A Mutational Analysis of *dishevelled* in Drosophila Defines Novel Domains in the Dishevelled Protein as Well as Novel Suppressing Alleles of *axin*

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ABSTRACT

Drosophila *dishevelled* (*dsh*) functions in two pathways: it is necessary to transduce Wingless (Wg) signaling and it is required in planar cell polarity. To learn more about how Dsh can discriminate between these functions, we performed genetic screens to isolate additional *dsh* alleles and we examined the potential role of protein phosphorylation by site-directed mutagenesis. We identified two alleles with point mutations in the Dsh DEP domain that specifically disrupt planar polarity signaling. When positioned in the structure of the DEP domain, these mutations are located close to each other and to a previously identified planar polarity mutation. In addition to the requirement for the DEP domain, we found that a cluster of potential phosphorylation sites in a binding domain for the protein kinase PAR-1 is also essential for planar polarity signaling. To identify regions of *dsh* that are necessary for Wg signaling, we screened for mutations that modified a *GMR-GAL4;UAS-dsh* overexpression phenotype in the eye. We recovered many alleles of the transgene containing missense mutations, including mutations in the DIX domain and in the DEP domain, the latter group mapping separately from the planar polarity mutations. In addition, several transgenes had mutations within a domain containing a consensus sequence for an SH3-binding protein. We also recovered second-site-suppressing mutations in *axin*, mapping at a region that may specifically interact with overexpressed Dsh.

WNT signaling molecules are crucial for cell-cell communication, cell fate specification, embryonic axis formation, and growth control during development of vertebrates and invertebrates (Wodarz and Nusse 1998; Yamaguchi 2001). Genes that transduce signals from Wnt proteins are conserved across species (Cadigan and Nusse 1997; Peifer and Polakis 2000). In Drosophila, *wg* is required during embryogenesis for segmental patterning, muscle development, and midgut formation and to specify appendage primordia. Frizzled (Fz) and Frizzled-2 (DFz2), two seven-pass transmembrane proteins with cysteine-rich extracellular domains, act as Wg receptors (Bhanot *et al.* 1996). Wg binds to these receptors and to the Arrow LDL-related coreceptor (Bhanot *et al.* 1996; Tamai *et al.* 2000; Wehrli *et al.* 2000), resulting in the activation of Dishevelled (Dsh). *dsh* encodes a cytoplasmic phosphoprotein (Klingensmith *et al.* 1994; Theisen *et al.* 1994; Yanagawa *et al.* 1995) and its activity is required for the transduction of the Wg signal (Noordermeer *et al.* 1994; Siegfried *et al.* 1994). It is thought that Dsh acts by binding to and inhibiting the Axin protein that negatively regulates Wg signaling (Kishida *et al.* 1999; Li *et al.* 1999a; Smalley *et al.* 1999; Salic *et al.* 2000). Axin is a scaffolding protein that binds to APC, another negative regulator of Wg signaling, and to Armadillo (Arm), a homolog of β-catenin (Behrens *et al.* 1998; Hart *et al.* 1998; Ikeda *et al.* 1998; Sakanaka *et al.* 1998). Axin negatively regulates Arm by facilitating the action of Zeste white-3 (Zw-3; Siegfried *et al.* 1992), a homolog of the vertebrate serine/threonine kinase GSK3-β (Hart *et al.* 1998; Ikeda *et al.* 1998). Zw-3 phosphorylates Arm, leading to its destruction by the ubiquitin pathway (Peifer and Polakis 2000). Inhibition of Zw-3 results in the stabilization of Arm. Stabilized Arm forms a complex with the HMG-box DNA-binding protein dTCF/Pangolin (Brunner *et al.* 1997; van de Wetering *et al.* 1997) and activates transcription of Wg target genes.

In addition to mediating Wg signaling events, *dsh* is required to generate planar polarity, also known as tissue polarity, by regulating both the correct orientation of ommatidia within the Drosophila eye and the alignment of bristles on the adult epidermis (Theisen *et al.* 1994; Krasnow *et al.* 1995; Strutt *et al.* 1997). This second function of *dsh* appears to be distinct from its role in Wg signaling and may result in the activation of a Jun-N-terminal kinase pathway (Boutros *et al.* 1998; Li *et al.* 1999b). *dsh* acts downstream of *frizzled* (*fz*) in planar polarity signaling (Krasnow *et al.* 1995; Strutt *et al.* 1997). Although *fz* acts redundantly with *Dfz2* during Wg signaling, it is required for planar polarity (Adler *et al.* 1990; Bhanot *et al.* 1999; Chen and Struhl 1999). In contrast, there is no evidence for an involvement of

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Dfz2 in planar polarity signaling. Recent studies suggest that Fz and Dfz2 have different affinities for Wg and also differ in signaling specificity in regions other than the ligand-binding domain (Boutros *et al.* 2000; Rulifson *et al.* 2000; Strapps and Tomlinson 2001). Thus, Dsh may interact with Dfz2 to regulate events that are associated with Wg signaling and with Fz to regulate planar polarity.

Three conserved domains have been identified in the Dsh protein, including an amino-terminal DIX (*Dis*hevelled, Axin) domain, a central PDZ (*P*ostsynaptic density 95, *D*iscs Large, *Z*onula occludens-1) domain, and a C-terminal DEP (*D*ishevelled, *E*gl-10, *P*leckstrin) domain (Boutros and Mlodzik 1999; Figure 4A). The DEP domain is required for planar polarity signaling. A viable *dsh* allele, dsh^1, possesses a lesion within this domain (Axelrod *et al.* 1998; Boutros *et al.* 1998) and disrupts planar polarity but not Wg signaling (Axelrod *et al.* 1998; Boutros *et al.* 1998; Figure 3, C and D). While the structure of the DEP domain has been elucidated (Wong *et al.* 2000), it is not known how this module works. Interestingly, the DEP domain is necessary and sufficient to translocate the Dsh protein to the membrane when Frizzled proteins are expressed (Axelrod *et al.* 1998).

Although the functions of these domains have been examined by deletion analysis, rigorous mutagenesis studies have not been undertaken *in vivo* (Boutros and Mlodzik 1999). We designed genetic screens to identify additional *dsh* alleles that are deficient for planar polarity signaling and to find *dsh* alleles that are deficient in Wg signaling. These studies led to the identification of a putative src homology 3 (SH3)-binding motif in Dsh that is essential for Wg signaling, suggesting that SH3 proteins may be important regulators of Wg signaling. We also investigated the role of potential phosphorylation sites for the function of Dsh in Wg and planar polarity signaling by site-directed mutagenesis. Besides mutations in *dsh* itself, we isolated mutations that act as second-site modifiers of the phenotype caused by overexpression of Dsh in the eye. Some of these modifiers encode novel mutations in *axin*.

MATERIALS AND METHODS

Planar polarity screens: A total of 23 rounds of mutagenesis were performed for both the planar polarity screens and the *dsh* misexpression screen. w^{1118} males were isogenized on the X chromosome. In rounds 1–9 of the mutagenesis these males were mutagenized overnight with a 15–30 mm solution of EMS (Sigma, St. Louis) in 1% sucrose using established procedures (Ashburner 1989). Mutagenized males were crossed to *y w* dsh^{v26}*/FM7, TW9* virgin females (Wieschaus *et al.* 1984; Perrimon and Mahowald 1987) and 20,000 F_1 *B*+ females were screened for planar polarity defects (Figure 1A). During subsequent rounds of the mutagenesis 0.5–3.3 mm of the mutagen 1-ethyl-1-nitrosourea (ENU) in 1% sucrose was used instead of EMS, because ENU rarely induces chromosomal rearrangements and produces a wider spectrum of mutations than does EMS (Ashburner 1989; Pastink *et al.* 1990). A total of 37,840 additional females were screened using this protocol. *v* males, isogenized on the X chromosome, were mutagenized as above with ENU and crossed to dsh^{A3} virgin females (Figure 1B). A total of 21,000 flies were screened and an additional *dsh* allele, dsh^{A21}, was identified. The mutation frequency of EMS was estimated to be between 23 and 30% and between 16 and 34% for ENU. The mutation frequency was determined by the frequency of sex-linked lethal mutations. To this end, individual *B* females of the genotype w^{1118}*/FM7, TW9* were crossed to wild-type males. Progeny from this cross were scored for the presence or absence of males. Since *FM7, TW9* is lethal in males, only males that have no lethality on the mutagenized w^{1118} chromosome were obtained.

***dishevelled* misexpression:** A *Bam*HI/*Eco*RI fragment of the dshmyc construct (Yanagawa *et al.* 1995) was cloned into the pUAST (Brand and Perrimon 1993) vector. Injection of *UAS-dshmyc* into *y w* flies was performed (Spradling 1986), and 46 lines were obtained. These lines were crossed to *GMR-Gal4* (Moses and Rubin 1991) and the phenotype of adult eyes was examined. Lines 3-8 and 1-16 produced a moderate phenotype when crossed to *GMR-Gal4* as compared to *GMR-Gal4; UAS-wg* flies or to other lines of *UAS-dsh* (Figure 2C; data not shown). The addition of the myc epitope did not interfere with *UAS-dsh* function in this assay when compared to a line containing *UAS-dsh* without the myc tag. The cytological insertion positions of these lines were determined. The insertion positions mapped to 64C, line 3-8, and 85B/C, line 1-16. Since lines 3-8 and 1-16 were homozygous viable, mapped to the third chromosome, and appeared to have single insertion sites, they were utilized for the misexpression screen.

A *UAS-*dsh^1 construct was generated from DNA that was PCR amplified from dsh^1 genomic DNA preparations (see below). A PFLM1/Blp1 fragment was isolated from this PCR product and cloned into PFLM1/Blp1-digested pBS-dshmyc. This construct was cloned into the pUAST vector and *UAS-*dsh^1*myc* flies were obtained as above. Twenty-four lines were isolated. Their phenotypes were similar to wild-type *UAS-dsh* when crossed to *GMR-Gal4*.

Mutant lines of *UAS-dsh* that showed a modification of the original eye phenotype were crossed to *da-Gal4*, which drives ubiquitous expression in the embryo, and to *69B-Gal4*, which drives expression in the wing (Brand and Perrimon 1993), to determine the effect of misexpression in these tissues.

