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Wg/Wnt Signal Can Be Transmitted through Arrow/LRP5,6 and Axin Independently of Zw3/Gsk3β Activity

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Summary

Activation of the Wnt signaling cascade provides key signals during development and in disease. Here we provide evidence, by designing a Wnt receptor with ligand-independent signaling activity, that physical proximity of Arrow (LRP) to the Wnt receptor Frizzled-2 triggers the intracellular signaling cascade. We have uncovered a branch of the Wnt pathway in which Armadillo activity is regulated concomitantly with the levels of Axin protein. The intracellular pathway bypasses Gsk3β/Zw3, the kinase normally required for controlling β-catenin/Armadillo levels, suggesting that modulated degradation of Armadillo is not required for Wnt signaling. We propose that Arrow (LRP) recruits Axin to the membrane, and that this interaction leads to Axin degradation. As a consequence, Armadillo is no longer bound by Axin, resulting in nuclear signaling by Armadillo.

Introduction

The Wnt/Wingless (Wg) signaling pathway plays an essential role in animal development. It functions to specify such diverse processes as embryonic segmentation, CNS organization, and limb development. Wnts are secreted glycoproteins that exert their effects on neighboring cells by binding to the Frizzled (Fz) transmembrane receptor family (Wodarz and Nusse, 1998). Single-pass transmembrane proteins of the LDL receptor-related protein family (LRP5 and -6, and Arrow) have also been implicated in the reception of the Wnt signal (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). It remains unclear how exactly these receptors receive the Wnt signal and in turn stimulate downstream components such as Dishevelled (Dvl/Dsh). Upon Wnt stimulation, Dsh binds to a complex containing Axin, APC, Zw3 (Shaggy, GSK3β), and Armadillo/β-catenin (Arm/β-cat). Such Dsh binding prevents Zw3 from phosphorylating Arm/β-catenin, blocking the degradation of Arm/β-catenin and allowing it to accumulate in the cytoplasm. As Arm levels increase, some protein enters the nucleus, where it binds to the transcriptional repressor TCF and activates transcription of specific target genes.

Although the Wnt signaling pathway has been the subject of intense investigation, the initiation of the intracellular signal at the receptor complex as well as the cytoplasmic events that transduce this signal and affect the transcriptional activity of Arm/β-catenin remain unclear. Much work has focused on the regulation of Arm/β-catenin levels, as this event is biochemically accessible and often the most obvious consequence of Wg/Wnt signaling. Zw3/GSK3β-mediated phosphorylation of Arm is required for Slimb/βTrcp to target Arm for degradation by the proteasome. Both the phosphorylation and the degradation of Arm require the activity of Axin (Hamada et al., 1999; Willert et al., 1999a) and APC (Ahmed et al., 1998, 2002), which function as a scaffold for Zw3 activity (Salic et al., 2000). Efforts to link the level of Zw3 activity directly to Wg signaling at the cell surface have been less successful. Recent evidence also shows that before Zw3 can phosphorylate Arm/β-catenin, Arm must first be primed through phosphorylation by casein kinase I (CKI). However, it remains controversial whether this priming phosphorylation is instructive, that is, subject to modulation by Wnt input, or constitutive in directing Arm/β-catenin for degradation (Amit et al., 2002; Liu et al., 2002). Moreover, none of these studies address whether the only means to control Wnt-dependent transcriptional output is through degradation of Arm/β-catenin.

In fact, certain well-characterized Wnt signaling pathways in both vertebrates and invertebrates show no obvious changes in the level of Arm/β-catenin in response to Wnt ligand, and appear to operate instead by controlling Arm/β-catenin nuclear localization (Schneider et al., 1996; Novak et al., 1998; Logan et al., 1999). In addition, the components that govern Arm phosphorylation might also affect its subcellular localization. APC has been proposed to function in the nuclear export of Arm/β-catenin (Henderson, 2000; Rosin-Arbesfeld et al., 2000), and Axin and TCF may play a role as cytoplasmic and nuclear anchors, respectively (Tolwinski and Wieschaus, 2001). In cell culture, phosphorylation of Axin both increases its affinity for Arm/β-catenin and Axin protein stability, whereas Wnt signaling induces Axin degradation (Willert et al., 1999b; Yamamoto et al., 1999; Mao et al., 2001b). These observations and previous work suggest that a reexamination of the mechanisms that control Arm activity in *Drosophila* is warranted.

Here we demonstrate in vivo that physical proximity of Frizzled and Arrow is sufficient to initiate the intracellular signaling cascades originating at the receptor. Mimicking such proximity by protein fusion produces a constitutively active receptor. Subsequently, Arrow interacts with Axin and posttranslationally downregulates Axin activity. We further find that the levels of Axin protein are modulated by Wg ligand. Finally, we investigate the role of Zw3 phosphorylation and Arm degradation in patterning the *Drosophila* epidermis. We show that in the

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absence of Zw3, Arm activity is still modulated in embryos. We propose a model where Wg signaling is controlled not by modulation of Zw3 kinase activity, but by negative regulation of Axin mediated by Arrow.

Results

Spatial Regulation of Wg Signaling without Zw3

One key step in Wnt/Wg pathway activation is believed to be the inhibition of Zw3-mediated degradation of Arm. The level of Arm protein observed in *zw3* mutant cells is much higher than that normally observed in Wg-responding cells. This loss-of-function phenotype argues that Zw3 is essential for maintaining low Arm levels (Peifer et al., 1994b; Siegfried et al., 1994), but does not of itself indicate whether regulation of Zw3 is an essential component of Wg signaling. To test whether cells could respond to Wnt signaling without modulating Zw3 activity, we induced germline clones in order to remove Zw3 function from embryos. These were combined with a weak allele of *arm*; we reasoned that this combination might approximate the normal physiological state of the cell. In wild-type cells, Arm activity is kept in check through the degradation machinery; in these germline clones, the high Arm protein levels should be balanced by the reduced activity of the mutant Arm protein.

In wild-type embryos, each parasegment is divided into two regions: a region of naked cuticle, derived from cells signaled by Wg, and denticles (Figure 1A). In *zw3* single mutants, Arm levels are high, and all epidermal cells make naked cuticle (Figure 1B). Reducing *arm* function suppresses this phenotype, and allows an underlying pattern of denticle fates to reemerge. The epistasis of *arm* over *zw3* was one of the first observations that identified Arm as a downstream target of Zw3 (Peifer et al., 1994a; Siegfried et al., 1994). In our current experiments, we used two weak hypomorphic *arm* alleles (arm^{F1a} or $arm^{H8.6}$ at the permissive temperature of 18°C), and found that the uniform lawn of denticles observed previously in *zw3 arm* mutants is now broken by regions of naked cuticle. The ability of cells to form naked cuticle reflects a residual activity provided by the hypomorphic alleles. Both alleles supply significant Arm activity, but to different levels, and consequently show different extents of naked cuticle in the double mutant (Figures 1C and 1D, respectively). The periodic nature of the pattern in the arm^{weak} *zw3* embryos resembles the normal arrangement of denticle bands and naked cuticle in wild-type embryos, and suggests that some aspects of the wild-type signal transduction system that normally lead to naked cell fates may still be functioning in such embryos. This conclusion was confirmed by examination of Wg and En expression patterns. In wild-type embryos, both genes are spatially restricted to single narrow stripes in each parasegment (Martinez-Arias et al., 1988). In *zw3* germline clones, the stripes of Wg and En expression expand, whereas in germline clones homozygous for strong *arm* alleles, expression of both markers is lost. In contrast to such results with single mutants, we find that germline clone embryos homozygous for *zw3* and arm^{weak} alleles maintain both genes in their normal wild-type expression pattern (Figure 1F and data not shown). This result is particularly remarkable in the arm^{F1a} *zw3* embryos, which show high uniform accumulation of Arm protein characteristic of *zw3* mutants (Figure 1F compared to Figure 1E). Such uniform accumulation of arm^{F1a} protein might be expected to induce an even level of signaling and a uniform cuticle fate a result that we did not observe (Figure 1C). This raised the possibility that the pattern in *zw3* arm^{weak} mutants either arose from differences already inherent in the cells, or that it reflects the ability of cells to respond to Wg signaling by some previously uncharacterized route.

