenin-TCF complex or by interfering with its transcriptional activator function.

While it is well established that the formation of the β-catenin-TCF complex is a prerequisite for the activation of Wnt targets, the mechanism of its action remains poorly understood. Recent advances include reports that β-catenin can interact in vitro with the TATA binding protein (TBP; Hecht et al., 1999), as well as with TIP49 (Bauer et al., 1998), a protein that interacts with TBP. Hence, β-catenin may activate gene expression by targeting components of the basal transcriptional machinery to promoters of its target genes. The situation in vivo, however, is probably more complex. Prieve and Waterman (1999), for example, reported that a direct complex of β-catenin with components of the basal transcriptional machinery is not sufficient to activate the expression of target genes in vivo, and that in addition to TCF and β-catenin, a third component is necessary.

Here, we use a genetic assay in *Drosophila* to identify mutations in two segment polarity genes, *legless* (*lgs*) and *pygopus* (*pygo*). We find that their products, like those of *pan* and *arm*, are required for the transmission of the Wg signal throughout development. We show that *lgs* encodes the structural and functional homolog of human BCL9, the product of the *B cell lymphoma 9* gene, and that *pygo* codes for a PHD finger protein. We further find that Lgs, and by inference BCL9, functions as an adaptor protein to physically link Pygo to the β-catenin-TCF complex and that this recruitment of Pygo is required for β-catenin to function as a transcriptional coactivator. Together, our results provide both genetic and molecular evidence for the existence of a

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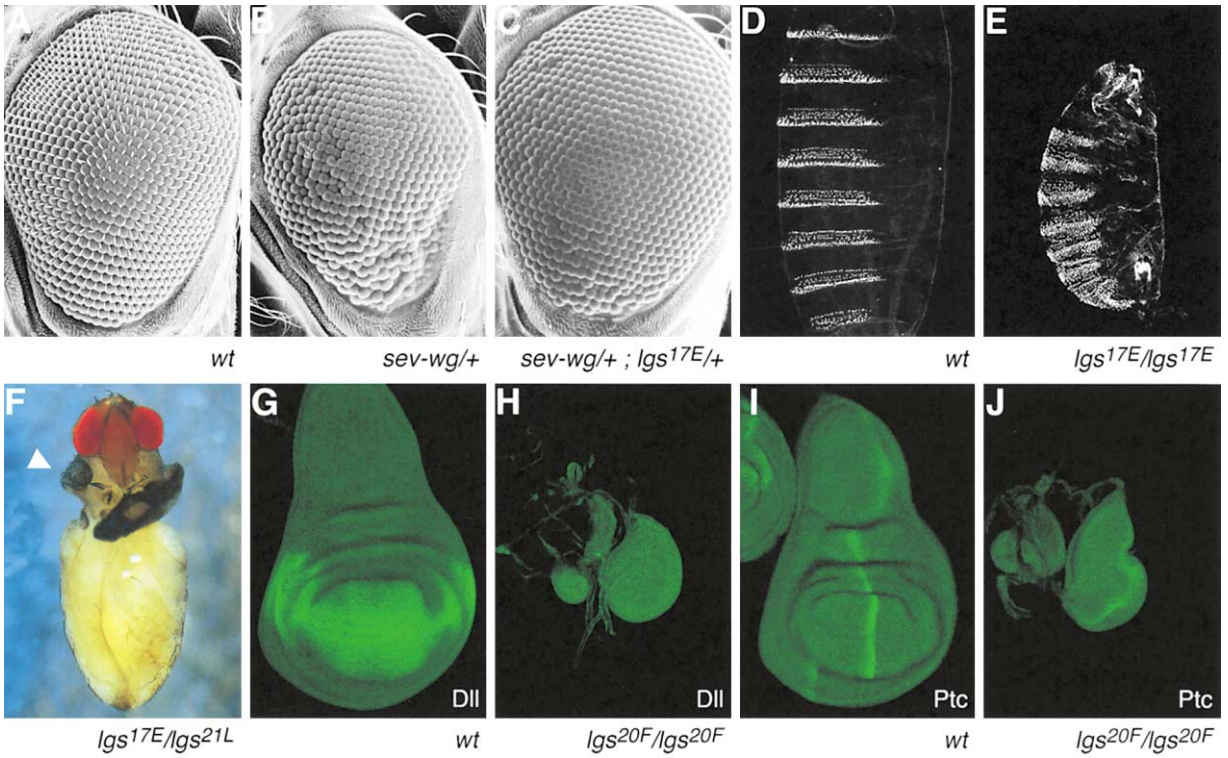

Figure 1. *lgs* Is Required for Wg Signaling throughout Development

(A–C) Eye phenotypes of wild-type (A), *sev-wg*/+ (B), and *sev-wg*/+, lgs^{17E}/+ (C) animals. Scanning electron micrographs are shown; anterior is to the left.

(D and E) Cuticle preparations of larvae derived from wild-type (D) and *lgs* mutant (E) embryos. The lgs^{17E}/lgs^{17E} embryo in (E) is derived from a homozygous lgs^{17E} mutant germline clone that was obtained by transplanting *lgs* mutant pole cells into embryos derived from *grandchildless* mutant females.

(F) lgs^{17E}/lgs^{21L} pharate adult removed from the pupal case. Legs and antennae are almost completely absent. One wing is transformed into a duplicated notum (arrowhead), and sternite tissue is replaced by pleura.

(G–J) Confocal images of third instar wing imaginal disc preparations stained with antibodies against Dll (G and H) and Ptc (I and J). Wild-type animals show normal expression of these genes (G and I). Discs derived from lgs^{20F} mutant larvae are abnormally small, yet express Ptc (J), but fail to express Dll (H).

previously unknown complex of nuclear proteins that endows β-catenin, and hence the Wnt/Wg signaling system, with the ability to induce target genes. They further raise the possibility that a deregulation of the Wnt pathway may, in some situations, cause B cell malignancies.

Results

Genetic Identification of *legless* as a Gene Required for Wg Signaling

In our attempt to identify new components of the Wnt signal transduction pathway, we performed a screen for dominant suppressors of the rough eye phenotype caused by a *sevenless-wg* transgene (*sev-wg*; Brunner et al., 1997) that drives ectopic expression of *wg* during eye development (Figures 1A–1C). The majority of suppressors found in this screen belonged to one of three complementation groups, two of which represent alleles of the previously identified loci *armadillo* (*arm*) and *pangolin* (*pan*), which encode the *Drosophila* homologs of β-catenin and TCF, respectively (Brunner et al., 1997). Six mutations, however, are recessive alleles of a locus on chromosome 4 that we designated *legless* (*lgs*). In transheterozygous situations, three *lgs* alleles interacted genetically with either *arm* or *pan* mutations, causing adult phenotypes characteristic of reduced *wg* activity (see below). These results suggested that *lgs*, like *arm* and *pan*, encodes a component of the Wg transduction pathway and raised the possibility that these components may participate in a common, critical task.

Animals transheterozygous for a strong and a weak allele of *lgs* die as pharate adults with a striking phenotype: they lack legs and antennae (Figure 1F), appendages whose growth and pattern are critically dependent on Wg activity (Peifer et al., 1991). These animals also show occasional wing-to-notum transformations, the founding phenotype of the *wg* gene (Sharma and Chopra, 1976) that reflects its role in defining the wing blade primordium (Ng et al., 1996). Strong *lgs* alleles cause larval lethality: homozygotes rarely reach the third instar stage, and the few that do so exhibit miniature imaginal discs that fail to express the Wg target gene *Distalless* (*Dll*) but show normal expression levels of the Hedgehog (Hh) target gene *patched (ptc*, Figures 1G–1J). To explore whether *lgs* may also be required for Wg signaling at embryonic stages, we sought to remove a potential maternal contribution of *lgs*. The

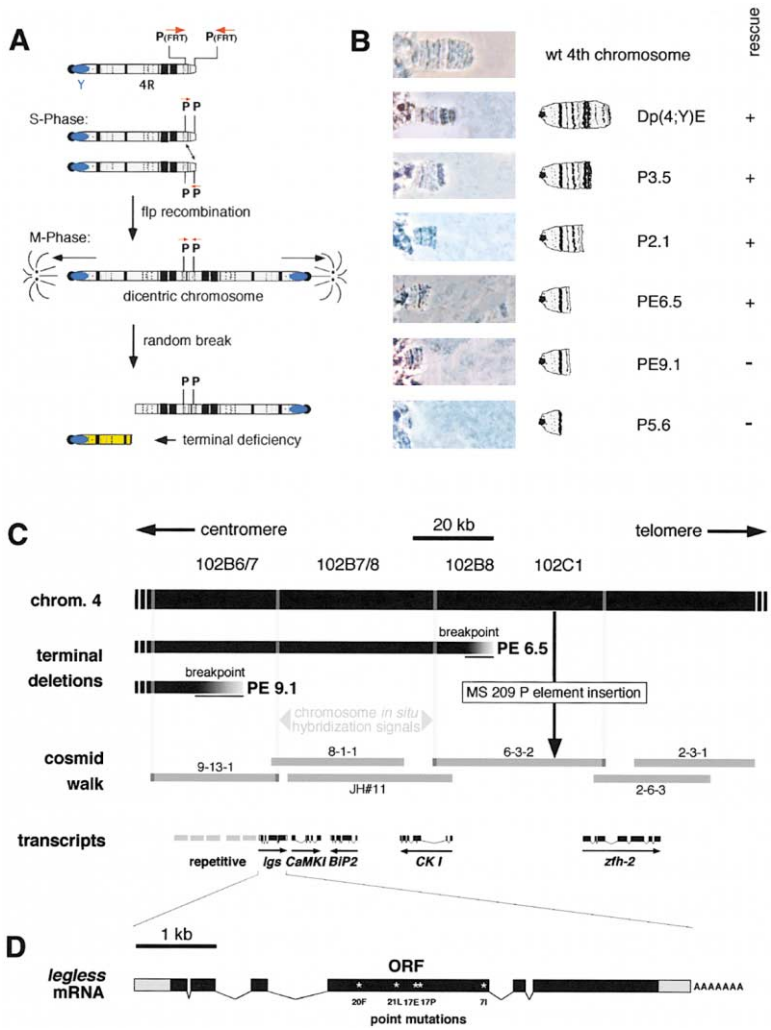

Figure 2. Isolation of the *lgs* Gene

(A) Schematic diagram illustrating the procedure to generate terminal deficiencies. A derivative of chromosome 4, which carries two FRT elements of inverse orientations (DcY; Ahmad and Golic, 1998), served as starting material. Using Flp-mediated recombination between sister chromatids, dicentric chromosomes were produced, each of which, upon mitosis, randomly broke into a pair of chromosomes with a duplicated or deleted terminal portion, respectively.

