**Identification and in vivo role of the Armadillo-Legless interaction**

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Summary

The Wnt signalling system controls many fundamental processes during animal development and its deregulation has been causally linked to colorectal cancer. Transduction of Wnt signals entails the association of β-catenin with nuclear TCF DNA-binding factors and the subsequent activation of target genes. Using genetic assays in *Drosophila*, we have recently identified a presumptive adaptor protein, Legless (Lgs), that binds to β-catenin and mediates signalling activity by recruiting the transcriptional activator Pygopus (Pygo). Here, we characterize the β-catenin/Lgs interaction and show: (1) that it is critically dependent on two acidic amino acid residues in the first Armadillo repeat of β-catenin; (2) that it is spatially and functionally separable from the binding sites for TCF factors, APC and E-cadherin; (3) that it is required in endogenous as well as constitutively active forms of β-catenin for Wingless signalling output in *Drosophila*; and (4) that in its absence animals develop with the same phenotypic consequences as animals lacking Lgs altogether. Based on these findings, and because Lgs and Pygo have human homologues that can substitute for their *Drosophila* counterparts, we infer that the β-catenin/Lgs binding site may thus serve as an attractive drug target for therapeutic intervention in β-catenin-dependent cancer progression.

Key words: *Drosophila*, Disease, Colorectal cancer, Wnt signalling, β-catenin

Introduction

Colorectal cancer is the second leading cause of cancer incidence and cancer death among adults in Europe. It is estimated for the year 2000 alone that 362,620 people were diagnosed with colorectal cancer, and 198,778 patients died owing to this disease (Ferlay et al., 2001). In more than 80% of the sporadic colorectal cancers, both alleles of the Adenomatous Polyposis Coli (*APC*) gene are inactivated (Kinzler and Vogelstein, 1996). The APC protein forms – together with Axin and GSK3β – a degradation complex for β-catenin. In this complex, GSK3β phosphorylates β-catenin, which in turn is ubiquitinated and thereby targeted for destruction. Wnt signalling inhibits the degradation complex and hence leads to the cytoplasmic accumulation and entry of β-catenin into the nucleus, where it forms a complex with members of the Pangolin(Pan)/TCF/Lef family of DNA-binding proteins, the putative adaptor protein Legless/BCL9 (Lgs) and the transcriptional regulator Pygopus (Pygo) (Behrens et al., 1996; Belenkaya et al., 2002; Brunner et al., 1997; Kramps et al., 2002; Parker et al., 2002; Riese et al., 1997; Thompson et al., 2002; van de Wetering et al., 1997). Loss of APC also causes an increase in β-catenin levels and thus leads to a constitutive activation of this pathway. The nuclear β-catenin complex activates transcription of known proto-oncogenes such as *Myc* and *cyclin D1* (He et al., 1998; Shutman et al., 1999). Preventing the formation of the TCF/β-catenin/Lgs/Pygo complex should halt the expression of these genes and thus alleviate the detrimental effects caused by the loss of the APC tumour suppressor. Indeed, overexpression of a dominant-negative form of TCF4 in colorectal cancer cells results in G1 cell cycle arrest (van de Wetering et al., 2002). Furthermore, reduction of human Pygo expression by means of RNA interference in colorectal cancer cells reduces the transcriptional output induced by the nuclear β-catenin complex (Thompson et al., 2002).

Here, we set out to characterize the interaction of β-catenin and Lgs. We report the identification of two amino acids of β-catenin that play an essential role in Lgs binding. This presumed binding site is specific for Lgs and is not required for APC, E-cadherin or TCF4. We show that Armadillo (Arm), the *Drosophila* homologue of β-catenin, depends on these amino acid residues for mediating Wnt/Wg signalling in vivo, but not for establishing functional adherens junctions. Together, our results indicate that the β-catenin/Lgs interaction may provide an attractive target for therapeutic intervention.

