Genetic Evidence That *Drosophila frizzled* Controls Planar Cell Polarity and Armadillo Signaling by a Common Mechanism

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ABSTRACT

The *frizzled* (*fz*) gene in *Drosophila* controls two distinct signaling pathways: it directs the planar cell polarization (PCP) of epithelia and it regulates cell fate decisions through Armadillo (Arm) by acting as a receptor for the Wnt protein Wingless (Wg). With the exception of *dishevelled* (*dsh*), the genes functioning in these two pathways are distinct. We have taken a genetic approach, based on a series of new and existing *fz* alleles, for identifying individual amino acids required for PCP or Arm signaling. For each allele, we have attempted to quantify the strength of signaling by phenotypic measurements. For PCP signaling, the defect was measured by counting the number of cells secreting multiple hairs in the wing. We then examined each allele for its ability to participate in Arm signaling by the rescue of *fz* mutant embryos with maternally provided *fz* function. For both PCP and Arm signaling we observed a broad range of phenotypes, but for every allele there is a strong correlation between its phenotypic strength in each pathway. Therefore, even though the PCP and Arm signaling pathways are genetically distinct, the set of signaling-defective *fz* alleles affected both pathways to a similar extent. This suggests that *fz* controls these two different signaling activities by a common mechanism. In addition, this screen yielded a set of missense mutations that identify amino acids specifically required for *fz* signaling function.

ANIMAL development requires the interplay between cell fate decisions and morphogenetic events. It is not well understood how cell fate decisions, which are the outcome of changes in gene regulation, are coordinated with changes in morphology, such as cell movement, shape change, and polarization. The *frizzled* (*fz*) gene in *Drosophila* has the remarkable ability to control both cell fate decisions and the planar polarization of epithelia. What makes *fz* exceptional is that it interacts with two distinct signaling cascades to accomplish these functions. We are trying to understand the mechanism that regulates the switch between these two pathways. Uncovering how *fz* chooses between these two functions will provide insight into how morphogenesis and cell fate choices are orchestrated.

During *Drosophila* development, one of the first functions of *fz* is to pattern the embryo. Cells of the embryonic epidermis secrete a cuticle that displays a reiterative pattern of denticle belts separated by regions of smooth (naked) cuticle. Wg, the *Drosophila* Wnt-1 ortholog, is responsible for specifying the naked cuticle cell fate. During signaling, Wg acts through the related receptors Fz and Frizzled-2 (Fz2), which are genetically redundant (BHAT 1998; KENNERDELL and CARTHEW

1998; BHANOT *et al.* 1999; CHEN and STRUHL 1999; MULLER *et al.* 1999). Frizzled proteins consist of an amino-terminal domain, situated outside of the cell, called the cysteine-rich domain (CRD), followed by a seven-transmembrane signaling moiety. Several studies have shown that the CRD is necessary and sufficient for Wnt binding (BHANOT *et al.* 1996; HSIEH *et al.* 1999; DANN *et al.* 2001). In combination with Arrow (Arr) (WEHRLI *et al.* 2000), another transmembrane protein, the Fz proteins transduce Wg signaling to a cytoplasmic complex among the proteins Axin (Axn), Armadillo (Arm, β -catenin), Adenomatous polyposis coli (APC), and Zw3 (Zw3), which regulates the stability of the Arm protein, normally leading to its degradation. Upon Fz-mediated activation of Dishevelled (Dsh), the degradation of cytoplasmic Arm is blocked. As Arm accumulates in the cytoplasm and nucleus, it interacts with Pangolin (Pan), a transcription factor from the T-cell factor/lymphoid enhancer factor family to regulate target gene expression (reviewed in LOGAN and NUSSE 2004; TOLWINSKI and WIESCHAUS 2004; BEJSOVEC 2005). In *wg*, *dsh*, *arm*, and *pan* mutants or in *fz*, *fz2* double mutants, naked cells are no longer specified and only denticle-producing cells remain, resulting in the characteristic “lawn of denticles” phenotype (NUSSLEIN-VOLHARD and WIESCHAUS 1980; WIESCHAUS and RIGGLEMAN 1987; PERRIMON *et al.* 1989, 1996; BRUNNER *et al.* 1997; VAN DE WETERING *et al.* 1997; CHEN and STRUHL 1999). Because of the crucial

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role of Arm, we refer to this cell fate mode of *fz* signaling as Arm signaling, a process essential for many developmental events in *Drosophila*.

fz has another, genetically distinct, and nonredundant function in *Drosophila*. It is required to direct polarization of epithelia covering the surface of the fly (VINSON and ADLER 1987). The adult cuticle contains numerous elements that are organized in a plane orthogonal to the apical-basal axis and that are oriented relative to the body axes. For example, wing hairs point toward the distal tip of the wing and hairs and bristles on the dorsal thorax point posteriorly. This type of epithelial polarity is referred to as PCP and has been reviewed recently (ADLER 2002; TREE *et al.* 2002a; STRUTT 2003; FANTO and McNEILL 2004).

Genetic screens in *Drosophila* have uncovered a core set of genes acting with *fz* to polarize tissues. These are *dsh* (KLINGENSMITH *et al.* 1994; THEISEN *et al.* 1994), *prickle* (*pk*) (GUBB *et al.* 1999), *van gogh/strabismus* (*vang*) (TAYLOR *et al.* 1998; WOLFF and RUBIN 1998), and *starry night/flamingo* (*stan*) (CHAE *et al.* 1999; USUI *et al.* 1999). Together, these genes act through the small GTPase *rhoA* (*rhoI*) and *rho-kinase* (*rok*) to regulate the cytoskeleton, leading to the polarization of cells (STRUTT *et al.* 1997; WINTER *et al.* 2001). While *fz* and *dsh* participate in both Arm and PCP signaling, none of the other Arm signaling components, most notably *arr*, *zw3*, and *arm*, have PCP phenotypes (AXELROD *et al.* 1998; WEHRLI *et al.* 2000).