(B) A representative series of terminal deficiencies of Dp(4;Y)E. Cytological analyses of polytene salivary chromosomes are shown on the left, accompanied by a schematic drawing of each chromosome in the center and the genetic complementation data on the right, which reflects their ability to rescue the lgs^{17E}/lgs^{21L} genotype. For comparison, a wild-type chromosome 4 is shown on top.

(C) Positional cloning of the *lgs* gene. Sequences flanking the MS209 P element insertion, which maps to 102C1, were used to initiate a bidirectional cosmid walk. Chromosome in situ hybridization of an end fragment of cosmid 9-13-1 to both Dp(4;Y)E terminal deletions, PE 9.1 and PE 6.5, determined the proximal end of the walk. The distal end was established with a fragment on cosmid 6-3-2 that hybridized only to the complete chromosome 4. PCR revealed the presence of *zfh-2* coding sequence on cosmids beyond that point. Transgenic copies of cosmids 9-13-1, 8-1-1, and 6-2-3, none of which contains a complete *lgs* gene, failed to rescue *lgs* mutant animals. Using subfragments from cosmids 9-13-1 and 8-1-1, transcripts of *Bip2*, *CaMKI*, *lgs*, as well as several repetitive transcripts were obtained from an embryonic cDNA library. *lgs* was identified among these genes by sequencing the *BiP2* and *lgs* coding regions from mutant lines, which revealed missense or nonsense mutations in five of the six *lgs* alleles, as indicated in (D).

(D) Structure of the *lgs* transcript. The 5.3 kb full-length cDNA clone LD02061 (Berkeley *Drosophila* Genome Project) is depicted with introns. Black boxes represent coding sequences; gray boxes represent UTR sequences.

location of *lgs* on chromosome 4 necessitated the use of pole cell transplantation to generate mosaic females with a wild-type soma and a *lgs* mutant germline. Homozygous mutant embryos derived from such a female display a segment polarity phenotype (Figures 1D and 1E) in which the larval epidermis forms a lawn of ventral denticles and lacks naked cuticle between the segmental denticle belts (Nüsslein-Volhard and Wieschaus, 1980). The similarities between the phenotypes of *lgs* and *wg* further support our conclusion that *lgs* encodes a component of the Wg signaling pathway.

Genetic Mapping and Positional Cloning of *lgs*

Chromosome 4 does not permit meiotic mapping because, unlike all other *Drosophila* chromosomes, it fails to undergo spontaneous recombination during female meiosis (Bridges, 1935). To genetically map *lgs*, we therefore had to use an alternative approach. We generated a series of 60 terminal deficiencies of Dp(4;Y)E, a Y chromosome that carries at its tip an extra copy of chromosome 4 (Muller and Edmondson, 1957). These deficiencies resulted from random breaks of dicentric Dp(4;Y)E chromosomes, which we produced by Flp-mediated unequal sister-chromatid exchange (Figure 2A; Ahmad and Golic, 1998). Each terminal deficiency of Dp(4;Y)E was analyzed cytologically and tested for its ability to rescue a homozygous *lgs* mutant genotype (a small number of those are shown in Figure 2B). Two terminal deficiencies were identified which barely differed cytologically, yet one, PE6.5, is lgs^{+} whereas the other, PE9.1, is lgs^{-} (Figure 2B). Based on these results, the *lgs* gene must be located at position 102B7.

A chromosomal walk was initiated with probes flanking P element MS209 to isolate a contiguous stretch of 150 kb DNA covering the breakpoints of both PE6.5 and PE9.1, as determined by chromosome in situ hybridization (Figure 2C). Because the region between these two breakpoints must contain at least an essential part of the *lgs* gene, we focused on transcribed regions within this interval and identified and compared their sequences with those derived from *lgs* mutant chromosomes. One transcript with an open reading frame en-

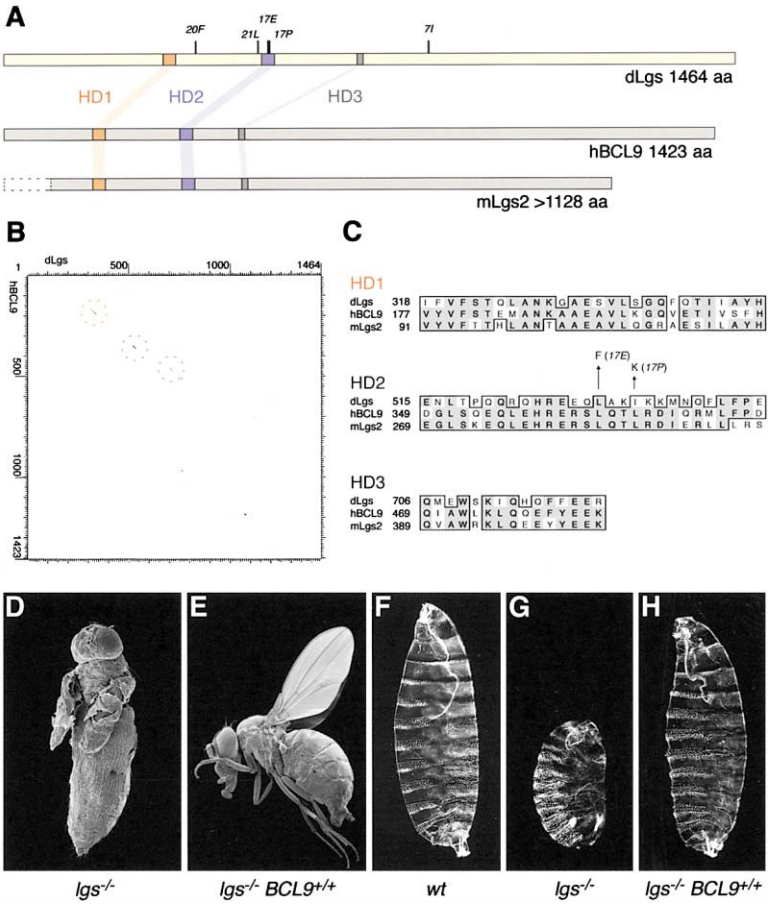

Figure 3. *lgs* Encodes the *Drosophila* Homolog of Human BCL9

(A) Schematic representation of the Lgs protein, human BCL9, and the BCL9-related protein Lgs2 (the mouse sequence of Lgs2 is shown because it is more complete, yet still lacks the N-terminal end). The only domains that show statistically significant sequence similarities between Lgs and its mammalian homologs are shown in colors (HD1, HD2, and HD3). Mutations found in our *lgs* alleles are indicated above the Lgs protein and affect the following amino acids: lgs^{20F}, Q384stop; lgs^{21L}, G509E; lgs^{17E}, L529F; lgs^{17P}, I532K; lgs^{7I}, Q850stop; lgs^{20K}, no nucleotide change was identified within the coding region.

(B) Protein matrix with Lgs on the horizontal and hBCL9 on the vertical axis. Although the overall similarity is low, several short regions of higher similarities can be found (circled) at comparable relative positions in both proteins. The matrix alignment tool of DNA Strider v.1.3f4 was used with the following parameters: BLOSUM62 matrix, stringency factor 7, 23 aa window.

(C) Alignment of amino acid sequences of HD1, HD2, and HD3 of Lgs, hBCL9, and mLgs2. Similarities are boxed, identities shaded in gray. The numbers to the left indicate the positions of HD1, HD2, and HD3 within their respective protein sequences.

(D and E) Scanning electron micrographs of a lgs^{21L}/lgs^{17E} pharate adult (D) and a rescued lgs^{21L}/lgs^{17E} *tubulinα1-BCL9* animal (E) are shown. lgs^{21L}/lgs^{17E} fail to hatch and show a severe lgs mutant phenotype (see also Figure 1F).

(F–H) Cuticle preparations of larvae derived from wild-type (F), *lgs* mutant (G), and *lgs* mutant *tubulinα1-BCL9* (H) embryos. The lgs^{20F}/lgs^{20F} embryo in (G) is derived from a homozygous lgs^{20F} mutant germline clone that was generated with the help of a *tubulinα1>lgs>Gal4* transgene (see Experimental Procedures). Rescue of the segment polarity phenotype is achieved by the maternal presence of a BCL9-expressing transgene (H).

coding a protein of 1464 amino acids contained point mutations in five out of six *lgs* alleles, two of which, lgs^{20F} and lgs^{7I}, are nonsense mutations predicted to cause a premature termination of translation (Figure 2D). This candidate gene must represent *lgs*, since a full-length cDNA driven by the ubiquitous promoter of the *tubulinα1* gene was found to completely rescue the lethality and other phenotypes associated with homozygous *lgs* mutations (data not shown).

lgs Encodes the *Drosophila* Homolog of Human BCL9

The Lgs protein sequence contains neither a recognizable protein motif nor does it show sequence homologies to any other *Drosophila* protein. Also, no proteins encoded in the genomes of *Caenorhabditis elegans*, mouse, and humans were predicted to share extensive sequence similarities with Lgs. However, we did identify a few stretches of ~30 amino acids in Lgs that show a statistically significant match to sequences in human and mouse BCL9 proteins (Figures 3A–3C; Willis et al., 1998). Although short, these patches of protein similarities are arranged in a colinear fashion in these proteins (Figure 3B). We refer to these regions as homology domains 1–3 (HD1–3). Intriguingly, all three missense mutations isolated in *lgs* map within, or in immediate vicinity to, HD2 (Figure 3A).

BCL9 was identified as the gene overexpressed in a cell line derived from a patient with precursor B cell acute lymphoblastic leukemia. It was found that a translocation caused the juxtaposition of the *BCL9* gene with regulatory elements of an immunoglobulin locus (Willis et al., 1998). In both mouse and humans there is an additional BCL9-related gene, referred to as *mLgs2* and *hLGS2*, respectively (Figure 3A). No data has been reported describing the normal function of these genes.