As Wg is expressed normally in such embryos (Figure 1F), we tested whether the naked regions of cuticle in the arm^{F1a} *zw3* double mutants reflected a response to Wg signaling by removing the wild-type *wg* allele. Triple mutant embryos, in which the arm^{F1a} *zw3* germline clones also lacked *wg* gene function zygotically (Figure 2C), lose naked cuticle, and approximate the *wg* null phenotype. Triple mutant embryos are not identical to arm^{F1a} *wg* embryos. This is expected, as the lack of Zw3 leads to much higher levels of Arm^{F1a} (Figure 2F). However, because *wg* exerts an effect when the downstream component *zw3* is absent, it is clear that there is a Zw3-independent Wg signal. This unexpected result argues that signaling in arm^{weak} *zw3* embryos can be further increased by exposure to Wg. Importantly, the target of this signal is Armadillo because the Wg response in double mutants is eliminated when a stronger *arm* allele is used ($arm^{H8.6}$ at 25°C, arm^{XM19} (Peifer et al., 1994a; Siegfried et al., 1994). This key result indicates the existence of a Wg-dependent mechanism regulating Arm activity that is independent of Zw3-mediated degradation of Arm.

Axin Levels Are Modulated across the Parasegment

The above results argue that Wg signaling can affect Arm activity and activate Arm transcriptional targets via a mechanism that does not require Zw3, and therefore does not appear to involve the canonical Zw3-mediated degradation of Arm. To investigate how this Wg signal might be transduced to Arm, we first considered Axin, as loss of Axin results in a naked cuticular phenotype similar to that of *zw3* germline clones (Figure 2J; Hamada et al., 1999). Here we show that cells of the double *wg axin* mutant adopt the smooth cuticle fate characteristic of *axin* null mutants (Figure 2I; see Experimental Procedures). This suggests that the *axin* gene functions downstream of Wg input and therefore cells could be subject to negative regulation of Axin by Wg. Such regulation could affect Arm activity, because we have previously shown that Axin affects Arm activity and intracellular localization in a way that cannot be attributed solely to its role as a scaffold for Zw3 phosphorylation (Tolwinski and Wieschaus, 2001).

We looked at the expression of Axin in wild-type embryos at stages when Wg signaling occurs. Axin antibodies (Willert et al., 1999a) were not strong enough to detect the endogenous protein, but worked well when Axin levels were increased using the UAS/GAL4 system (Brand and Perrimon, 1993). To our surprise, although the drivers we use express Axin RNA uniformly, the protein accumulates to high levels only in the anterior of each parasegment. Therefore, Axin does not accumulate at high levels in those cells that are exposed to the Wg ligand (Figure 2K).

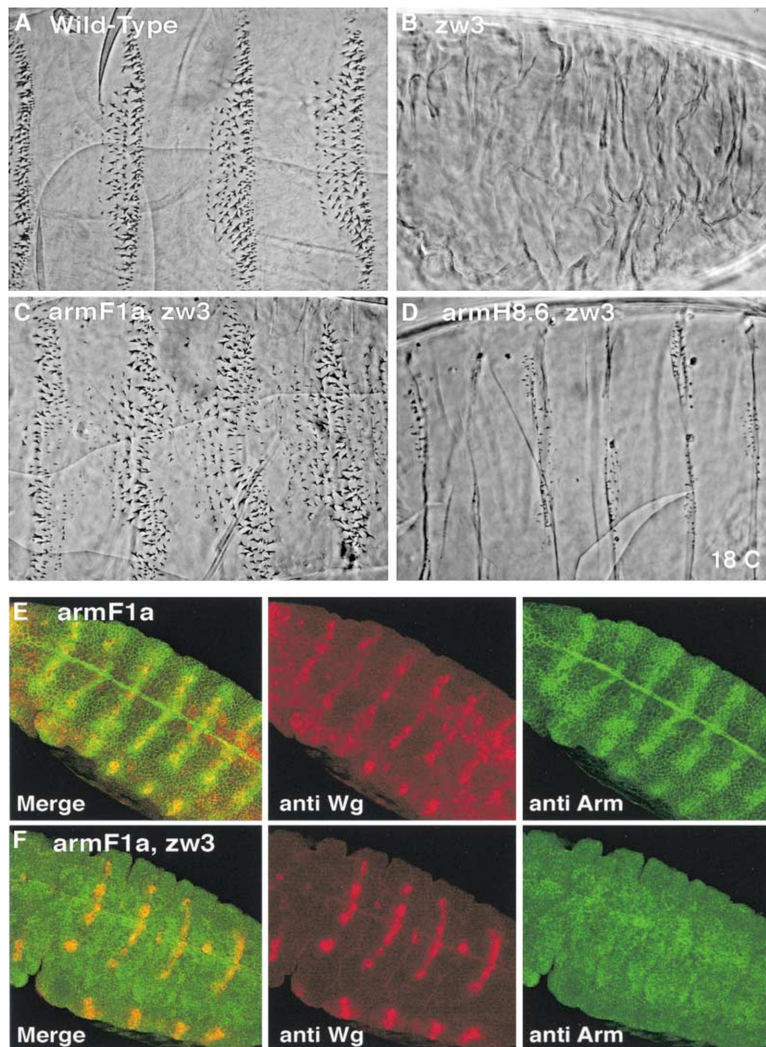

Figure 1. The Embryonic Cuticle Retains Periodic Patterning in the Absence of Zw3, Though Arm Degradation Is Off and the Protein Accumulates in All Cells

(A) Wild-type cuticular pattern, where each embryonic segment is subdivided into naked and denticle covered regions.

(B) *zw3*$^{M11\text{-}1}$ mutant embryo, showing the typical cell fate transformation associated with uniform Wg signaling; specifically, all cells adopt the naked cell fate.

(C) An embryo maternally and zygotically mutant (germline clone or glc) for *arm*^{F1a} *zw3*$^{M11\text{-}1}$ shows a reemergence of denticles in a spatially restricted pattern.

(D) *arm*$^{H8.6}$ zw3$^{M11\text{-}1}$ glc embryo at the permissive temperature (18°C) similarly shows a reemergence of denticles, but the naked region is expanded in comparison to *arm*^{F1a}, reflecting the higher activity of the *arm*$^{H8.6}$ allele.

(E) *arm*^{F1a} *zw3*$^{M11\text{-}1}$ glc embryo stained for Wg and Arm shows that the mutant Arm protein is expressed, and its degradation is still spatially regulated by Wg in discrete stripes corresponding to Wg expression (essentially a wild-type pattern).

(F) An *arm*^{F1a} *zw3*$^{M11\text{-}1}$ glc embryo stained for Arm and Wg, shows that although Wg stripes are maintained, Arm protein accumulates in all cells equally.

The registration of the Axin protein stripes suggested that this pattern depended on the periodic expression of Wg. To test this, we expressed Axin in both *wg* mutant embryos and in embryos that globally express Wg (UAS-Wg). In both cases, the Axin protein no longer accumulated in stripes (Figures 3A and 3B). Further, Western blots indicated that the two genotypes eliminate Axin striping by complementary mechanisms. Extracts from embryos expressing Wg ectopically show a sharp decrease in endogenous Axin levels (Figure 3C). Reciprocally, the absence of Wg leads to levels of expressed myc-Axin significantly higher that those observed in wild-type (Figure 3D). These data indicate that the striped pattern of Axin is Wg dependent and arises from a downregulation of Axin protein in cells that are exposed to Wg signal. These results extend previous studies in cell culture (Willert et al., 1999b; Yamamoto et al., 1999) by demonstrating that Wg signaling negatively regulates Axin accumulation in vivo and that downregulation of Axin is important in patterning.

We next tested whether Axin might account for the *zw3*-independent response to Wg signaling observed in *arm*^{weak} *zw3* embryos. To address this point, we first examined Axin protein expression and found that Axin stripes are present in such embryos (Figure 3E). This result argues that the periodic downregulation of Axin, although a response to Wg signal, does not require Zw3. To test whether Axin that accumulates outside the Wg expression domain contributes to the denticle fates observed in such embryos, we made triple mutants in which *arm*^{F1a}*, zw3* germline clones also lacked Axin function zygotically (but retains maternally contributed Axin). This reduction in Axin leads to an expansion of the naked cuticle region (compare Figure 2D to Figure 2B), consistent with the idea that Axin in this region downregulates Arm activity. We infer that the significant maternal Axin contribution, which is intact in these embryos, precludes a more dramatic shift toward naked cuticle fate. In sum, our results show that Wg signaling can control cell fate through Armadillo, but in a manner independent of *zw3* through modulation of Axin levels. In the absence of Axin, maximal signaling results. To address how Axin may be regulated by Wg signaling, we turn to Arrow.