Little is known about the activation of the Fz protein in both PCP and Arm signaling. Furthermore, it is not understood what regulates the separation into two signaling pathways downstream of Fz, sometimes called canonical and noncanonical signaling. Are there separate pools of Fz committed to each function or does the switch between the two pathways occur downstream of a common interaction? Understanding how these two pathways are controlled will provide insight into how cell fate choices are coordinated with tissue morphogenesis. A related question is how the differences in the two *fz* genes in *Drosophila*, *fz* and *fz2*, are translated into different functions: the Fz2 protein participates only in Arm signaling while Fz can regulate both Arm and PCP signaling (CHEN and STRUHL 1999). Initial experiments using chimeric molecules have indicated that the unique role of the Fz2 protein in mediating signaling from Wg to Arm is due to the high affinity of the ligand-binding domain, the CRD, for Wg (RULIFSON *et al.* 2000). The transmembrane region of Fz allows it to couple to PCP signaling (BOUTROS *et al.* 2000). Recently, it has also been suggested that different subcellular distributions between Fz and Fz2 proteins play an important role in the choice between Arm and PCP signaling (WU *et al.* 2004).

In this article, we have taken a genetic approach to examine the two functions of Fz, asking whether we could isolate specific alleles that affect *fz* signaling in either the PCP or the Arm pathway. Several alleles of *fz* that affect

PCP signaling are known, identifying residues in cytoplasmic loops and other domains (ADLER *et al.* 1994; JONES *et al.* 1996). In addition, various site-directed mutations in *fz* transgenes have been made to examine consequences for Wnt signaling (BOUTROS *et al.* 2000; RULIFSON *et al.* 2000; UMBHAUER *et al.* 2000; STRAPPS and TOMLINSON 2001; CHEN *et al.* 2004; CONG *et al.* 2004). These mutagenesis experiments have pointed at the need for conserved amino acids in the cytoplasmic tail. However, no study has measured how these mutations control one pathway or the other. Receptors that control different pathways often do so by using separate domains to interact with different downstream effectors. Such domains can be mapped by mutations that affect one pathway *vs.* another pathway (TALLQUIST *et al.* 2003).

In this work, we analyze a set of molecularly characterized *fz* alleles for signaling activity. We have restricted our survey to endogenous *fz* alleles, rather than generating ectopically expressed transgenes. By taking advantage of the ability of maternal *fz* to function in the *Drosophila* embryo as a receptor for Wg, we have directly compared how *fz* alleles that have PCP phenotypes in adult flies behave in the embryo to control Arm signaling. Moreover, by measuring signaling functions in a semiquantitative way, we compare allelic strength in both pathways.

MATERIALS AND METHODS

Isolation of *fz* alleles: Isogenic *w; P{neoFRT}/80B* males were mutagenized with ENU using standard protocols (ASHBURNER 1989; GREENSPAN 1997). Groups of males were mated *en masse* to *w; fz^{K21}/TM6C Sb Tb e* virgin females. Approximately 30,000 nonbalanced F₁ progeny were screened for a *fz* phenotype. The mutagenized chromosome was tested for germline transmission and isolated by mating F₁ mutants to *w; fz^{K21}/TM6C Tb Sb e* and rescanning for the *fz* phenotype. Forty alleles showed a PCP phenotype. Of the 40 potential alleles, 12 were transmitted to the germline. The remainder either were sterile or did not transmit the mutation due to mosaicism inherent in F₁ screens (GREENSPAN 1997). The sex ratio of germline transmission was 10 males and 2 females, in approximately equal proportion to the number originally isolated, 29 males and 11 females. Stocks were established for the mutants with germline transmission. We obtained from Paul Adler the 8 previously described missense alleles *fz^{HE11}*, *fz^{K22}*, *fz^{F31}*, *fz^{R53}*, *fz^{GL31}*, *fz^{R54}*, *fz^{HD21}* and *fz^{R52}* and 3 amorphic alleles *fz^{D21}* (deletion), *fz^{K21}* (inversion), and *fz^{P21}* (frameshift causing a premature stop) (JONES *et al.* 1996).

Polarity signaling strength of *fz* alleles: Polarity signaling strength was determined in animals *trans*-heterozygous for the allele to be counted and for *fz^{K21}*, an amorphic allele. We counted the number of MHCs in a compartment on the ventral surface of the wing delimited in the anterior-posterior axis by the first and second wing veins and in the proximal-distal axis by the first 15 stout margin bristles on the dorsal surface of the wing margin, similar to WINTER *et al.* (2001). Ten wings of individual animals were counted for each wing. MHCs were also counted using *fz^{GL31}* as a reference allele with similar results.

Wingless transduction strength of *fz* alleles: Embryos were collected from a cross of *fz*^{*}/*fz*^{GL31} *fz*² females and *fz*^{GL31} *fz*²/*TM3* males. Twenty-five percent of the progeny are homozygous *fz*^{GL31} *fz*² and receive a maternal contribution of *fz*^{*}, the allele to be tested, and *fz*^{GL31} (JONES *et al.* 1996). For alleles displaying a strong or moderate phenotype, a representative cuticle was photographed. In these two phenotypic classes, ~25% of the cuticles displayed a phenotype. For alleles with a weak phenotype, the number of cuticles with an observable defect was <25%. For this class we photographed cuticles showing defects.