To explore the possibility that, despite the low degree of sequence similarity, BCL9 could represent the functional homolog of Lgs, a full-length BCL9 cDNA was assembled from human EST clones and used to generate a *tubulinα1* promoter-driven transgene. As illustrated in Figure 3E, this transgene was able to rescue viability and limb pattern of lgs^{17E}/lgs^{21L} animals, which normally die as pharate legless adults (Figure 3D). Even animals homozygous for the putative null allele lgs^{20F}, which causes larval lethality and encodes a severely truncated protein (amino acids 1–383), are rescued to adults, although the majority of these individuals (80%) fail to hatch from their pupal cases. Finally, we observed that the segment polarity phenotype of embryos lacking

both maternal and zygotic *lgs* function (see below) is fully rescued by expression of BCL9 (Figures 3F–3H). Together, these results provide convincing evidence that *lgs* encodes the *Drosophila* homolog of human BCL9 and suggest that the role of these two proteins in Wnt signaling is evolutionarily conserved.

Lgs Is a Nuclear Protein that Functions Downstream of Arm to Transduce the Wg Signal

To determine whether *lgs* is required for the generation of active Wg signal or for transduction of this signal, we assayed Wg target gene expression in genetic mosaics. Clones of cells lacking *lgs* function were generated with a *tubulinα1>lgs>Gal4* transgene in a lgs^{20F} homozygous background. Excision of the *>lgs>* Flp-out cassette generates *lgs* mutant cells that are marked by the gain of *Gal4* expression, which can be monitored by the activity of a *UAS-GFP* reporter gene. Typically, clones of *lgs* mutant cells underwent only a few divisions after they were generated in the presumptive wing blade; then, the mutant cells stopped proliferating and either died or were actively eliminated from the disc epithelium (data not shown). When stained for the presence of *Dll* expression 48 hr after mitotic recombination was induced, *lgs* mutant cells exhibited reduced levels of Dll protein (Figures 4A and 4B). This cell-autonomous behavior indicates that Lgs acts in Wg-receiving cells, permitting their survival and the expression of target genes.

To map the position of Lgs action along the Wg transduction pathway, we used Shaggy/Zw3 (Sgg) as a reference for an epistasis analysis (Siegfried et al., 1994). The *sgg* gene encodes the *Drosophila* homolog of GSK3, which plays a critical role in the β-catenin/Arm destruction complex (Wodarz and Nusse, 1998). Loss of *sgg* function causes the constitutive activation of the Wg pathway, which results in an embryonic phenotype opposite to that of *wg* or *lgs* mutations. Since *sgg*, as well as *lgs*, provides some of its function maternally, we sought to remove the maternal and zygotic products of both genes simultaneously. A *tubulinα1>lgs,sgg*$^{+}$*>Gal4* transgene was introduced into a *lgs*, *sgg* null mutant background to generate double-mutant female germ cells (Figure 4E). The *sgg*, *lgs* double-mutant embryos derived from these cells were identified by the use of three marker genes (see legend to Figure 4E). Such embryos displayed a "lawn of denticles" phenotype (Figure 4I), indistinguishable from that of *lgs* single-mutant embryos (Figure 4G), but in direct contrast to the "naked" phenotype of *sgg* single-mutant animals (Figure 4H). We interpret this result to indicate that *lgs* acts downstream of *sgg* in the Wg pathway.

To further narrow down the point of *lgs* action, we asked whether the constitutive signaling activity of N-terminally truncated Arm is dependent on *lgs* function. We used the *tubulinα1>lgs>Gal4* system described above to generate embryos devoid of wild-type *lgs* product, yet instead ubiquitously expressing a *UAS-*arm^{S10} transgene (Figure 4F), which encodes a mutant Arm protein with a 54 amino acid deletion in the N-terminal domain (Pai et al., 1996). Expression of arm^{S10} resulted in a "naked cuticle" phenotype in the presence of *lgs* (Figure 4K) but not in its absence (Figure 4L). Therefore, *lgs* function is required even for the activity of a stabilized form of Arm.

Finally, we localized the *lgs* gene product in cells with an antiserum directed against the N-terminal half of Lgs and found that wild-type cells, but not lgs^{20F} mutant cells, express Lgs protein in the nuclei (Figures 4C and 4D). Both subcellular localization and Lgs protein expression levels are invariant throughout the disc, suggesting that neither of these properties is regulated by the activity of the Wg signaling pathway. Nuclear localization of Lgs appears to be essential for its signaling activity, since a membrane-localized form of Lgs (CD2-Lgs), which does not have access to the nucleus, cannot rescue *lgs* mutant animals (data not shown).

Together, these results indicate that Lgs acts at the very bottom of the Wg signal transduction cascade, in conjunction with, or downstream of, nuclear Arm.

Lgs and BCL9 Bind Arm/β-Catenin via Their HD2 Domain

We used the yeast two-hybrid system and GST pull-down assays to examine the possibility that Lgs physically interacts with Arm. Indeed, we found that the N-terminal half of Lgs binds to the Arm protein (Figure 5A). We subsequently fine-mapped the Arm binding domain of Lgs to the HD2 region (Figures 5A and 5B) and the Lgs binding domain of Arm to armadillo repeats 1–4 (Figure 5C). Consistent with these results, we found that BCL9 also binds to Arm, as well as to β-catenin, and that the domain required for the interactions with β-catenin again maps precisely to the conserved HD2 sequence (Figures 5A and 5B).

Since lgs^{17P} and lgs^{17E} encode amino acid substitutions in HD2, we tested whether their protein products can bind to Arm protein in vitro. The binding of Lgs^{17P} (Figure 5D) and Lgs^{17E} (not shown) to Arm is reduced at least 10-fold compared to wild-type Lgs protein. We interpret this finding, which reinforces our observations of genetic interactions between *arm* and *lgs* alleles, as evidence that Wnt/Wg signal transduction normally depends on molecular interactions between the Lgs/BCL9 and Arm/β-catenin proteins.

Molecular Identification of Pygo as a Second Binding Partner of Lgs

A mutant form of Lgs protein from which HD2 was deleted exhibited a strong dominant-negative effect on Wg-dependent patterning processes when expressed from a transgene in wild-type larvae (data not shown). We took this observation as an indication that Lgs normally interacts not only with Arm, but also with at least one additional component. In an effort to identify such components, we carried out yeast two-hybrid screens for interacting proteins. In two independent screens in which either the entire protein or the N-terminal half of Lgs was used as a bait, we identified a novel PHD finger protein as a Lgs binding protein (referred to as Pygopus, a legless lizard with scaly skin; Figure 6A). The 815 amino acid residue Pygo protein carries a C-terminal domain of 60 amino acids (Figure 6B) that shows extensive homologies to the PHD (plant homology domain) finger, also known as LAP (leukemia-associated protein) do-

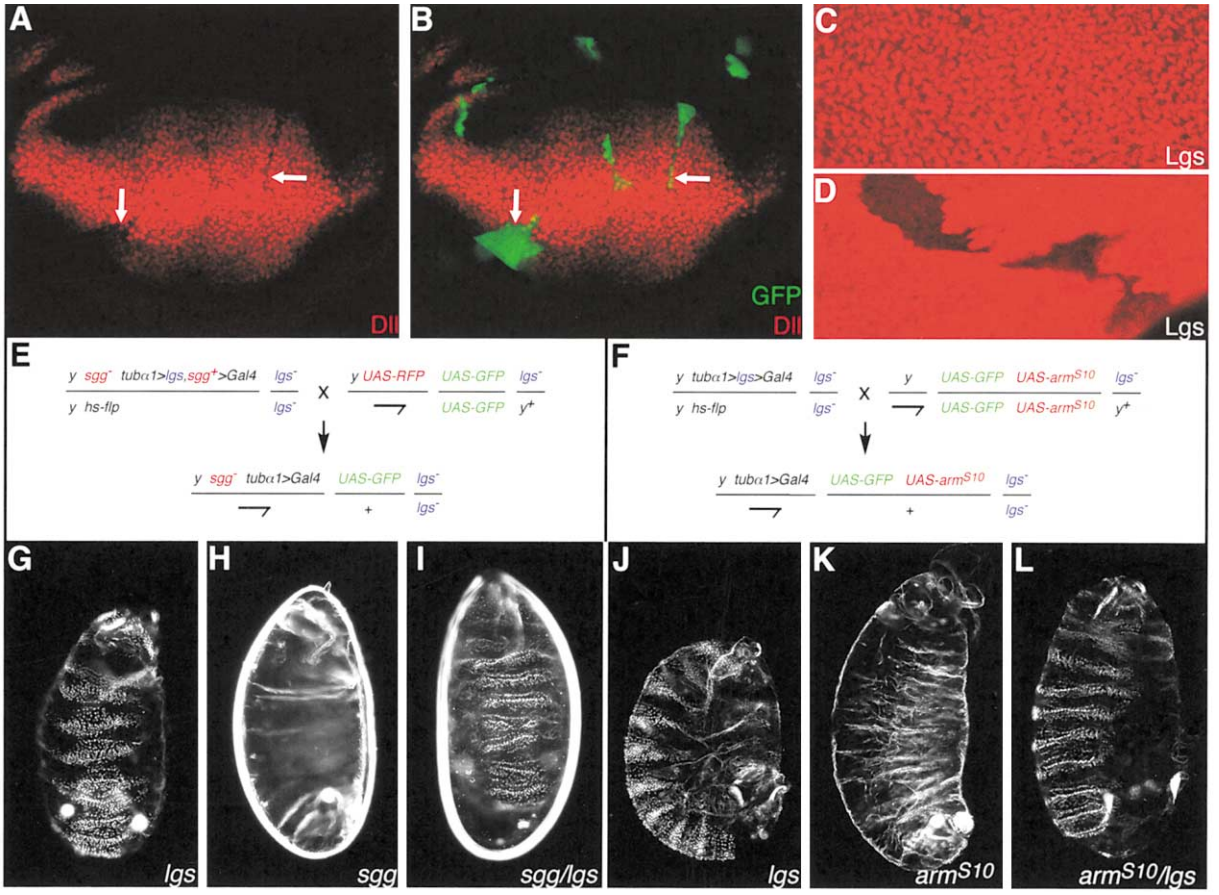

Figure 4. Lgs Is Required in Wg-Receiving Cells, Downstream of *sgg*, for the Activity of Arm^{S10} Protein

(A and B) *lgs* mutant clones were generated and marked by the Flp-induced loss of a *lgs*$^{+}$ transgene and simultaneous gain of *Gal4* expression (visualized with *UAS-GFP*, in green, [B]) using a *tubulinα1>lgs>Gal4* rescue construct in a *lgs*^{20F} homozygous mutant background. Clones were induced during second instar and analyzed in late third instar larvae by staining for the expression of the Dll protein (in red). Single channel view in (A), merged image in (B).