***dishevelled* misexpression screen:** *w/Y; Sp/Cyo; UAS-dsh* males were mutagenized overnight with a 25 mm solution of EMS in rounds 6–9 of the mutagenesis. In subsequent rounds, 0.5–2 mm solution of ENU (Sigma) in 1% sucrose was used. These males were isogenized on the third chromosome. Mutagenized males were crossed to *w; GMR-Gal4* virgin females and the eyes from ~90,000 F_1 flies were screened (Figure 1C).

Of these 90,000 flies, 104 mutant lines were generated (Table 1). Twenty-five lines were isolated with apparently wild-type eyes (Figure 2F), 68 lines had eyes larger than those of parental lines but smaller than those of wild-type flies (Figure 2D), and 11 lines had eyes that were almost wild type in size but rough, suggesting that they retained less function in Wg signaling than did the previous class (Figure 2E; Brunner *et al.* 1997). A total of 101 of these lines map to the third chromosome as would be expected if they contain lesions within the *UAS-dsh* transgene. Three lines map to the fourth chromosome and could not be mapped further due to the absence of recombination on the fourth chromosome.

These screens were designed to identify specific amino acid changes rather than large deletions or truncations of the Dsh protein and we therefore examined the expression of the Dsh protein encoded by the transgene in 49 lines using anti-myc antibodies. Twenty-eight lines expressed Dsh protein at robust

levels and 26 of these lines were sequenced. A total of 36 lines have disruptions either in Dsh protein expression or in sequence of the *UAS-dsh* transgene. Included in this group are all 25 lines that possess wild-type eyes and 11 lines that contain partial loss-of-function mutations. Twelve lines do not contain disruptions in Dsh protein expression or in the sequence of the *UAS-dsh* transgene, suggesting that they possess second-site modifiers of the *GMR-GAL4; UAS-dsh* eye misexpression phenotype. Five of these lines were mapped (see below). The remaining 55 lines have not been analyzed further but the data are available on request.

Generation of *dsh* mutants lacking potential phosphorylation sites or protein domains: To generate mutants of *dsh* in which conserved serine or threonine residues were substituted by alanines, we used the oligonucleotide-mediated site-directed mutagenesis method of Kunkel (Sambrook *et al.* 1989). The mutagenesis reactions were carried out using single-stranded DNA prepared from the myc-tagged, full-length dsh cDNA cloned in pBluescript (Yanagawa *et al.* 1995) and the following mutagenesis primers: Dsh-ST1 CTCGCATATCAAGCG GCAGCCGTGCTCGCCGCCGATCTCGAGTCG (T178A, S181A, S183A, S186A, S187A); Dsh-ST2 GATCTCGAGGCGGCCGC TCTCTTTGGCGCCGAAGCCGAGCTCACG (S191A, T192A, S193A, T197A, S199A); Dsh-ST4 TCGCGCGCCGCGGCGTAC GCCGCCATAGCCGACTCG (T235A, S236A, S237A, S239A, S240A, T242A); Dsh-ST5 CCGACGCGGCCATGGCCCTAAA TATTATTGCCGTCGCCATCAAC (S244A, T245A, S247A, T252A, S254A); Dsh-CK2 CTCTTTGGCGCCGAATCC (T197A); Dsh-CK3 TCGTACAGCGCTATAACCGAC (S240A); Dsh-CK4 GAGAACATGGCCAACGACGAG (T314A). The resulting mutations are given in parentheses. The mutated Dsh cDNAs were cut out of the Bluescript vector with *Sal*I and *Xba*I and were ligated into pCasPeR-hs cut with *Xba*I and *Stu*I after filling in the *Sal*I site of the Dsh fragment. The same strategy was used to subclone the deletion mutants DshΔbasic, DshΔPDZ, and DshΔDEPD-2 (Yanagawa *et al.* 1995) into pCasPeR-hs.

Rescue of *dsh* loss-of-function alleles: Hemizygous dsh^{v26} animals die at late third instar or early pupal stages (Klingensmith *et al.* 1994). To test for rescuing capacity of the transgenes, we crossed heterozygous *y* dsh^{v26}*/FM7* females with males carrying a heat-shock-inducible *dsh* construct on one of the autosomes. Developing larvae and pupae were exposed to a 30-min heat shock every 24 hr until hatching of adult flies. Rescued dsh^{v26} hemizygous males were easily identified by yellow body color and eyes with wild-type shape (B^+).

To score the rescuing capacity of our transgenes with respect to the tissue polarity phenotype of dsh^1, we analyzed the orientation of wing hairs and thoracic bristles of hemizygous dsh^1 males carrying a *dsh* transgene on one of the autosomes.

Mapping of *UAS-dsh* modifiers: *UAS-dsh* males that suppressed the Dsh eye and wing misexpression phenotype but that did not encode mutations within *UAS-dsh, UAS-dsh M,* were crossed to *ru h th st cu sr e ca/TM3 Sb* females. *UAS-dsh M/ru h th st cu sr e ca* females were crossed to *ru h th st cu sr e Pr ca/TM6B* males and recombinant males were scored for these recessive markers. Three individual males of each recombinant class (for example *ru* or *ru h* or *ru h th st*) and the reciprocal class were crossed to *w; GMR-Gal4* females and *Pr*+ flies were scored for the presence of *UAS-dsh* and for the modifier. *If UAS-dsh* was not present then *w; GMR-Gal4/*+; recombinant/+ males were crossed to *w; GMR-Gal4/Cyo; UAS-dshT/TM6B* females and *w; GMR-Gal4/*+; *UAS-dsh/*(+ or recombinant) flies were scored for the presence of the modifier. Of these flies, 50% are expected to contain the modifier. Five lines, *UAS-dsh* $M^{8\text{-}3}$, *UAS-dsh* $M^{8\text{-}4}$, *UAS-dsh* $M^{8\text{-}13}$, *UAS-dsh* $M^{8\text{-}66}$, and *UAS-dsh* $M^{16\text{-}21}$, were strong suppressors and these mapped to *ca* at position 3-100.7. We more finely mapped three of them, *UAS-dsh* $M^{8\text{-}13}$, *UAS-dsh* $M^{8\text{-}66}$, and *UAS-dsh* $M^{16\text{-}21}$, by crossing *w; ra M/TM6B* males to *w;* $P\{w(+mC)\}dco^{j3B9}$*/TM3, Sb* females. *w; ra M/*$P\{w(+mC)\}dco^{j3B9}$ females were crossed to *Pr ra ca/TM6B* males and males that recombined in the interval between *ra* and *dco* of the genotype *ra* $P\{w(+mC)\}dco^{j3B9}$*/Pr ra ca* or + +*/Pr ra ca* were crossed to *w; GMR/Cyo; UAS-dsh/TM6B* and the presence or absence of the modifier was noted. *ra* maps at position 3-97.3 and *dco* maps to the right of *ca* at cytological position 100B2-4. Recombinants of the genotype *ra M* $P\{w(+mC)\}dco^{j3B9}$ were obtained. Interestingly, these flies suppressed the *GMR-Gal4; UAS-dsh* eye phenotype more strongly than did *ra M* flies, suggesting that *dco* interacts in this assay. *dco* encodes Drosophila casein kinase I and its mammalian homolog is implicated in Wnt signaling (Kloss *et al.* 1998; Peters *et al.* 1999; Sakanaka *et al.* 1999). *w; ra M/TM6B* males were crossed to *P{w(+mC)}axin/TM6B* females and recombinants were mapped in the same manner as the above cross. From these two crosses we concluded that *M* lies near *axin* and proximal of *dco* in the interval 99D4-5 to 100B2-4. We were unable to obtain recombinant *ra M P{w(+mC)}axin* flies. This indicated that this modifier might be *axin* and the *axin* gene in three of these lines, *UAS-dsh* $M^{8\text{-}13}$, *UAS-dsh* $M^{8\text{-}66}$, and *UAS-dsh* $M^{16\text{-}21}$ was sequenced (see below). These lines did indeed contain novel *axin* mutations.

Sequence analysis: *dsh* is a single exon gene, facilitating sequencing of alleles. Genomic DNA of adult flies of the genotype dsh^1, dsh^{A3}, or dsh^{A21} was isolated and the entire coding region was sequenced. PCR primers from positions 870, 5′ of the coding region, and 2914, 3′ of the coding region, (Berkeley *Drosophila* Genome Project) were used to amplify genomic DNA and these PCR products were directly sequenced using the ABI system.

Isolates that affected the phenotype of *UAS-dsh* generated in the misexpression screen were crossed to *GMR-Gal4* and third instar eye discs were dissected from these flies. These discs were stained with the 9E10 anti-myc antibody obtained from the hybridoma facility at The University of Wisconsin using established protocols (Blair 1992). Since the myc tag in UAS-dsh is at the carboxy end of the protein, *UAS-dsh* alleles that encode truncations within the Dsh protein would not be labeled with the anti-myc antibody. The entire coding region was sequenced from lines that produced intact protein. Genomic DNA from these lines was isolated and DNA was amplified using PCR. Primers were designed to sequences within the *UAS-dsh* transgene to ensure that the *UAS-dsh* gene but not endogenous *dsh* gene would be amplified. PCR-amplified *UAS-dsh* products were reamplified using the original 5′ or 3′ primer along with an internal primer to obtain enough DNA for sequencing. Sequencing was done as above.

Genomic DNA was isolated from lines *UAS-dsh* $M^{8\text{-}13}$, *UAS-dsh* $M^{8\text{-}66}$, and *UAS-dsh* $M^{16\text{-}21}$. These lines are now called $axin^{8\text{-}13}$, $axin^{8\text{-}66}$, and $axin^{16\text{-}21}$. DNA fragments were PCR amplified using primers to *axin* genomic sequence (Berkeley *Drosophila* Genome Project) at positions 740 and 1660, 1310 and 3455, 2640 and 4046, and 3432 and 4805. These fragments represent the entire coding region of axin and were sequenced as above.

Adult wing mounting: Wings were mounted in Euparal and incubated overnight at 65°.