Materials and methods

β-catenin and Arm mutants

The crystal structure of β-catenin (Protein Data Bank accession code 2BCT) was used in conjunction with the PDB viewer (http://au.expasy.org/spdbv) to select amino acid residues of β-catenin that are exposed and have more than 30% accessibility. Twenty-five human β-catenin mutants (in pGAD424) were obtained from von Kries et al. (von Kries et al., 2000). These constructs comprised only Arm repeats 3-12 and the C terminus, and were extended with Arm repeat 1 and 2, such that all final constructs extended from amino acids 129 to 781. Another 14-point mutations were introduced in repeats 1-3 by site-directed mutagenesis (Quickchange-Kit, Stratagene). Human LGS1 (amino acids 199-392), human TCF4 (amino acids 1-130), mouse Apc (amino acids 1152-1393), mouse E-cadherin (amino acids 773-885) and mouse α-catenin (amino acids 1-750) were cloned into pBTM116 (Bartel and Fields, 1997). For interactions with α-catenin the mutations were brought into the context of full length β-catenin constructs. All constructs were verified by sequencing.

A subset of the β-catenin mutations were also introduced into Arm.
For simplicity, we use the amino acid numbering of β-catenin for both β-catenin and Arm throughout the text. β-Catenin D162, E163, D164 and K435 would correspond to Arm D170, E171, D172 and K443.

Yeast two-hybrid assays
The yeast two-hybrid system as described previously (Bartel and Fields, 1997) was used. Interactions between proteins were measured using the quantitative ‘Liquid Culture Assay Using ONPG as Substrate’ (Clontech, 2001).

Transgenes
For embryonic experiments arm transgenes were expressed from UAS-constructs under control of the daughterless-Gal4 driver (Wodarz et al., 1995). Three independent lines were established and tested for the arm<sup>310-wt</sup>, arm<sup>310-D164A</sup> and arm<sup>310-K435E</sup> constructs, and two independent lines for Δarm-wt, Δarm-D164A and Δarm-K435E; in all cases, different integrations of the same construct yielded similar effects. For rescue experiments full-length arm transgenes were driven by the tubulin<sup>1A1</sup> (tub) promoter (Basler and Struhl, 1994). All arm-coding regions used contain a Myc epitope in their C-terminal region at the same position as the arm<sup>310</sup> construct used by Pai et al. (Pai et al., 1997).

Germline clones
To obtain arm germline clones, second and third instar larvae generated from a cross between arm<sup>2a9</sup> FRT101; tub-arm<sup>-wt</sup> or -D164A/+ virgins with ovo<sup>D1</sup> FRT101/Y; hs-flp[F38]/hs-flp[F38] were heat-shocked at 38°C for 1.5 hours. After hatching, the fertile females would produce only progeny from arm<sup>2a9</sup> mutant germlines. Embryos bearing the tub-arm-wt transgene were crossed to y w males and laid embryos that all contain maternal tub-arm-wt product (otherwise no eggs would be generated). Only 25% of these embryos will inherit neither the rescuing transgene nor the paternal arm<sup>+</sup> allele, and these embryos resemble zygotic arm<sup>-</sup> embryos. The observed number for such embryos was 90 out of 385. Females that were arm<sup>2a9/arm<sup>2a9</sup></sup>, tub-arm-D164A/+ were crossed with y w males and laid embryos that all contain maternal tub-arm-D164A product (otherwise no eggs would be generated). Three classes of embryos are expected (50% class I, 25% class II, 25% class III): while all embryos are maternally mutant for arm<sup>2a9</sup>, 50% of them (class I) are zygotically arm<sup>+</sup>, and hence rescued (from the paternal X chromosome), and the other 50% (classes II and III) are also zygotically mutant for arm<sup>2a9</sup>. Half of these (class II), however, inherit the tub-arm-D164A transgene, and show a slightly weaker segment polarity phenotype (Fig. 5C) compared with the other half (class III) that does not (Fig. 5D). The observed numbers for these three classes were 33, 12 and 14, respectively. For the generation of lgs germline clones see Kramps et al. (Kramps et al., 2002).

Disc clones
Mutant imaginal disc clones were generated by crossing arm<sup>2a9</sup> FRT181/FM7 females with hs-flp hs-GFP FRT18; tub-arm<sup>-wt</sup> or -D164A/TM6b. Ninety-six hours after egg laying, larvae were heat shocked at 38°C for 1 hour. Female larvae that did not carry TM6b were dissected 48 hours after the heat shock. Imaginal discs were fixed and stained by standard techniques. Antibodies used were mouse monoclonal anti-DII (gift from I. Duncan), rat monoclonal anti-De-cadherin (DCAD2, gift from T. Uemura) and rabbit polyclonal anti-Lgs (Kramps et al., 2002).