(C and D) High-power magnification of a fraction of a wild-type wing blade primordium stained with an anti-Lgs antiserum (C). To demonstrate the specificity of the antiserum, two *lgs* mutant clones located at the periphery of the wing primordium are shown (D). These clones were generated by the method described above for (A) and (B).

(E) Genetic scheme describing how embryos that simultaneously lack maternal and zygotic *sgg* and *lgs* functions were generated and marked. These embryos must (1) express GFP, indicating that they derive from a *sgg*^{M11} *lgs*^{20F} double-mutant female germ cell, (2) lack RFP expression, indicating that they are male embryos without a paternally contributed *sgg*$^{+}$ X chromosome, and (3) have a *yellow* (*y*) mutant cuticle, indicating that they are homozygous for the *lgs*^{20F} mutation on chromosome 4.

(F) Genetic scheme describing how embryos that simultaneously lack maternal and zygotic *lgs* functions but zygotically express *arm*^{S10}, which encodes a constitutively active form of Arm (Pai et al., 1996), were generated and marked.

(G–I) Epistasis analysis for *lgs* and *sgg*. *lgs* single-mutant embryos give rise to short larvae, which form a lawn of ventral denticles (G). Larvae derived from embryos lacking only *sgg* function show the characteristic naked phenotype associated with constitutive Wg signaling (H). The naked phenotype is reversed and the larvae show a lawn phenotype in the double-mutant situation (I).

(J–L) Epistasis analysis for *lgs* and *arm*^{S10}. In contrast to *lgs* single-mutant embryos (J), larvae derived from embryos in which the *UAS-arm*^{S10} transgene was ubiquitously expressed under the control of the maternally provided *tubulinα1>Gal4* transgene show a naked phenotype (K). The naked phenotype is reversed to a lawn phenotype in the double-mutant situation (L).

main. This domain comprises a cysteine-rich Zn binding motif, which has been associated with proteins involved in chromatin-mediated regulation of transcription (Aasland, et al., 1995). The PHD finger of Pygo is necessary and sufficient to mediate the interaction with Lgs (Figure 6C). We mapped the region of Lgs responsible for Pygo binding to the HD1 sequence (Figure 6D). Moreover, we identified and isolated two human homologs of the *Drosophila pygo* gene (Figure 6A). The protein products of both human genes, hPYGO1 and hPYGO2, possess a highly conserved PHD finger that interacts with the HD1 of BCL9 (Figure 6D). The only other domain in *Drosophila* Pygo and hPYGO1/hPYGO2 that shows significant sequence homology is a 50 amino acid stretch in the N-terminal region, which we refer to as "N-terminal homology domain" (NHD; Figures 6A and 6B).

The interaction with Pygo appears to be relevant for the in vivo function of Lgs, since a mutant form of Lgs with a deletion of HD1 was unable to rescue *lgs*^{20F} mutant animals (not shown). Consistent with this finding, we observed that epitope-tagged forms of Pygo localize to the nuclei of imaginal disc cells (data not shown).

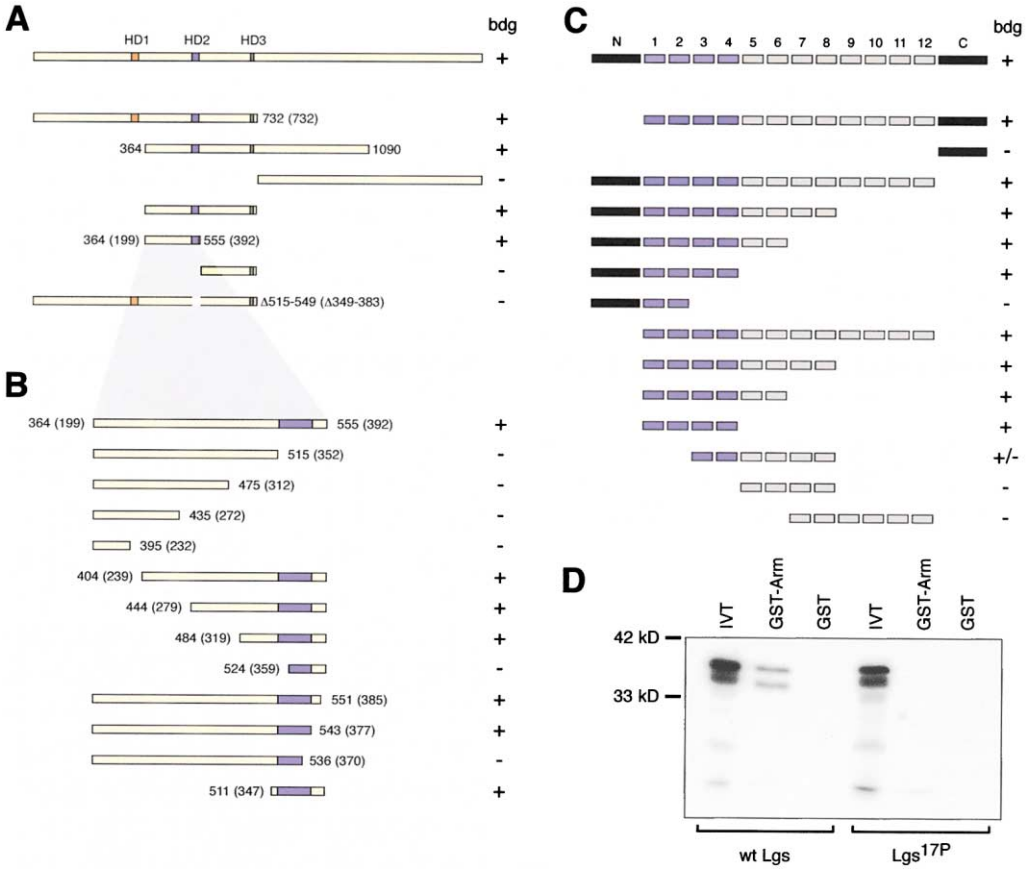

Figure 5. The HD2 of Lgs and BCL9 Binds to Arm and β-Catenin

(A and B) The molecular interaction between Lgs/BCL9 and Arm/β-catenin was mapped by yeast two-hybrid (A) and GST pull-down assays (B). Full-length Lgs/BCL9 protein is schematically represented on top, and a subset of their indicative derivatives is shown below. The numbers indicate the positions of the N or C termini of the Lgs/BCL9 fragments. All fragments shown were tested for the case of Lgs and are marked by numbers without parentheses. A subset of the fragments in (A) and all fragments in (B) were also tested for BCL9 and are marked with numbers in parentheses that refer to the sequence of BCL9. The results of the interaction studies are indicated to the right (bdg). Fragments in (B) were synthesized in vitro as fusion proteins with an N-terminal GFP moiety. Proteins were pulled down with GST-Arm$^{\Delta N}$ (aa 154–843) or GST-β-catenin.

(C) The domains of Arm required for Lgs binding were mapped by GST pull-down assays. A schematic representation of the full-length Arm protein (top) and a subset of its derivatives (below) is shown. Numbers 1–12 refer to the "arm repeats" (Peifer et al., 1994). Proteins were pulled down with GST-Lgs^{N} (aa 1–732).

(D) GST pull-down experiment is shown for a fragment of Lgs^{17P}. Identical results were obtained for the equivalent fragment of Lgs^{17E} (not shown). Full-length Lgs^{17E} protein also loses its ability to bind to Arm when tested by coimmunoprecipitation from lysates of HEK 293 cells expressing both proteins (not shown). GST-Arm$^{\Delta N}$ fusion protein (aa 154–843) and in vitro translated wild-type (wt) and mutant (Lgs^{17P}) protein fragments (aa 364–555) were used. Abbreviations: IVT, in vitro translated protein; GST, negative control with GST protein alone.

pygo Is a Segment Polarity Gene Required for Wg Signaling

The physical association of Pygo and Lgs suggests that Pygo, like Lgs, may be required for Wg signaling in vivo. To explore this hypothesis, we searched our collection of suppressors of the *sev-wg* phenotype for mutations that map to the tip of the right arm of chromosome 3, the position of the *pygo* gene. One such suppressor, *Sup^{130}*, mapped to this position and intriguingly, it shows dominant lethality in combination with the *lgs* allele *lgs^{17E}* (*Sup^{130}*/+ *lgs^{17E}*/+ transheterozygous animals do not survive). We sequenced the *pygo*-coding region using genomic DNA from homozygous *Sup^{130}* mutant larvae and identified a 14 bp deletion starting at amino acid position 751. Hence, this allele is referred to as *pygo^{130}* and encodes a truncated Pygo protein lacking the C-terminal PHD finger. The lethality caused by the homozygous *pygo^{130}* genotype can be fully rescued by a *tubulinα1* promoter-driven transgene that contains either the coding region of the *Drosophila pygo* gene or that of one of its two human homologs hPYGO1 and hPYGO2.