Western blot of *fz* alleles: Embryos were collected from a sibling cross of *fz*^{*}/*fz*^{P21} *Df(3L)fz2* flies. The only Fz in these animals will be translated from *fz*^{*} since *fz*^{P21} is a protein null allele. Embryos were lysed using a Dounce homogenizer with a tight-fitting pestle in TNT buffer (150 mM NaCl, 50 mM Tris pH 7.5, and 1% Triton-X100) supplemented with protease and phosphatase inhibitors. Fz was immunoprecipitated from 300 µg of extract using 2 µl of a polyclonal antibody, NFzD, directed against amino acids 158–231 and 5 µl of a 1:1 slurry of protein-A sepharose (Pharmacia) in TNT buffer. Immunoprecipitated material was Western blotted. Fz was detected using a monoclonal antibody, clone 1C11 obtained from the Developmental Studies Hybridoma Bank (<http://www.uiowa.edu/~dshbwww/>).

Molecular characterization *fz* alleles: The *fz* genomic locus is large with the coding region distributed over 90 kb in five exons (ADLER *et al.* 1990). We sequenced RT-PCR products produced from the 12 alleles that we isolated and *fz*^{GL31} (JONES *et al.* 1996). The sequenced allele was isolated either from homozygous flies or in *trans* with *fz*^{K21}, an amorphic allele that is a euchromatic inversion breaking within a 46-kb region critical for *fz* function (ADLER *et al.* 1990). Total RNA was isolated from three female flies using Trizol (GIBCO, Gaithersburg, MD). Approximately 2 µg of total RNA was primed with oligo(dT) and reversed transcribed with Thermoscript (Invitrogen, San Diego). Two microliters of the resulting cDNA was used in a 50-µl PCR reaction. Because the *fz*^{K21} allele does not contribute to the reaction, amplified products result exclusively from the mutant chromosome. The product was purified using a PCR purification column (QIAGEN) and ~100 ng per reaction was used as a template for fluorescent dye terminator sequencing.

The RT-PCR primers used were:

sense, GATCGAGAAAAAGCCCCAAAA
antisense, GGAGTAAGGGCGTGGACTTG.

The sequencing primers used were:

P1, GGAAGTAGTATGTGGCGTCAAATCCTG
P2, ATGGAGGCGAGGATTGTG
P3, CCTTCTTGATTGACTCTGCG
P4, TTATTCCACCTGGTTGCCTG
P5, TGGGCTGCTTGTCTACGAG
M1, ACAACTGGAGGTCATCACTGC
M2, TTTAGTTCGTAGCCATTCCC
M3, CAAAACAGGCGAATGC
M4, AAGCAGCCCAGTAATCCAC
M5, GCTCTAGACTAGACGTACGCCTGCGC.

Isolation of *fz2* alleles: Isogenic *fz*^{GL31} *th st*/*TM6b Tb* males were mutagenized with EMS using standard protocols (ROBERTS 1986; ASHBURNER 1989). Groups of males were mated *en masse* to virgin females *ftz e*/*TM6C Sb Tb*. In the F₁ generation, single males *fz*^{GL31} *th st*/*TM6C Sb Tb* (asterisk denotes mutation induced by EMS) were mated to *fz*^{R52} *Df(3L)fz2*/*TM6b Tb* females. The F₂ generation were screened for the absence of the *fz*^{GL31} *th st* */*fz*^{R52} *Df(3L)fz2* progeny and, if absent, the mutagenized chromosome was isolated from the *fz*^{GL31} *th st* */*TM6b Tb e* siblings. A total of 8500 chromosomes were

screened and 34 lethal mutations were isolated in three complementation groups. Group I had six members that stained negatively for Fz2 protein in embryos (MULLER *et al.* 1999) and displayed a *wg* phenotype when tested for the ability to pattern embryos. All but one had mutations in the *fz2* ORF. Group II contained the gene *reptin*, a negative regulator of Wg signaling (BAUER *et al.* 2000). Group III remains uncharacterized.

Molecular characterization of *fz2* alleles: To characterize the mutations in *fz2*, we amplified the coding exon of *fz2* from genomic DNA by PCR using primers 1 (sequence CCA AGG GGT TCC AAG TCA) and 2 (sequence ACC GTG CTG CTG CTC ATC). For each mutation the ORF was subcloned into pCR2.1 using the TopoTA system (Invitrogen). Each ORF was fully sequenced on both strands and a double peak indicated the presence of a mutation.