Results
Identification and characterization of the Lgs binding site of β-catenin
The primary structure of β-catenin consists of acidic N and C termini, and a highly basic central region containing 12 imperfect sequence repeats that are known as Armadillo repeats (Arm repeats). These repeats pack against each other to form a continuous superhelix, which features a positively charged groove (Huber et al., 1997). The Arm repeat domain provides binding sites for APC, Axin, E-cadherin, TCF4 and human LGS1 (Fig. 1A). Despite lack of significant sequence homology, APC, E-cadherin and TCF4 are known to bind competitively to β-catenin (Hulsken et al., 1994; Omer et al., 1999). Structural studies have shown that APC, E-cadherin and TCF4 bind to largely overlapping regions of the positively charged groove of β-catenin (Eklof Spink et al., 2001; Graham et al., 2000; Graham et al., 2001; Huber and Weis, 2001; Xing et al., 2003). Lgs instead requires the first four Arm repeats for binding to Arm (Kramps et al., 2002). We set out to map and characterize the human LGS1-β-catenin interaction by performing an alanine mutagenesis scan.

A set of 39 β-catenin mutants containing substitutions of single, exposed amino acid residues (mostly with basic and aromatic side chains, Fig. 1C,D) was tested for the ability to bind human LGS1 in a yeast two-hybrid system (see Materials and methods). Two of these mutants showed a reproducible reduction in binding: the D162A mutation reduced binding by fourfold compared with wild-type β-catenin and D164A even by 25-fold. In the initial screen, R386A also showed a reduced binding to human LGS1 (two-fold, Fig. 1E), but this reduction was variable (Fig. 1F) and is not considered to be significant.

In addition to its role in Wnt signalling, β-catenin is also a component of the cadherin-based cell adhesion system, linking the transmembrane protein E-cadherin to α-catenin, thereby connecting adherens junctions to the cytoskeleton (reviewed by Pokutta and Weis, 2002). β-Catenin is also part of its own degradation complex consisting of APC, Axin and GSK3β. In order to evaluate the specificity of the mutations that disrupt β-catenin/human LGS1 binding, mutations D162A and D164A were tested for their effect on the interactions between β-catenin and APC, E-cadherin, TCF4 and α-catenin. As a control, we also included in this analysis E163A, which had virtually no effect on the β-catenin/human LGS1 interaction even though it also reduces the negative charge at this region of the protein. None of the three mutations affected the binding of β-catenin to APC, E-cadherin or TCF4. Binding to α-catenin, however, was reduced approximately twofold by D162A and D164A (Fig. 1F). This was unexpected, as the region comprising amino acids 120-151 of β-catenin has been shown to be necessary and sufficient for binding to α-catenin (Aberle et al., 1994). Amino acids D162, E163 and D164 form an acidic knob in repeat 1 of β-catenin (Fig. 1B, white arrow), on the side opposite to the basic groove. From these results we conclude: (1) that β-catenin binds human LGS1 and APC/E-cadherin/TCF4 on opposite sides; and (2) that the binding to human LGS1 but not to APC/E-cadherin/TCF4 is disrupted by the mutations D162A and D164A.

Arm-D164A fails to rescue armadillo null mutant animals
Armadillo (Arm) is the Drosophila homologue of β-catenin. The two proteins show high sequence similarity, especially in the Arm repeat region (Peifer and Wieschaus, 1990; Peifer et al., 1994). To investigate whether a mutant form of Arm, which can no longer bind Lgs, has impaired transcriptional activity in Drosophila, we first analyzed whether β-catenin and Arm use
equivalent sites for binding human and Drosophila Lgs. The D164A mutation – and as a negative control the E163A mutation – were introduced into Arm and found to affect the Arm/Lgs interaction to the same extent as the corresponding mutations in catenin (not shown). We then tested whether Arm-D164A can substitute for the wild-type form of Arm in vivo by performing a rescue assay with the arm 2a9 allele, which has a frameshift mutation in Arm repeat 3 (Fig. 1A) and is the strongest arm allele known. Hemizygous arm 2a9 males die as embryos but can be rescued by tubulinα1-promoter-driven arm-wt or arm-E163A transgenes to adulthood with no obvious phenotypes (Table 1). By contrast, arm 2a9 males die as embryos or early larvae when these transgenes contain the D164A mutation or K435E, which affects TCF/Pan binding (Graham et al., 2000). We interpret these results to indicate that