To assay the possible role of Pygo in Wg signal transduction during development, we generated embryos homozyous for the *pygo^{130}* mutation that derived from female germ cells equally mutant for *pygo*. Such embryos are devoid of any wild-type Pygo activity and die with a severe segment polarity phenotype (Figures 7A–7C). Mutant individuals lacking only zygotic function survive until early pupal stages and exhibit imaginal discs that are abnormally small. The Hh target gene *ptc* was expressed at wild-type levels in these discs; however, no expression of the Wg target *Dll* could be detected (Fig-

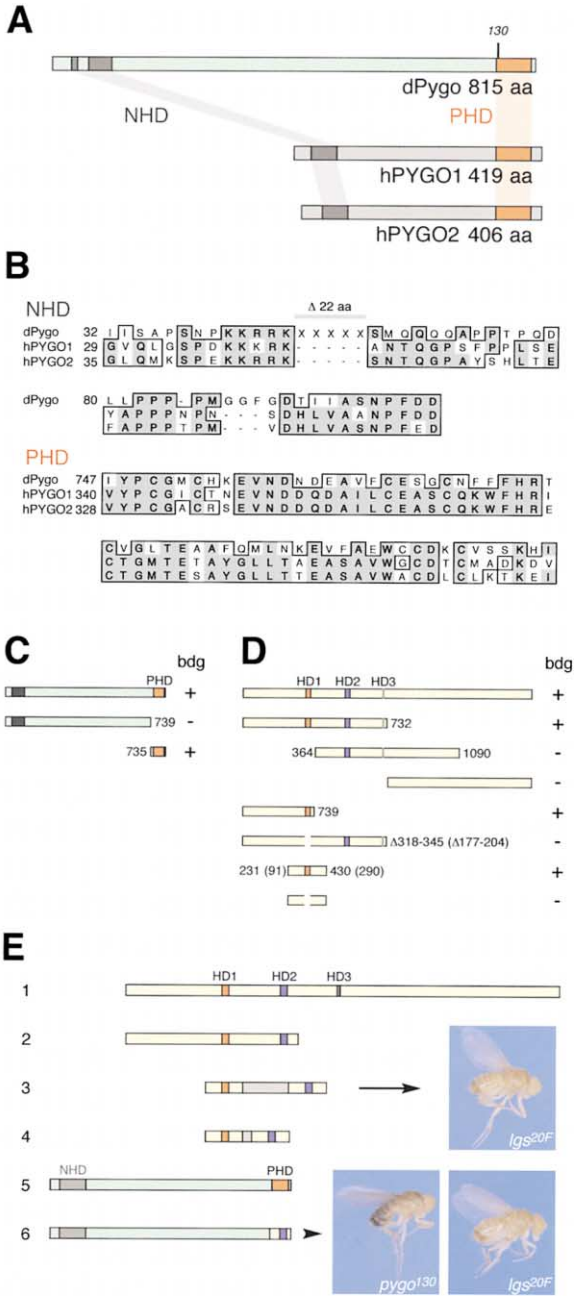

Figure 6. *Drosophila* and Human Pygo Proteins Contain a PHD Finger Motif with Which They Bind to the HD1 of Lgs/BCL9

(A) Schematic representation of Pygo, human PYGO1, and human PYGO2. The two domains that show high sequence similarities are highlighted in dark gray (NHD, N-terminal homology domain) and red (PHD, plant homology domain). The NHD appears to be unique, as we failed to find a similar sequence in other *Drosophila* or human proteins.

(B) Alignment of amino acid sequences of NHD and PHD in Pygo and its human homologs. Similarities are boxed, identities shaded in gray. The numbers to the left indicate the positions of NHD and PHD within their respective protein sequences. For the NHD alignment, a gap of 22 aa has been introduced in the *Drosophila* NHD [represented as $(X)_5$].

(C) Schematic representation of the proteins tested in the yeast two-hybrid assay for their interactions with Lgs and BCL9. Results are indicated to the right (bdg).

(D) Schematic representation of the proteins tested in the yeast two-hybrid assay for their interactions with Lgs. The two proteins shown at the bottom were tested by a pull-down assay for both Lgs (numbers without parentheses) and BCL9 (numbers in parentheses), with the same result (bdg). The deletion removing HD1 comprises aa 318–345 for Lgs and aa 177–204 for BCL9. Fusion proteins used were S-Tag-Pygo (aa 542–815) and GST-hPYGO2 (aa 301–406).

(E) Lgs and the PHD finger of Pygo serve to assemble Pygo and Arm. Schematic representation of Lgs (yellow) and Pygo (light green) constructs that were used in transgene assays to assess their ability to rescue *lgs* or *pygo* mutant animals. (1) Full-length Lgs (pOP216, aa 1–1464); (2) C-terminally truncated Lgs (pTK131, aa 1–583); (3) HD1-Gal11-HD2 (pTK153, aa 268–395 [HD1], aa 369–500 [Gal11], aa 465–596 [HD2]); (4) HD1-$(HA)_3$-HD2 (pTK143, aa 268–395 [HD1], aa 465–596 [HD2]); (5) full-length Pygo (pTK56, aa 1–815); (6) Pygo[ΔPHD]-HD2 [pTK135, aa 1–740 (Pygo[ΔPHD]), aa 483–561 (HD2)]. Transgenes 1–4 are able to rescue lgs^{20F} homozygotes. An example for an adult animal rescued by transgene 3 is shown on the right. Transgene 5 can rescue $pygo^{130}$ homozygotes. Transgene 6 can rescue $pygo^{130}$ as well as lgs^{20F} homozygotes (photographs on the right).

ures 7F–7I). These discs appear to lack the presumptive wing blade field and possess two primordia for the notum (Figures 7G and 7I). From these results we conclude that *pygo*, like *arm*, *pan*, and *lgs*, is required for Wg signal transduction in vivo.

From the results presented so far, it can be concluded that Lgs and Pygo are required for the signaling activity of Arm, and that this function depends on the ability of Lgs to interact molecularly with Arm and on the ability of Pygo to molecularly interact with Lgs. Based on their subcellular localization and epistatic relationship with Arm^{S10}, Lgs and Pygo are unlikely to exert their function by impeding proteasome-mediated degradation of Arm. They could play a role in permitting nuclear import or preventing nuclear export of Arm. However, we think this is also unlikely, because we fail to detect any difference in subcellular localization of Arm in *lgs* mutant embryos (not shown). An alternative possibility is that Lgs and BCL9, respectively, function to tie Pygo to the β-catenin-TCF complex, perhaps to allow Pygo to activate and sustain the expression of Wnt target genes. This hypothesis raises several predictions that will be addressed below. First, this model implies that the main task of Lgs/BCL9 is to serve as an adaptor to tether Pygo to Arm/β-catenin. Thus, most of the Lgs/BCL9 protein should be dispensable, as long as HD1 is covalently linked to HD2, allowing the formation of a bridge between Arm/β-catenin and Pygo. Lgs might even be entirely superfluous if Pygo was endowed with the ability to directly bind β-catenin. Second, this model would require that Arm/β-catenin is able to bind simultaneously, and stably, with both Pan/TCF and Lgs/BCL9. This in turn would necessitate separate binding sites on Arm/β-catenin for Pan/TCF and Lgs/BCL9. Finally, Pygo proteins would have to possess the ability to stimulate transcription when recruited to promoters of Wnt target genes.

The Principal Role of Lgs Is to Recruit Pygo to Arm

We have already presented evidence that Lgs function critically depends on the integrity of its HD1 and HD2 domains. We have not yet addressed, however, the role of HD3, nor that of any other part of the protein. A

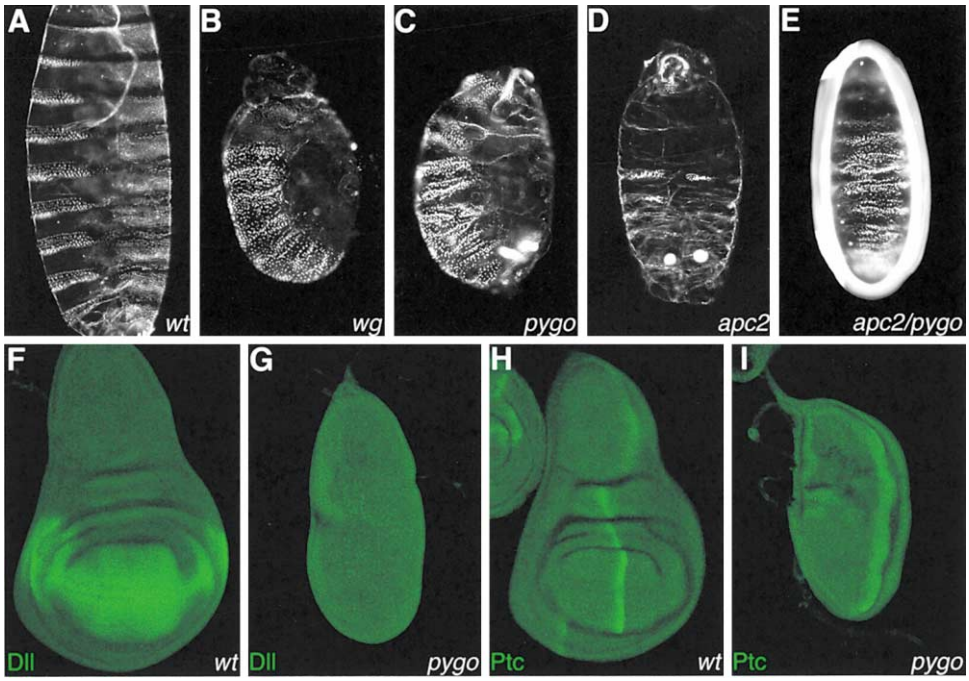

Figure 7. *pygo* Is a Segment Polarity Gene Required for Wg Signaling

(A–C) Cuticle preparations of larvae derived from wild-type (A), *wg* mutant (B), and *pygo* mutant embryos (C). The $pygo^{130}/pygo^{130}$ embryo in (C) is derived from a homozygous $pygo^{130}$ mutant germline clone (see Experimental Procedures) and displays a *wg*-like phenotype.

(D and E) *pygo* functions downstream of *dAPC2*. Two cuticle preparations are shown from larvae that developed in the absence of maternal and zygotic wild-type *dAPC2* function (McCartney et al., 1999). The embryo in (E) additionally lacks the maternal and zygotic function of *pygo* (see Experimental Procedures). In contrast to *dAPC* single-mutant animals, which have strongly reduced denticle belts, double-mutants display a *pygo*-like phenotype.

(F–I) Confocal images of third instar wing imaginal disc preparations stained with antibodies against Dll (F and G) and Ptc (H and I). Wild-type animals show normal expression of these genes (F and H). Discs derived from $pygo^{130}$ mutant larvae are small, yet express Ptc (I), but fail to express Dll (H). Lack of Dll expression may be an indirect consequence of the earlier wing-to-notum transformation in $pygo^{130}$ larvae. However, we also see a strong reduction of Dll expression in $pygo^{130}$ mutant cells of mosaic animals (not shown).

key result was obtained with a transgene encoding a truncated Lgs protein (amino acids 1–583) that lacks HD3 and all sequences C-terminal of HD3 (Figure 6E). This transgene fully rescued homozygous lgs^{20F} animals. We next tested if HD1 and HD2 were sufficient for Lgs function and used a 150 amino acid stretch of the unrelated nuclear yeast protein Gal11 to join HD1 and HD2 (see Figure 6E). Surprisingly, expression of this hybrid protein fully rescued patterning and growth of lgs^{20F} null mutant animals. Although some of these animals failed to hatch from their pupal cases, more than 50% did hatch, and hence represent fully rescued animals (Figure 6E). An even smaller protein, consisting only of HD1 and HD2 connected with three copies of the HA tag, could also substitute for Lgs protein at a rescuing efficiency only slightly lower than that of the HD1-Gal11-HD2 protein. Finally, we asked whether even HD1 might be dispensable if Pygo was directly equipped with HD2 and hence with the ability to bind to Arm. A *tubulin*α*1* promoter-driven transgene was constructed in which the PHD finger of Pygo was replaced by the HD2 domain of Lgs (Figure 6E). Remarkably, this fusion protein rescued both lgs^{20F} as well as $pygo^{130}$ mutant animals (Figure 6E). Thus, we conclude that the PHD finger has no other essential role besides binding Pygo to Lgs, and that the primary, and possibly sole, function of Lgs is the recruitment of Pygo to Arm.