Arrow Binds and Downregulates Axin

Arrow is a single-pass transmembrane protein of the LDL receptor-related protein family (LRP), which is an essential Wg pathway component. It acts downstream

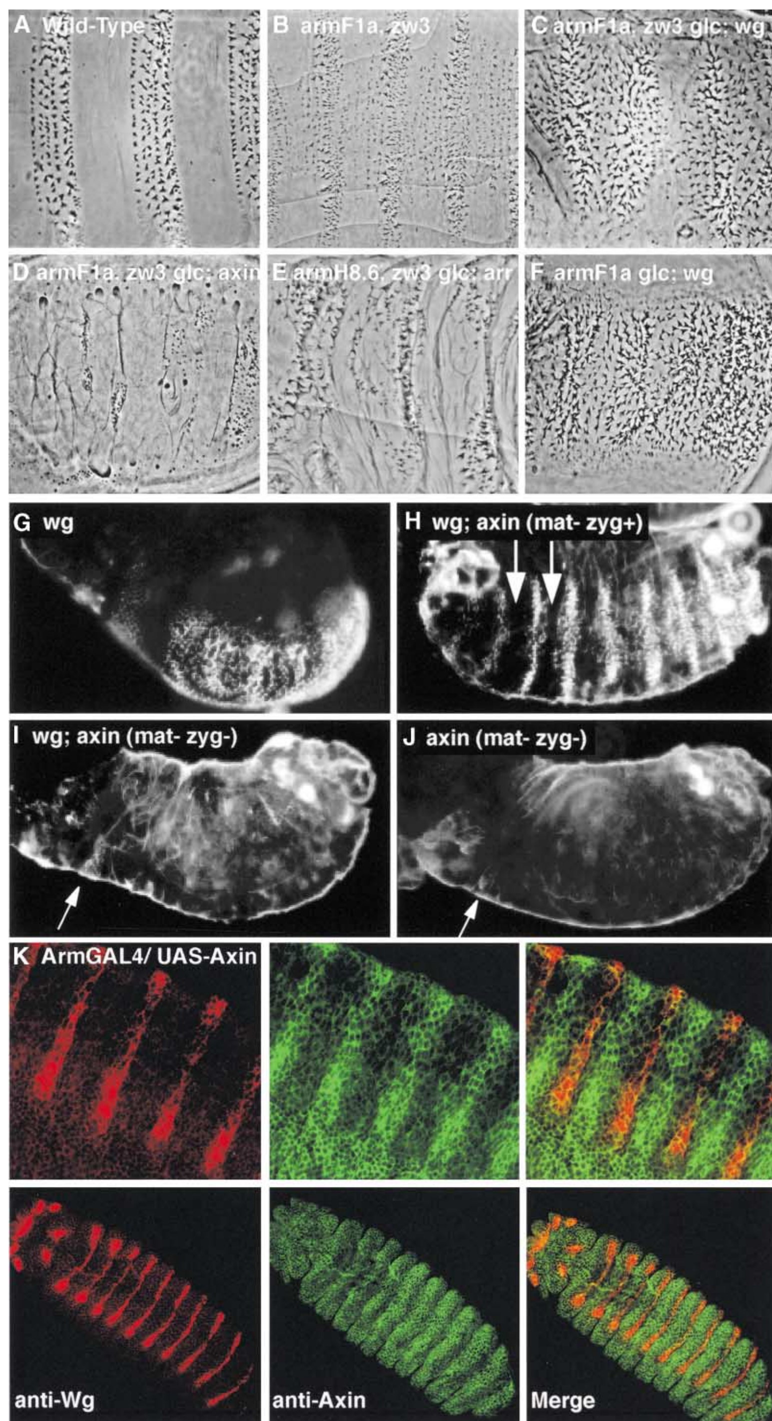

Figure 2. The Periodic Pattern of the Cuticles of arm^{F1a} $zw3^{M11-1}$ Mutant Embryos Is Dependent upon Proper Expression of Wg, Axin, and Arrow

(A) Wild-type.

(B) An arm^{F1a} $zw3^{M11-1}$ glc embryo.

(C) An arm^{F1a} $zw3^{M11-1}$ glc embryo also lacking all wg ***function*** (wg^{IG22}), showing an effect on the periodic pattern of the cuticle.

(D) A glc embryo for arm^{F1a} $zw3^{M11-1}$ that lacks zygotic Axin function ($axin^{S044230}$) but retains maternally contributed Axin. The cuticle loses much of its periodic character tending toward naked cell fates.

(E) An $arm^{H8.6}$ $zw3^{M11-1}$ glc that lacks zygotic Arrow function ($arrow^{2}$) but retains maternally contributed Arrow. The cuticle loses much of its periodic character tending toward denticle cell fates (compare to Figure 1D).

(F) An arm^{F1a} glc lacking all *wg* function (wg^{IG22}), which is essentially the *wg* phenotype.

(G) *wingless* (*wg*) mutant leads to a lack of smooth cuticle.

(H) *wg*; $axin^{mat-\ zyg+}$. The *wg* phenotype is partly suppressed by the lack of maternally supplied axin, because some smooth cuticle is induced and these embryos have a significant segmentally repeating pattern (arrows).

(I and J) *wg*; $axin^{mat-\ zyg-}$ (I). If both maternal and zygotic Axin is removed from a *wg* mutant embryo, then the suppression of the *wg* phenotype is complete and only smooth cuticle is produced. Such embryos are indistinguishable from the *axin* null mutant embryos shown in (J).

(K) Embryos expressing Axin stained with anti-Wg in red and anti-Axin in green. Axin expression appears in stripes that correspond to cells not receiving Wg ligand, even though the ArmGAL4 driver drives uniform expression of all markers tested (not shown).

of Wg but upstream of Dsh, and, as a membrane protein, these epistasis data placed Arrow in the cell membrane to interact with Frizzleds to initiate signaling (Wehrli et al., 2000).

In a yeast two-hybrid screen of ten million transformants using the Arrow cytoplasmic domain as bait, we identified 15 Axin clones (Figure 4A). Our analysis largely agrees with the finding of Mao and coworkers (2001b) that the mouse ortholog of Arrow, mLRP5, interacts with mouse Axin; however, we find that the putative Zw3 binding domain of fly Axin does not contribute in binding to Arrow (Figure 4A). In addition, we find that full-length Axin does not interact with Arrow, because the N-terminal half of Axin prevents interaction with Arrow (Figure 4A).

Based on the Axin-Arrow interaction detected in yeast and the role of Arrow in regulating Wg signaling (Wehrli et al., 2000), we tested whether Arrow influences the cuticle cell fates of arm^{weak} *zw3* embryos. In $arm^{H8.6}$ *zw3* embryos, most cells tend toward the naked cell fate

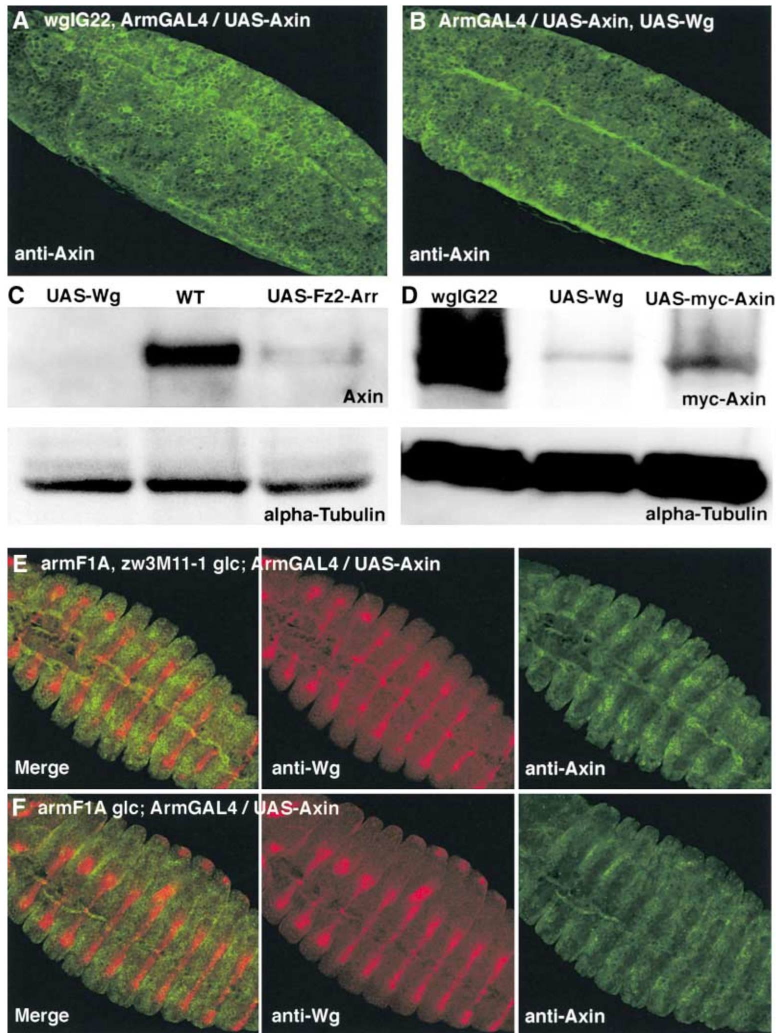

Figure 3. Axin Is Posttranslationally Regulated in a Wg-Dependent Manner

(A) Axin staining is uniform in an embryo that is a *wg*^{IG22} null mutant.