RESULTS

Screen for planar polarity alleles of *dsh*: The viable dsh^1 allele causes planar polarity defects, resulting in aberrant orientation of bristles and hairs if present in hemizygous male adults or in combination with a *dsh* null allele (dsh^{v26}; Figure 3, C and D). Since dsh^1 survives over dsh^{v26}, planar polarity mutants will produce surviv-

ing adults and can be identified in an F_1 screen. We used dsh^{v26} heterozygotes to screen for new *dsh* alleles (Figure 1A). Using the mutagen EMS, we screened through 20,000 flies and obtained 1 *dsh* allele, dsh^{A3} (Table 1). We then used the mutagen ENU to screen an additional 37,840 flies. Although a total of 15 potential positive *dsh* alleles were observed in the F_1 generation, only 1 transmitted to the F_2. We reasoned that alleles of *dsh* may have a higher probability of being recovered if we screened over a *dsh* allele that retained wg signaling function. dsh^{A3} flies are viable and fertile in contrast to dsh^{1} flies that are only semiviable. Thus an additional screen was performed in combination with dsh^{A3} using the mutagen ENU. A total of 21,000 flies were screened, and 20 potential new *dsh* alleles were observed, but only 1 allele, dsh^{A21}, was recovered in the F_2. dsh^{A3} and dsh^{A21}

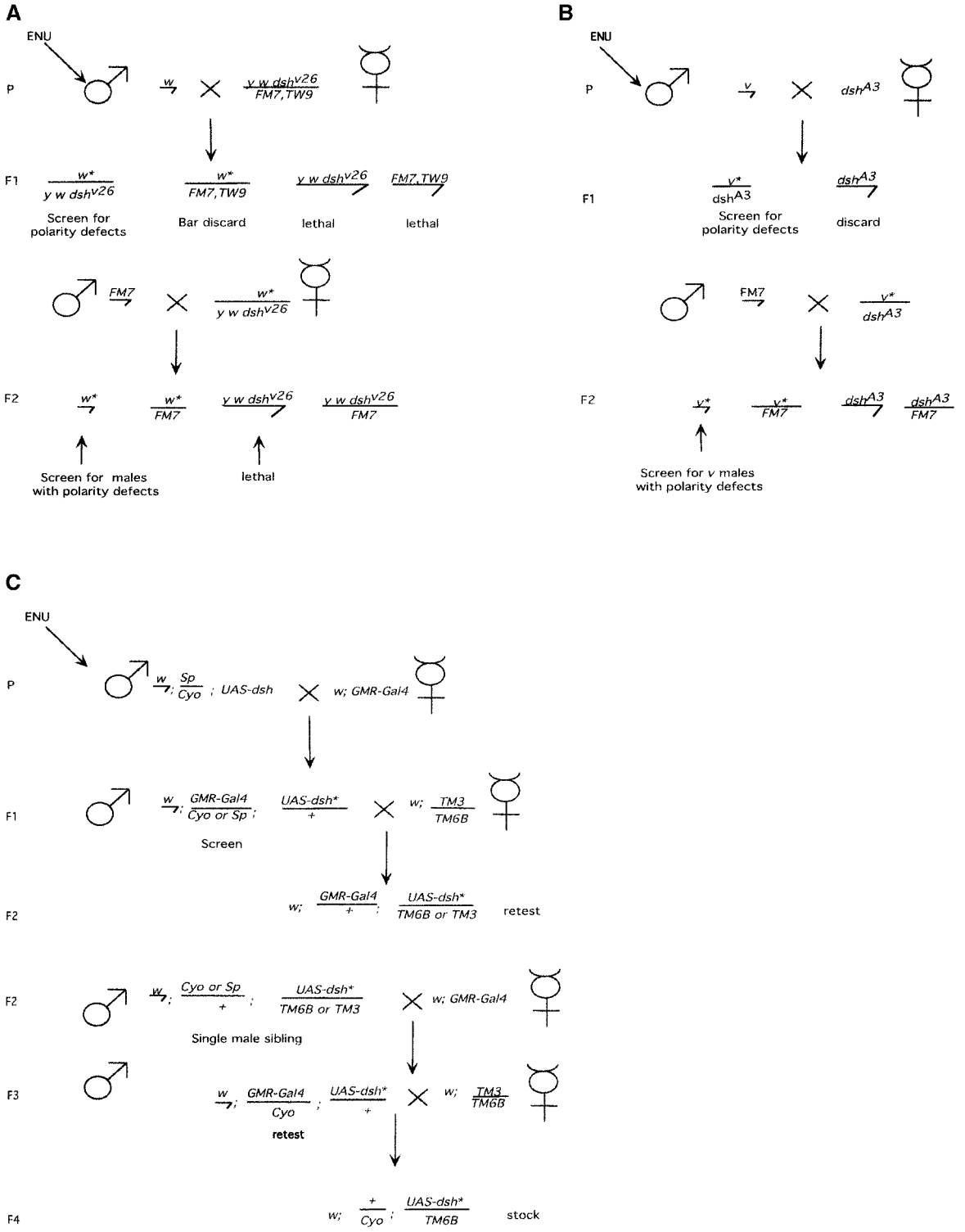


TABLE 1

Statistics of the mutagenesis screens

Planar polarity screens	No. screened	No. new *dsh* alleles
dsh^{v26}	57,840	1 (dsh^{A3})
dsh^{A3}	21,000	1 (dsh^{A21})

Wg signaling screen (Dsh overexpression in the eye)	No. screened	L	PL	Lro
Line UAS-Dsh 3-8	65,000	19	53	9
Line UAS-Dsh 1-16	25,000	6	15	2
Total	90,000	25	68	11

Wg signaling activity of the *UAS-dsh* transgene was assayed by crossing these mutant lines to *GMR-Gal4* and comparing their phenotypes with parental lines (Figure 2). L, eyes wild type, indicating a loss of *UAS-dsh* function; PL, eyes larger than those of parental lines, indicating a partial loss of *UAS-dsh* function; Lro, eyes are almost normal in size but rough in appearance, indicating a severe but not complete loss of *UAS-dsh* function.

have phenotypes similar to dsh^{1} (Figure 3, C and D) and display planar polarity abnormalities in the wing and thorax. The defects of dsh^{A21} flies are variable, indicating that this allele has variable expressivity. dsh^{A3} and dsh^{A21} encode mutations within the DEP domain like dsh^{1} (Table 2 and Figure 4).

Screen for *dsh* alleles that disrupt Wg signaling: *dsh* alleles interfering with Wg signaling are hemizygous and homozygous lethal, complicating the isolation of large numbers of these alleles. We used the GAL4:UAS (Brand and Perrimon 1993) system to produce mutations in a *UAS-dsh* transgene that are compromised in their ability to activate Wg signaling when overexpressed. *dsh* was tagged with the myc epitope in the carboxy terminus to distinguish it from the endogenous gene, and *UAS-dsh* transgenic flies were generated. The GMR enhancer drives expression of the GAL4 protein in cells in developing eyes (Moses and Rubin 1991). If expression of *UAS-wg* or *UAS-dsh* is driven by *GMR-GAL4*, then adult eyes are dramatically reduced in size (Figure 2, B and C). This effect occurs during pupation since *GMR-Gal4; UAS-dsh* third instar eye discs have no abnormalities (data not shown). Flies that carry a $UAS\text{-}dsh^{1}$ construct exhibit the same reduced eye phenotype as flies that carry a wild-type *UAS-dsh* construct (data not shown), indicating that this phenotype is not generated by planar polarity signaling.

To obtain mutations in the transgene, males carrying the *UAS-dsh* transgene were mutagenized with ENU and crossed to *GMR-GAL4* females (Figure 1C). We examined F_1 progeny for either wild-type eyes (indicating an inactivating mutation in the transgene) or eyes that were larger than those of the parental lines (indicating a partial loss of function in the transgene). Tables 1 and 2 present the results.

Characterization of *UAS-dsh* alleles: *UAS-dsh* mutant lines were analyzed further to determine whether these mutations attenuate Wg signaling in other developmental processes. These lines were crossed to *69B-Gal4*, which is expressed in the ectoderm during embryonic and larval development (Brand and Perrimon 1993). When parental *UAS-dsh* lines were crossed to *69B-Gal4*, most of the offspring animals died during pupal stages. Severely abnormal wings were observed in adult survivors (Figure 3E). These wings had ectopic margin bristles in the interior of the wing, a defect that is caused by excessive Wg signaling (Diaz-Benjumea and Cohen 1995). We observed other defects that included venation abnormalities, blisters, and disruptions in planar polarity, although the latter was difficult to assay because the wing was severely deformed. The process of planar polarity is sensitive to levels of signaling since both a lack of signaling and a gain of signaling cause pattern disruptions. Since *dsh* transduces both Wg and planar polarity signaling events, these defects were expected. Many *UAS-dsh* lines displayed wings that either were wild type or carried planar polarity disruptions when crossed to *69B-Gal4* (Figure 3, F and H; Table 2). Pupal lethality was also suppressed.

UAS-dsh lines were also crossed to *da-Gal4*, which is

Figure 1.—Screens for new *dsh* alleles that affect planar polarity and Wg signaling. (A) *dsh* is located on the X chromosome at cytological position 10B6. w^{1118} males were mutagenized and mated to virgin females that are heterozygous for a *dsh* null allele ($dshv^{26}$) and for the balancer chromosome (*FM7, TW9*). F_1 males were not recovered because they were null for *dsh* or possessed the *FM7, TW9* balancer chromosome that is lethal in hemizygous flies (see materials and methods). The thoraces of F_1 females were examined for polarity defects that occur in combination with dsh^{v26}. They were distinguished from flies carrying the *FM7, TW9* chromosome because they were *Bar*+. Females that had planar polarity phenotypes were mated to *FM7*/Y males. Surviving males in the F_2 generation were screened for planar polarity defects, visible in hemizygous males. Stocks were established from these males. (B) Male flies marked with *vermillion* (*v*) were mutagenized and mated to female flies homozygous for the dsh^{A3} mutation. Thoraces of female flies were screened for polarity defects. Females that possessed defects were mated to *FM7*/Y males and *v* F_2 males were screened for polarity defects. *v* maps at position 1-33 and *dsh* maps at 1-34.5, making recombination between *v* and dsh^{A3} unlikely, and permitting us to distinguish the dsh^{A3} chromosome from new *dsh* mutations. (C) Males of the genotype *w; Sp/Cyo; UAS-dsh* were mutagenized and crossed to *w; GMR-Gal4* females. The eyes of F_1 flies were screened to determine if they were larger or smaller than eyes from the parental lines (Figure 2). Candidate F_1 males were crossed to *w; TM3, Sb/TM6B* females and *w; GMR-Gal4/+; UAS-dsh*/TM3, Sb* or *w; GMR-Gal4/+; UAS-dsh*/ TM6B* flies in the F_2 generation were analyzed to determine whether the modified phenotype was still present. If so, individual sibling males were crossed to *w; GMR-Gal4* females. The *UAS-dsh* transgene was marked with the *mini-(w)*+ gene, which allowed its detection in the F_2 and F_4 generations.