A Trimolecular Complex of Lgs/BCL9, Arm/β-catenin, and Pan/TCF

The primary structure of Arm and β-catenin consists of an N-terminal and a C-terminal tail flanking a central domain of ~500 residues composed of 12 armadillo repeats (Peifer et al., 1994). These repeats pack against one another to form a superhelix that features a positively charged groove (Huber et al., 1997). The armadillo repeat domain mediates the binding of β-catenin to cadherins, APC, Axin, and TCF. Despite the lack of significant sequence homologies, these proteins bind competitively to β-catenin, presumably because they contact the same surface area of β-catenin (reviewed by Daniels et al., 2001). If Lgs/BCL9 also bound to this surface, it would be expected to compete with Pan/TCF for the interaction with β-catenin and could not be recruited to Wnt target genes. We therefore sought to address this issue by using peptide competition and coimmunoprecipitation experiments.

First we used biotinylated peptides representing the N-terminal domains of hTCF4 (or Pan) to pull down labeled β-catenin (or Arm protein) with avidin beads (Figures 8A and 8B). This peptide-protein interaction could be effectively disrupted by an excess of nonbiotinylated TCF or Pan peptides, but not by an excess of HD2 peptides (Figures 8A and 8B). We then used GST-BCL9 protein to pull down labeled hTCF4 in the presence of

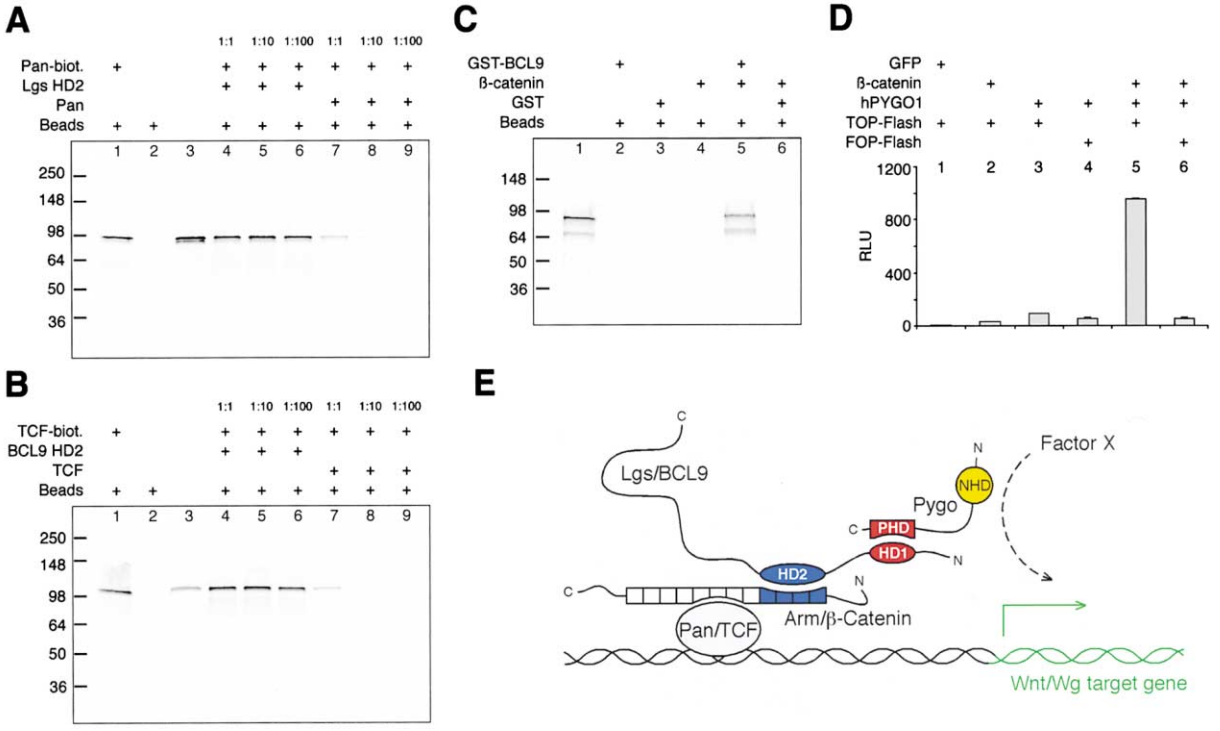

Figure 8. Lgs/BCL9 and Pan/TCF Do Not Compete for Binding to, But Can Form a Complex with, Arm/β-Catenin

(A and B) Peptide competition experiments. In vitro translated [^{35}S]methionine Arm$^{\Delta N}$ ([A], aa 154–843) or β-catenin (B) protein (lanes 3) was pulled down with biotinylated peptides derived from Pan ([A], aa 1–53) or from hTCF4 ([B], aa 1–53) and avidin beads (lanes 1). In parallel, the binding reaction was carried out in the presence of increasing amounts of the respective nonbiotinylated peptides (lanes 4–6), or Arm/β-catenin binding peptides derived from Lgs HD2 ([A], aa 510–549) or BCL9 HD2 ([B], aa 344–383) in lanes 7–9. Ratios of biotinylated to competing peptides are indicated on top of the figures.

(C) Trimolecular interaction between TCF, β-catenin, and BCL9. In vitro translated, labeled hTCF4 protein (lane 1) was pulled down in the presence of the components indicated above the gel. Only in the presence of β-catenin (aa 136–671) can hTCF4 be pulled down with GST-BCL9 (aa 128–433).

(D) Effects of hPYGO1 on TCF-mediated transcription. 293 cells were transiently transfected with the pTOP-Flash or pFOP-Flash luciferase and different effector plasmids, as indicated. ΔN-β-catenin (50 ng) or hPYGO1 (350 ng) activate the pTOP-Flash reporter; cotransfection of hPYGO1 with ΔN-β-catenin strongly enhances the response. RLU: relative light units.

(E) Model for the activation of Wnt target genes by Lgs/BCL9 and Pygo. Lgs/BCL9 tethers Pygo to β-catenin, which is bound to regulatory regions of Wnt targets by Pan/TCF. Protein-protein interaction domains are highlighted in blue and red. For details see Discussion.

β-catenin protein. hTCF4 is efficiently retained on BCL9-charged glutathion beads in the presence, but not absence, of β-catenin, which apparently can function as a bridge between the two proteins (Figure 8C). These results indicate that Lgs/BCL9 and Pan/TCF do not compete for their interaction with Arm/β-catenin, but rather bind it simultaneously. This in turn suggests that Lgs/BCL9 is recruited to TCF binding sites of Wnt target genes.

Transcriptional Upregulation of a Wnt Reporter by Pygo

To address the role of Pygo in β-catenin-mediated transcription, we used a TCF reporter gene (TOP-Flash) in immortalized human embryo kidney cells (HEK 293 cells). Low levels of a stable mutant form of β-catenin (ΔN-β-catenin; van de Wetering et al., 1997) were introduced into these cells to partially stimulate the pathway. The additional expression of hPYGO1 (Figure 8D) or hPYGO2 (not shown) led to a large increase in luciferase activity (30-fold). These levels are significantly higher than the sum of those produced by either treatment alone (Figure 8D). This potentiation of β-catenin activity by hPYGO1 and 2 appears to be mediated by the interaction of endogenous TCF protein with its DNA target sites, as it is only observed with TOP-Flash, which contains five optimal TCF binding sites, but not with the control reporter FOP-Flash, which contains five mutated sites.

Although less powerful per se than our genetic arguments, this experiment adds supportive evidence to the notion that Pygo proteins transduce Wnt signals by activating TCF target genes in a β-catenin-dependent manner.

Discussion

Wnt proteins control a multitude of fundamental patterning processes during animal development by regulating the transcription of target genes. The key component of this pathway, β-catenin, transduces Wnt signals by virtue of its posttranscriptional upregulation, permitting its translocation to the nucleus and the association with DNA binding proteins of the TCF family. These β-catenin-TCF complexes have been proposed to func-

tion as bipartite transcription factors that activate the expression of Wnt targets (van de Wetering et al., 1997). However, the mechanism by which these complexes activate gene expression is poorly understood. Here we used genetic methods in *Drosophila* to discover and validate two components of the Wnt pathway. Our results indicate that TCF and β-catenin function in a tetrapartite complex with Lgs/BCL9 and Pygo to transcriptionally activate target genes in response to Wnt signals.

Nuclear β-Catenin as Part of a Universal Complex of Dedicated Core Components

Key to our findings was a sensitized morphological reporter system for Wg signaling with which we identified a new *Drosophila* segment polarity gene, *lgs*. We showed that it is required in Wg-receiving cells for the expression of Wg target genes and that it encodes a nuclear protein with slight, but significant, structural similarities to human BCL9. Genetic epistasis experiments and protein-protein interaction assays indicated that Lgs and BCL9 bind to Arm and β-catenin, respectively. The identification of Lgs permitted the isolation of Pygo, which like Lgs, is required for Wg signaling. Pygo and its human homologs bind to Lgs and BCL9, respectively. We showed that Lgs/BCL9 and the DNA binding proteins Pan/TCF can bind simultaneously to Arm/β-catenin. Together, these findings provide the basis for postulating the existence of a nuclear multiprotein complex, in which β-catenin, as appropriately concealed in its name, ties Lgs/BCL9 and thus Pygo to Wnt-inducible promoters (Figure 8E).