RESULTS

Screen and molecular characterization of *fz* alleles:

The analysis described here is based on comparing the strength of *fz* alleles in PCP and Arm signaling, initially by isolating and characterizing *fz* alleles affecting PCP. Mutations in *fz* are viable and result in the disruption of PCP of tissues, including the hairs on the wing and bristles on the dorsal thorax (GUBB and GARCIA-BELLIDO 1982; VINSON and ADLER 1987). By screening for a PCP phenotype on the thorax and wing due to lack of complementation of *fz*^{K21}, a null inversion allele (ADLER *et al.* 1990), we isolated 12 ENU-induced *fz* alleles (see MATERIALS AND METHODS). We combined these with a set of 9 previously isolated EMS-induced *fz* alleles (ADLER *et al.* 1987; JONES *et al.* 1996) for a total of 21. The new alleles were sequenced and the molecular lesion associated with each is presented in Table 1 and schematically in Figure 1. Of notable interest, there are 12 missense mutations, which are located throughout the transmembrane portion of the receptor (Figure 1). We found no missense mutations in the CRD, suggesting that this region of the Fz protein is dispensable for PCP signaling. Since the CRD is responsible for Wnt binding, this result is consistent with recent work suggesting that indeed Fz may not utilize a Wnt ligand in PCP signaling (ADLER 2002; LAWRENCE *et al.* 2002; AMONLIRDVIMAN *et al.* 2005). In parallel to our screen for *fz* alleles, we also conducted a screen for novel alleles of *fz2*, the other Wg receptor in *Drosophila*. Fz2 is primarily involved in Arm signaling (CHEN and STRUHL 1999). Thus, we reasoned that screening for alleles affecting its function might generate mutations in the CRD. We recovered 5 new alleles of *fz2* (Table 2); however, none have mutations within the CRD and these alleles are not considered further.

PCP signaling function of *fz* alleles: Having collected a significant set of *fz* alleles, we first assessed their relative signaling strength in PCP. Each cell of the wing epithelium secretes a single hair (WONG and ADLER 1993). In addition to having abnormal hair polarity, *fz* mutations cause cells to secrete multiple hairs (Figure 2) (ADLER *et al.* 1987). Quantifying the occurrences of

TABLE 1
Summary of *fz* alleles

Allele	Mutation	Location of mutation	Abundance of Fz protein	Apparent mass of Fz protein	PCP signaling phenotype	Arm signaling phenotype
<i>fz</i> ^{P21} (JONES <i>et al.</i> 1996)	Insertion, X	CRD	None	—	ND	ND
<i>fz</i> ^{SN}	C134X	CRD	ND	ND	Strong	Strong
<i>fz</i> ^{CL}	K184X	Extracellular “hinge”	ND	ND	Strong	ND
<i>fz</i> ^{MG}	Novel splice acceptor, X	Extracellular hinge	None	—	Strong	Strong
<i>fz</i> ^{HE11} (JONES <i>et al.</i> 1996)	C229Y	Extracellular hinge	Normal	Larger	Weak	Weak
<i>fz</i> ^{I22} (JONES <i>et al.</i> 1996)	P278L	First intracellular loop	Normal	Normal	Moderate	Moderate
<i>fz</i> ^{I31} (JONES <i>et al.</i> 1996)	P278S	First intracellular loop	Normal	Normal	Weak	Weak
<i>fz</i> ^W	Q322X	First extracellular loop	Lower	Smaller	Strong	Strong
<i>fz</i> ^{RN}	W354X	Third transmembrane domain	Lower	Smaller	Weak	Strong
<i>fz</i> ^{JB}	W355X	Third transmembrane domain	None	—	Strong	Strong
<i>fz</i> ^{CK}	W362X	Third transmembrane domain	Lower	Smaller	Weak	Strong
<i>fz</i> ^{R53} (JONES <i>et al.</i> 1996)	A374G	Second intracellular loop	Normal	Normal	Weak	Weak
<i>fz</i> ^{TT}	H383Y	Fourth transmembrane domain/second intracellular loop	Normal	Normal	Weak	Weak
<i>fz</i> ^{SY}	G405D	Second extracellular loop	ND	ND	Weak	Weak
<i>fz</i> ^{ED}	C412R	Second extracellular loop	Normal	Normal	Weak	Weak
<i>fz</i> ^{GL31} (JONES <i>et al.</i> 1996)	P429L, V454F	Fifth transmembrane domain, third intracellular loop	Lower	Larger	Moderate	Strong
<i>fz</i> ^{R54} (JONES <i>et al.</i> 1996)	M469R, W500R	Third intracellular loop, third extracellular loop	Lower	Larger	Moderate	Moderate
<i>fz</i> ^{KW}	I472K	Sixth transmembrane domain/third intracellular loop	Lower	Normal	Moderate	Moderate
<i>fz</i> ^{HD21} (ADLER <i>et al.</i> 1994)	G485R	Sixth transmembrane domain	Lower (18°), very low (25°)	Larger	Moderate (18°), weak (25°)	Moderate (18°), moderate (25°)
<i>fz</i> ^{R52} (JONES <i>et al.</i> 1996)	W500X	Third extracellular loop	Very low	Smaller	Moderate	Moderate
<i>fz</i> ^{MP}	G545E	Seventh transmembrane domain	Lower	Normal	Moderate	Moderate

multiple hair cells (MHCs) provides an informative measure of *fz* signaling strength (ADLER *et al.* 1987; KRASNOW and ADLER 1994). By this analysis, strong hypomorphic alleles can be distinguished from null alleles in cases in which studying hair polarity alone cannot (ADLER *et al.* 1987).

We assayed the number of MHCs for all of the *fz* alleles in *trans* over a null allele, *fz*^{K21} (Figure 3A). The alleles fall into a broad range. Significantly, there is a direct correlation between the number of MHCs and the extent of the hair polarity disruption that is observed in wing hairs (data not shown) or bristles on the dorsal thorax (Figure 3, B–E). Mutations that lead to only a few MHCs have a weak polarity phenotype (Figure 3C).