TABLE 2

Missense mutations that disrupt *dsh* or *UAS-dsh* function

	Allele	Mutation	Eye	Wing	Embryo
DIX domain	*UAS-dsh*$^{8-65}$	F40S	L	1	Viable
	UAS-dsh$^{8-9}$	V43E	PL	3	Pupal lethal
	***UAS-dsh*$^{8-16}$**	G64V	L	2	Viable
	***UAS-dsh*$^{16-43}$**	G64V	L	2	Viable
	UAS-dsh$^{8-19}$	V66A	L	2	Viable
	UAS-dsh$^{8-80}$	N80I	PL	3	Pupal lethal
	UAS-dsh$^{16-1}$	R82Q	L	3	Pupal lethal
PDZ domain	*UAS-dsh*$^{8-1}$	V253D	PL	6	Lethal
SH3 domain	*UAS-dsh*$^{8-12}$	P358L	Lro	3	Pupal lethal
	UAS-dsh$^{8-79}$	D360V	Lro	3	Pupal lethal
	UAS-dsh$^{8-68}$	G362D	PL	5	Not tested
DEP domain	*dsh*^{A3}	R413H			
	dsh^{1}	K417M			
	UAS-dsh$^{8-64}$	V440D	L	2	Viable
	UAS-dsh$^{16-37}$	A446V	L	3	Viable
	UAS-dsh$^{8-43}$	I459N	L	2	Viable
	dsh^{A21}	C472R			

Positions of mutations within *dsh* or *UAS-dsh* are indicated. The allele number is followed by the mutation and its position. *UAS-dsh*$^{8-16}$ and *UAS-dsh*$^{16-43}$, boldface type, encode identical mutations. The phenotype of *UAS-dsh* alleles crossed to *GMR-Gal4* is indicated in the eye column, *UAS-dsh* alleles crossed to *69B-Gal4* in the wing column, and *UAS-dsh* alleles crossed to *da-Gal4* in the embryo column. Numbers in the wing column indicate the severity of wing phenotypes from *69B-Gal4; UAS-dsh* individuals. L, eyes wild type in size, indicating a loss of *UAS-dsh* function; PL, eyes larger than those of parental lines, indicating a partial loss of *UAS-dsh* function; Lro, eyes are almost normal in size but are rough in appearance, indicating a severe but not complete loss of *UAS-dsh* function; 1, wild-type wing; 2, slight planar polarity phenotype; 3, moderate planar polarity phenotype; 4, planar polarity phenotype and <10 ectopic wing margin bristles, 5, planar polarity phenotype and >10 ectopic wing margin bristles; 6, planar polarity phenotype, ectopic wing margin bristles, and severe blistering and venation defects.

expressed ubiquitously in the embryo (Brand and Perrimon 1993; Wodarz *et al.* 1995). Ectopic Wg signaling is known to cause lethality and cuticular abnormalities during embryogenesis (Noordermeer *et al.* 1992) and parental lines displayed embryonic or larval lethality when crossed to *da-Gal4*. In contrast, most *UAS-dsh* lines either were viable or displayed pupal lethality when they were crossed to *da-Gal4* (Table 2). Thus mutations within *UAS-dsh* that attenuated Wg signaling during eye development also attenuated Wg signaling in other developmental processes.

UAS-dsh mutations map to several domains. Seven *UAS-dsh* alleles encode missense mutations within the DIX domain and these alleles all strongly disrupt Wg signaling in our assays (Table 2). We identified only one *UAS-dsh* mutation in the PDZ domain, *UAS-dsh*$^{8-1}$, (Table 2), which only mildly disrupts ectopic Wg signaling during eye development. It is lethal when crossed to *da-Gal4* and semilethal when crossed to *69B-Gal4*, and surviving adults have wings resembling those of the parental lines (Figure 3G).

Three *UAS-dsh* alleles possess mutations within a novel domain that is located between the PDZ and the DEP domains (Figure 4A). Interestingly, this domain contains a motif that encodes a class I consensus core sequence for an SH3 protein-binding site (Figure 4B; Kay *et al.* 2000). The consensus site consists of the sequence RTEPVRP at position 352–358 and is surrounded by proline-rich sequence. These *UAS-dsh* alleles strongly disrupt Wg signaling events indicating that this domain may be important for Wg signaling (Table 2; Figures 2E and 3F).

Three alleles of *UAS-dsh* map to a region within the DEP domain, which extends from position 440 to 459 (Table 2; Figure 4). These alleles abrogate Wg signaling (Table 2) implying that a subregion of the DEP domain might be utilized in Wg signaling events. This is in contrast to studies that show that the DEP domain is dispensable for Arm accumulation in Drosophila cl-8 cells (Yanagawa *et al.* 1995).

***In vitro* mutagenesis of potential phosphorylation sites of Dsh:** While a forward mutational screen for novel alleles is powerful, it is not feasible to uncover mutations in multiple sites. In the case of phosphorylation, proteins are often modified at several sites and such sites could be redundant. Several protein kinases are known

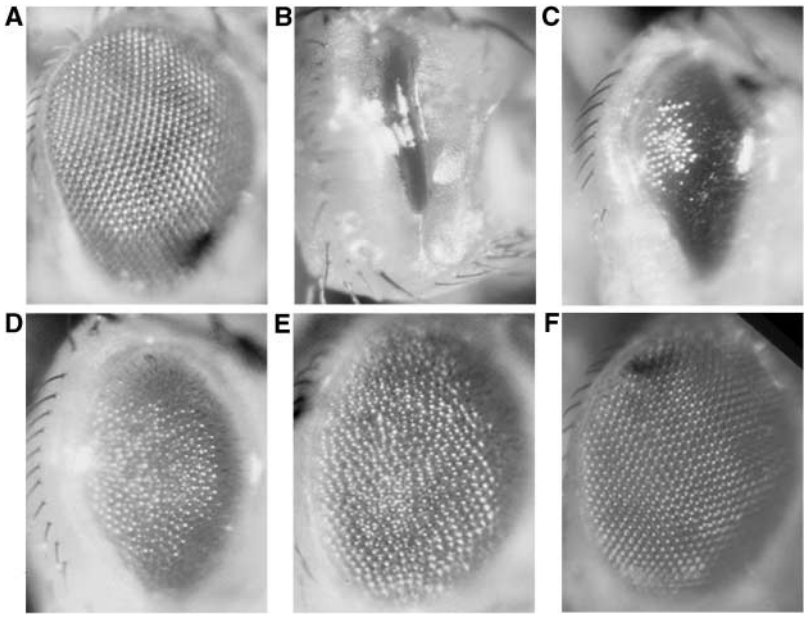


Figure 2.—Eye phenotypes of *UAS-dsh* isolates when crossed to *GMR-Gal4*. Drosophila wild-type eyes (A) are characterized by 750 ommatidia that develop during the third larval instar as the morphogenetic furrow progresses from posterior to anterior in the eye disc (Bate and Martinez Arias 1993). The *GMR* promoter is expressed posteriorly to the morphogenetic furrow in developing photoreceptor cells (Moses and Rubin 1991). When *GMR-Gal4* is crossed to *UAS-wg* (B) or *UAS-dsh* (C), adult eyes that are severely reduced in size result. This defect is caused by degeneration that occurs during pupal development. One class of *UAS-dsh* isolates (D) possesses eyes that are larger than those of parental lines (C) but smaller than those of wild type (A), indicating a partial loss of function in *UAS-dsh*. Another class of *UAS-dsh* isolates possesses eyes that are almost wild type in size (E) but rough, suggesting that very little signaling function of *UAS-dsh* remains. The remaining *UAS-dsh* isolates appear to lack signaling since their eyes are wild type in appearance (F).

to interact with Dsh. In particular the area surrounding a basic region in Dsh (amino acids 178–254) is phosphorylated by CK1, CK2, and PAR-1 (Willert *et al.* 1997; Peters *et al.* 1999; Sun *et al.* 2001). This region contains 27 serine and threonine residues and many of these are conserved (Figure 4C). To uncover potential phosphorylation sites, we replaced most of the conserved serine and threonine residues in the region from amino acids (aa) 178 to 254 by alanine using *in vitro* mutagenesis. This was done in clusters of 4–6 serine and threonine residues at a time (Figure 4C). Subsequently, several of these mutated clusters were combined to generate larger regions devoid of serine and threonine residues. In addition, constructs were generated that lacked the basic domain, the PDZ domain, or the complete C terminus including the DEP domain (Yanagawa *et al.* 1995).

The mutagenized Dsh constructs were introduced into flies and subjected to a series of functional assays. We first asked whether the different transgenes were able to rescue the loss-of-function phenotype of the amorphic allele dsh^{v26} (Klingensmith *et al.* 1994). Developing larvae and pupae were exposed to a 30-min heat shock every 24 hr until hatching of adult flies. With this regimen, rescue of dsh^{v26} to viability was observed for all Dsh transgenes except for DshΔPDZ and DshΔDEPD-2 (Table 3). Rescue was also observed in dsh^{v26} hemizygous males derived from germline clones (data not shown). From these results we conclude that those transgenes that rescue lethality are fully functional in the absence of endogenous Dsh.

In a second assay, we tested the transgenes for rescue of the tissue polarity phenotype of dsh^{1} (Perrimon and Mahowald 1987; Axelrod *et al.* 1998; Boutros *et al.* 1998). Our results are summarized in Table 3. Six of the 11 transgenes fully rescued the tissue polarity phenotype of dsh^{1}, while 5 did not show any rescue. The five constructs that failed to rescue the dsh^{1} phenotype are DshΔPDZ, DshΔDEPD-2, DshST124, DshST4, and DshST45 (see Figure 4C). The latter three constructs all have in common the substitution of serine/threonine residues by alanines in the highly conserved cluster ST4 (Figure 4C). Intriguingly, cluster ST4 is located within the region of Dsh required for binding of and phosphorylation by the protein kinase PAR-1 (Sun *et al.* 2001). As PAR-1 is essential for polarity signaling during oogenesis in Drosophila (Shulman *et al.* 2000; Tomancak *et al.* 2000; Cox *et al.* 2001; Huynh *et al.* 2001), it may also affect planar polarity by phosphorylation of sites located in cluster ST4. This cluster also contains a consensus phosphorylation site for CK2 (Figure 4C), but this serine residue (S240) is probably not required for tissue polarity signaling. A mutant Dsh protein carrying a substitution of serine 240 by alanine fully rescues tissue polarity signaling in a dsh^{1} mutant background (Table 3). The same is true for the point mutants changing the other three potential CK2 consensus phosphorylation sites in the region between aa 178 and 320 to alanines (Table 3; see Figure 4C).