In vivo evidence for this model is provided by the nature of the mutations recovered in our screen. First, the two alleles found in the *pan* gene (Brunner et al., 1997) both encode proteins with N-terminal mutations that have a deleterious effect on the affinity to β-catenin (Brunner et al., 1997). Second, the *lgs*^{17E} allele encodes a protein with a mutation in the HD2 domain that abolishes the interaction between Lgs and Arm. And third, the *pygo*^{130} allele encodes a protein lacking its PHD finger and hence the ability to bind to Lgs. In all cases, the disruption of only one of the three protein-protein interactions within the tetrapartite β-catenin complex leads to a common outcome, namely a decrease in Wg signal response.

All previously identified proteins that have been implicated in mediating β-catenin-dependent activation of Wnt target genes, such as TATA binding protein (TBP; Hecht et al., 1999), TIP49 (Bauer et al., 1998), p300/CBP (Hecht et al., 2000; Takemaru and Moon, 2000), or Brg-1 (Barker et al., 2001), have pleiotropic roles, and their specific in vivo contribution to Wnt transduction cannot be assessed by genetic means. In sharp contrast, the newly identified proteins Lgs/BCL9 and Pygo appear to represent components that are dedicated to the Wnt/Wg signaling pathway. This argument is based largely on our inability to detect *lgs* or *pygo* phenotypes that differ from those caused by reduction of Wg activity. The argument is reinforced, however, by our observation that the PHD finger of Pygo and all but HD2 of Lgs are dispensable if Pygo is allowed to directly interact with β-catenin in the form of a hybrid Pygo[ΔPHD]-HD2 protein.

The activities of Pygo and Lgs are specific to the Wg signaling pathway, where they appear to be universally required to mediate all apparent Wg responses during normal *Drosophila* development. We are not currently aware of any tissue in which Arm transduces a Wg signal in the absence of Pygo and Lgs. However, we note that our results do not rule out the possibility that there may be situations in mammalian systems where Pygo or BCL9 proteins are specifically absent or limiting (see below).

Our results suggest that the primary function of Lgs/BCL9 is to recruit Pygo into the Arm/β-catenin complex. Our understanding is less complete regarding the mechanism of action of Pygo. In reporter gene assays, Pygo effectively enhances the transcription of TCF reporter genes, indicating that it possesses properties of a transcriptional activator. The human and *Drosophila* Pygo proteins share two domains of structural similarities, the N-terminal homology domain (NHD) and the C-terminal PHD finger. The PHD finger appears to mediate the binding of Pygo to the HD1 domain of Lgs/BCL9. Hence, it is likely the NHD motif by which Pygo exerts its transcriptional activator function. While we do not yet know which proteins are targeted by the NHD, plausible candidates include TBP-associated factors (TAFs), histone acetyl transferases, and components of chromatin-remodeling complexes. A recent study suggested a role for the Brahma chromatin-remodeling complex in repressing Wg target genes (Collins and Treisman, 2000), indicating that altering chromatin conformation may be an obligatory step when activating these genes. Firm answers regarding the mode of action of Pygo will have to await the identification of NHD target proteins.

A Possible Role for Wnt Signaling in B Cell Malignancies

BCL9 has been identified by the translocation t(1;14)(q21;q32) from a patient with precursor-B-cell acute lymphoblastic leukemia (ALL; Willis et al., 1998). B-cell malignancies and, in particular, the B-cell non-Hodgkin's lymphomas are often associated with chromosomal translocations in which certain genes become overexpressed due to a juxtaposition to immunoglobulin loci (Look, 1997; Willis and Dyer, 2000). BCL9 transcript levels are normally very low in B cells, but 50-fold higher in the CEMO-1 cell line from which the translocation break point was cloned (Willis et al., 1998). Our finding that the *Drosophila* homolog of BCL9, Lgs, is a component of the Wg signaling pathway raises the possibility that activation of the Wnt pathway may be causally linked to certain forms of B-cell leukemia or lymphoma.

The formation of mature B- and T-lymphocytes and other hematopoetic lineages is guided by complex genetic and environmental cues. Some recent reports have implicated Wnt signaling in these processes. Proliferation and differentiation of $CD4^{-}CD8^{-}$ double-negative thymocytes (Staal et al., 2001; Ioannidis et al. 2001) and pro-B cells (Reya et al., 2000) require an intact Wnt pathway. Hence, in both pro-T and pro-B cells, Wnt signals might provide important mitogenic stimuli at central developmental stages, although some of the effects of these signals may depend on inputs from additional receptor systems (Gounari et al., 2001). Further

support for the notion that deregulated Wnt signaling could play a role in blood cell cancers comes from recent findings that Wnt3A has a mitogenic effect on pro-B cells (Reya et al., 2000) and that in pre-B cell ALL patients Wnt 16 was overexpressed (McWhirter et al., 1999).

However, in *Drosophila* the mere overexpression of Lgs does not lead to an activation of the Wg pathway, and compared to hPYGO1 and 2, expression of BCL9 has only a mild stimulatory effect in TCF reporter assays (not shown). It is possible, though, that the expression of high BCL9 levels may render certain cell types more responsive to Wnt inputs. Hence, an additional event, such as the upregulation of Wnt expression, could create conditions in which the high levels of BCL9 cause a deregulated cellular behavior resulting in pre-B ALL. Such a scenario could explain the apparent low incidence of deregulated BCL9 expression in tumor samples (Willis and Dyer, 2000).

An interesting observation in this respect was recently described by Prieve and Waterman (1999), who found that Jurkat T cells and normal T lymphocytes exhibit a vast difference in their responsiveness to β-catenin. Overexpression of β-catenin led to a 150-fold induction of reporter gene expression in Jurkat cells but had no discernible effect in normal T cells, although both cells showed a comparable response to a VP16 control. This result was interpreted to indicate that normal T lymphocytes lack a component necessary for gene activation by nuclear β-catenin. If such a situation occurred in vivo where the amounts of either BCL9 or Pygo were limiting, a transcriptional upregulation of the respective gene might have a significant impact.

BCL9 and Pygo: New Opportunities for the Development of Anticancer Drugs

The most prevalent activation of the Wnt pathway in cancer is caused by the loss of APC. Mutations in APC occur in >85% of inherited and sporadic colorectal cancers (Kinzler and Vogelstein, 1996). Such mutations result in the accumulation of nuclear β-catenin and the concomitant overexpression of Wnt target genes. The β-catenin-TCF complex has therefore emerged as an attractive target for anticancer drugs (Daniels et al., 2001). Our discovery that Lgs/BCL9 and Pygo are required for the transcriptional activity of Arm/β-catenin raises the possibility that the protein-protein contacts between the three components may represent additional targets for anticancer intervention. As a proof of principle we tested the effect of disrupting one such interaction on the consequences of mutations in *Drosophila* APC2. Embryos devoid of wild-type maternal and zygotic *dAPC2* function die during embryogenesis with a naked cuticle phenotype caused by the constitutive activation of the Wg pathway (Figure 7D; McCartney et al., 1999). This situation is reverted to a lawn-of-denticle phenotype (loss of Wg signaling) if $dAPC2^{\Delta S}$ embryos are also mutant for $pygo^{130}$ (Figure 7E). The $Pygo^{130}$ protein lacks the C-terminal PHD finger. Hence, in $dAPC2^{\Delta S}$ animals, the loss of wild-type APC2 function is largely, if not completely, ineffective if Pygo can no longer interact with Lgs.

Any drug designed to disrupt a protein-protein interaction involving β-catenin must be highly specific and should not interfere, for example, with the binding of E-cadherin to β-catenin. E-cadherin has properties of a tumor suppressor, and its loss has been implicated in the transition from adenoma to carcinoma (Perl et al., 1998). Recent structural studies indicated that E-cadherin and TCF use many of the same protein-protein contacts to bind to β-catenin (Huber and Weis, 2001), complicating the strategy to identify substances that specifically disrupt the β-catenin-TCF interaction. Our observation that TCF and BCL9 do not compete for their binding to β-catenin suggests that the β-catenin-BCL9 interaction may provide an attractive alternative for therapeutic intervention.

Experimental Procedures

Isolation and Mapping of *lgs* and *pygo* Alleles

EMS-treated genomes (2×10^5) were screened for suppressors of the *sev-wg* rough eye phenotype (Brunner et al., 1997). Third chromosomal suppressors were coarsely mapped by meiotic recombination using a panel of P[y^+] insertions. Terminal deficiencies of the chromosome 4 derivative Dp(4;Y)E were generated by heat-shocking progeny of w^{1118}; P[ry^+,*70FLP*]3A; *ci gvl ey sv* females and w^{1118}/*DcY*; *ci gvl ey sv* males (Ahmad and Golic, 1998) at 48 hr for 1 hr at 38.5°C. Mosaic F_1 males were individually crossed to w^{1118}; *ci gvl ey sv* females. The recessive markers uncovered by a terminal deficiency serve as a crude indication of the extent of the deficiency, as they are evenly spread over chromosome 4.

Transgenes

All *lgs* transgenes are based on the 5.3 kb cDNA clone LD02061. A full-length *BCL9* cDNA was assembled from ESTs that contained *BCL9*-cDNA and from PCR products generated on human cDNA and genomic DNA. The translated sequence of our full-length *BCL9* clone (pTK1) was found to deviate slightly from the sequence previously published by Willis et al. (1998) due to a single additional base pair at position 4171 extending the open reading frame from 4185 to 4281 bp. *tubulinα1>lgs>Gal4* and *tubulinα1>lgs,sgg*$^+$*>Gal4* flp-out constructs are based on a *sgg*$^+$ minigene (*sgg10*, obtained from M. Bourouis) and components described previously. *UAS-RFP* is based on the Ds-Red red fluorescent protein cDNA from Clontech (Invitrogen). Lgs-CD2 protein was expressed from a cDNA coding for the full-length rat CD2 transmembrane protein (aa 1–344) was fused to the 5′ end of the *lgs* cDNA. A full-length *Drosophila pygo* cDNA (pTK45.1) was assembled from plasmid DNA of the Lgs-interacting yeast two-hybrid clone TK5.35h, which contained bp 749–2448 of the predicted *pygo* open reading frame, and a genomic PCR fragment, which covered bp 1–975. Full-length cDNA clones for hPYGO1 and hPYGO2 were isolated from a human placenta library (Invitrogen). Wild-type *lgs* (Figure 6E1), *lgs aa1–583* (Figure 6E2), wild-type *pygo* (Figure 6E5), *pygo[ΔPHD]-HD2* (Figure 6E6), *hPYGO1*, and *hPYGO2* transgenes were driven by the *tubulinα1* promoter. The *HD1-Gal11-HD2* (Figure 6E3), *HD1-HA-HD2* (Figure 6E4), and *Lgs-CD2* transgenes were expressed from UAS-constructs under the control of a ubiquitously expressed *actin5c-Gal4* driver.

