The EMBO Journal (2005) 24, 3493–3503 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
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THE EMBO JOURNAL

The role of the cysteine-rich domain of Frizzled in Wingless-Armadillo signaling

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The Frizzled (Fz) receptors contain seven transmembrane helices and an amino-terminal cysteine-rich domain (CRD) that is sufficient and necessary for binding of the ligands, the Wnts. Recent genetic experiments have suggested, however, that the CRD is dispensable for signaling. We engineered *fz* CRD mutant transgenes and tested them for Wg signaling activity. None of the mutants was functional in cell culture or could fully replace *fz in vivo*. We also show that replacing the CRD with a structurally distinct Wnt-binding domain, the Wnt inhibitory factor, reconstitutes a functional Wg receptor. We therefore hypothesized that the function of the CRD is to bring Wg in close proximity with the membrane portion of the receptor. We tested this model by substituting Wg itself for the CRD, a manipulation that results in a constitutively active receptor. We propose that Fz activates signaling in two steps: Fz uses its CRD to capture Wg, and once bound Wg interacts with the membrane portion of the receptor to initiate signaling.

The EMBO Journal (2005) **24,** 3493–3503. doi:10.1038/sj.emboj.7600817; Published online 15 September 2005
Subject Categories: development
Keywords: Frizzled; signaling; Wingless; Wnt

Introduction

Owing to their fundamental role in development and disease, it is of great interest to elucidate how Wnt proteins activate intracellular signaling. Recently, much progress has been made in identifying cytoplasmic signaling components that act downstream of the Wnt receptor Frizzled (Fz), which is responsible for regulating the stability of β-catenin, the main effector of Wnt signaling. In comparison, relatively little is known about the initial events that occur at the membrane, including which structural features of Fz are required to activate the cytoplasmic signaling machinery (Veeman *et al*, 2003a; Logan and Nusse, 2004).

In *Drosophila*, Wg signaling through Armadillo (Arm, *Drosophila* β-catenin) is required for patterning embryos (reviewed in Logan and Nusse, 2004; Tolwinski and Wieschaus, 2004; Bejsovec, 2005). The embryonic epidermis secretes a cuticle with groups of cells that produce hair-like projections, called denticles, separated by those that produce smooth or 'naked' cuticle. *wg* signaling promotes the formation of naked cuticle by promoting the post-translational stabilization of the transcriptional coactivator, Arm. As Arm protein accumulates in the cytoplasm and nucleus, it interacts with the TCF/LEF transcription factor Pangolin (Pan) to regulate target genes responsible for cell fate changes. In *Drosophila*, *fz* acts redundantly with another member of the Fz family, *frizzled2* (*fz2*) as a receptor for Wg. Either *fz* or *fz2* (Bhanot *et al*, 1996; Kennerdell and Carthew, 1998; Bhanot *et al*, 1999; Chen and Struhl, 1999), together with the membrane component, *arrow* (*arr*), form a receptor complex for the Wg protein (Wehrli *et al*, 2000). Embryos lacking *wg*, *arr* or both *fz* and *fz2* cannot initiate Arm signaling and, thus, are completely covered by denticles.

All Fz receptors contain within their extracellular portion a region called the cysteine-rich domain (CRD), named for its invariant pattern of 10 cysteine residues. Furthermore, a domain with homology to the CRD is present on a receptor involved in Hedgehog signaling, Smoothened (Smo; Alcedo *et al*, 1996; van den Heuvel and Ingham, 1996) and in the receptor tyrosine kinase Ror2, which is proposed to activate Arm/β-catenin-independent (noncanonical) Wnt signaling (Oishi *et al*, 2003). The CRD of Fz has been crystallized (Dann *et al*, 2001) and binds Wnt proteins with nanomolar affinity (Hsieh *et al*, 1999b; Wu and Nusse, 2002). Several mutations in a *fz* CRD have been engineered that affect Wnt binding (Hsieh *et al*, 1999b). Given that CRD domains confer Wnt binding, it was unexpected that *fz* transgenes lacking the CRD were reported to respond normally to Wg and activate Arm signaling *in vivo* (Chen *et al*, 2004). What is the function of this highly conserved portion of *fz*? We addressed this question by testing a set of CRD variants for Arm signaling in cell culture and *in vivo*.

Results

Generating fz transgenes to test for CRD requirements

Studies in different experimental systems have addressed the role for the CRD in Wnt signaling, but with conflicting outcomes. It was initially found that the CRD of the *fz* receptor is necessary for Wnt binding (Bhanot *et al*, 1996; Hsieh *et al*, 1999b; Dann *et al*, 2001; Wu and Nusse, 2002). In addition, expression of the CRD alone acts as a dominant negative, indicating that this portion of *fz* can nonproductively bind Wnt ligands, limiting the available pool for endogenous receptors (Cadigan *et al*, 1998). However, the role of the CRD as the sole determinant of Wnt binding has been recently challenged by experiments showing that Fz transgenes lacking a CRD are still capable of functioning in Arm signaling (Chen *et al*, 2004).

To address the function of the CRD, we made a series of modified *fz* transgenes (Figure 1A). We engineered two

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Received: 20 June 2005; accepted: 5 August 2005; published online: 15 September 2005

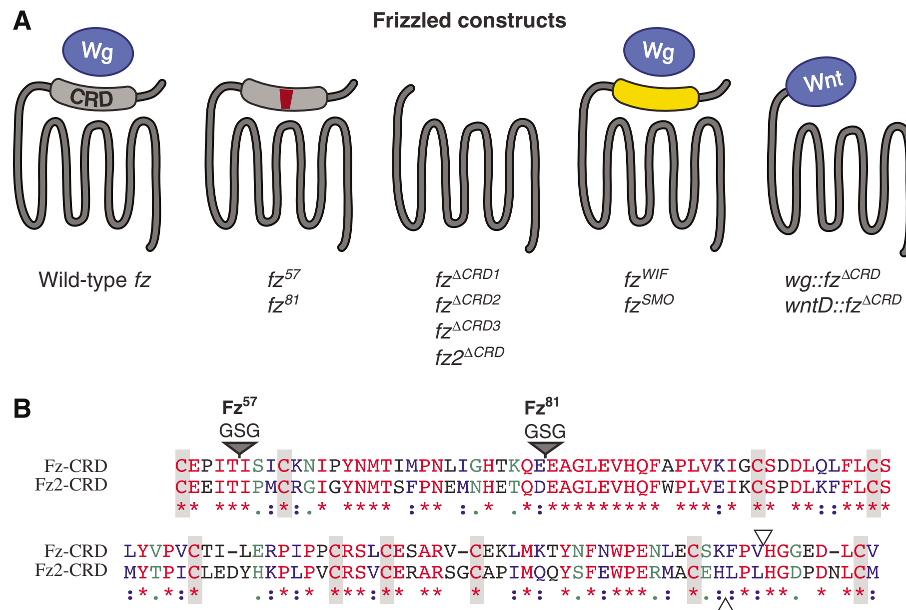

Figure 1 Design *fz* CRD variants. (**A**) To test the role of the CRD in Arm signaling, we engineered the following *fz* constructs into a vector for constitutive expression in *Drosophila* via the *tubulin-α1* promoter (pTub): Wild-type *fz* (581 amino acids). fz^{57} and fz^{81} insert three amino acids (GSG) between residues in the CRD and are predicted to interfere with Wg binding (Hsieh *et al*, 1999b). $fz^{\Delta CRD1}$ specifically deletes the CRD in-frame (amino acids 53–164). This leaves the remainder of the receptor intact, including the native signal sequence and the entire extracellular 'hinge' region lying between the CRD and the first transmembrane domain. $fz^{\Delta CRD2}$ and $fz2^{\Delta CRD}$ replace the entire N-terminal portions (up to the open triangles shown in panel B), including the native signal sequences of *fz* and *fz2* with the *wg* signal sequence and three Flu epitopes (Chen *et al*, 2004). $fz^{\Delta CRD3}$ specifically removes the eight amino acids of the CRD present in $fz^{\Delta CRD2}$ but absent in $fz^{\Delta CRD1}$, fz^{WIF} and fz^{SMO} specifically exchange the CRD with the WIF domain of hWIF (Hsieh *et al*, 1999a) or the CRD of *Drosophila smoothened* (Alcedo *et al*, 1996; van den Heuvel and Ingham, 1996). $wg::fz^{\Delta CRD}$ and $wntD::fz^{\Delta CRD}$ fusions join the full-length Wg or WntD protein in-frame with the region of Fz C-terminal to the CRD in $fz^{\Delta CRD1}$ (amino acids 165–581). Wnt-Fz fusions use the signal sequence native to their respective Wnt protein. (**B**) Alignment of the *Drosophila* Fz and Fz2 amino-acid sequence within the CRD. The 10 cysteine residues defining this domain are indicated by shaded vertical bars. Identical amino acids are indicated in red (*). Amino acids with conserved properties are indicated in purple (:) and green (.). Two mutations previously characterized to disrupt the binding between *Xenopus* Wnt8 and Fz2 CRD (Hsieh *et al*, 1999b) were engineered into a homologous position in the Fz CRD (gray triangles). $Fz^{\Delta CRD1}$ removes the entire region shown, leaving the flanking N- and C-terminal sequences unmodified. The breakpoints of $Fz^{\Delta CRD2}$ and $Fz2^{\Delta CRD}$ are indicated with open triangles. $Fz^{\Delta CRD2}$ includes eight amino acids not present in $Fz^{\Delta CRD1}$. These eight amino acids are deleted in $Fz^{\Delta CRD3}$ with the remainder of the construct identical to $Fz^{\Delta CRD2}$.