Constitutive signalling activity of ArmS10 depends on D164

Mutations in the N terminus of catenin that impede its phosphorylation and subsequent degradation cause – like loss of APC or Axin – constitutive activation of the Wnt/Wg pathway and are found in 10% of sporadic colorectal cancers (Sparks et al., 1998; Korinek et al., 1997; Morin et al., 1997). In Drosophila, embryonic overexpression of N-terminally truncated forms of Arm mimics this situation and leads to a naked cuticle phenotype, owing to overactivation of the pathway. ArmS10 is one such form, owing to an in-frame deletion that removes the GSK3β phosphorylation sites but
to indicate that ArmS10 largely depends on Lgs binding for its biological activity. As a control, we also overexpressed a form of ArmS10 that is affected in Pan binding (ArmS10-K435E); the K435E mutation also efficiently reverts the gain-of-function of ArmS10 that is affected in Pan binding (ArmS10-K435E); the biological activity. As a control, we also overexpressed a form of ArmS10 that is affected in Pan binding (ArmS10-K435E); the K435E mutation also efficiently reverts the gain-of-function activity of ArmS10 (Fig. 2D). Thus, the constitutive activity of ArmS10 depends on the binding of both Lgs and Pan.

Another stable and thus constitutively active form of Arm is ΔArm, in which a large N-terminal region comprising the GSK3β phosphorylation and α-catenin-binding sites is replaced by a myristoylation signal (Zecca et al., 1996). Its biological activity depends on the presence of wild-type cellular Arm (Tolwinski and Wieschaus, 2001; Tolwinski and Wieschaus, 2004). It has been shown that expression of membrane-tethered forms of β-catenin leads to the nuclear localization of endogenous β-catenin (Miller and Moon, 1997). If ΔArm signalling is mediated by wild-type cellular Arm, then disrupting the binding to Lgs or Pan should not affect its ability to activate the Wg pathway. Indeed, we find that overexpression of ΔArm-wt, ΔArm-D164A and ΔArm-K435E all resulted in a completely naked cuticle phenotype (Fig. 2F-H), suggesting that ΔArm signals via cellular Arm, which is wild-type and hence able to recruit Lgs.

Reduced expression levels of Wg targets in arm-D164A cells

To assess the role of the D164 site in the transcriptional function of Arm, we analyzed Wg target gene expression in

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Rescue (%)</th>
<th>n</th>
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<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>150</td>
</tr>
<tr>
<td>tub-arm-wt</td>
<td>96</td>
<td>166</td>
</tr>
<tr>
<td>tub-arm-E163A</td>
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<td>133</td>
</tr>
<tr>
<td>tub-arm-K435E</td>
<td>0</td>
<td>66</td>
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D164 is required for Arm function. Females heterozygous for arm269 were crossed with males containing different tubulin1 promoter-driven rescue constructs. The percentages of rescued males containing both the arm269 allele and the tubulin1 rescue constructs are shown. n indicates the male progeny that contain the tubulin1 rescue construct but inherited the wild-type arm allele from the balancer chromosome instead of the arm269 allele and therefore corresponds to the expected number of arm269 males with the tubulin1 rescue construct.
sites to link E-cadherin and diffuse in arm
Drosophila E-cadherin discs with an antibody directed against 1996). In order to visualize adherens junctions we stained wing neighbour cells (Dahmann and Basler, 2000; Uemura et al., adherens junctions or different E-cadherin levels sort out from suggest that Arm-D164A can confer appear to restore the function of adherens junction. This indicates that the loss or reduction of Dll expression in arm null and arm-D164A clones is due to the inability of these cells to respond efficiently to Wg rather than due to a general effect caused by cellular decay. It further rules out the possibility that the nuclei of mutant cells have escaped the apicobasal plane at which target gene expression was optically recorded.