Mutations that produce more MHCs lead to more pronounced disruptions of the thorax bristles (Figure 3, D and E). Similar results were obtained when we counted the number of MHCs in *trans* over a hypomorphic allele, *fz*^{GL31}. Despite small changes in the order, the alleles fell into approximately the same range that *fz*^{K21} did and showed a good correlation between the number of MHCs and the PCP phenotype on the dorsal thorax (data not shown). Thus, the MHC phenotype is useful as a semiquantifiable measure of PCP signaling in *fz* mutants and allowed us to rank the *fz* alleles on the basis of their PCP signaling strength.

Arm signaling function of *fz* alleles: Next we asked whether the new and previously isolated *fz* alleles also

FIGURE 1.—Schematic of *fz* alleles. Mutations found in *fz* alleles isolated in PCP screens are located throughout the transmembrane portion of the receptor. There are no missense mutations located in the CRD, shaded in gray. The cold-sensitive mutation encoded by the *fz^{HD21}* allele is green. With the exception of *fz^{HD21}*, all other missense mutations (yellow) affect amino acids that are conserved in the frizzled family. Nonsense mutations are red. Mutations that insert amino acids are indicated with open triangles. All insertion mutations encounter a stop codon that results in the synthesis of a truncated protein.

exhibit a range of phenotypes when assayed for their Arm signaling function. In embryos, both *fz* and *fz2* can function as a Wg receptor (BHAT 1998; KENNERDELL and CARTHEW 1998; BHANOT *et al.* 1999; CHEN and

STRUHL 1999; MULLER *et al.* 1999). Because of its redundancy with *fz2*, the Arm signaling function of *fz* missense mutations has not been analyzed. We devised an assay to measure the Arm signaling function of the *fz* alleles by taking advantage of the fact that the maternal contribution of one copy of *fz*, but not of *fz2*, is sufficient to specify the normal pattern of naked cuticle in zygotic *fz fz2* mutants (BHANOT *et al.* 1999; CHEN and STRUHL 1999) (Figure 4A). This assay made use of a chromosome carrying both the strong, but not null, *fz^{GL31}* allele (Figures 3 and 4B) (JONES *et al.* 1996) and the *fz2²* allele (Table 2), which encodes an extracellular stop codon before the first transmembrane domain. A relatively simple crossing scheme yielded informative mutant embryos that displayed a range of cuticle phenotypes (Figure 4B). The alleles broadly fall into three phenotypic

TABLE 2
Summary of *fz2* alleles

Allele	Mutation	Location of mutation
<i>fz2¹</i>	Q268X	Extracellular hinge
<i>fz2²</i>	W285X	Extracellular hinge
<i>fz2³</i>	P487L	Fifth transmembrane domain
<i>fz2⁴</i>	W603X	Seventh TM/cytoplasmic
<i>fz2⁵</i>	W603X	Seventh TM/cytoplasmic
<i>fz2⁶</i>	None detected	NA

FIGURE 2.—The MHC phenotype of *fz* mutants. This portion of the wing contains the region of the anterior ventral surface used to quantify the MHCs of the *fz* alleles (see MATERIALS AND METHODS). (A) Cells in a wild-type wing each secrete a single hair. (B) In a weak *fz* mutant, *fz*^{SY}, some cells may secrete more than one hair (red arrows). The number of MHCs increases in moderate (*fz*^{MP}) (C) and strong (*fz*^{JB}) (D) *fz* mutants. The number of MHCs observed in this region of the wing is a strong predictor of the severity of the phenotype observed in other tissues, such as the bristles on the dorsal thorax.

classes: (1) strong alleles that cause dramatically shortened embryos and the absence of naked cuticle; (2) moderate alleles that result in modestly shortened embryos and display mild patterning defects such as denticle belt fusions or numerous denticles in regions that should be entirely composed of naked cuticle; and (3) weak alleles that do not affect the length of the embryos and have minor, if any, patterning defects. Thus, as with PCP signaling, the *fz* alleles also display a range of phenotypes in Arm signaling.

We then compared the phenotypic strength of each *fz* allele in both pathways. Interestingly, there is an overall correlation between the strength of the two phenotypes (Figures 3A and 4B). Alleles with the greatest disruption of PCP signaling clustered together and showed the strongest defect in Arm signaling. The alleles with moderate and weak PCP phenotypes likewise had moderate and weak Arm signaling phenotypes, respectively.

Two alleles seem to break the correlation between the PCP and Arm signaling phenotypes: *fz*^{RN} and *fz*^{CK}. Both alleles encode nonsense mutations that would truncate the Fz protein within the third transmembrane domain, likely generating a strong loss-of-function allele. These alleles indeed have a moderate-to-strong cuticle phenotype (Figure 4B); however, their PCP defect is unexpectedly weak (Figure 3A). We attribute the relatively weak PCP phenotype to translational readthrough of the stop codon, resulting in the synthesis of full-length Fz protein. Indeed, as we show below, both *fz*^{RN} and *fz*^{CK} produce some full-length Fz protein.

We conclude that the strength of *fz* signaling in PCP is related to its strength in the Arm pathway, suggesting that these two readouts of receptor activity are mechanistically similar.

Western blot analysis of Fz mutant proteins: The loss of function of mutant alleles can be due to a variety of defects in the protein, ranging from misfolding to quicker turnover and lack of proper transport. We tested the properties of the various mutant proteins by examining the abundance and mobility by gel electrophoresis. The endogenous Fz protein is present at a low level (KRASNOW and ADLER 1994). Therefore, to determine the abundance and mobility of the mutant Fz proteins, we concentrated them from embryos by immunoprecipitation and then performed Western blot analysis.