mutations into the CRD of *fz* that are predicted to disrupt specifically Wnt binding, but otherwise leave the protein intact (Hsieh *et al*, 1999b). These mutations (fz^{57} and fz^{81}) insert three amino acids (GSG) (Figure 1B) that map to the surface of the CRD folded structure (Dann *et al*, 2001). We also constructed a form of *fz* in which the entire CRD was deleted ($fz^{\Delta CRD1}$) and two forms where it was replaced by another domain. In one case, we exchanged the *fz* CRD with the CRD of *Drosophila smoothened* (fz^{SMO}), a domain that does not bind Wg (Wu and Nusse, 2002). In the other case, we substituted the Wnt inhibitory factor (WIF) domain of the human WIF (fz^{WIF}), a secreted molecule that can bind to Wnt proteins, including Wg (Hsieh *et al*, 1999b). In addition, we generated transgenes where the *Drosophila* Wnt genes, *wg* and *wntD* (CG8458 formerly *wnt8*) (Ganguly *et al*, 2005; Gordon *et al*, 2005), are substituted for the CRD ($wg::fz^{\Delta CRD}$ and $wntD::fz^{\Delta CRD}$). Lastly, we obtained two previously described CRD deletions of *fz2* and *fz* ($fz2^{\Delta CRD}$ and $fz^{\Delta CRD2}$) (Chen *et al*, 2004). The $fz^{\Delta CRD2}$ transgene differs from the version we engineered ($fz^{\Delta CRD1}$) in three ways: $fz^{\Delta CRD2}$ uses the signal sequence of *wg*, it is tagged with three copies of the Flu epitope and leaves eight amino acids of the CRD including the 10th conserved cysteine residue. For comparison, we made a modified form of $fz^{\Delta CRD2}$ that eliminates the eight amino acids, but preserves the *wg* signal sequence and Flu epitopes ($fz^{\Delta CRD3}$) (Figure 1B).

Expression of fz transgenes in S2 cells

To characterize initially our constructs, we expressed them in *Drosophila* S2 cells. S2 cells are well suited for studying *fz*-mediated Arm signaling since they lack endogenous *fz* and *wg* expression, providing a clean background (Bhanot *et al*, 1996; Sato *et al*, 1999). We performed a Western blot of whole-cell extracts made from cells transfected with *fz* variants to determine whether the transgenes produce the expected proteins. Probing the samples with an antibody directed against an epitope in the extracellular 'hinge' region present in all variants (except for $fz2^{\Delta CRD}$) showed that all proteins are synthesized (Figure 2A). When we compared abundance for the three $fz^{\Delta CRD}$ variants, we observed there was less $Fz^{\Delta CRD2}$ protein synthesized compared to either $Fz^{\Delta CRD1}$ or $Fz^{\Delta CRD3}$. The same difference in abundance between $Fz^{\Delta CRD2}$ and $Fz^{\Delta CRD3}$ was observed when extracts were probed with an anti-Flu antibody, which additionally shows that $fz2^{\Delta CRD}$ is abundantly produced (Figure 2A).

To determine whether the *fz* transgenes we constructed produce proteins that are localized to the plasma membrane, we transfected cells and immunostained them with our extracellular *fz* antibody (Figure 2B). There is no specific signal observed in untransfected or mock-transfected cells (Figure 2B). Like wild-type Fz, all Fz variants are targeted to the cell surface. The abundance of each Fz protein on the membrane correlates with the protein level observed in whole-

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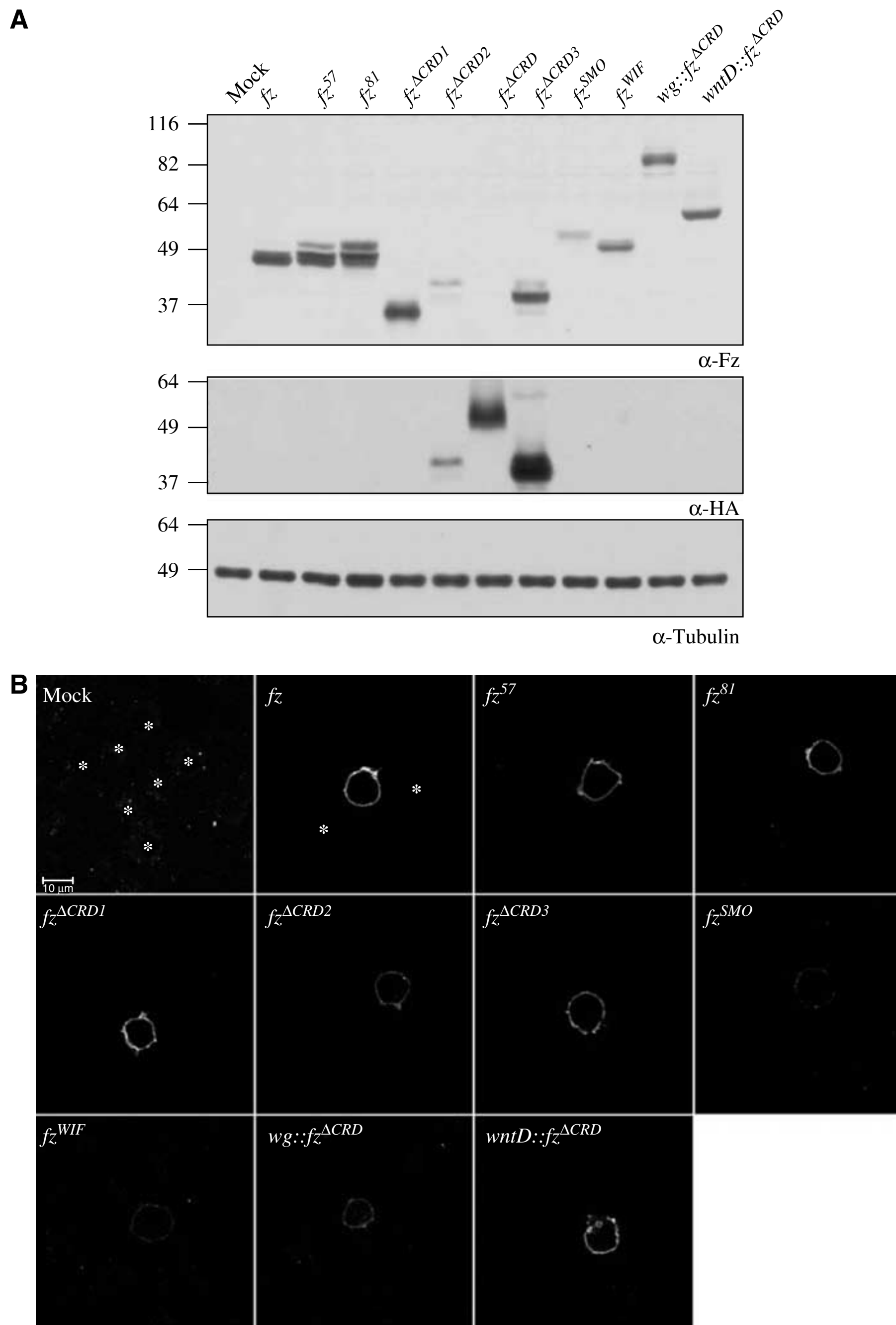