(B) Coexpression of UAS-Wg and UAS-Axin leads to uniform Axin staining throughout the epidermis.

(C) Western blot analysis showing a decrease in the endogenous levels of Axin present in embryos uniformly expressing Wg or the activated receptor chimera Fz2-Arr[intra] as compared with wild-type.

(D) Western blot analysis of total extract to compare expression levels of a myc-tagged Axin expressed in the absence of Wg (*wg*^{IG22} mutant), uniformly expressed Wg (UAS-Wg), and wild-type embryos.

(E) *arm*^{F1a} *zw3*$^{M11-1}$ glc mutants expressing UAS-Axin show Axin levels to be higher in cells not exposed to Wg ligand.

(F) *arm*^{F1a} glc shows higher levels of Axin in cells not exposed to Wg. The uniform driver Arm-GAL4 was used to expresses UAS-Axin at even levels.

(Figure 1D), but when the zygotic contribution of *arrow* is removed, there is a shift to denticle cell fates (Figure 2E), suggesting that Arrow is still required for such embryos to respond to the Wg signal. The significant maternal contribution of *arrow* may prevent a more dramatic shift toward the denticle cell fate. *arr* germline clone embryos lack all Wg signal transduction, resulting in a lawn of denticles (Wehrli et al., 2000), and we find that such embryos also fail to accumulate Axin protein in stripes (Figure 4B). To test whether the denticle fates in *arr* germline clones reflect a failure to downregulate Axin, with resulting inactivation of Armadillo, we used RNA interference (RNAi) to reduce both Axin and Arrow levels. *arrow*^{RNAi}-treated embryos exhibited a loss of naked cuticle (Figure 4F) and most cells formed denticles. However, when the *axin* levels are also reduced in these embryos by coinjecting both axin^{RNAi} and arrow^{RNAi}, then the *arrow* phenotype was significantly suppressed (Figure 4H). This is consistent with the notion that in *arrow*^{null} mutants, excess denticle fates result from the failure to inhibit Axin accumulation. We also infer from this that Arrow may be the link between Wg input and Axin regulation. We tested this next, by exploring the relationship between Arrow and initiation of signal transduction at the membrane.

A Chimeric Fz2-Arrow Fusion Potentiates Signal Transduction

Arrow/LRP5,6 may act as a coreceptor with Frizzled class molecules (Tamai et al., 2000; Wehrli et al., 2000). The simplest version of the coreceptor model suggests that for initiation of signal transduction, the cytoplasmic domains of Arrow and Frizzled come into proximity, bringing together associated proteins to initiate signaling. To test this, we generated transgenic flies that express a fusion of the cytoplasmic domain of Arrow to the C terminus of Fz2 (Fz2-Arr[intra]; Figure 5E). Our reasoning was that such a chimeric protein might bypass the need for ligand-induced association, and would generate ligand-independent signal transduction. We tested this in the developing wing, where increased Wg signaling induces ectopic bristle formation. We found that the Fz2-Arr[intra] chimera generates many more bristles, and these bristles are located further away from the wing margin than those generated by expression of either Arrow alone, Frizzled2 alone, or both Arrow and Frizzled2 (Figures 5A and 5B, and data not shown). Thus, the chimeric protein potentiates signaling much more strongly than overexpression of the individual proteins. The chimera also potentiated signaling in the embryonic epidermis, where it resulted in a change of fate from

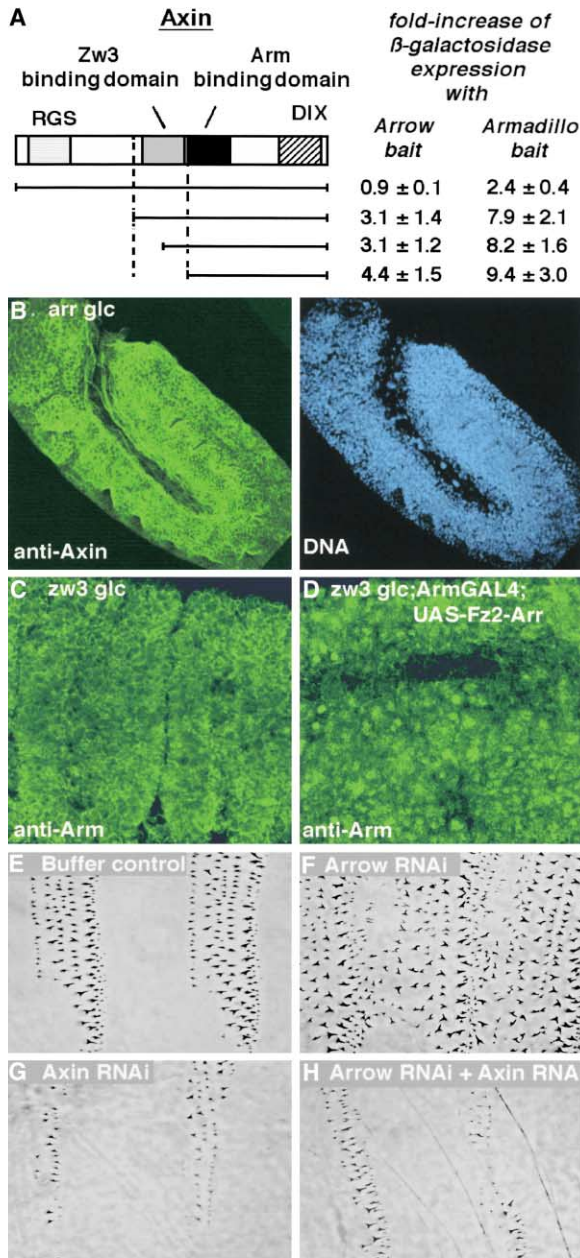

Figure 4. Arrow Interacts with Axin and Controls Axin Stability

(A) Schematic representation of Axin (not to scale) indicating the RGS domain that binds APC, the binding domains for Zw3/Sgg kinase and Armadillo, and the DIX dimerization domain. The brackets underneath represent GAL4 activation domain fusions to C-terminal Axin fragments (isolated in our yeast two-hybrid screen) and full-length Axin (top bracket). The fusion points of all 15 isolated clones reside in the bracketed region centered around the Zw3 binding domain. Four were tested to determine whether the Zw3 binding domain contributes to binding the Arrow bait. β-galactosidase activity was used to quantify the interaction (right) with both the Arrow bait and Armadillo bait. Fusion points shown are Axin amino acid Met1, Thr353, Ser401, and Gln418.

(B–D) Arrow is required for Axin striping, and affects Arm intracellular localization.

(B) In embryos lacking all Arrow function (*arrow* glc), UAS-Axin expressed with the ArmGAL4 driver does not produce stripes.

(C) In $zw3^{M11-1}$ glc, Arm levels are greatly increased, and the Arm protein appears throughout the cells.

(D) *zw3* glc that express the Fz2-Arr[intra]; Arm protein stains more intensely in the nucleus.

(E–H) A knockdown of Axin function suppresses the Arrow loss-of-function phenotype by RNA interference. Ventrolateral views of cuticle preparations scored after injection of interfering RNAs (RNAi) prior to cellular blastoderm. Anterior is to the left.

(E) Wild-type (WT) injected with buffer alone.

(F) WT injected with Arr RNAi exhibited loss of smooth cuticle, as in *arr* null mutants (not shown).