Yeast Two-Hybrid and GST Pull-Down Experiments

Yeast two-hybrid interaction studies and screens were carried out as described by Paroush et al. (1994). Two alternative Lgs-LexA-baits were used: *pLexA-lgsFL* coded for the full-length Lgs protein and *pLexA-lgsN* coded for the N-terminal 732 amino acids of the Lgs protein fused to LexA. Both bait constructs were used for screening a *Drosophila* 0–12 hr embryonic cDNA library (RFLY-1), fused to the AB42 activation domain. Three independent library cDNA clones that all coded for C-terminal parts of the Pygo protein (starting at aa 251, 731, and 742), were found to interact specifically with both of these baits and with comparable BCL9 baits. GST pull-down experiments were carried out according to standard protocols (Sambrook and Russell, 2001).

Immunohistochemistry

Antibodies were mouse monoclonal anti-Ptc (gift from I. Guerrero), mouse monoclonal anti-Dll (gift from I. Duncan), mouse monoclonal anti-Arm 7A1, and rabbit polyclonal anti-Lgs^N (antiserum prepared against GST-Lgs^N, aa 1–732).

Germline and Disc Clones

Flp-out recombination for germline clones was induced in females during pupal stages by a 60 min heat shock at 37°C for the *lgs* clones and by a 30 min heat shock at 36°C for the *lgs sgg* clones. GFP-expressing embryos were selected for cuticle preparations 24 hr after egg deposition. Imaginal disc clones were generated essentially by the method described in Figure 4F, except that no *UAS-arm^{S10}* transgene was present and the progeny rather than the parental generation was heat shocked (30 min at 36°C, 48–60 hr before dissection). *pygo* and *dAPC2 pygo* double-mutant germline clones were generated with FRT82 *pygo^{130}* and FRT82 *dAPC2$^{\Delta S}$ pygo^{130}* chromosomes. In both cases we used an FRT82 *ovo^{D1}* chromosome to select for mutant germ cells.

Peptide Competition Experiments

N-terminally biotinylated hTCF4 and Pan peptides (as specified in legend to Figure 8) were incubated with [^{35}S]methionine β-catenin and Arm, respectively, for 1.5 hr at 4°C in the presence of Avidin-agarose (Sigma). For competition experiments, competing nonbiotinylated hTCF4/Pan and noncompeting Lgs/BCL9 HD2 peptides were added during the incubation reaction at 1, 10, and 100 times the molar concentrations of the biotinylated peptides.

Luciferase Assays

HEK 293 cells were transfected with 0.4 μg of reporter plasmid (pTOP-Flash or pFOP-Flash; van de Wetering et al., 1997) and 0.4 μg of expression plasmid. As an internal control to monitor transfection, 10 pg of a UbC promoter-humanized Renilla plasmid (phRG-B, Promega) were added. cDNAs encoding hPYGO1 and ΔN-β-catenin were expressed in pCDNA3.1. Luciferase activities were determined 24 hr after transfection using the Dual-Luciferase Assay System (Promega).

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References

Aasland, R., Gibson, T.J., and Stewart, A.F. (1995). The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem. Sci. *20*, 56–59.

Ahmad, K., and Golic, K.G. (1998). The transmission of fragmented chromosomes in *Drosophila melanogaster*. Genetics *148*, 775–792.

Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M., and Clevers, H. (2001). The chromatin remodelling factor Brg-1 interacts with β-catenin to promote target gene activation. EMBO J. *20*, 4935–4943.

Bauer, A., Huber, O., and Kemler, R. (1998). Pontin52, an interaction partner of β-catenin, binds to the TATA box binding protein. Proc. Natl. Acad. Sci. USA *95*, 14787–14792.

Bridges, C.B. (1935). The mutants and linkage data of chromosome four of *Drosophila melanogaster*. Biol. Zh. *4*, 401–420.

Brunner, E., Peter, O., Schweizer, L., and Basler, K. (1997). pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in *Drosophila*. Nature *385*, 829–833.

Collins, R.T., and Treisman, J.E. (2000). Osa-containing Brahma chromatin remodeling complexes are required for the repression of wingless target genes. Genes Dev. *14*, 3140–3152.

Daniels, D.L., Eklof Spink, K., and Weis, W.I. (2001). β-catenin: molecular plasticity and drug design. Trends Biochem. Sci. *26*, 672–678.

Gounari, F., Aifantis, I., Khazaie, K., Hoeflinger, S., Harada, N., Taketo, M.M., and von Boehmer, H. (2001). Somatic activation of β-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development. Nat. Immunol. *2*, 863–869.

Hecht, A., Litterst, C.M., Huber, O., and Kemler, R. (1999). Functional characterization of multiple transactivating elements in β-catenin, some of which interact with the TATA-binding protein in vitro. J. Biol. Chem. *274*, 18017–18025.

Hecht, A., Vleminckx, K., Stemmler, M.P., van Roy, F., and Kemler, R. (2000). The p300/CBP acetyltransferases function as transcriptional coactivators of β-catenin in vertebrates. EMBO J. *19*, 1839–1850.

Huber, A.H., and Weis, W.I. (2001). The structure of the β-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by β-catenin. Cell *105*, 391–402.

Huber, A.H., Nelson, W.J., and Weis, W.I. (1997). Three-dimensional structure of the armadillo repeat region of β-catenin. Cell *90*, 871–882.

Hülsken, J., Vogel, R., Erdmann, B., Cotsarelis, G., and Birchmeier, W. (2001). β-catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell *105*, 533–545.

Ioannidis, V., Beermann, F., Clevers, H., and Held, W. (2001). The β-catenin-TCF-1 pathway ensures CD4(+)CD8(+) thymocyte survival. Nat. Immunol. *2*, 691–697.

Kinzler, K.W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell *87*, 159–170.

Look, T.A. (1997). Oncogenic transcription factors in the human acute leukemias. Science *278*, 1059–1064.

McCartney, B.M., Dierick, H.A., Kirkpatrick, C., Moline, M.M., Baas, A., Peifer, M., and Bejsovec, A. (1999). *Drosophila* APC2 is a cytoskeletally-associated protein that regulates wingless signaling in the embryonic epidermis. J. Cell Biol. *146*, 1303–1318.

McWhirter, J.R., Neuteboom, S.T., Wancewicz, E.V., Monia, B.P., Downing, J.R., and Murre, C. (1999). Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia. Proc. Natl. Acad. Sci. USA *96*, 11464–11469.

Muller, H.J., and Edmondson, M. (1957). Transposition of entire 4-euchromatin into a fully functional Y. Drosoph. Inf. Serv. *31*, 140–141.

Ng, M., Diaz-Benjumea, F.J., Vincent, J.P., Wu, J., and Cohen, S.M. (1996). Specification of the wing by localized expression of wingless protein. Nature *381*, 316–318.

Nüsslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. Nature *287*, 795–801.

Pai, L.M., Kirkpatrick, C., Blanton, J., Oda, H., Takeichi, M., and Peifer, M. (1996). *Drosophila* α-catenin and E-cadherin bind to distinct regions of *Drosophila* Armadillo. J. Biol. Chem. *271*, 32411–32420.

Paroush, Z., Finley, R.L., Jr., Kidd, T., Wainwright, S.M., Ingham, P.W., Brent, R., and Ish-Horowicz, D. (1994). Groucho is required for *Drosophila* neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. Cell *79*, 805–815.

Peifer, M., and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. Science *287*, 1606–1609.

Peifer, M., Rauskolb, C., Williams, M., Riggleman, B., and Wieschaus, E. (1991). The segment polarity gene armadillo interacts with the wingless signaling pathway in both embryonic and adult pattern formation. Development *111*, 1029–1043.

Peifer, M., Berg, S., and Reynolds, A.B. (1994). A repeating amino

acid motif shared by proteins with diverse cellular roles. Cell *76*, 789–791.

Perl, A.K., Wilgenbus, P., Dahl, U., Semb, H., and Christofori, G. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature *392*, 190–193.

Polakis, P. (2000). Wnt signaling and cancer. Genes Dev. *14*, 1837–1851.

Prieve, M.G., and Waterman, M.L. (1999). Nuclear localization and formation of β-catenin-lymphoid enhancer factor 1 complexes are not sufficient for activation of gene expression. Mol. Cell. Biol. *19*, 4503–4515.

Reya, T., O'Riordan, M., Okamura, R., Devaney, E., Willert, K., Nusse, R., and Grosschedl, R. (2000). Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. Immunity *13*, 15–24.

Sambrook, J., and Russell, D.W. (2001). Molecular Cloning—A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sharma, R.P., and Chopra, V.L. (1976). Effect of the Wingless (wg1) mutation on wing and haltere development in *Drosophila melanogaster*. Dev. Biol. *48*, 461–465.

Siegfried, E., Wilder, E.L., and Perrimon, N. (1994). Components of wingless signalling in *Drosophila*. Nature *367*, 76–80.

Staal, F.J., Meeldijk, J., Moerer, P., Jay, P., van de Weerdt, B.C., Vainio, S., Nolan, G.P., and Clevers, H. (2001). Wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription. Eur. J. Immunol. *31*, 285–293.

Taipale, J., and Beachy, P.A. (2001). The Hedgehog and Wnt signalling pathways in cancer. Nature *411*, 349–354.

Takemaru, K.I., and Moon, R.T. (2000). The transcriptional coactivator CBP interacts with β-catenin to activate gene expression. J. Cell Biol. *149*, 249–254.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., et al. (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. Cell *88*, 789–799.

Willis, T.G., and Dyer, J.S. (2000). The role of immunoglobulin translocations in the pathogenesis of B-cell malignancies. Blood *96*, 808–822.

Willis, T.G., Zalcberg, I.R., Coignet, L.J., Wlodarska, I., Stul, M., Jadayel, D.M., Bastard, C., Treleaven, J.G., Catovsky, D., Silva, M.L., and Dyer, M.J. (1998). Molecular cloning of translocation t(1;14)(q21;q32) defines a novel gene (BCL9) at chromosome 1q21. Blood *91*, 1873–1881.

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. Annu. Rev. Cell Dev. Biol. *14*, 59–88.

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