Figure 2 Fz protein abundance and surface localization in S2 cells. (**A**) Whole-cell protein extracts of S2 cells transfected with *fz* transgenes probed with a polyclonal antibody directed against the 'hinge' region of Fz (α-Fz, top panel), an epitope, which Fz2 lacks. When the same samples are probed with an HA antibody, we observed that $Fz2^{\Delta CRD}$ is expressed comparably to $Fz^{\Delta CRD3}$, which are both expressed at higher levels than $Fz^{\Delta CRD2}$. The molecular mass in kDa of a known protein standard is indicated. (**B**) Cell surface localization of Fz protein was assayed in S2 cells transfected with *fz* transgenes and stained using an antibody directed against the 'hinge' region of Fz. Fz2 was not tested as it lacks this epitope. The same settings were used for all samples aside from mock-transfected cells where the gain was intentionally set higher to emphasize lack of signal. Staining is specific, as untransfected cells (*) are not detectable. The scale bar in upper left panel is 10 μm.

cell extracts. Therefore, similar to wild type, all Fz variants are synthesized and targeted to the plasma membrane.

fz transgenes with CRD mutations are compromised for Arm signaling in cell culture

As a measure of signaling, we tested transcriptional activation of Arm signaling by a reporter assay (Figure 3A). S2 cells were transiently transfected with a TCF/LEF-dependent luciferase reporter (Veeman *et al*, 2003b) and an *fz* transgene alone or together with a *wg* transgene. None of the *fz* transgenes activated the reporter in the absence of *wg* showing, as reported previously, that the Fz protein does not activate signaling in the absence of a Wnt (Bhanot *et al*, 1996). Expression of *wg* alone did not substantially activate the reporter (1.7 ± 0.3-fold). Cells transfected with both wild-type *fz* and *wg* potently activated the reporter (80.6 ± 0.3-fold). In comparison, cells expressing *wg* and either fz^{57} or fz^{81} show a dramatic reduction in their response, with fz^{81}

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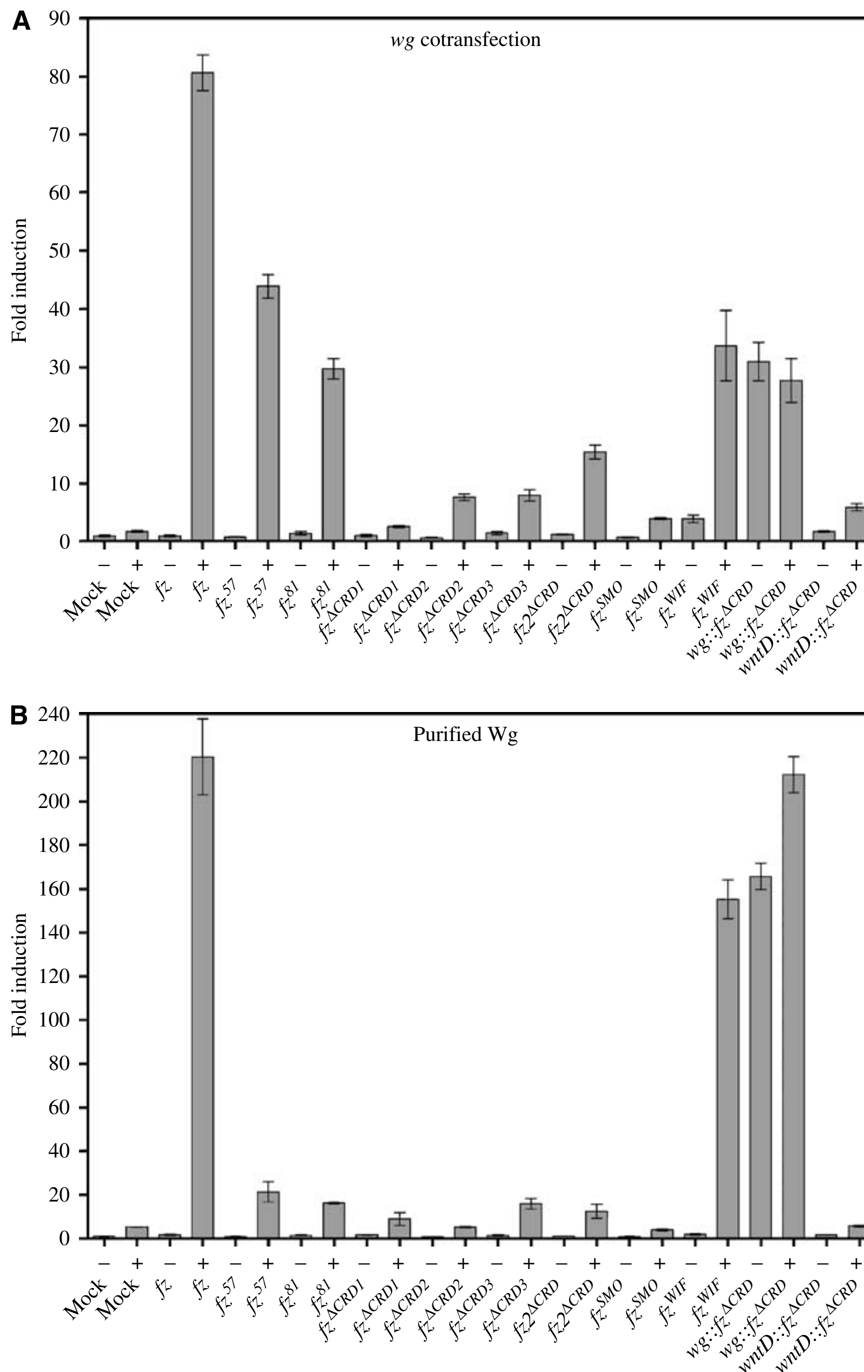

Figure 3 Arm signaling of *fz* CRD variants in S2 cells. (**A**) To measure Arm signaling, TCF/LEF-dependent luciferase reporter assays were performed on cells cotransfected with pTub (Mock) or a pTub-*fz* variant and empty pTub (−) or pTub-*wg* (+). (**B**) TCF/LEF-dependent luciferase reporter assays were performed on cells cotransfected with pTub (Mock) or a pTub-*fz* variant and challenged with plain medium (−) or medium containing purified Wg (+).

being more compromised than fz^{57} (29.8±3.1- and 43.9±3.4-fold, respectively). Cells expressing $fz^{\Delta CRD1}$ or fz^{SMO} only modestly elevated the reporter in the presence of *wg* (2.6±0.3- and 3.9±0.3-fold). The $fz2^{\Delta CRD}$ moderately activated the reporter when cotransfected with *wg* (15.4±2.1-fold). Interestingly, $fz^{\Delta CRD2}$ displayed a marked increase in *wg*-dependent reporter activation compared to $fz^{\Delta CRD1}$ (7.6±1.0- versus 2.6±0.3-fold). Either the *wg* signal sequence or Flu tags are responsible for the enhanced *wg* responsiveness since $fz^{\Delta CRD3}$ shows a similar elevation (8.0±1.7-fold) compared to $fz^{\Delta CRD1}$. Taken together, these data show that the CRD of *fz* is required for robust signaling.

Importantly, expression of fz^{WIF}, which adds back to the $fz^{\Delta CRD1}$ receptor a Wnt-binding domain, partially restored *wg* responsiveness (33.7±10.5-fold). Expression of the $wg::fz^{\Delta CRD}$ fusion transgene alone led to the activation of the reporter (31.0±5.8-fold) that was not enhanced by co-transfection of *wg* (27.7±6.6-fold). Activation is specific to the $wg::fz^{\Delta CRD}$ since another wnt-fz fusion, $wntD::fz^{\Delta CRD}$, did not activate the reporter on its own (1.7±0.2-fold) and