Fz protein produced from wild-type embryos migrates with a similar mobility as Fz produced in cultured *Drosophila* S2 cells stably expressing a *fz* transgene (Figure 5). The Fz^{TT} and Fz^{ED} mutant proteins are indistinguishable from wild-type Fz, as are the previously described Fz^{J22}, Fz^{F31}, and Fz^{R53} mutants (Figure 5) (JONES *et al.* 1996). We believe that another allele, *fz*^{SY}, which was not assayed by Western blot, also belongs in this class, given that it is one of the weakest alleles in the collection. Thus, six alleles encode amino acid changes that do not appreciably disrupt the synthesis or processing of the Fz protein. In contrast, the Fz^{GL31}, Fz^{R54}, Fz^{HD21}, Fz^{KW}, and Fz^{MP} mutants have a markedly lower abundance than wild-type Fz (Figure 5). Each immunoprecipitation started with an equal amount of embryo extract. Therefore, the observed decrease in Fz abundance reveals that these mutations cause increased protein turnover. In addition to changes in abundance, the mobility of the Fz^{R54}, Fz^{HD21}, and Fz^{MP} mutants is reduced compared to that of wild-type Fz (Figure 5) (ADLER *et al.* 1994; JONES *et al.* 1996). The truncation mutants Fz^{MG} and Fz^{JB} are not detectable (Figure 5). Truncated proteins that migrate at the predicted size are present for the nonsense mutants Fz^{JW}, Fz^{RN}, and Fz^{CK} (Figure 5). A slower mobility protein that migrates in a similar position as wild-type Fz is also present for these three alleles. We interpret the slower mobility form to be translational readthrough of the introduced stop codon and, as previously mentioned, the production of some full-length Fz protein as the underlying reason that *fz*^{CK} and *fz*^{RN} have a weak PCP

FIGURE 3.—PCP signaling strength of *fz* alleles. (A) The number of MHCs was counted in a section of the ventral wing between the first and second wing veins in a region defined by the first 15 stout margin bristles (inset). All alleles (*fz**) were assayed in *trans* with *fz^{K21}*, a null allele. The number of MHCs was plotted for each of 10 wings (solid circle) along with their average (plus symbol). Some alleles appear to have <10 data points because the dots overlap due to identical values. The number of MHCs correlates with the severity of the polarity disruptions in other tissues, such as the dorsal thorax. Alleles that display the greatest numbers of MHCs have the strongest polarity phenotypes in other tissues. *fz^{D21}* is an amorphic deletion allele (JONES *et al.* 1996). (B) Photomicrograph of the dorsal thorax of a wild-type fly. Note the parallel arrangement and uniform posterior polarity of the bristles. (C) Alleles with the fewest number of MHCs have near wild-type bristle polarity. Only slight deviations from the normal posterior alignment are observed in *fz^{SY}/fz^{K21}*. (D) Alleles with an intermediate number of MHCs also exhibit a mild bristle phenotype on the thorax. The majority of the lateral bristles in *fz^{MP}/fz^{K21}* deviate from wild type by 45° or less. (E) Lateral bristles in *fz^{JB}/fz^{K21}* that normally point posteriorly frequently deviate from their normal orientation up to 90°, pointing straight toward the middle of the thorax. When all the alleles were assayed in *trans* with the hypomorphic allele *fz^{GL31}*, the ranking was similar with only minor differences (data not shown). In addition, when *fz^{GL31}* was used as the reference allele, the correlation remained between the number of MHCs and the polarity phenotypes observed in other tissues, such as the thorax (data not shown).

phenotype. We analyzed Fz protein produced in embryos, which raises the question, Why does translational readthrough suppress only the PCP phenotype? A likely explanation is that there is a differential threshold for *fz* in PCP and Arm signaling, where PCP signaling requires less *fz* to function. This hypothesis is supported by previous work showing that *fz* can function in PCP signaling even when present at extremely low levels (KRASNOW and ADLER 1994). Not all of the alleles that are subject to stop suppression result in functional proteins. The full-length protein produced by readthrough of the *fz^{JW}* stop codon is not functional in PCP or Arm signaling since this allele has strong phenotypes in both pathways. It is possible that the amino acid replacing the stop itself

affects signaling or folding of the full-length Fz^{JW} protein. Finally, consistent with previous observations that stop suppression is context dependent, not all of the nonsense alleles produce full-length Fz protein (CHAO *et al.* 2003). These data are summarized in Table 1.

DISCUSSION

In this article, we have surveyed a representative set of new and existing *fz* mutant alleles and asked whether the two pathways to which *fz* can contribute, PCP and Arm signaling, can be separated genetically. Such an approach has been taken for other receptors, including

FIGURE 4.—Arm signaling strength of *fz* alleles. (A) Cross to test the Arm signaling strength of *fz* alleles where *fz*^{*} denotes the allele tested. (B) The *fz* alleles exhibit a spectrum of Arm signaling phenotypes in embryos. Representative mutant cuticles from a cross of *fz*^{*}/*fz*^{GL31} *fz*²^{e2} mothers and *fz*^{GL31} *fz*²^{e2}/*TM3* fathers are shown. Twenty-five percent of the progeny are homozygous for the *fz*^{GL31} *fz*²^{e2} chromosome and receive maternal *fz*^{*} and *fz*^{GL31} transcripts. The order from upper left to lower right was determined by PCP signaling strength (Figure 3). Approximately 25% of the progeny from strong and moderate alleles display a cuticle similar to that shown. For the weak class of mutants, <25% of the progeny displayed a phenotype.

the platelet-derived growth factor receptor, where a set of site-directed mutants discriminates between the various downstream effectors (TALLQUIST *et al.* 2003). Here, we took advantage of the relative ease by which new endogenous alleles of genes can be isolated in *Drosophila*. This allowed us to examine the defects caused by mutant alleles without relying on gene transfer methods. Moreover, we tested for *in vivo* gene function rather than for cell culture, the only approach by which *Fz* mutations have previously been examined for Wnt signaling function.