All the constructs that rescued the tissue polarity defects of dsh^{1} also showed a wild-type tissue polarity pattern in rescued dsh^{v26} hemizygous males (data not shown). From this result we conclude that these transgenes are sufficient to rescue all aspects of Wingless signaling and tissue polarity signaling in the complete absence of endogenous Dsh. Interestingly, with respect to tissue polarity signaling, full rescuing activity of the heat-inducible transgenes was obtained at basal expression levels without heat shock. Transgenes that did not rescue the tissue polarity defects without heat shock also failed to rescue when raised under the heat-shock regimen described for rescue of dsh^{v26} (data not shown).

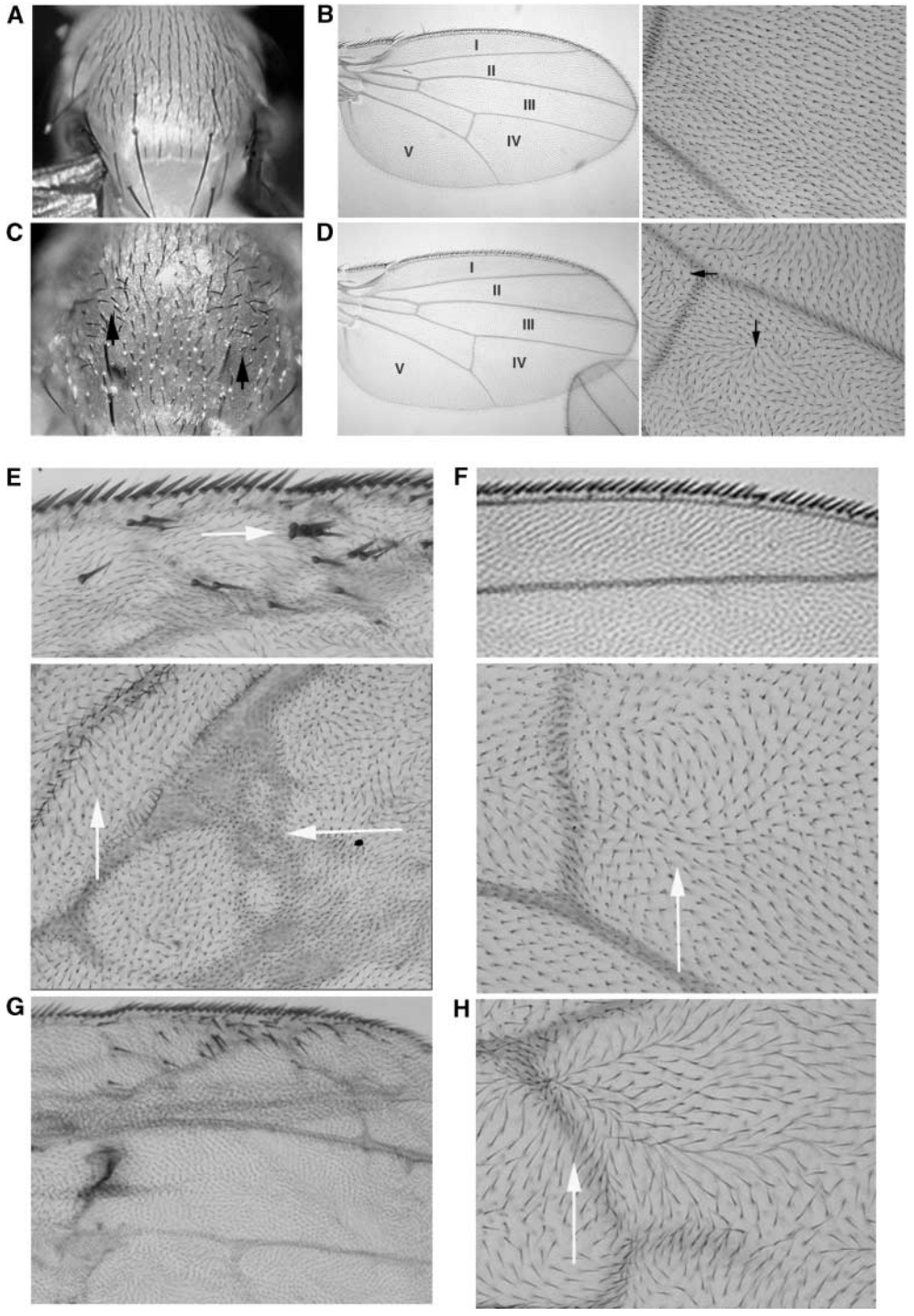
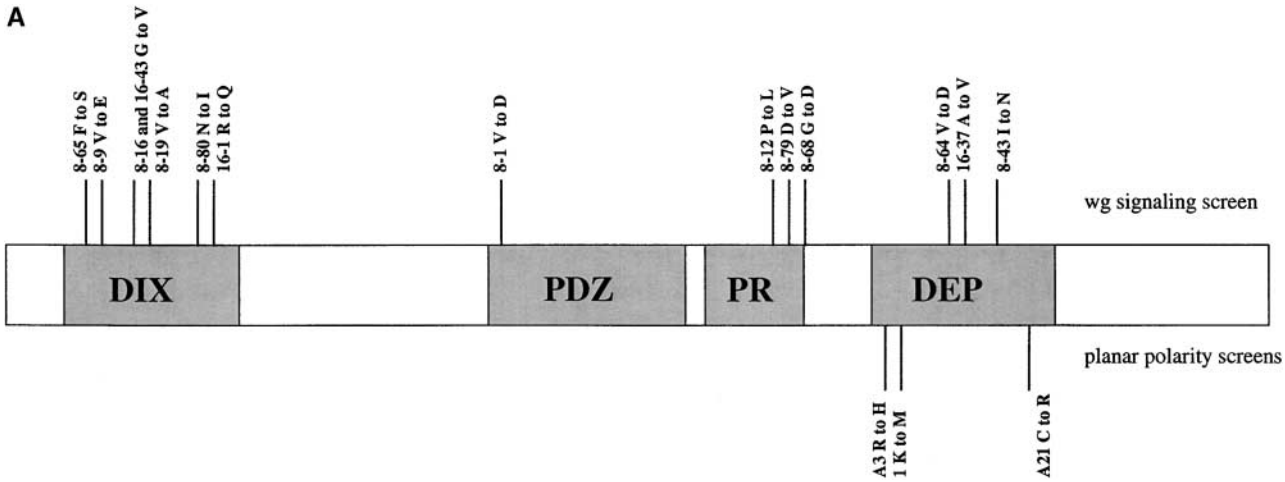


FIGURE 3.—The planar polarity phenotype and wing phenotypes of *UAS-dsh* isolates when crossed to *69B-Gal4*. The hairs and bristles of wild-type flies are oriented in a regular manner such that all hairs or bristles in a particular region are pointing in the same direction. The bristles of wild-type thoraces point from the anterior direction to the posterior direction (A) and the hairs of wild-type wings point in a proximal-to-distal direction (B). In flies that are mutant for dsh^{1} (C and D) the orientation of bristles and hairs is disorganized and irregular whorls are seen on the thorax (C, arrows) and wings (D, arrows). B and D (left) show a ×5 magnification of wings from a wild-type and a dsh^{1} mutant fly, respectively. The five regions of the wing are indicated, region I between the anterior wing margin and L2, region II between L2 and L3, region III between L3 and L4, region IV between L4 and L5, and region V between L5 and the posterior margin. B and D (right) show a ×40 magnification of wings from a wild-type and a dsh^{1} mutant fly. In this and all subsequent figures, region IV is characterized for planar polarity defects. Anterior is up, posterior is down (A and C). Proximal is left and distal is right (B–H). L is longitudinal vein. The phenotype of parental line *UAS-dsh* (E) is quite severe when crossed to *69B-Gal4*. Survivors from this cross have ectopic mechanosensory bristles near the wing margin (top, arrow), severe vein and planar polarity defects (bottom, arrows) and some individuals have blisters (data not shown). $UAS\text{-}dsh^{8\text{-}12}$ and $UAS\text{-}dsh^{16\text{-}1}$ flies have planar polarity defects (F and H, arrows), but no ectopic bristles and only mild venation defects when crossed to *69B-Gal4*. $UAS\text{-}dsh^{8\text{-}1}$ flies have a partial loss-of-function phenotype when crossed to *GMR-Gal4*, but their phenotype resembles parental lines when crossed to *69B-Gal4* (G).

Thus, the amount of Dsh required for its function in tissue polarity signaling is apparently lower than the amount required for function in Wg signaling.