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showed only minimal activation in the presence of *wg* (5.9±1.0-fold). To determine whether the *fz* CRD variants can interact with Wg protein, we transfected the receptor transgenes with a TCF/LEF-dependent reporter, and after allowing time for protein expression, we challenged the cells with Wg protein purified from conditioned medium of S2 cells constitutively expressing *wg* (Figure 3B) (Willert *et al*, 2003). We observed a similar trend with cotransfection. However, whereas the cells transfected with wild-type *fz* more robustly activated Arm signaling in this assay (220.5±24.4-fold), cells transfected with fz^{57} and fz^{81} showed a diminished capacity to activate the reporter (21.4±6.5- and 16.2±0.4-fold, respectively). Importantly, all recombinant Fz proteins localize to the cell surface (Figure 2B, $fz2^{\Delta CRD}$ was not tested), which suggests that in the cotransfection assay, the Fz^{57} and Fz^{81} proteins might in part interact with Wg protein in an intracellular compartment during synthesis, which might augment an otherwise weak interaction. The CRD deletion variants and fz^{SMO} behave similarly in this assay and the cotransfection assay. Strikingly, cells expressing fz^{WIF} are significantly more responsive to purified soluble Wg protein than cotransfected *wg* (155±12.7 and 33.7±10.5, respectively). wg::$fz^{\Delta CRD}$-expressing cells showed a modest increase in reporter activation in the presence of soluble Wg protein (165.9±8.4 versus 212.3±11.7). Cells expressing $wntD$::$fz^{\Delta CRD}$ failed to activate the reporter in the absence or presence of Wg protein.

wg::*fz*$^{\Delta CRD}$ requires arr to activate Arm signaling

Wg is thought to activate Arm signaling by bridging the two membrane coreceptors Fz and Arr and bringing together their associated intracellular signaling components (Tamai *et al*, 2000; Tolwinski *et al*, 2003; Cong *et al*, 2004). It has been reported that lowering the amount of Arr protein by RNA interference (RNAi) interferes with Arm signaling in cell culture (Schweizer and Varmus, 2003). As shown above, expression of wg::$fz^{\Delta CRD}$ constitutively activates Arm signaling. We therefore asked whether the Wg::$Fz^{\Delta CRD}$ protein requires Arr to elicit signaling. We performed TCF/LEF reporter assays on S2 cells expressing either wg::$fz^{\Delta CRD}$ or $wntD$::$fz^{\Delta CRD}$ and lowered the amount of Arr protein by RNAi (Figure 4). This results in a 3.5-fold reduction in reporter activation in cells expressing wg::$fz^{\Delta CRD}$. Thus, the Wg::$Fz^{\Delta CRD}$ protein requires the Arr coreceptor to constitutively activate Arm signaling.

fz transgenes with altered CRDs are compromised for Arm signaling in vivo

To assess whether CRD mutations interfere with Arm signaling mediated by Wg *in vivo*, we tested a subset of the transgenes to determine whether they could rescue *fz,fz2* mutant embryos. We removed *fz* and *fz2* by making germline clones (Perrimon *et al*, 1996). These mutant embryos exhibit defects in the specification of naked cuticle, indicating loss of Arm signaling (compare Figure 5A and B). When ubiquitously expressed throughout the embryo, wild-type *fz* is fully capable of restoring normal patterning to the mutant embryos (Figure 5C). In fz^{57}, fz^{81} and $fz^{\Delta CRD}$ rescue crosses, denticles are present in regions that should be entirely naked. We also found fusions of denticle belts (Figure 5D–F). Consistent with the TCF/LEF reporter assays for *fz* function (Figure 3), the ability of fz^{57} to rescue the mutant embryos was stronger than

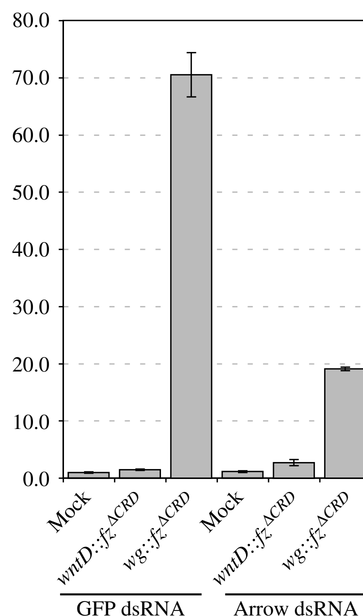

Figure 4 wg::$fz^{\Delta CRD}$ requires *arr* to activate Arm signaling in S2 cells. TCF/LEF reporter assays on two sets of cells transfected with empty plasmid (Mock), $wntD$::$fz^{\Delta CRD}$ or wg::$fz^{\Delta CRD}$ transgenes. One set was cotransfected with control double-stranded RNA (GFP dsRNA) and the second set was cotransfected with *arr* dsRNA (Arrow dsRNA). There is a 3.7-fold reduction in signaling when cells are cotransfected with *arr* dsRNA compared to the control dsRNA.

the two other transgenes, but rescue was not complete (Figure 5D). fz^{81} and $fz^{\Delta CRD1}$ gave incomplete rescue, although in each case the resulting cuticle phenotype was less severe than the *fz,fz2* mutant.

We also tested whether the transgenes could rescue lethality caused by the absence of endogenous *fz*-mediated Arm signaling. Only a wild-type *fz* transgene is capable of rescuing *fz,fz2* germline clones to adulthood (Figure 5C, Table I). In contrast, the CRD mutant transgenes were incapable of restoring viability to *fz,fz2* mutants (Table I).

In addition, we tested whether *fz* transgenes carrying CRD mutations can rescue Arm signaling in the *Drosophila* wing. Activation of Arm signaling by *wg* and *fz* is required for the formation of bristles that normally pattern the margin of the wing (Chen and Struhl, 1999). In addition, *fz* has another, *wg*-independent function that is unrelated to Arm signaling. It is responsible for the planar cell polarization (PCP) of *Drosophila* tissues including the hairs and bristles of the wing and clones of cells mutant for *fz* also nonautonomously affect the polarization of surrounding wild-type cells (Vinson and Adler, 1987). Wing margin bristles made by *fz* mutant clones have an abnormal elevation, rotated away from the margin (Figure 6, compare panel C with A and B). Homozygous clones for *fz* and *fz2* are identifiable by the recessive pigmentation marker, *yellow* (*y*) (open arrows), and develop unpigmented (yellow) margin bristles where heterozygous tissue produces darker pigmented bristles (closed arrows). As in the embryo, *fz* and *fz2* act redundantly as the receptor for Wg. Similar to control clones (Figure 6A), clones of cells mutant for either *fz* or *fz2* alone (Figure 6B and C) are still capable of Wg-mediated activation of Arm signaling as evidenced by numerous yellow bristles. Clones of cells lacking both *fz* and *fz2* fail to generate yellow bristles and

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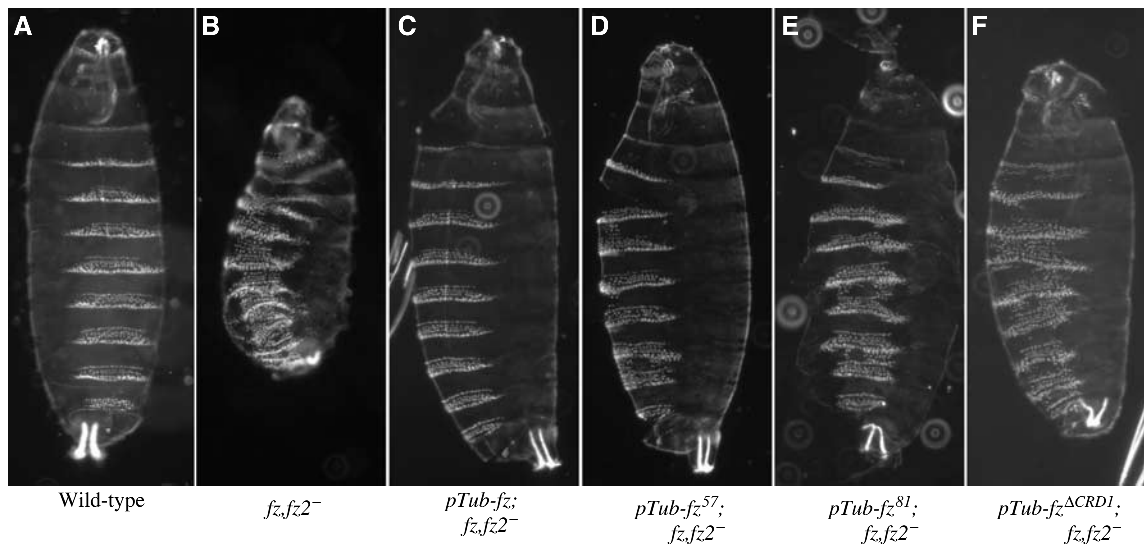

Figure 5 *fz* CRD mutations show Arm signaling defects in embryos. (**A**) Cuticle of a wild-type *Drosophila* embryo. (**B**) *fz,fz2* germline clone zygotically mutant for *fz* and *fz2*. The following panels are the same genotype as in (B), zygotically expressing via the *tubulin-1*α promoter (*pTub*) one copy of (**C**) wild-type *fz*, (**D**) fz^{57}, (**E**) fz^{81} and (**F**) $fz^{\Delta CRD1}$. For each of the transgenes, one insert line was tested.