Our main finding is that we cannot separate PCP and Arm signaling activity. While we cannot exclude that

pathway-specific alleles would be uncovered in a larger series, our data support the view that the mechanism by which *Fz* operates is similar in both pathways. This conclusion is based on the correlation in signaling activity, in particular the finding that several alleles behave as intermediate or weak in both assays. In general, such a correlation suggests that one activity of a gene is related to another activity in a mechanistic and functional way. We restricted our analysis to measurements *in vivo*. Therefore, we cannot quantify the data in such a way that would allow us to state that the two functions are linearly (or otherwise) related. However, the data suggest that the phenotypes display, at least by approximation, a

FIGURE 5.—Western blot of *fz* alleles. Characterization of protein abundance and mobility of Fz isolated from embryos produced by a sibling cross of *fz*^{*}/*fz*^{P21} *Df*(3L)*fz*2, where *fz*^{*} denotes the allele tested. The only *fz* contribution in the resulting embryos is *fz*^{*} or *fz*^{P21}. Since *fz*^{P21} encodes a protein null allele (JONES *et al.* 1996), the only detectable Fz protein is translated from *fz*^{*} transcripts. Fz protein cannot be observed in whole-embryo extracts (data not shown). Therefore, Fz protein was concentrated from total embryo extract by immunoprecipitation using an antibody that recognizes the hinge portion of the receptor, which is located between the end of the CRD and the first transmembrane domain. An equal amount of protein (300 μ g) was used for each immunoprecipitation. Fz was detected in the immunoprecipitated material by probing Western blots with a monoclonal antibody that recognizes an epitope in the CRD (PARK *et al.* 1994). Drosophila S2 cells do not express *fz* and were used as a negative control. Probing an extract prepared from S2 cells stably transformed with a *fz* transgene (*pMKfz*) marks the position of Fz with a relative molecular mass slightly >50 kDa. Fz protein isolated from embryos having a single copy of wild-type *fz* comigrates with Fz produced in S2/*pMKfz* cells. The *fz*^{MG} and *fz*^{JB} truncation alleles do not express detectable levels of protein. The nonsense mutations *fz*^{RN} and *fz*^{CK} produce both the predicted size truncated protein and an additional product that migrates near the position of wild-type Fz. The *fz*^{JW} also has a larger protein product. However, it migrates more slowly than wild-type Fz and the comparable bands in *fz*^{RN} and *fz*^{CK}. Slower-mobility products result from improper processing in the endoplasmic reticulum, which is likely the result of misfolding. The missense alleles *fz*^{J22}, *fz*^{F31}, *fz*^{R53}, *fz*^{TT}, and *fz*^{ED} all have an abundance and mobility similar to that of wild-type Fz. The remaining alleles all display a marked decrease in protein level with *fz*^{R54}, *fz*^{HD21}, and *fz*^{MP} also having a reduced mobility.

semilinear relationship to each other (Figures 3A and 4B). Therefore, we propose that Fz engages a component shared by both PCP and Arm signaling.

Elucidating the identity of this shared component is crucial to understanding how this receptor activates signaling. Defining amino acids in Fz specifically required for signaling could potentially provide a valuable means to probe Fz interactions. Previous attempts to generate signaling-compromised mutations in Fz have been restricted to examining only the intracellular amino acids (UMBHAUER *et al.* 2000; CONG *et al.* 2004). Because we made alleles of the endogenous gene, we were able to sample the entire Fz protein. Importantly, we found signaling-specific mutations in the extracellular loops and transmembrane helices in addition to in the in-

FIGURE 6.—Model of Fz activation in PCP and Arm signaling. In the absence of a Wnt ligand, Fz has an intrinsic ability to signal to the PCP pathway. This may occur via a direct interaction with Dsh. When Wg is present, the coreceptor Arr is brought into proximity to Fz, which results in the close apposition of Dsh and Axn. This combination of intracellular components switches the output of Fz from PCP to Arm signaling.

tracellular loops. Because these mutations are located throughout the entire protein, we argue that Fz is a dynamic molecule that changes conformation to relay its activation to a shared intracellular signal component. There are two major candidates for this shared component: Dsh and $G\alpha_o$ (KATANAIEV *et al.* 2005). Both proteins have been implicated in PCP and in Arm signaling and the Dsh protein has been shown to bind Fz (CHEN *et al.* 2003; WONG *et al.* 2003; CONG *et al.* 2004). How Dsh and $G\alpha_o$ are coupled to Fz is not yet clear.