Mutations in *axin* act as suppressors of Dsh overexpression phenotypes: Twelve *UAS-dsh* stocks with attenuated misexpression phenotypes in the eye did not disrupt Dsh protein expression and had no mutations within the *UAS-dsh* transgene. These stocks are referred to as *UAS-dsh M* (for modifier of *UAS-dsh*). Five lines, including $UAS\text{-}dsh\ M^{8\text{-}3}$, $UAS\text{-}dsh\ M^{8\text{-}4}$, $UAS\text{-}dsh\ M^{8\text{-}13}$, $UAS\text{-}dsh\ M^{8\text{-}66}$, and $UAS\text{-}dshM^{16\text{-}21}$, acted as strong suppressors and were mapped to region 3-100.7 (Figure 5A). Three of these lines, $UAS\text{-}dsh\ M^{8\text{-}13}$, $UAS\text{-}dsh\ M^{8\text{-}66}$, and $UAS\text{-}dsh\ M^{16\text{-}21}$, were mapped more finely to region 99D4-5 to 100B4 (see MATERIALS AND METHODS). Since the Drosophila *axin* gene maps at 99D4-5 and is an important



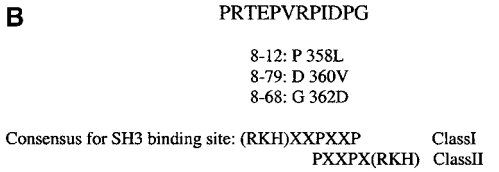


FIGURE 4.—Positions of *dsh* and *UAS-dsh* mutations generated in the above screens. (A) The domain structure of *dsh* is indicated. The PDZ domain and the DEP domain follow the amino terminal DIX domain. A newly identified domain called PR for proline rich lies between the PDZ and the DEP domains. Mutations that disrupt Wg signaling functions of *UAS-dsh* are indicated above the schematic diagram and mutations that specifically disrupt planar polarity signaling are indicated below. (B) The *UAS-dsh*$^{8\text{-}12}$, *UAS-dsh*$^{8\text{-}79}$, and *UAS-dsh*$^{8\text{-}68}$ mutations cluster in the proline-rich domain. This region contains a class I core consensus binding site for an SH3 protein. The positions of the mutations *UAS-dsh*$^{8\text{-}12}$, *UAS-dsh*$^{8\text{-}79}$, and *UAS-dsh*$^{8\text{-}68}$ are indicated. (C) Protein sequence alignment of Dsh with murine Dvl1. Numbering of amino acids according to the published sequences is shown. Identical or highly conserved amino acids are indicated in gray. The DIX, PDZ-binding, proline-rich, and DEP domains are boxed. *dsh* and *UAS-dsh* mutations are shown above the sequence and amino acids that are altered by these mutations are boxed. The underlined regions designated as ST1, -2, -4, and -5 have been subjected to *in vitro* mutagenesis, leading to exchange of every serine and threonine residue within each cluster for alanine. Consensus phosphorylation sites for Casein kinase 2 are designated as CK1–4 and are marked by asterisks on top of the alignment. (D) The structure of the DEP domain of DVL1 (the mammalian homolog of Dsh established by WONG *et al.* (2000); downloaded from NCBI http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=15530 and visualized using the Cn3D program). The position of the mutations is in white.

regulator of Wg signaling, we sequenced *axin* from these three lines and from the *UAS-dsh* parental strain. All three lines possess mutations in *axin* (Figure 5B). The *UAS-dsh M*$^{16\text{-}21}$ and *UAS-dsh M*$^{8\text{-}66}$ mutations change a threonine residue into an asparagine residue and an arginine into a lysine residue, respectively. *UAS-dsh M*$^{8\text{-}13}$ maps to the GSK3-β binding domain of Axin and changes a conserved proline into a serine residue at position 421. These *axin* mutant lines are homozygous viable and have no phenotypes by themselves. They suppress Dsh misexpression phenotypes but do not suppress Wg or DFz2 misexpression phenotypes (MATERIALS AND METHODS; data not shown; see DISCUSSION).

DISCUSSION

By using three independent experimental approaches, we have identified a set of new mutations in Dsh that disrupt two distinct signaling events. These new mutations map to specific regions within Dsh and thus provide important information on the function of the different protein domains. We screened for mutations in the endogenous *dsh* gene that disrupt planar polarity (Figure 1, A and B) and utilized a misexpression phenotype to screen for mutations that affect Wg signaling (Figure 1C). Mutations that disrupt the Wg signaling function of Dsh occur throughout the protein while mutations that disrupt planar polarity signaling are confined to the DEP domain (Figure 4, A and C). However, we also demonstrate that mutation of potential phosphorylation sites positioned between the basic region and the PDZ domain of Dsh (cluster ST4) specifically disrupts the ability of Dsh transgenes to rescue the tissue polarity phenotype. We discuss the mutations uncovered in this work by domains, beginning at the N terminus.

DIX domain mutations: Seven *UAS-dsh* alleles encode missense mutations that map to the DIX domain and all reduce or abrogate Wg signaling in three separate assays (Table 2). These results clearly demonstrate that the DIX domain is required for Wg signaling and agree with other studies in Drosophila (YANAGAWA *et al.* 1995; AXELROD *et al.* 1998; BOUTROS *et al.* 1998). Furthermore, studies of the mammalian homolog of Dsh, Dvl1, show that the DIX domain of Dvl can interact directly with itself and that it binds to axin (KISHIDA *et al.* 1999; LI *et al.* 1999a). The N-terminal DIX domain in Dsh shares 37% amino acid identity with the C-terminal DIX domain

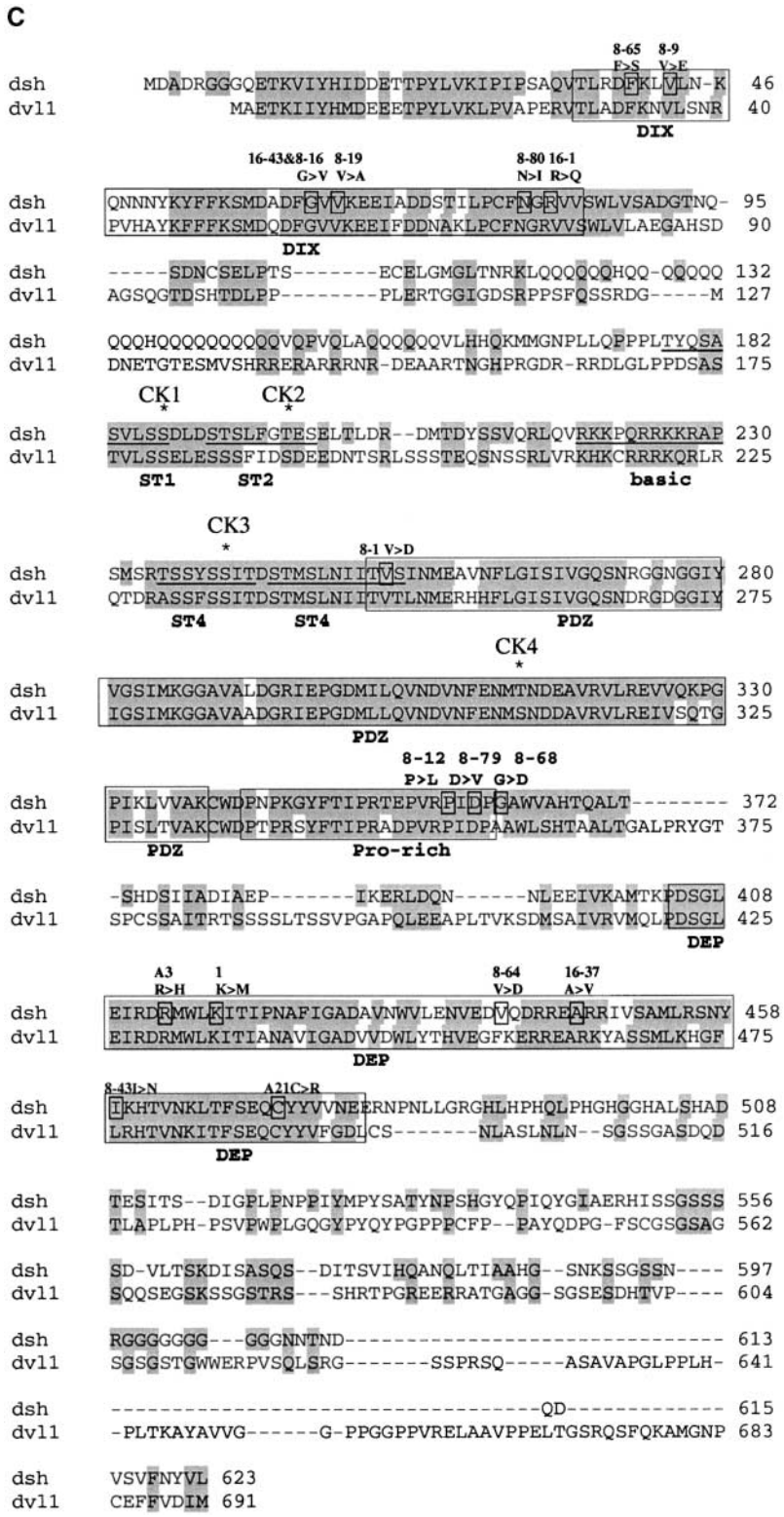


FIGURE 4.—*Continued.*

of Axin. The Axin DIX domain also interacts with itself and is necessary to regulate the stability of β-catenin in SW48O cells (KISHIDA *et al.* 1999). This indicates that DIX domains are important for protein-protein interactions and that the DIX domain of Dsh may mediate Wg signaling by binding to and inhibiting Axin.

The basic region: Dsh possesses a highly conserved basic region (aa 219–228; Figure 4C) of unknown function. We did not isolate any point mutations in this region in our genetic screens. Moreover, deletion of the basic region compromises neither the function of Dsh in Wg signaling nor its function in planar polarity

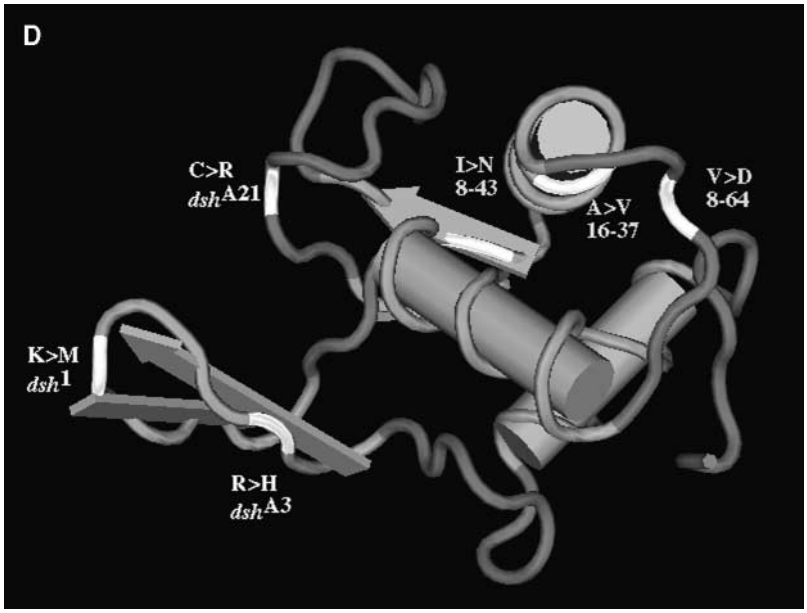


FIGURE 4.—*Continued.*

signaling. We note, however, that two of the transgenic lines carrying the DshΔbasic construct under control of the hsp70 heat-shock promoter rescue the lethality of the dsh^{v26} null allele even in the absence of heat shock, in contrast to all other lines we tested (Table 3). This result points to a potential role of the basic region as a negative regulator of the signaling function of Dsh.