Table I Rescue of viability in $fz,fz2^{-}$ germline clones

Rescue construct	*n*	%
pTub-fz	71	11
$pTub\text{-}fz^{57}$	43	0
$pTub\text{-}fz^{81}$	42	0
$pTub\text{-}fz^{\Delta CRD1}$	60	0

often lack bristles altogether (Figure 6D) (Chen and Struhl, 1999). Loss of Arm signaling, such as what occurs in *fz,fz2* mutant clones, results in the ectopic expression of *wg* (Rulifson *et al*, 1996), which can ultimately result in the formation of an ectopic bristle due to the inappropriate activation of Arm signaling in heterozygous cells neighboring the clone (arrowheads) (Chen and Struhl, 1999). Uniform expression of wild-type *fz* in the wing completely restores the patterning to *fz fz2* mutant cells present at the margin (Figure 6E). Importantly, in contrast to wild-type *fz*, expression of the CRD mutants fz^{57}, fz^{81} and $fz^{\Delta CRD1}$ does not fully restore patterning to *fz,fz2* mutant clones (Figure 6F–H). In addition to polarization defects, the Arm signaling defects manifest as abnormal bristle spacing and ectopic bristles near the margin. However, the order of the Arm signaling strength of the CRD mutants deviated from that observed in the embryo and cell culture. In the latter assays, rescue of Arm signaling by fz^{81} was less efficient than either fz^{57} or $fz^{\Delta CRD1}$, which rescue Arm signaling to a similar degree. As was the case in the embryo, all of the CRD mutants were able to significantly, but not fully rescue Arm signaling.

fz transgenes with CRD mutations have Arm signaling defects. Does adding back a Wnt-binding domain restore full function? Expression of fz^{WIF} rescues *fz,fz2* clones, although rescue is incomplete, with observable defects in the patterning of the margin (Figure 6I). In some cases, a bristle within a clone of mutant tissue is missing from the margin, appearing to leave a gap. However, these instances differ qualitatively from gaps seen with the expression of the CRD mutants since in clones rescued by fz^{WIF} the socket for the absent bristle is formed (*). Therefore, fz^{WIF} is capable of restoring Arm signaling to cells mutant for *fz* and *fz2* and rescues more efficiently than the *fz* mutants, but it is not as efficient as wild-type *fz*.

Overexpresion of fz^{WIF} and $wg::fz^{\Delta CRD}$ activate Arm signaling in vivo

In addition to rescue of *fz,fz2* mutant clones in the wing, we overexpressed fz^{WIF} in the wing using the Gal4/UAS system (Brand and Perrimon, 1993) to assess whether it is capable of signaling *in vivo*. Interestingly, overexpression of fz^{WIF} throughout the wing blade results in a global disruption of PCP and the formation of ectopic bristles near the wing margin, a gain-of-function Arm signaling phenotype (Figure 7A and B). Bristle formation near the source of Wg suggests that fz^{WIF} requires a Wnt ligand to activate Arm signaling *in vivo*, as it does *in vitro* (Figure 3). Interestingly, the phenotypes observed following fz^{WIF} overexpression are the composite of the individual overexpression phenotypes of wild-type *fz* and *fz2*, which lead exclusively to disruption of PCP and generation of ectopic bristles, respectively (Figure 7C and D) (Zhang and Carthew, 1998). The presence of ectopic bristles shows that fz^{WIF} is capable of activating Arm signaling via Wg *in vivo* as it does in cell culture.

Overactivation of Arm signaling throughout the entire wing, as caused by overexpression of *wg* and $wg::fz^{\Delta CRD}$ using the same driver as above, leads to an overall disruption of normal development preventing the assay of ectopic bristle formation (data not shown). Therefore, we returned to *Drosophila* embryos to ask whether overexpression of $wg::fz^{\Delta CRD}$ activates Arm signaling *in vivo*. $wg::fz^{\Delta CRD}$ and $wntD::fz^{\Delta CRD}$ were expressed in the domain of the gene *hairy* (*h*), which is expressed in alternating segments. Importantly, similar to overexpression of wild-type *wg*, overexpression of $wg::fz^{\Delta CRD}$ leads to the activation of Arm signaling resulting in the formation of ectopic naked cuticle (Figure 8B and C). As a control, expression of *wntD* or $wntD::fz^{\Delta CRD}$ did not promote naked cuticle formation (Figure 8D and E). Thus,

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both fz^{WIF} and $wg::fz^{\Delta CRD}$ can activate Arm signaling when overexpressed *in vivo*. However, whereas fz^{WIF} requires the endogenous Wg protein (in cell culture and in the wing tissue, where ectopic bristles form close to th Wg source at the margin), $wg::fz^{\Delta CRD}$ constitutively activates Arm signaling.

Discussion

What is the function of the Fz CRD in Arm signaling?

Our principle finding is that the Fz CRD is required for efficient Arm signaling. *Fz* transgenes carrying CRD mutations have compromised Arm signaling function in cell culture and cannot fully restore Arm signaling to *fz,fz2* mutants *in vivo*. In addition, we found that adding a heterologous Wnt-binding domain (WIF) to a CRD-deleted *fz* restores its ability to activate Arm signaling via Wg in cell culture. Based on the manipulations and results, we hypothesized that the function of the CRD is to bring Wg in close proximity with the membrane portion of the receptor, a function that can be taken over by other Wnt-binding domains. We tested this idea by creating a transgene fusing Wg to Fz, eliminating the CRD in the process, which results in a constitutively active receptor.

While both *in vivo* and *in vitro* tests reveal that mutants with a defective Wnt interaction domain are compromised for Arm signaling, the requirement for the CRD is most evident in cell culture where all of the mutants show a reduced activity, particularly the one where the entire CRD is lacking. In the cell culture experiments, where we can measure the Wg signaling in a quantitative manner, we find a range of responses to the CRD mutants (Figure 3) corresponding to the differences in Wnt-binding strength (Hsieh *et al*, 1999b). *In vivo*, by examining cuticles and the abilities of the CRD mutants to restore signaling, we also notice a range of phenotypes (Figure 5). While these rescue data are more difficult to measure, the phenotypes correspond in strength to the *in vitro* signaling levels. We infer from this relationship that signaling operates through the same mechanism *in vivo* as in cell culture. As an extension of this argument, we suggest that the CRD plays a similar role in cell culture as in the embryo. However, signaling *in vivo* is less stringently dependent on the presence of the CRD, suggesting that its absence is being compensated for by other factors. If the function of the CRD (or other Wnt-binding domains such as the WIF) is, as we propose, to bring Wg in close proximity to the membrane domain of Fz, it is possible this function is taken over by other molecules acting in trans and that these factors are not present *in vitro*. Candidates for such molecules are members of the CRD containing ROR family and the RYK receptor tyrosine kinase, which has a WIF domain (Yoshikawa *et al*, 2003; Forrester *et al*, 2004; Inoue *et al*, 2004; Lu *et al*, 2004). It is also possible that extracellular matrix molecules provide such an accessory function, by presenting or concentrating Wg close to the Fz signaling domain.