(G) WT injected with Axin RNAi exhibited loss of denticles, as in *axin* mutants (not shown).

(H) WT coinjected with a mixture of Arr and Axin RNAi exhibited loss of denticles (an *axin* phenocopy).

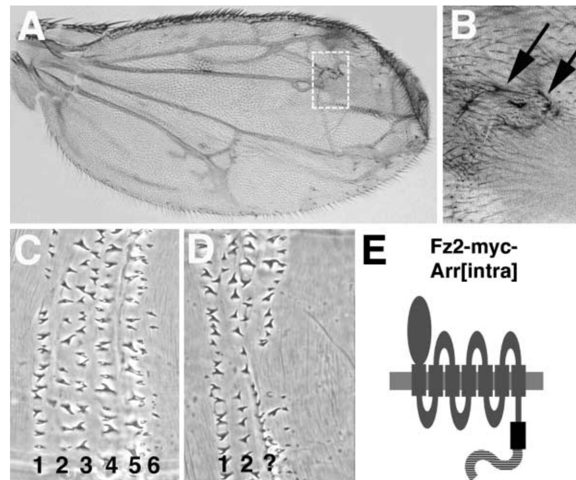

Figure 5. Fz2-Arr[intra] Potentiates Wg Signaling in Embryos and in Wings

(A and B) Wing. The GAL4 driver line MS1096 was used to drive expression of UAS-Fz2-Arr[intra] in the developing wing. Ectopic wing margin bristles (arrows) and ectopic vein tissue (arrowheads) are apparent, indicative of increased Wg signaling.

(B) A magnified view of the white box in (A) shows ectopic margin bristles that are distant from the wing margin. Expression of Fz2-Arr[intra] produces a stronger effect than that of the individual subunits, Arrow or Fz2, whether expressed separately or together in the same animal (Wehrli et al., 2000, and data not shown).

(C and D) Embryonic cuticle preparations; anterior to the left.

(C) Ptc-GAL4/UAS-Fz2-myc: little or no disruption to normal ventral cuticle pattern is observed expressing an Fz2 construct with the myc tag in the same position as in the activated chimera (see [D]). Numbering in the panel shows the six denticle rows. The differentiation of denticles requires that there is no Wingless pathway activity in these cells.

(D) Ptc-GAL4/UAS-Fz2-myc-Arrow[intra]: loss of several rows of ventral denticles is observed. This phenocopies ectopic Wingless pathway activation. This phenotype is more penetrant in the more posterior parasegments.

(E) Schematic representation of Fz2-Arr[intra]. Fz2 seven transmembrane domain protein (dark gray); a 6× myc-tag (black box) was fused to the Fz2 C terminus, followed by the Arrow cytoplasmic domain (Arr[intra]; hatched).

denticle to smooth cuticle (Figure 5D), a hallmark of Wg signal transduction.

Ligand Independence of Fz2-Arr[intra]

We next asked whether the chimera initiates signaling in a ligand-independent manner or simply potentiates an already present Wg signal. When expressed in the

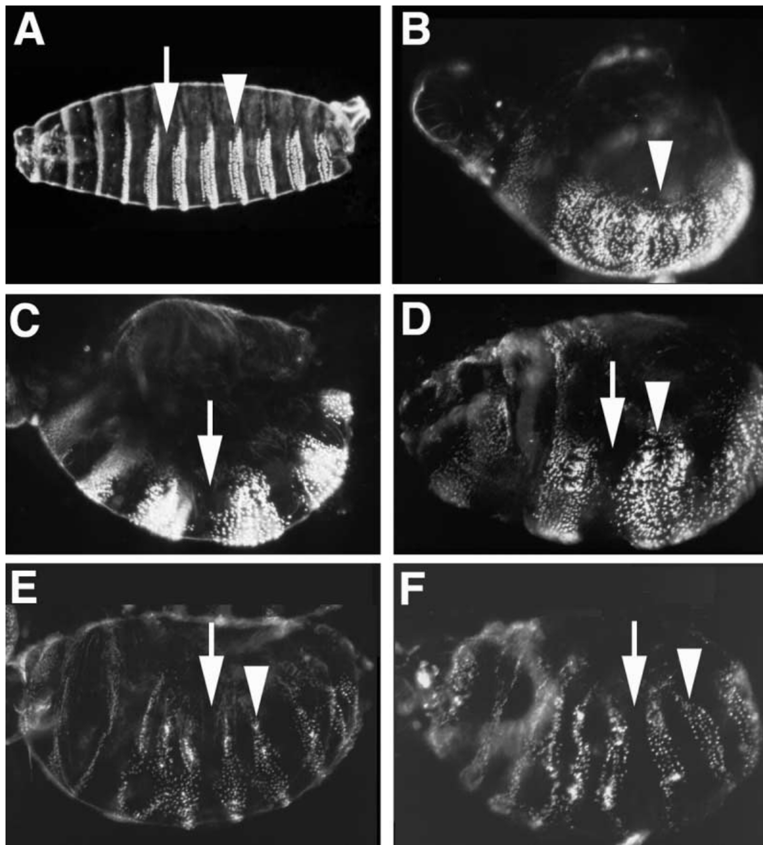

Figure 6. Fz2-Arr[intra] Is an Activated Receptor

(A) Wild-type (WT) embryo with alternating bands of denticles (arrowhead) and smooth cuticle (arrow).

(B) A *wg* mutant embryo lacks all smooth cuticle.

(C) *wg*; Prd-GAL4/UAS- Fz2-Arr[intra]. Smooth cuticle is restored (arrow) in a *wg* mutant embryo by expression of Fz2-Arr[intra].

(D) *arr*; Prd-GAL4/UAS- Fz2-Arr[intra]. Smooth cuticle is also restored in an *arrow*^{null} mutant embryo after expression of Fz-Arr[intra]; rescue is clearly visible in the anterior half of the embryo (arrow) but incomplete posteriorly.

(E) En-GAL4/UAS- Fz2-Arr[intra]; *fz fz2*. Fz2-Arr[intra] expression restores smooth cuticle.

(F) En-GAL4/UAS- Arm*(S10); *fz fz2*. For comparison, the rescue (smooth cuticle) observed in *fz fz2* null embryos expressing activated Armadillo. The GAL4 driver in (C and D) was Paired-GAL4 and in (E and F) Engrailed-GAL4, resulting in a different pattern of rescue.

absence of Wg ligand (*wg* null embryos; Figure 6B), Fz2-Arr[intra] is able to induce smooth cuticle (Figure 6C; Table 1). This demonstrates that Fz2-Arr[intra] initiates signaling in the absence of ligand. Importantly, ligand-independent signaling does not occur with overexpression of Fz2 alone, nor with tagged versions of Fz2 (Fz2-Myc; Fz2-eGFP; Table 1). This argues against the possibility that simply overexpressing Fz2 allows it to engage another *Drosophila* Wnt in place of absent Wg. Furthermore, overexpression of Arr[intra] as a membrane-tethered form (myristoylated Arr[intra]) fails to induce signaling in the absence of, or to potentiate signaling in the presence of, ligand (Table 1 and data not shown). These controls underscore that Fz2-Arr[intra] initiates signaling independently of ligand and therefore constitutes a constitutively active Wg/Wnt receptor.