Arm-D164A can restore adherens junctions of arm mutant cells
We noticed that arm2a9 clones are not only smaller than their twin-spots but that their shape is round compared with the irregular outline of normal clones (Fig. 3B). On the contrary, arm clones that express arm-wt or arm-D164A transgenes are similar in size to their twin-spots and have an irregular shape (Fig. 3C,D). These differences hint at an adherens junction defect of arm null mutant cells, as β-catenin is required at these sites to link E-cadherin and α-catenin, and cells with defective adherens junctions or different E-cadherin levels sort out from neighbouring cells (Dahmann and Basler, 2000; Uemura et al., 1996). In order to visualize adherens junctions we stained wing discs with an antibody directed against Drosophila E-cadherin (Uemura et al., 1996). Indeed, E-cadherin distribution is diffuse in arm null clones (Fig. 4A). As arm-wt and arm-D164A transgenes rescue the abnormal distribution of dE-cadherin in arm cells (Fig. 4B,C), and no longer cause aggregation into non-intermingling cell groups, their products appear to restore the function of adherens junction. This suggests that Arm-D164A can confer β-catenin function at adherens junctions, and hence is able to sufficiently tether E-cadherin and α-catenin.

Replacement of maternal and zygotic Arm by Arm-D164A is equivalent to lack of Lgs function
Although both the failure to rescue zygotically mutant animals and the failure to rescue gene expression in arm mutant disc cells indicate a requirement for the D164 site and hence the Arm/Lgs interaction, neither of these assays provides the means to compare the reduction of Wg transduction to that caused by the genetic removal of Lgs. Additionally both assays may be influenced by the perdurance of wild-type arm product. The most stringent test for the role of D164 in Wg signalling is the creation of embryos in which both the maternal and the zygotic contribution of wild-type arm product are replaced by Arm-D164A. To achieve this situation, we generated germline clones using the arm2a9 allele. Such germline clones fail to produce eggs because of junctional defects (Peifer et al., 1993). However, arm2a9 germline clones do give rise to eggs and phenotypically normal larvae in the maternal and zygotic presence of a tubulin1-arm-wt transgene (not shown). When only maternal arm-wt product is contributed, eggs are laid from mutant germline clones and the resulting embryos resemble zygotic arm2a9 embryos. Eggs were also laid from clones expressing the tubulin1-arm-D164A transgene, corroborating our conclusion that Arm-D164A protein can restore functional adherens junctions (Fig. 5C,D). Embryos whose sole source of Arm, both maternally and zygotically, was the tubulin1-arm-D164A transgene (Fig. 5C) died with a segment polarity phenotype characterized by an excess of ventral denticles at the expense of naked cuticle. This phenotype, which is weaker than that of wg null mutants (Nüsslein-Volhard et al., 1984; Bejsovec and Wieschaus, 1993), closely resembles the phenotype of embryos devoid of maternal and zygotic lgs function (Fig. 5B). Because the arm-D164A phenotype is not notably weaker than that of lgs embryos, we conclude that the D164A mutation effectively eliminates in vivo – as in the yeast assay – most or all Lgs function. Moreover, as we failed to observe any phenotypes of arm-D164A embryos that surpassed those of lgs embryos, we also conclude that the D164 site is unlikely to serve any critical function other than recruitment of Lgs.

Discussion
The Wnt signalling pathway not only controls a multitude of fundamental patterning processes during animal development (reviewed by Wodarz and Nusse, 1998), its deregulation is also
responsible for an increasing number of cancers. Hence, major efforts strive towards the identification of all protein components involved in this pathway and also for the detailed characterization of their molecular interactions. We have recently identified two novel genes involved in the transduction of the Wg signal, lgs and pygo, and found that their products serve as adaptor proteins to convert nuclear β-catenin/Arm activity into transcriptional activation of target genes (Kramps et al., 2002). Here, we are concerned with the question of how β-catenin and Lgs interact molecularly with each other. Our analysis addressed three issues: localization of the binding site on β-catenin, specificity of this site vis-à-vis other partners of β-catenin and in vivo significance of this interaction for Wg signal transduction.

Binding site
By means of site-directed mutagenesis we assayed the role of conspicuous β-catenin residues in the binding to human LGS1. Two amino acids, D162 and D164, were identified that are both necessary for human LGS1 binding. Because substitutions of these residues with other amino acids did not affect the binding of several other proteins to β-catenin, we interpret their role as contact sites for human LGS1, rather than a structural function enhancing stability and/or three-dimensional conformation of β-catenin. This conclusion, however, will need to be confirmed by determining the crystal structure of the β-catenin/human LGS1 complex.