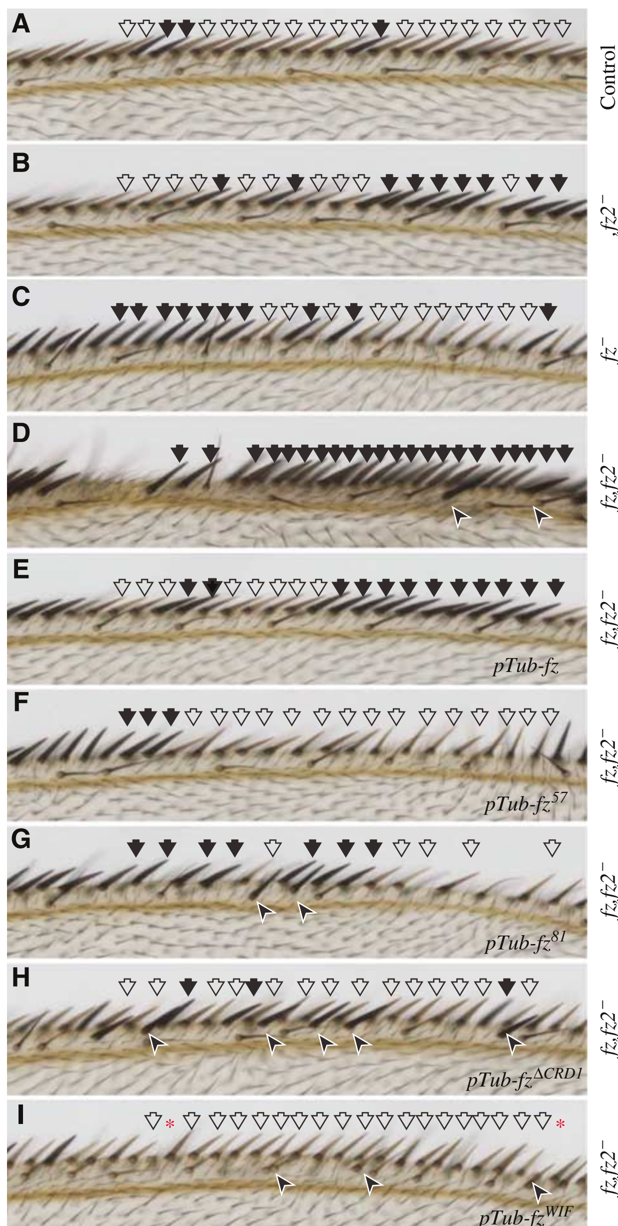

Figure 6 *fz* CRD mutations show Arm signaling defects in wings. Shown here are the wing margins of flies possessing different clones as indicated by the vertical label to the right of each panel. Clones that cross the margin elaborate yellow bristles due to the loss of the y^+ marker and are indicated with open arrows. Darker bristles that are elaborated by heterozygous cells are indicated with closed arrows. Arrowheads indicate the presence of a bristle formed inappropriately away from the margin. (**A**) Control clones that do not carry any mutations produce numerous yellow bristles, similar to clones of either $fz2^-$ (**B**) or fz^- (**C**). (**D**) $fz,fz2^-$ clones do not generate yellow margin bristles and have large patches along the margin lacking bristles. The bristles generated by surrounding heterozygous tissue show tighter spacing and occasional ectopic bristles that form away from the margin (arrowheads). (**E**) The margin gaps and ectopic bristle phenotypes of $fz,fz2^-$ clones are fully rescued by the ubiquitous expression of one copy of wild-type *fz*. These wings generate many yellow margin bristles. (**F**) $fz,fz2^-$ clones rescued by fz^{57} have subtle defects in the spacing of the margin bristles. (**G**) $fz,fz2^-$ clones rescued by fz^{81} have fewer yellow margin bristles and more severe bristle spacing problems. (**H**) $fz,fz2^-$ clones rescued by $fz^{\Delta CRD1}$ have ectopic margin bristles, which are almost invariably dark, indicating they are derived from nearby heterozygous cells. (**I**) $fz,fz2^-$ clones rescued by fz^{WIF} produce numerous yellow bristles. There are places along the margin where a bristle appears to be missing. However, unlike the defects in bristle spacing seen with fz^{57} and fz^{81} rescued clones, in fz^{WIF} rescued clones a structure resembling a bristle socket forms in the place of the apparently missing bristle (*). For each of the transgenes, two separate insert lines were tested.

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Is the only function of the CRD (or another Wg-binding domain, such as WIF) to capture Wg and to present it to the coreceptor Arrow? In that view, there would be no need for the seven-transmembrane domain of the Fz receptors; Fz would solely act to promote Wg interacting with Arrow. We find this unlikely; there are several studies that point to a requirement of specific residues in the Fz membrane domain in signaling (Umbhauer *et al*, 2000; Cong *et al*, 2004; Toomes *et al*, 2004; Povelones *et al*, 2005). Mutations in those residues, either engineered or present in natural alleles, disrupt signaling. In addition, it has been recently proposed that in *Drosophila*, *fz* activates PCP and Arm signaling through heterotrimeric G proteins (Katanaev *et al*, 2005). Finally, expressing the CRD on the cells surface as a GPI-linked membrane molecule does not promote signaling, but instead acts as a dominant negative (Cadigan *et al*, 1998). Taken together, these data suggest that the transmembrane portion of *fz* is a dynamic signal activating molecule and not merely a Wg presentation module.

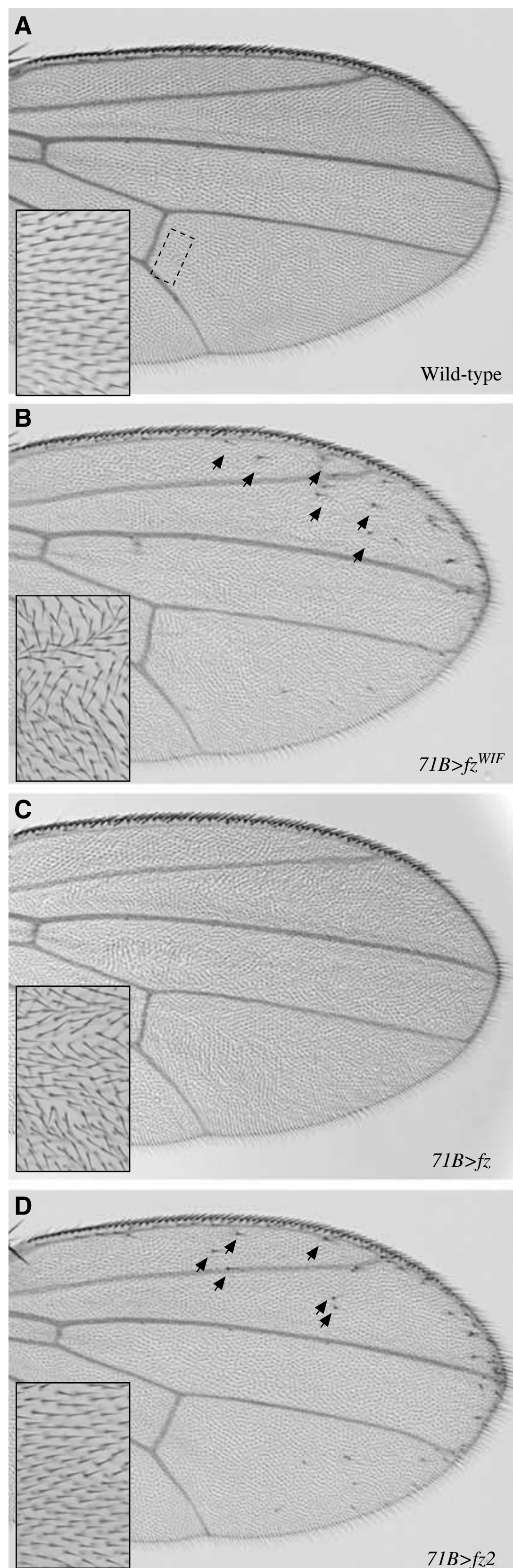

Figure 7 fz^{WIF} activates Arm signaling *in vivo*. (**A**) Picture of a wild-type *Drosophila* wing. The boxed region distal to the posterior crossvein indicates the location of the inset in all panels. Note all wing hairs uniformly point towards the distal portion of the wing. (**B**) Overexpression of fz^{WIF} generates both Arm-dependent ectopic bristles near the wing margin (arrows) and Arm-independent PCP defects throughout the wing. (**C**) Overexpression of *fz* alone only leads to a PCP disruption throughout the wing. (**D**) Overexpression of *fz2* only produces ectopic bristles near the wing margin (arrows).

Planar cell polarity phenotypes

As shown in Figure 7, overexpression of fz^{WIF} in the *Drosophila* wing leads to both gain-of-function PCP and Arm signaling phenotypes. This is the composite of the consequences of *fz* and *fz2* overexpression, which individually activate PCP and Arm signaling, respectively (Zhang and Carthew, 1998). There is much interest in determining how each receptor couples to a particular pathway (Adams *et al*, 2000; Boutros *et al*, 2000; Rulifson *et al*, 2000; Wu *et al*, 2004). Although there is some disagreement in these studies, it is generally concluded that the transmembrane portion of *fz*, including the cytoplasmic tail, couples it to PCP signaling. Since fz^{WIF} contains this portion of *fz*, it is not surprising that it too affects PCP signaling. What structural feature of *fz2* is responsible for coupling it exclusively to Arm signaling? We found that specifically replacing the *fz* CRD with the WIF domain results in a receptor that, like *fz2*, can activate Arm signaling. This finding is consistent with a study of *fz*/*fz2* chimeras where the ability to activate Arm signaling was shown to be a property of the *fz2* CRD (Rulifson *et al*, 2000). It was proposed that the feature conferring Arm coupling was the 10-fold higher affinity of the *fz2* CRD for the Wg protein. By analogy, the WIF domain, like the *fz2* CRD, may have a higher affinity for Wg than the *fz* CRD.