We then considered the possibility that signaling by the chimeric protein could depend on endogenous Frizzleds. In *Drosophila*, two Frizzleds, Fz and Fz2, function as redundant Wg receptors (Bhanot et al., 1999; Chen and Struhl, 1999); when both are removed, the embryo forms a lawn of denticles. When we removed *fz* and *fz2* from embryos and expressed Fz2-Arr[intra] using En-GAL4, we observed completely penetrant rescue of naked cuticle fates in the Engrailed domain (Figure 6E; Table 1). We then removed *arrow* and expressed Fz2-Arr[intra] using Prd-GAL4. These experiments also revealed significant rescue, although there was some residual dependence on *arrow* function (Figure 6D). These experiments illustrate that signaling is indeed initiated by the chimeric protein, and that it does so without relying at all on endogenous Frizzleds, or, largely, on

Table 1. Frequency with which Fz2mycArr[intra] or Control Constructs Rescues the Cuticle Phenotype of Embryos Mutant for Various Components of the Wg Pathway

	Rescue Construct				
Mutant Background	Fz2mycArr[intra]	Fz2	Fz2myc	Fz2-eCFP	Myr-Arr[intra]
wingless	92/310 (29.6%)	0/190 (0%)	0/163 (0%)	0/177 (0%)	0/297 (0%)
fz fz2 (maternal/zygotic)	46/184 (25.0%)	ND	ND	ND	ND
arrow (maternal/zygotic)	57/368 (15.5%)	ND	ND	ND	ND
dishevelled (maternal/zygotic)	0/294 (0%)	ND	ND	ND	ND
armadillo (maternal/zygotic)	0/127 (0%)	ND	ND	ND	ND

Constructs were introduced into mutant embryos using the UAS/GAL4 system and the number of embryos in which Wg signaling was rescued (indicated by the presence of smooth cuticle as illustrated in Figure 6) was determined. Only 25% of embryos have the combination of the GAL4 driver line and the test construct, and thus 25% is the expected frequency if full rescue occurs. For crosses, see Experimental Procedures. ND, not determined.

endogenous Arrow. Finally, we found that the constitutively activated receptor acts through the canonical Wg signal transducers Dsh and Arm, respectively, because no signaling is observed in their absence (Table 1). The requirement for Dsh is particularly revealing, because epistasis and biochemical tests place its activity at the highest point in the intracellular signal transduction pathway. Thus, the reliance on Dsh function implies that the events occurring at Fz2-Arr[intra] are the earliest intracellular Wnt signaling events characterized to date. We conclude that the initiation of Wnt/Wg signal transduction may require the association of Arrow and Frizzled.

The Fz2-Arr[intra] Chimera Destabilizes Axin and Induces the Nuclear Relocalization of Arm

We then asked whether the constitutively activated receptor might signal by destabilizing Axin. Therefore, we compared the overall levels of endogenous Axin by Western blot of extracts from wild-type embryos to those from embryos ubiquitously expressing the activated receptor (Figure 3D). We see a reduction, but it is less strong than that observed with UAS-Wg. The less dramatic effect is consistent with the lower level of signaling induced by Fz2-Arr[intra] compared to that generated by ectopic expression of Wg. The following taken together, the physical interaction of Arrow and Axin, the involvement of Arrow in signal initiation, and the effect of an activated receptor on Axin accumulation, suggests that Arrow is the link between Wg input and signal transduction that was revealed in *arm*^{weak} *zw3* embryos.

In wild-type embryos, Axin functions in part as an anchor for cytoplasmic Armadillo (Tolwinski and Wieschaus, 2001). If Wg signaling in *arm*^{weak} *zw3* embryos depends on local Axin downregulation, we might expect a periodic nuclear localization of Arm protein. We tested for, but did not observe, the predicted pattern of nuclear Arm accumulation in *arm*^{F1a} *zw3*, nor in *arm*$^{H8.6}$ *zw3* embryos. This result is perhaps not surprising, given that it is similarly difficult to see periodic nuclear accumulation of Arm within the Wg-responding cells of wild-type embryos (data not shown; Peifer et al., 1994b). In *arm*^{F1a} *zw3* embryos, the detection may be even more difficult, as subtle nuclear accumulation may be obscured by the increased levels of *arm*^{F1a} protein caused by *zw3*. Detecting modulation in *arm*$^{H8.6}$ genotypes has proven equally difficult because this protein has decreased stability, which precludes detecting even cytoplasmic striping under permissive conditions where embryos are wild-type in pattern and hatch.

We have, however, found that the Fz2-Arr[intra] activated receptor will drive Arm into the nucleus. In these experiments, we expressed Fz2-Arr[intra] in *zw3* mutant embryos, and examined the subcellular localization of Arm. In *zw3* mutant embryos, Arm localizes diffusely throughout the cell with some enrichment at the membrane (Peifer et al., 1994b; Figure 4C). However, when Fz2-Arr[intra] is expressed in *zw3* mutants, Arm localizes to the nuclei of cells (Figure 4D). Thus, activating signaling can mobilize Arm in a manner similar to that which occurs when a membrane-tethered form of Arm (ΔArm) is expressed in *zw3* mutants (Tolwinski and Wieschaus, 2001). We infer that a similar process occurs after signal initiation, because Arrow interacts with Frizzleds during normal development after engaging the Wg ligand.

Discussion

Our data argue for a different regulatory mechanism of Wg signal transduction, proceeding through the inhibition of the protein Axin, rather than through the inhibition of Zw3/GSK3β. Axin was identified in both vertebrates and invertebrates as a negative component of the pathway (Zeng et al., 1997; Hamada et al., 1999). Later work established Axin as a critical scaffold protein required for the assembly and function of the degradation complex (Salic et al., 2000). This complex functions in the destruction of Arm/β-catenin by bringing the kinase Zw3 and Arm into close proximity, leading to the phosphorylation of Arm, and thereby targeting it to the proteasome for degradation. For efficient Arm degradation, both Axin and APC must be present in the complex (Salic et al., 2000). How Wg input controls activity of the degradation complex has never been properly established, although most models have focused on the inhibition of the kinase Zw3 (Polakis, 2002). It is also unclear whether Arm degradation always plays a central role in converting Wnt input into transcriptional responses. In sea urchins and mammals, the most obvious response to Wnt signaling is a relocalization of Arm protein from the cytoplasm to the nucleus (Schneider et al., 1996; Novak et al., 1998; Logan et al., 1999), and we have shown that both Axin and APC have a profound effect on Arm localization that cannot be explained by their interaction with Zw3 or the degradation complex alone (Tolwinski and Wieschaus, 2001; Ahmed et al., 2002). In contrast to this work, a recent study using a membrane-tethered, activated form of Arm suggested that cytoplasmic activation of the pathway is the key step in transcriptional activation (Chan and Struhl, 2002), and that Arm's nuclear localization may be unimportant. The authors' failure to eliminate activity of the endogenous Arm allele weakens their conclusion. In previous studies (Tolwinski and Wieschaus, 2001), we have shown that the same membrane-tethered form drives the endogenous Arm into the nucleus, and that introduction of even hypomorphic mutations in the endogenous allele substantially eliminates the ectopic signaling effects of the membrane-tethered form. Although we therefore favor a nuclear role for Arm, the pathway identified in the present study could regulate Arm activity regardless of where it occurred.

Here we present evidence that the Wg signal can be transmitted through a posttranslational regulation of Axin accumulation. Despite uniform transcription of Axin, using the UAS/GAL4 system, Axin accumulated to different levels in different cells across each parasegment. Cells with lower steady-state levels of Axin were those exposed to Wg input, and this was strictly dependent on Wg. Loss of Wg causes excess accumulation of Axin, whereas uniform Wg expression (and therefore signaling) lowers total Axin levels. The phenomena we observe in embryos parallel earlier reports showing that Axin accumulation is affected by Wnt signaling in tissue culture cells. Those initial experiments showed that

GSK3β phosphorylation of Axin led to its stabilization (Willert et al., 1999b; Yamamoto et al., 1999). However, the actual role that phosphorylation plays appears to be more complex, as further work contradicted this finding (Mao et al., 2001b). In our experiments, we have not examined the phosphorylation state of Axin in cells responding to Wg (those with low Axin levels), nor in those not exposed to Wg (high Axin levels). Therefore, we cannot distinguish whether modification may inactivate Axin or whether modification leads to removal of Axin by degradation. We do, however, find that Zw3 kinase activity is not necessary for the reduction in Axin accumulation that we observe and that the Axin striping pattern was maintained in embryos that lacked Zw3 function. These results argue for a link between Wg signaling and Axin accumulation that is independent of the Zw3-mediated degradation complex.

Arrow Interacts with Axin and Is Required for Its Destabilization

Although Zw3 does not appear to be required for Axin degradation, we find that the more upstream component, Arrow, appears to be important for this mode of Wg signal transduction. We found that the cytoplasmic domain of Arrow interacts with Axin in the yeast two-hybrid system, an interaction also identified for one of the mammalian LRPs, mLRP5, whose rapid binding of mAxin is ligand stimulated (Mao et al., 2001b). Our binding data are largely in agreement with theirs, except that we find no contribution of the Zw3 binding region of Axin in binding of Arrow bait (Figure 4A). Interestingly, full-length Axin fails to interact significantly with the Arrow C terminus in yeast and all the Axin clones we isolated in the library screen lacked sequences N-terminal to position 353. This finding suggests that an inhibitory domain is present in Axin, N-terminal to the Zw3 binding domain, and that this inhibitory domain prevents Axin from binding Arrow. It is possible that the Wnt signal necessary for the mouse Axin interaction with LRP5 (Mao et al., 2001a) induces a conformational change in Axin that removes, modifies, or otherwise displaces the inhibitory domain. In contrast, Armadillo bait significantly binds both full-length and truncated Axin (Figure 4A). These data taken together with our demonstration here that signaling leads to loss in Axin striping and a lowered steady-state level of Axin, suggest that the Arrow/LRP5 interaction with Axin induces a change in activity and/or stability of Axin.