Specificity
We showed that neither D162 nor D164 is required for binding to APC, E-cadherin or TCF4. Substitutions of these amino acids did reduce binding to α-catenin twofold, but our in vivo data suggest that this reduction does not prevent the assembly of adherens junctions. The specificity of the β-catenin/human LGS1 interaction vis-à-vis that of β-catenin and APC, E-cadherin or TCF4 is consistent with their respective locations on the surface of β-catenin. While crystallographic studies showed that APC, E-cadherin and TCF4 all bind to a common, extended surface within the groove of β-catenin formed by Arm repeats 3-10 (reviewed by Daniels et al., 2001), our analysis indicates that human LGS1 binds an acidic knob in Arm repeat 1. This knob is not only located more N terminally, it is also situated on the side of β-catenin, which is opposite to the groove (Fig. 1B). The spatial separation of these binding sites is in agreement with their separable functions observed in our yeast binding assays, as well as with previous GST pull-down assays, in which we observed simultaneous binding of TCF4 and human LGS1 to β-catenin (Kramps et al., 2002).

In vivo significance
To assess the role of D162 and D164 in Wg transduction, we
subjected mutant forms of Arm to various assays designed to reveal their in vivo function. Simple rescue and overexpression experiments showed that transgenic Arm-D164A cannot substitute for endogenous Arm, and that the D164A mutation significantly reduces the constitutive signalling activity associated with N-terminal deletions of Arm. When tested in more advanced assays, we find that D164 is required by wing disc cells to maintain Wg target gene expression and by developing embryos for segmentation. Together, these experiments support the conclusion that Arm signalling function relies on its capability to bind to Lgs throughout development.

Although it is straightforward to interpret our results as a qualitative indication for the significance of the Arm/Lgs interaction, it is more difficult to assess their outcome in a quantitative manner. For example, the apparent residual expression of Dll in Arm-D164A cells may reflect perduance of wild-type Arm or Dll proteins, but it could also indicate that a fraction of the Wg signal can be transmitted despite the D164A mutation. This latter scenario could in turn be attributed to some residual binding of Arm to Lgs, but it could also be explained by a partial redundancy of Lgs function. Lgs may be required for efficient Arm-mediated activation of Wg targets, but some activation may also occur in its absence. Consistent with this latter view, we have observed that animals lacking maternal and zygotic lgs product exhibit phenotypes equivalent to animals in which the sole source of Arm is the D164A transgene, yet neither of the two phenotypes are quite as severe as that of wg-null mutants.

Possible relevance for human cancer

The Wnt pathway is highly conserved between Drosophila and vertebrates. The human homologues of Lgs (LGS1/BCL9) and Pygo (PYGO1 and PYGO2) can rescue lgs and pygo mutant flies, respectively (Kramps et al., 2002). This suggests that these proteins have the same function in vertebrates and in Drosophila. It is possible therefore, that our in vivo data can be extrapolated to Wnt signalling in mammals.

Mutations in APC occur in more than 80% of inherited and sporadic colorectal cancers (Kinzler and Vogelstein, 1996). These mutations lead to accumulation of free β-catenin and as a result to overexpression of Wnt target genes. A chemical compound that interferes with the formation of the nuclear TCF/β-catenin/Lgs/Pygo complex should in theory halt the progression of cancer. Such an anti-cancer drug must be highly specific though, as it should only disrupt the nuclear β-catenin/Lgs interaction. Weis, 2001; Xing et al., 2003). Therefore, designing an inhibitor that specifically disrupts the β-catenin/TCF interaction is a difficult task (Daniels et al., 2001; Lepourculet et al., 2004). On the contrary, our mapping and specificity results indicate that the β-catenin/Lgs interaction site could be targeted without interfering with the binding of β-catenin to APC and E-cadherin. Moreover, our analysis shows that genetic disruption of the Arm/Lgs interaction leads to severely reduced Wg signalling, suggesting that the protein-protein interaction between β-catenin and Lgs may provide an attractive target for therapeutic intervention.

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Arm/Armadillo-Legless interaction

Development and disease