Materials and methods

Construction of pTub-fz transgenes

Wild-type *Drosophila fz* was PCR amplified using the following primers containing restriction sites for cloning:

5′-*fz* Spe I: GGACTAGTATGTGGCGTCAAATCCTG
3′-*fz* Xba I: GCTCTAGACTAGACGTACGCCTGCGC

The fz^{57}, fz^{81} and $fz^{\Delta CRD}$ transgenes were constructed using Splicing by Overlapping Extension PCR (SOE–PCR). Two overlapping fragments (frag 1 and frag 2) with the specific lesion were generated in separate reactions using wild-type *fz* as a template. Frag 1 was generated using 5′-*fz* Spe I and 3′-*fz* frag 1 primers

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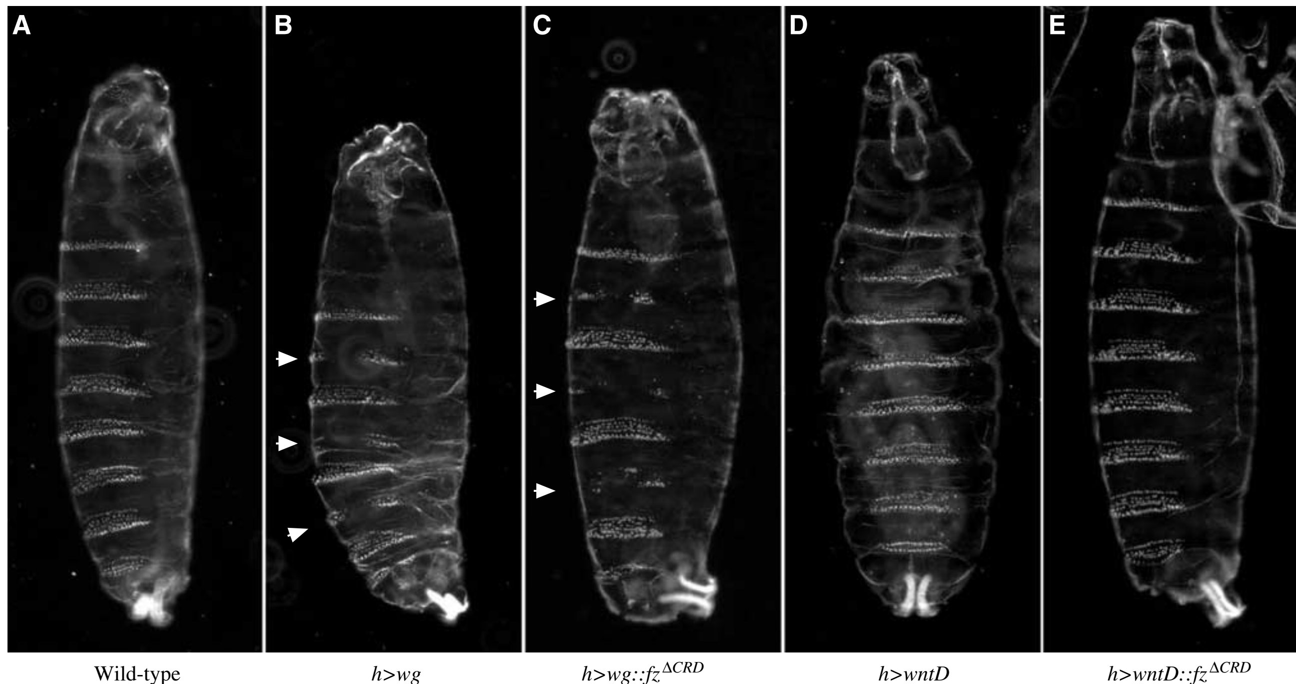

Figure 8 *wg*::$fz^{\Delta CRD}$ activates Arm signaling *in vivo*. (**A**) Wild-type cuticle pattern displayed by an embryo with one copy of the *hairy-gal4* (*h*) driver but lacking a UAS transgene. (**B**) Expression of *wg* in alternating segments results in the formation of ectopic naked cuticle (arrows). (**C**) Expression of *wg*::$fz^{\Delta CRD}$ results in the formation of ectopic naked cuticle (arrows). Either expression of *wntD* (**D**) or *wntD*::$fz^{\Delta CRD}$ (**E**) does not result in naked cuticle formation.

(below). Frag 2 was generated using 5′-*fz* frag 2 and 3′-*fz* Xba I primers. The two fragments were combined as a template in a third reaction with the primers 5′-*fz* Spe I and 3′-*fz* Xba I:

3′-fz^{57} frag 1 CTTGCAGATCGATATTCCGGATCCGGTGATGGGTTCACAGCG
5′-fz^{57} frag 2 CGCTGTGAACCCATCACCGGATCCGGAATATCGATCTGCAAG

3′-fz^{81} frag 1 CCTCCAGACCCGCCTCTCCGGATCCCTCCTGCTTGGTATGGCC
5′-fz^{81} frag 2 GGCCATACCAAGCAGGAGGGATCCGGAGAGGCGGGTCTGGAGG

3′-$fz^{\Delta CRD1}$ frag 1 GGTATTCTCCGCGCGATTGTGATGTGGCAGTCC
5′-$fz^{\Delta CRD1}$ frag 2 CATCACAATCGCGCGGAGAATACCACATCATCG

We obtained the previously described pTub-$fz^{\Delta CRD}$, pTub-$fz2^{\Delta CRD}$ (Chen *et al*, 2004). The $fz^{\Delta CRD}$ transgene constructed by Chen *et al* (2004) (in this work called $fz^{\Delta CRD2}$) differs from ours in three ways: it utilizes the signal sequence of wg, contains three Flu epitopes and includes eight more residues, including the 10th cysteine residue of the CRD. We modified the $fz^{\Delta CRD2}$ transgene to remove the eight *fz* residues (157–164) and generate a molecule with the identical C-terminal portion as $fz^{\Delta CRD1}$ ($fz^{\Delta CRD3}$). The $fz^{\Delta CRD3}$ transgene was constructed by SOE–PCR using $fz^{\Delta CRD2}$ as a template. Since this construct uses the *wg* signal sequence, frag 1 was produced with 5′-*wg* Spe I and the 3′-frag 1 primer (below). Frag 2 was amplified using the 5′ primers listed below and 3′-*fz* Xba I. The full-length product was amplified with 5′-*wg* Spe I and 3′-*fz* Xba I:

3′-$fz^{\Delta CRD3}$ frag1 GGTATTCTCCGCGAGGGGCAAGCTAGCGTAATC
5′-$fz^{\Delta CRD3}$ frag2 AGCTTGCCCCTCGCGGAGAATACCACATCATCG

The *wg*::$fz^{\Delta CRD}$ and *wntD*::$fz^{\Delta CRD}$ transgenes were constructed by SOE–PCR using *wg* and *wntD* as a template for frag 1 and unique cloning primers containing a *Spe*I site. *fz* was the template for frag 2 using the primers below and the same 3′-*fz* Xba I primer as above:

5′-*wg* Spe I: GGACTAGTATGGATATCAGCTATATC
3′-*wg*::$fz^{\Delta CRD}$ frag1: GGTATTCTCCGCCAGACACGTGTAGATGACC
5′-*wg*::$fz^{\Delta CRD}$ frag2: TACACGTGTCTGGCGGAGAATACCACATCATCG

5′-*wntD* Spe I: GGACTAGTATGATTTTTGCCATCACATTC
3′-*wntD*::$fz^{\Delta CRD}$ frag1: GGTATTCTCCGCGTAGCAGGAGTACTGCCTTTC
5′-*wntD*::$fz^{\Delta CRD}$ frag2: TACTCCTGCTACGCGGAGAATACCACATCATCG

The fz^{WIF} and fz^{SMO} transgenes were constructed by SOE–PCR using three overlapping fragments. The 5′-*fz* Spe I and 3′-*fz* Xba I primers were used to generate frag 1 and 3, respectively. These same primers were used to amplify the full-length product:

3′-fz^{WIF} frag1: GGCCTCCGCCCGGCGATTGTGATGTGGCAGTCC
5′-fz^{WIF} frag2: CATCACAATCGCCGGGCGGAGGCCGGGCCGCCG
3′-fz^{WIF} frag2: GGTATTCTCCGCACATGTTTTAAAGAAGATAGC
5′-fz^{WIF} frag3: CTTTAAAACATGTGCGGAGAATACCACATCATCG

3′-fz^{SMO} frag1: GGTGGGGTAGCAGCGATTGTGATGTGGCAGTCC
5′-fz^{SMO} frag2: CATCACAATCGCTGCTACCCCACCTCGAACGCAACC
3′-fz^{SMO} frag2: GGTATTCTCCGCACACTGGCCAGTTCCGTTG
5′-fz^{SMO} frag3: ACTGGCCAGTGTGCGGAGAATACCACATCATCG

All final PCR products were cut with *Spe*I and *Xba*I and inserted into *Xba*I cut P-element vector pTub for constitutive expression in *Drosophila* via the *tubulin-α1* promoter (Chen *et al*, 2004).