We propose the following model as the way in which the Fz protein controls two different signaling pathways (Figure 6). Fz signals in a ground state to the PCP pathway in the absence of a Wnt. Consistent with this model, we found that none of the *fz* missense mutations, which were all isolated on the basis of their PCP phenotype, are located in the CRD. This model is also supported by recent work suggesting that Fz does not require a Wnt ligand in PCP signaling (LAWRENCE *et al.* 2002; AMONLIRDVIMAN *et al.* 2005) but that its activity is regulated by interactions between neighboring cells and differential levels of the cytoplasmic mediators Pk and Dsh (TREE *et al.* 2002b; AMONLIRDVIMAN *et al.* 2005). Furthermore, experiments with chimeric *fz* transgenes have shown that the transmembrane portion of Fz, not the CRD, is responsible for coupling it to PCP signaling (RULIFSON *et al.* 2000; WU *et al.* 2004). In this context, it should also be mentioned that global signaling during PCP may be under the control of differential expression of molecules involved in cell adhesion (YANG *et al.* 2002; MA *et al.* 2003; SIMON 2004).

In our model, the presence of a Wnt ligand switches Fz to the Arm signaling pathway by recruiting the membrane protein Arr to it. It has been previously shown that *arr* mutants disrupt Arm signaling but do not have a PCP phenotype (WEHRLI *et al.* 2000). While the exact

mechanism of Arr-Fz-Wg interactions is not clear, a currently favored hypothesis is that Arr acts together with Fz as a coreceptor for Wg, generating a ternary complex (TAMAI *et al.* 2000; LIU *et al.* 2005). On the cytoplasmic side, Arr recruits Axn to the membrane (MAO *et al.* 2001; TOLWINSKI *et al.* 2003; TAMAI *et al.* 2004). We speculate that the membrane localization of Axn via Arr brings it in close proximity with Dsh, resulting in the inactivation of Axn and the stabilization of Arm. However, it is also possible that recruiting Axn to the membrane via Arr is sufficient to drive signaling (CLIFFE *et al.* 2003; TOLWINSKI *et al.* 2003). It has also been proposed that Wg initiates signaling by recruiting Axn to the membrane via Dsh and that once at the membrane Axn is inactivated, allowing Arm to accumulate (CLIFFE *et al.* 2003). Axn is a negative regulator of Arm signaling and, like *arr*, *axn* mutants have no polarity phenotype (HAMADA *et al.* 1999). During PCP signaling, in the absence of Wg, a trimeric complex between Wg, Fz, and Arr would not be assembled and Fz functions independently of Arr but also of Axn.

We postulate that the presence of ligand-unbound Fz facilitates Dsh activation in PCP signaling. In contrast to *arr* and *axn*, *dsh* is required for both the Wg and the PCP pathway, making *fz* and *dsh* the only two genes shared between Wg and PCP signaling (Figure 6). The pathways are genetically distinct, however, in that there are three specific alleles of *dsh* that prevent it from participating in the PCP pathway but allow it to function normally in Arm signaling (AXELROD *et al.* 1998; PENTON *et al.* 2002). These *dsh* alleles contain missense mutations that map to the same domain of the protein (AXELROD *et al.* 1998; PENTON *et al.* 2002). Here we have shown that, unlike *dsh*, the two functions of *fz* are not separable by specific mutations. This suggests that Fz activates a common component and that the switch between the two pathways occurs downstream of *fz*.

There is one other series of endogenous *fz* mutant alleles: mutations in the human Frizzled 4 (FZD4) gene lead to familial exudative vitreoretinopathy (FEVR) (ROBITAILLE *et al.* 2002; KONDO *et al.* 2003; OMOTO *et al.* 2004; TOOMES *et al.* 2004b; XU *et al.* 2004). The signaling pathway used by FZD4 is not entirely clear. Some studies suggest that FZD4 signals through an alternative (Ca²⁺) Wnt pathway (ROBITAILLE *et al.* 2002). However, the finding that the mutations in the Wnt coreceptor LRP (JIAO *et al.* 2004; TOOMES *et al.* 2004a), which is implicated in Arm/ β -catenin signaling and not in the Ca²⁺ pathway, also cause FEVR does not support this model. Moreover, FZD4 in collaboration with LRP5 can be stimulated to activate Arm/ β -catenin signaling by Norrin, a non-Wnt ligand that binds to the CRD (XU *et al.* 2004). Of the 12 FEVR alleles, 4 result in mutations in the CRD (ROBITAILLE *et al.* 2002; KONDO *et al.* 2003; OMOTO *et al.* 2004; TOOMES *et al.* 2004b; XU *et al.* 2004). Of the remaining alleles, one is located N terminal to the CRD (G36D), thus potentially interfering with the

processing of the hydrophobic signal peptide (TOOMES *et al.* 2004b). Another mutation (C181R) affects a conserved cysteine residue in the "hinge" region (OMOTO *et al.* 2004). There is a mutation (R417Q) in a conserved arginine in the third intracellular loop and a stop codon (W319X) identical to the truncation allele *fz^{RN}* described in this study (KONDO *et al.* 2003). Interestingly, there is a FEVR allele (G488D) that affects a conserved glycine residue in the seventh transmembrane domain that is also altered in the *fz* alleles *fz^{MP}* (G545E, this article) and *fz^{HC52}* (G545R) (JONES *et al.* 1996). Three FEVR alleles, S495F and Q505X (TOOMES *et al.* 2004b) and the in-frame deletion of MW493-4 (ROBITAILLE *et al.* 2002), are located at the junction of the seventh transmembrane domain and the cytoplasmic tail near a conserved motif that has been shown to be required for Arm/ β -catenin signaling function (UMBHAUER *et al.* 2000). The existence of CRD mutations in FEVR alleles of FZD4 demonstrates that mutations in this domain can indeed have a functional consequence and highlights the role of the CRD in FZD4 function in the retina. It also supports the hypothesis that CRD mutations have not been recovered in any of the PCP screens for *fz* alleles because this domain is dispensable in that process.

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