The prevailing view on Wnt reception states that Arrow/LRP5,6 function as coreceptors together with Frizzled proteins. It is well established that Frizzled proteins bind Wnt ligands and that this interaction is essential for Wnt signal transduction. Initial work on LRP6 extended this model in suggesting that Wnt provides a bridging function in assembling a complex of Frizzleds and Arrow, at least for the particular combination mFz8/mWnt-1/mLRP6 (Tamai et al., 2000; Semenov et al., 2001). However, biochemically, such complex formation has also not always been confirmed (Mao et al., 2001a). In addition, the functional significance for signaling of the observed ternary complex has not been demonstrated in vivo. We therefore designed an experiment that tested whether, in vivo, physical proximity of Arrow and Frizzled-2 is sufficient for signaling. In fact, we found that it can initiate ligand-independent signal transduction. The constitutive activity of the Fz2-Arr[intra]chimeric protein is significant, as only expression of the fusion protein but not expression of the individual components (Fz2 and Arr[intra]) activated signal transduction (Figures 5 and 6; Table 1; data not shown). We infer that association of Frizzled2 with the Arrow C terminus is indeed a key step in signal initiation in vivo, and that the proximity afforded here by protein fusion also occurs during normal signaling. The Fz2-Arr[intra] chimera is uncoupled from the need for ligand to trigger the intracellular signal transduction cascade. Therefore, we cannot address whether the Arrow extracellular domain participates in a true "reception" complex with Fz2 in Wg binding. Nevertheless, Arrow, or at least its C terminus, likely interacts intimately with Fz2 during signal initiation at the membrane. In addition, activation of the pathway by the Fz2-Arr[intra] chimera proceeds through canonical pathway components, most notably requiring Disheveled, a result consistent with our previous finding that Dsh functions downstream of Arrow (Wehrli et al., 2000). In cultured vertebrate cells, one report suggested that in some circumstances, LRP6 could induce Wnt signal transduction independently of Disheveled (Li et al., 2002). In contrast, our rather different experiment of overexpressing biologically active Arrow cytoplasmic sequences in the form of the Fz2-Arr[intra] chimera revealed a strict Dsh dependence (Table1), suggesting instead an obligate role for the Dsh protein at signal initiation by Arrow, and by extension, presumably by vertebrate LRP5,6.

We note that though the Fz2-Arr[intra] chimera clearly signals, it is not as active as a Wg-stimulated endogenous receptor complex. Presumably, and not surprisingly, the protein fusion will present a distorted topology to cofactors required in the signal initiation complex, and therefore is not optimally configured for initiating signal transduction. This may explain why the chimera retains some measure of reliance on endogenous Arrow, as is apparent from a reduced level of signaling in its absence (Figure 6D).

It may be surprising that overexpression of myristoylated-Arr[intra] in transgenic flies neither potentiates Wg signaling nor induces de novo signaling (Table 1 and data not shown). This suggests that binding of Axin by Arr[intra] does not induce Wg signal transduction. It suggests further that the contribution of the Arrow cytoplasmic domain in the Fz2-Arr[intra] chimera is not only to bind Axin but to present it in the context of other proteins necessary to initiate signaling. In this, our results differ from transfection experiments with myristoylated LRP5[intra] that produced a transcriptional response in transiently transfected NIH3T3 cells (Mao et al., 2001a). Such different findings may be due to a more complex situation in the mammalian cell line used, differences in levels of the expressed protein, or the presence of modifying factors (such as Wnts) in the culture medium.

The Role of Zw3/GSK3β in Wnt Signaling Responses

Previous work has shown that the removal of Zw3 leads to a uniform activation of the Wg signal transduction

pathway throughout the embryonic epidermis, with all the cells assuming the naked cell fate. Crucially, signal transduction was shown in this case to be independent of Wg input (Siegfried et al., 1992). This epistasis experiment was among the fundamental observations that established the order of gene action in the Wg signaling pathway. In addition, because Zw3 is required for Arm destruction, it suggested that a key aspect of signal transduction would be to modulate the activity of Zw3, and, thereby, the Arm destruction complex. However, removal of Zw3 leads to unphysiologically high levels of Arm in all cells, resulting in excess signaling by Arm. To bypass this problem, we compromised Arm activity by introducing weak (hypomorphic) Arm mutations into the *zw3* null mutant background. By using this genetic combination, we found that the artificially high levels of Armadillo in previous experiments obscured the fact that Wg input was still registering and modulating signal transduction in cells that have no Zw3 activity. Thus, we conclude that the embryonic epidermis can still be patterned in the absence of Zw3, and have shown that this patterning is dependent on Wg pathway signal transduction and is initiated by Wg input. This implies strongly that modulating the activity of the destruction complex is not the sole path for Wg signal transduction in the cell. We cannot eliminate the possibility that another kinase might substitute for Zw3 in our experiments, or that some remaining activity from the *zw3* allele accounts for the patterning that we see. The allele we used behaves genetically as an amorphic mutation (Ruel et al., 1993b). Further, we see no striped modulation in Arm staining pattern that would suggest residual modulated activity of the destruction complex in response to Wnt signaling. The ultimate evaluation of our proposed Axin-dependent, Zw3-independent pathway would require eliminating Axin without affecting Zw3 (not possible with the current technology), and simultaneous production of *axin* and *arm* germline clones (difficult because they are on different chromosomes).

Recent evidence also shows that before Zw3 can act, Arm/β-catenin must first be primed through phosphorylation by CKI. However, it remains controversial whether this priming phosphorylation is instructive, that is, subject to modulation by Wnt input, or constitutive in directing Arm/β-catenin for degradation (Amit et al., 2002; Liu et al., 2002). In either case, however, both CKI and Zw3/GSK3β must be present for efficient degradation of Arm/β-catenin and consequent inhibition of signaling. Importantly, Axin is a key component in this process, because both kinases require Axin in order for Arm/β-catenin to be presented. Therefore, if Axin stability is downregulated by Wnt signaling, no phosphorylation of Arm/β-catenin will occur when the Wnt/Wg pathway is active. Because Axin also functions as an anchor retaining Arm protein in the cytoplasm (Tolwinski and Wieschaus, 2001), its degradation will not only stabilize Arm, but will enhance its nuclear accumulation.

Certain key components in the Wnt signaling pathway appear to be more dedicated to that pathway than others. The kinase Zw3/GSK3β is not exclusively a Wg component but rather functions in other pathways including Insulin, Notch, and Hh signaling (Woodgett, 1990; Ruel et al., 1993a; Jia et al., 2002; Kang et al., 2002; Price and Kalderon, 2002). Recently, Zw3/GSK3β has also been shown to function, together with Presenilin, in fine tuning low β-catenin levels in cells (Kang et al., 2002). Therefore, the role of Zw3 in the Wg pathway may be to maintain Arm levels low and in a critical range amenable to rapid change in response to signaling. Such a mechanism may be critically important in Wg signaling, where subtle differences in level can have a major impact on cell fate. In such a model, Zw3/GSK3β is primarily a "permissive" component that prevents signaling in the absence of Wg ligand, and downmodulation of its activity would not be a direct target of Wg input. Because Zw3/GSK3β acts in other pathways also, and its activity is subject to regulation by those pathways, such signaling pathways may indirectly affect transduction of the Wg signal by their modulation of Zw3/GSK3β activity. In contrast, other components, such as Axin, may be more exclusively used and regulated by Wg/Wnt signaling. This finding has obvious therapeutic implications, because targeting specific components such as Axin might offer advantages over the more promiscuous component Zw3/GSK3β.

In summary, we have demonstrated that Arrow and the Frizzled family of Wnt receptors function in a protein complex that triggers the intracellular signaling cascade. By binding to and causing a reduction in steady-state levels of Axin, Arrow provides a pivotal link between the receptor complex on the cell surface and the downstream events that control Arm activity. One consequence of Axin degradation may reflect its role as a scaffold for Zw3-mediated degradation of Arm. However, because we show that $zw3^{-}$ embryos still respond to Wg input though they fail to degrade Arm, regulation of the degradation complex cannot be the only target of Wg signaling. We therefore propose a Zw3-independent branch in the Wg pathway, one that might regulate the release of Armadillo from Axin, resulting in nuclear accumulation and signaling.