Arm signaling reporter assays

Drosophila S2 cells were transfected with the Arm signaling reporter superTOPFLASH (Veeman *et al*, 2003b), a loading control pIB/His/lacz (Invitrogen) and a *fz* transgene in combination with a *wg* transgene or empty vector. *wg* and *fz* variant transgenes were expressed via the constitutive *tubulin-α1* promoter (Chen *et al*, 2004). Transfection complexes were formed using 1.5 μl of Fugene6 (Roche) in 50 μl of DES serum-free medium + 2 mM GlutaMAX (Invitrogen) and 250 ng superTOPFLASH, 125 ng pIB/lacZ, 385 ng each of *fz* and *wg* or empty vector. Complexes were divided into three wells of a 96-well plate containing 2×10^5 cells/well. Cells were disrupted in 20 μl of lysis buffer and luciferase activity was measured in 5 μl of lysate 40 h post-transfection using the Dual-Light Assay System (Applied Biosystems) and a LB960 Luminometer (Berthold). β-Galactosidase activity was used to normalize the values. In experiments using purified Wg, 40 h post-transfection with receptor, superTOPFLASH and pIB/lacZ, 2×10^5 cells were placed into wells of a 96-well plate in 50 μl of medium. A measure of

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50 µl of culture medium containing purified Wg diluted at 1:125 (a final concentration of 120 ng/ml) or vehicle (PBS + 1% CHAPS + 250 mM NaCl). Each sample was done in duplicate. Cells were incubated for an additional 20 h and reporter assays were done as above.

arr and GFP RNAi

Double-stranded *arr* and *GFP* RNA was prepared from a PCR product using previously described primers (Schweizer and Varmus, 2003) that incorporate a binding site for the T7 polymerase (GGATTAATACGACTCACTATAGGGAGA). RNA was synthesized from PCR products using the MEGAScript RNAi kit (Ambion). S2 cells were transfected with 250 ng of *pTub*, *pTub-wg*::*fz*$^{\Delta CRD}$ or *pTub-wntD*::*fz*$^{\Delta CRD}$, 250 ng superTOPFLASH, 125 ng pIB/lacZ and 500 ng of double-stranded RNA of *arr* or *GFP*. Complexes were formed using 3 µl of Fugene6 and 100 µl of serum-free medium and were divided into three wells of a 24-well plate containing 1×10^6 cells/well. Reporter assays were performed as described above:

dsRNA *arr* (700 bp product),

5′- GGATTAATACGACTCACTATAGGGAGACAGATCGATGTGATCGTTAGG
3′- GGATTAATACGACTCACTATAGGGAGACCATGCTCTGACAGAGTTCG

dsRNA *GFP* (700 bp),

5′- GGATTAATACGACTCACTATAGGGAGAATGAGTAAAGGAGAAGAACTTTTC
3′- GGATTAATACGACTCACTATAGGGAGATTATTTGTATAGTTCATCCATGCC

Purification of Wg

Wg protein was purified according to the Wnt purification method previously described (Willert *et al*, 2003) from 6 l of conditioned medium taken from S2 cells stably expressing *wg*. The concentration of the purified protein is approximately 15 ng/µl.

Western blot and surface staining of fz variants

For Western blots, cells transfected with *fz* transgenes alone, identical to reporter assays with purified Wg and were lysed with TNT buffer (150 mM NaCl, 50 mM Tris (pH 7.5) and 1% Triton X-100). Protein was run on a 10% SDS–PAGE gel and probed with an N-terminal rabbit anti-Fz antibody (NFz) that recognizes the 'hinge' region of Fz, present on all constructs except *fz2*$^{\Delta CRD}$. Identical blots were probed with affinity-purified 16B12 mouse anti-HA and 3A5 mouse anti-Tubulin antibodies. For surface staining, cells from the same transfection were allowed to adhere to glass coverslips for 10 min. They were washed once with PBS and fixed for 10 min at room temperature in PBS + 4% formaldehyde. The cells were blocked in PBS + 10% normal donkey serum and incubated with NFz antibody in block buffer overnight at 4°C. Cells were visualized by incubation with Alexa488-labeled donkey anti-rabbit secondary antibody (Molecular Probes).

Rescue of Arm signaling in embryos

Germline clones of the genotype *fz*^{GL31}*th st fz2*^{e2}*FRT2A* were induced using the *FLP/ovoD/FRT* system (Perrimon *et al*, 1996). Adler *et al* (Jones *et al*, 1996) described the *fz*^{GL31} allele. The *fz2*^{e2} allele is a nonsense mutation (W285*) in the extracellular 'hinge' region. Clones were made by heat-shocking *yw hs-FLP/w; ovo*^{D}*w*$^{+}$*FRT2A/fz*^{GL31}*th st fz2*^{e2}*FRT2A* females every 12 h following a 24 h egg lay. Females with germline clones were crossed to males of the genotype *w; pTub-fz*^{rescue}*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*. The ability to fully rescue Arm signaling was measured by the determining the percentage of non-*TM6B* flies to eclose. The genotype of these flies was confirmed by scoring *th*, a recessive marker mapping between *fz* and *fz2*. There was also a residual PCP phenotype in a small compartment previously shown to be refractory to rescue using a different *fz* transgenes (Krasnow and Adler, 1994). We interpret this as incomplete rescue by *pTub-fz* of the PCP phenotype in the of *fz*^{GL31} background:

w; fz^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*
w; pTub-fz$^{WT.7}$*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*
w; pTub-fz$^{57.8}$*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*
w; pTub-fz$^{81.3}$*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*
w; pTub-fz$^{\Delta CRD}$*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*

Rescue of Arm signaling in the wing

Males from following stocks were crossed to *y;vg*^{Q1206}*-Gal4 UAS-flp; hsCD2,y*$^{+}$*ri FRT2A/TM2* and raised at 25°C to generate marked clones in adult wings as previously described (Strapps and Tomlinson, 2001). Mutants clones located at the margin are positively marked by the loss of y^{+}, which makes the bristles yellow. Heterozygous tissue produces dark bristles. For each of the transgenes, two separate insert lines were tested:

yw hsflp;	*nGFP FRT2A*
w;	*th st fz2*^{e3}*FRT2A/TM3*
w;	*fz*^{GL31}*FRT2A/TM6B*
w;	*fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM3*
w;	*pTub-fz*$^{WT.3}$*/CyOZ; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM3Z*
w;	*pTub-fz*$^{WT.7}$*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*
w;	*pTub-fz*$^{57.7}$*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM3Z*
w;	*pTub-fz*$^{57.8}$*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*
w;	*pTub-fz*$^{81.2}$*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM3Z*
w;	*pTub-fz*$^{81.3}$*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*
w;	*pTub-fz*$^{\Delta CRD}$*; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*
w;	*pTub-fz*$^{WIF.5}$*/CyO; fz*^{GL31}*th st fz2*^{e2}*FRT2A/TM6B*

Overexpression in the wing and in embryos

fz variants were cloned into a UAS expression vector and transformed into *Drosophila* by standard techniques. UAS lines were crossed to the *71B*-Gal4 and *h*-Gal4 (Flybase IDs: FBst0001747 and FBst0001734) drivers for expression in the wing and embryo, respectively. All crosses were raised at 25°C.

Acknowledgements

We would like to acknowledge Catriona Logan and Michael Gordon for insightful discussions and comments during the preparation of this manuscript and Matt Fish for help in making transgenic lines. The Wingless protein was kindly provided by Kim Harnish in our lab. This work was supported by the Howard Hughes Medical Institute and by a grant from the National Institutes of Health (R01 GM60388).

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