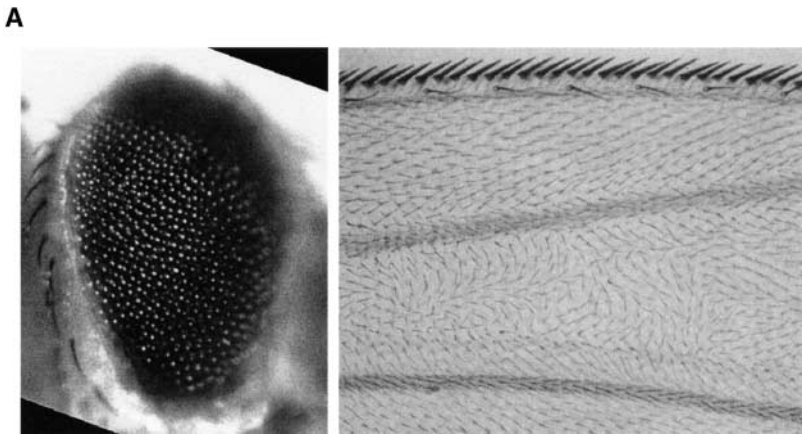
PDZ domain mutations: Surprisingly, we recovered only one allele, *UAS-dsh*$^{8\text{-}1}$, that maps to the PDZ domain. It only mildly attenuates the Dsh eye misexpression phenotype and does not attenuate the Dsh wing misexpression phenotype at all. While this result might suggest that there is only a minor requirement for this domain in Wg signaling, it could also mean that single point mutations have little effect on the function of the PDZ domain. In our hands, deletion of the PDZ domain completely abolishes Dsh function in both Wg signaling and tissue polarity. Our result contrasts with studies by AXELROD *et al.* (1998), which show that the PDZ domain is not necessary to rescue the embryonic lethality of dsh

TABLE 3

Rescue of dsh^{v26} and dsh^{1} phenotypes by Dsh transgenes

	No. of lines that rescue lethality *of* dsh^{v26}		No. of lines that rescue the tissue polarity phenotype of dsh^{1}	
Construct	No heat shock	Heat shock	No heat shock	No. of lines tested
Dshfull	0	14	14	14
DshΔBasic	2	10	10	10
DshΔPDZ	0	0	0	8
DshΔDEPD-2	0	0	0	7
DshST1	0	5	5	5
DshST124	0	10	0	13
DshST4	0	6	0	6
DshST45	0	4	0	5
DshCK2	0	13	11	13
DshCK3	0	8	8	8
DshCK4	0	5	5	5

All transgenes were expressed under the control of the hsp70 heat-shock promoter. Animals not exposed to heat shock were raised at a constant temperature of 25°. To expose developing larvae and pupae to heat shock, fly vials were submerged in a 37° water bath for 30 min every 24 hr until hatching of adult flies. For rescue of the dsh^{1} phenotype the basal expression level of the transgenes without heat shock was sufficient to obtain complete rescue of tissue polarity defects. Each experiment was done in duplicate and for each construct at least five independent transgenic lines were tested. In construct Dsh ST124 all serine and threonine residues in clusters ST1, ST2, and ST4 have been mutated to alanines, and in construct Dsh ST45 all serine and threonine residues in clusters ST4 and ST5 have been mutated to alanines.



B

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Daxin      MSGHPSGIRKHD-------DNECSGPRPPVPGEESR--------------------VKKM   33
Axin       MQSPKMNVQEQGFPLDLGASFTEDAPRPPVPGEEGELVSTDSRPVNHSFCSGKGTSIKSE   60
Conductin  MSSAVLVTLLP----DPSSSFREDAPRPPVPGEEGE-----TPPCQPSV--GKVQSTKPM   49

Daxin      TEGVA------------DTSKNSSPSYLNWARTLNHLLEDRDGVELFKKYVEEEAPAYND   81
Axin       TSTATPRRSDLDLGYEPEGSASPTPPYLRWAESLHSLLDDQDGISLFRTFLKQEGCA--D  118
Conductin  PVSSNARRNEDGLG-EPEGRASPDSPLTRWTKSLHSLLGDQDGAYLFRTFLEREKCV--D  106
                                                  RGS
Daxin      HLNFYFACEGLKQ----QTDPEKIKQIIGAIYR-FLRKSQLSISDDLRAQIKAIKTNPEI  136
Axin       LLDFWFACSGFRKLEPCDSNEEKRLKLARAIYRKYILDSNGIVSRQTKPATKSFIKDCVM  178
Conductin  TLDFWFACNGFRQMNLKDT---KTLRVAKAIYKRYIEN-NSVVSKQLKPATKTYIRDGIK  162
                      RGS
Daxin      --PLSPHIFDPMQRHVEVTIRDNIYPTFLCSEMYILYIQQMSAQQERCT--SSGA-TGSG  191
Axin       KQQIDPAMFDQAQTEIQSTMEENTYPSFLKSDIYLEYTRTGSESPKVCSDQSSGSGTGKG  238
Conductin  KQQIGSVMFDQAQTEIQAVMEENAYQVFLTSDIYLEYVRSGGENTAYMSN--GGLGSLKV  220

Daxin      SAGSSGSGGSSLAGACALPPTTASGKQQLPQLVPPGAFINL---PVSSVSGPPAGTCSA-  247
Axin       MSGYLPTLNEDEEWKCDQDADEDDGRDPLPPSRLTQKLLLETAAPRAPSSRRYNEGRELR  298
Conductin  LCGYLPTLNEEEEEWTC---ADLKCKLSPTVVG-LSSKTLRATASVRSTETAENG----FR  272

Daxin      ------SGSVYGPSTSASSSGSISATDTLPRSSTLPTLHEDSVLSLCDDFEKVQMQEGGG  301
Axin       YGSWREPVNPYYVNSGYALAPATSANDSEQQSLSSDADT----LSLTD------------  342
Conductin  SFKRSDPVNPYHVGSGYVFAPATSANDSE---LSSDALTDDS-MSMTD------------  316
                                  16-21  8-66
                                  T≥N   R>K
Daxin      SLGSGSVGAGARAPDYPIRLTRDLLIATQKRRLEIRPPGAHGYVYNPSTTNTSYVPNSRV  361
Axin       ---------SSVDGIPPYRI-------------------------------------------  353
Conductin  ---------SSVDGVPPYRMGS-----------------------------------------  329
                                                                  8-13P>S
Daxin      DSERASVSSGGRTDSDTMSISSCSMDGRPYIQRRHSSTESKAIRQSAMANKETNTFQVIP  421
Axin       ----------RKQHRREMQESIQVNGRVPLPHIPRTYRMPKEIR------------VEP  390
Conductin  ----------KKQLQREMHRSVKANGQVSLRHFPRTHRLPKEMT------------PVEP  367
             GSK3β/putative  Zw3  binding
Daxin      RTQRLHSNEHRPLKEEELVSLLIPKLEEVKRKRDLEERARERNPGAALLTNERSSASDRA  481
Axin       QKFAEELI--HRLEAVQRTREAEEKLEERLKRVRMEEEGEDGEMPSGPMASHKLPSVPAW  448
Conductin  AAFAAELI--SRLEKLKLELESRHSLEERLQQIREDEEKEGSEQ-----ALSSRDGAPVQ  420
                      β-catenin/Armadillo  binding
Daxin      FAEAIREKFAL-------DEDNDQDILDQHVSRVWKDQTPHRS-PGTMSPCPPIPSRRRT  533
Axin       HHFPPRYVDMGCSGLRDAHEENPESILDEHVQRVMRTPGCQSPGPG-----HRSPDSGHV  503
Conductin  H--PLALLPSGS------YEEDPQTILDDHLSRVLKTPGCQSPGVGRYSPRSRSPDHHHQ  472

Daxin      ATHDSGMVSDGAMSL-----------------------------------------------  548
Axin       AKTAVLGGTASGHGKHVPKLGLKLDTAG-----LHHHRHVHHHVHHNSARPK--EQMEAE  556
Conductin  HHHHQQCHTLLSTGGKLPPVAACPLLGGKSFLTKQTTKHVHHHYIHHHAVPKTKEEIEAE  532

Daxin      ------------------------------------------------------------  548
Axin       VARRVQSSFSWGPETHGHAK----PRSYSENAGTTLS---AGDLP-FGGKTSAPSKRNTK  608
Conductin  ATQRVRCLCPGGTDYYCYSKCKSHPKAPEPLPGEQFCGSRGGTLPKRNAKGTEPGLALSA  692

Daxin      ----------------------------------------SGHSMKHSKSMPDH-SSC  565
Axin       KAESGKNANAEVPSTT-EDAEKNQKIMQWIIEGEKEISRHRKAGHGSSGLRKQQAHESSR  667
Conductin  R-DGGMSSAAGGPQLPGEEGDRSQDVWQWMLESERQ---SKSKPHSAQSIRKSYPLESAR  648

Daxin      SRKLTNKWPSMNTDSGIS---MFSADTVTKYKDASSRSGSSTASKLEEAKRRLEDEPRRS  622
Axin       PLSIERPGAVHPWVSAQLRNSVQPSHLFIQDPTMPPNPAPNPLTQLEEARRRLEEEEKRA  727
Conductin  AAPGERVSRHHLLGASGHSRSVARAHPFTQDPAMPPLTPPNTLAQLEEACRRLAEVSK--  706

Daxin      RRYAQPPMQHLSQQPLASFSSSSSGGSISLP-----------------HQPPPLPAKPPE  665
Axin       NKLPSKQRYVQAVHQRGRTCVRPACAPVLSVVPAVSDLELSETETKSQRKAGGGSAPPCD  787
Conductin  ---PQKQRCCVASQQRD----RNHSAAGQAGASPFANPSLAPEDHKEPKKLASVHALQAS  759