Experimental Procedures

Fly Strains

Oregon R, wild-type. See Flybase for details on mutants used (http://flybase.bio.indiana.edu). All mutants used were complete loss of function except for the partial loss-of-function arm^{XM19}, $arm^{H8.6}$, and arm^{F1a}, which contains a change of G2990 to A, or Arg294His (this study). arm^{F1a} functions as a weak loss-of-function mutant, which is pupal lethal with readily detectable protein levels.

Crosses and Expression of UAS Constructs

armadillo Mutants

Maternally mutant eggs were generated by the dominant female sterile technique (Chou and Perrimon, 1992). For all Axin expression experiments, the ArmGAL4 driver was used, because earlier or stronger drivers turn off early Wg striping, which leads to a lack of Axin stripes. All X chromosome mutants use FRT 101.

$zw3^{M11-1}$ (maternal)/Y (zygotic)
arm^{F1a} $zw3^{M11-1}$ (maternal)/Y (zygotic)
$arm^{H8.6}$ $zw3^{M11-1}$ (maternal)/Y (zygotic)
arm^{F1a} (maternal)/Y (zygotic); wg^{IG22}/wg^{IG22} (zygotic)
arm^{F1a}zw3^{M11-1} (maternal)/Y (zygotic); wg^{IG22}/wg^{IG22} (zygotic)
arm^{F1a} $zw3^{M11-1}$ (maternal)/Y (zygotic); $axin^{S044230}/axin^{S044230}$ (zygotic)
$arm^{H8.6}$ $zw3^{M11-1}$ (maternal)/Y (zygotic); arr^{2}/arr^{2} (zygotic)
arm^{F1a} (maternal)/Y (zygotic); Arm-GAL4/UAS-Axin (zygotic)
arm^{F1a} $zw3^{M11-1}$ (maternal)/Y (zygotic); Arm-GAL4/UAS-Axin (zygotic)
arr^{2} (maternal)/arr^{2} (zygotic) ; Arm-GAL4/UAS-Axin (zygotic)
$zw3^{M11-1}$ (maternal)/Y (zygotic); Arm-GAL4/UAS-Fz2-Arr[intra] (zygotic)

Embryos Expressing Fz2 and Arrow Constructs

For mutations present on the first or second chromosomes (*arm*, *dsh*, and *wg*), the Prd-GAL4 driver, located on the third chromosome, was used to drive expression of the UAS constructs (Table 1). For *fz fz2*, located on the third chromosome, the En-GAL4 driver on the second chromosome was used. Embryos were collected at 30°C to ensure optimal GAL4 activity. Third chromosome FRT 2A, and second chromosome FRT 42B were used. The relevant genotypes are as follows.

wg^{CX4}/wg^{CX4}; Prd-GAL4/UAS-Fz2-Arr[intra]
dsh^{V26}/Y (zygotic) ; UAS-Fz2-Arr[intra]/+ ; Prd-GAL4/+
arm^{XM19} glc/Y (zygotic); UAS-Fz2-Arr[intra]/+ ; Prd-GAL4/+
arr^2/arr^2 (zygotic) ; Prd-GAL4/UAS-Fz2-Arr[intra]
En-GAL4/UAS-Fz2-Arr[intra]; fz^{H51} $fz2^{C1}$/fz^{H51} $fz2^{C1}$ (zygotic)
En-GAL4/UAS-Arm*[S10]; fz^{H51} $fz2^{C1}$/fz^{H51} $fz2^{C1}$ (zygotic)
wg^{CX4}/wg^{CX4}; Prd-GAL4/UAS-Fz2
wg^{CX4}/wg^{CX4}; Prd-GAL4/UAS-Fz2-eCFP
wg^{CX4}/wg^{CX4}; Prd-GAL4/UAS-Fz2-6x-myc
wg^{CX4}/wg^{CX4}; Prd-GAL4/UAS-Myr-Arr[intra]

Wings

MS1096-GAL4 was used to drive expression of UAS constructs in third instar wing imaginal discs at 30°C (Wehrli et al., 2000).

UAS-Transgenes and GAL4 Driver Lines

Previously published transgenes used were UAS-Arm*[S10] (Pai et al., 1996), UAS-Axin (Hamada et al., 1999), UAS-myc-Axin (Willert et al., 1999a), UAS-Wg (Hays et al., 1997), Arm-GAL4 (Sanson et al., 1996), Prd-GAL4 (Brand and Perrimon, 1993), and En-GAL4.

wg^- $axin^-$ Embryos

Germline clones for *axin* were induced in females of the genotype y w hs-flipase; wg^{CX4}/CyO; FRT82B $axin^{S044230}$/FRT82B ovo^D and mated to y w ; wg^{CX4}/CyO; FRT82B $axin^{S044230}$/TM6B at 25°C and cuticles prepared. Six hundred twenty-two embryos displayed only smooth cuticle (with exception of the beard; maternal/zygotic *axin* mutants), in 413 embryos at least some denticles were visible (maternal axin mutants incompletely rescued by zygotic axin+), and 142 embryos displayed a weak wingless phenotype, that is, smooth cuticle bands were visible and segmentation was clearly apparent (Figure 2H; wg^{CX4}/wg^{CX4}; $axin^{S044230}$ maternal/axin+ zygotic). No phenotypic *wg* null embryos were observed presumed to be wg^{CX4}/wg^{CX4}; $axin^{S044230}$ maternal/$axin^{S044230}$ zygotic, and these naked embryos contribute to the pool of smooth embryos ($n = 1177$; $P < 0.0001$, $\chi 2 = 0.00037$, $df = 1$).

Antibodies and Immunofluorescence

Embryos were treated and stained as described (Tolwinski and Wieschaus, 2001), except that they were fixed with heptane/4% formaldehyde in phosphate buffer (0.1 M $NaPO_4$ [pH 7.4]). The antibodies used were: anti-Wg (mAb 4D4, Developmental Studies Hybridoma Bank), anti-Engrailed (mAb 4D9, DSHB), anti-Armadillo (mAb N2 7A1, DSHB), rabbit anti-Armadillo (Peifer et al., 1994b), rabbit anti-c-Myc (Santa Cruz Biotechnology), anti-α-tubulin (Sigma), and anti-Sexlethal (mAb M-14, DSHB). Staining, detection, and image processing was done as described (Tolwinski and Wieschaus, 2001).

Western Blotting

Embryos were lysed in extract buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol, Complete Mini Protease [Sigma]), the extracts were separated on 8% SDS-PAGE, and blotted as described in Peifer et al. (1994a). Immunoprecipitations were preformed as described in Willert et al. (1999b). Embryos were used to make an extract from which Axin was immunoprecipitated with a rabbit polyclonal antibody. The immune complexes were probed with a rat monoclonal antibody against Axin, or c-*myc* rabbit polyclonal. As a loading control, small samples of the original extracts were used to compare the α-tubulin levels between extracts.

Plasmid Construction and Yeast Two-Hybrid Screen

Plasmids were constructed using conventional techniques. Fz2-myc contains a 6×-myc tag fused to the Fz2 C terminus; Fz2-Arr[intra] carries a 6×-myc tag followed by Arr[intra]; Arr[intra] contains the C-terminal 207 amino acids starting at Phe1472. Myr-Arr[intra] contains a myristoylation signal followed by a 6× FLAG tag and Arr[intra]. In a yeast two-hybrid screen, 12 million GAL4 activation domain fusion clones of an embryonic 3–8 hr library were screened (Luo et al., 1997) using a fusion of the Arrow cytoplasmic domain to GAL4-DNA binding domain as bait. Fifteen Axin fusion clones were isolated.

RNA Interference

dsRNA (2 μM) was injected into wild-type embryos prior to cellularization. With Arrow dsRNAi, 25 of 50 injected embryos (50%) exhibited loss of smooth cuticle, as in *arr* null mutants (not shown). With Axin dsRNA, 18 of 47 injected embryos (38%) exhibited an *axin* loss-of-function phenotype. Coinjection of Arr and Axin dsRNAi (2 μM of each) resulted in loss of denticles in 14 of 44 injected embryos (32%), an *axin* loss-of-function phenotype with the same range in phenotype as with Axin dsRNA alone. None of the embryos exhibited an *arr* loss-of-function phenotype.

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