Daxin      TIVV-FSFCEEPVPYRIKIPGTQPFLRQFKDYLPRRGHFRFFFKTHCEDPDSPVIQEEIV  724
Axin       SIVVGYYFCGEPIPYRTLVRGRAVTLGQFKELLTKKGSYRYYFKKVSDEFDCGVVFEEVR  847
Conductin  ELVVTYFFCGEEIPYRRMLKAQSLTLGHFKEQLSKKGNYRYYFKKASDEFACGAVFEEIW  819
           DIX
Daxin      NDSDILPLFGDKAMGLVKPSD                                         745
Axin       EDEPVLPVFEEKIIGKVEKVD                                         868
Conductin  DDETVLPMYEGRILGKVERID                                        840
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FIGURE 5.—Novel *Daxin* alleles suppress *UAS-dsh* misexpression phenotypes. (A) *Daxin*$^{16\text{-}21}$ suppresses *GMR-Gal4; UAS-dsh* phenotypes in the eye (left) and *69B-Gal4; UAS-dsh* phenotypes in the wing (right). (B) Protein sequence alignment of DAxin, mouse Axin, and Conductin. Numbering of amino acids according to the published sequences is shown. Amino acids that are identical or conserved with respect to DAxin are highlighted in gray. The RGS, DIX, putative GSK3-β-binding site and β-catenin-binding sites are boxed. DAxin mutations are indicated and altered amino acids are boxed.

null embryos. This discrepancy may be due to the fact that different types of rescue assays were used to study the domain requirements of Dsh. We used heat-shock-inducible transgenes that allowed complete rescue to adulthood under nearly physiological conditions, whereas Axelrod *et al.* overexpressed different mutant versions

of Dsh by RNA injection into embryos. The construct we used in our experiments deletes amino acids 287–336, which lie within the PDZ domain, while the construct that Axelrod used removes amino acids 152–333, consisting of the basic domain, CK1–CK4 and ST1–ST5. Thus an alternative explanation is that the construct utilized by Axelrod *et al.* deletes a region that inhibits the activity of Dsh. This is consistent with our observations implicating the basic domain as a negative regulator of dsh activity. Other studies have found a requirement for the PDZ domain in regulating β-catenin stability and in regulating transcription of LEF reporter constructs (Yanagawa *et al.* 1995; Li *et al.* 1999b; Yamamoto *et al.* 1999). In addition, the PDZ domain binds to Axin, FRAT, CK1, CK2, PP2A, and IDAX, proteins that regulate Wg signaling (Willert *et al.* 1997; Li *et al.* 1999a; Peters *et al.* 1999; Sakanaka *et al.* 1999; Strovel *et al.* 2000; Hino *et al.* 2001). Thus, while the PDZ domain may be dispensable for the function of Dsh under certain experimental conditions, it appears to be essential for Wg signaling and tissue polarity under physiological conditions.

SH3-binding domain mutations: We isolated three new *UAS-dsh* alleles that carry mutations in a novel domain of Dsh. This region lies between the PDZ domain and the DEP domain, is proline rich, and possesses a consensus sequence for a class I core SH3 protein-binding motif, RTEPVRP at position 352–358 (Figure 4B; Kay *et al.* 2000). Proline-rich sequences that contain this core domain mediate the binding of these proteins to SH3 proteins. This core motif is conserved in the mammalian homologs of *dsh* and so are the surrounding prolines. *UAS-dsh$^{8\text{-}12}$* mutates the last proline in this core binding motif into a leucine at position 358, and *UAS-dsh$^{8\text{-}79}$* and *UAS-dsh$^{8\text{-}68}$* mutate an aspartic acid at position 360 to valine and a glycine at position 362 to aspartic acid, respectively (Table 2; Figure 4). The proline and aspartic acid residues are conserved between *dsh* and its *dvl* homologs, while the glycine residue is replaced by an alanine residue in the *dvl* genes. *UAS-dsh$^{8\text{-}12}$* and *UAS-dsh$^{8\text{-}79}$* disrupt but do not completely abolish Wg signaling while *UAS-dsh$^{8\text{-}68}$* possesses more Wg signaling activity than does either *UAS-dsh$^{8\text{-}12}$* or *UAS-dsh$^{8\text{-}79}$* (Table 2). This could be because *UAS-dsh$^{8\text{-}68}$* maps further from the core SH3-binding site motif or because the amino acid that it mutates is not conserved. Although the mutations encoded by *UAS-dsh$^{8\text{-}79}$* and *UAS-dsh$^{8\text{-}68}$* do not map within the core SH3-binding consensus motif, it is known that amino acids that surround this motif are important for optimal ligand preference. Studies utilizing combinatorial peptide libraries have defined binding sequences for SH3 proteins and show that proline-rich sequences around the core domain are important for binding (Kay *et al.* 2000).

The identification of mutations in a putative SH3-binding domain is intriguing since these proteins have not been implicated in Wg signaling events. Interestingly, the cytoplasmic tails of Arrow and DFz2 also contain putative SH3-binding domains (Bhanot *et al.* 1996; Wehrli *et al.* 2000). In addition, D-Axin contains putative SH3-binding domains in the RGS domain, the β-catenin-binding domain, and immediately amino terminal to the DIX domain (Hamada *et al.* 1999; Willert *et al.* 1999). SH3 proteins act as adaptors linking signaling molecules into complexes and localizing proteins to the cell membrane (Mayer 2001). Hence it is tempting to speculate that proteins with multiple SH3 domains participate in Wg signaling events, perhaps to localize Dsh and Axin in proximity to the Arrow and DFz2 cell surface receptors. Indeed, Dsh is localized to the cell membrane when it is coexpressed with Fz1 in Xenopus oocytes. It has also been suggested that the DEP domain is important for this localization during planar polarity signaling (Axelrod *et al.* 1998).

DEP domain mutations: The isolation of two planar polarity alleles that encode mutations within the DEP domain agrees with other studies that demonstrate a requirement for this domain in planar polarity signaling (Axelrod *et al.* 1998; Boutros *et al.* 1998). The *dsh^{A3}* mutation maps four amino acids distal to the previously isolated *dsh^{1}* allele and encodes an arginine-to-histidine mutation at position 413 (Table 2; Figure 4). The *dsh^{A3}* and *dsh^{1}* mutations replace a positively charged amino acid with a neutral amino acid, suggesting that Dsh may contact negatively charged proteins during planar polarity signaling or bind to membrane phospholipids. *dsh^{A21}* encodes a cysteine-to-arginine mutation at position 472. Cysteine residues can be palmitoylated and such a lipid modification can be important for cell membrane attachment (although we tried to incorporate labeled palmitate in Dsh and failed to detect any). Thus it is possible that this mutation disrupts the ability of the DEP domain to become membrane localized during planar polarity signaling. On the recently established structure of the DEP domain (Wong *et al.* 2000), the *dsh^{A3}* and *dsh^{1}* mutations are located fairly close to the cysteine that is mutated in *dsh^{A21}*, suggesting that these three residues are collectively involved in the function of the DEP domain (Figure 4D).

Despite the fact that the DEP domain is viewed to be specific for polarity signaling, we did obtain three new *UAS-dsh* alleles that disrupted Wg signaling. These mutations clustered to a region of the DEP domain extending from position 440 to 459, away from the *dsh* planar polarity alleles (Table 2; Figure 4). This indicates that the DEP domain is required for Wg signaling, in agreement with findings that *dsh* constructs that lack the DEP domain cannot rescue the embryonic lethality of a *dsh* null allele (Axelrod *et al.* 1998; Boutros *et al.* 1998; Li *et al.* 1999b; this study).

Although all *UAS-dsh* alleles that were tested lost or attenuated the Wg signaling function, nearly all caused planar polarity defects when expressed in the wing. Both gain and loss of signaling activity alter planar cell polar-

ity. Therefore, it is hard to determine whether these alleles contain mutations that are specific for Wg signaling and that leave planar polarity functions intact, or if they act as dominant negatives for this function. Another possibility is that the Wg signaling function of *dsh* is more sensitive to perturbations in *dsh* activity than is the planar polarity function and that these alleles behave as hypomorphs. Indeed we find that lower levels of a *dsh* transgene are required to rescue planar polarity functions of *dsh* than to rescue Wg signaling functions (Table 3). Three *dsh* alleles exist that specifically perturb planar polarity, however, arguing that planar polarity functions and Wg signaling functions are separable. In addition, when constructs that contain the dsh^1 mutation are overexpressed they cannot rescue the endogenous dsh^1 mutation, arguing that dsh^1 is not a hypomorph and that Dsh acts as a modular protein (Boutros *et al.* 1998).

Phosphorylation mutants: Dsh is a phosphoprotein and Wg signaling generates hyperphosphorylated forms of Dsh, which are enriched in membrane fractions in biochemical assays (Yanagawa *et al.* 1995). While this finding suggests that the phosphorylation state of Dsh may regulate its activity, we did not isolate any mutations in putative phosphorylation sites in the forward screens. Furthermore, *in vitro* mutagenesis of putative phosphorylation sites of Dsh in a region spanning amino acids 178–254 did not reveal a requirement for these sites in the Wg signaling function of Dsh. Thus, phosphorylation sites in Dsh may be redundant and more than one site may need to be mutated to produce a phenotype. This conclusion is supported by phosphotryptic mapping experiments, which identified at least three phosphorylation sites in Dsh (Willert *et al.* 1997).

Surprisingly, however, region ST4 is essential for planar polarity signaling (Figure 4C; Table 3). This region was previously shown to bind the protein kinase PAR-1, which is thought to act in Wg signaling rather than in tissue polarity (Sun *et al.* 2001). We note, however, that the PAR-1 kinase is implicated in generating cell asymmetry in *Caenorhabditis elegans* (Guo and Kemphues 1995) and in the Drosophila oocyte (Shulman *et al.* 2000; Tomancak *et al.* 2000; Cox *et al.* 2001; Huynh *et al.* 2001). These results point to a potential role of PAR-1-mediated Dsh phosphorylation in planar polarity.

Second-site suppressors; mutations in *axin*: In addition to mutations in the *UAS-Dsh* transgene itself, the *UAS-dsh* misexpression screen yielded second-site modifiers on the third and fourth chromosome. Modifiers on the first and second chromosome could not be recovered due to the strategy of our screen (Figure 1C). Five of the second-site modifiers map near *axin* and were indeed found to contain mutations within the *axin* gene (Figure 5B). They behave as dominant suppressors of Dsh misexpression phenotypes in both the wing and eye (Figure 5A) but do not modify Wg or DFz2 misexpression phenotypes (data not shown). In addition, these alleles are homozygous viable and have no phenotype when they are recombined away from *UAS-dsh*. Since Axin normally suppresses Wg signaling, and null *axin* alleles do not interact with *UAS-dsh*, we infer that these alleles specifically suppress overexpressed forms of Dsh but do not affect Dsh that is regulated by Wg signaling. This would imply that overexpressed Dsh works through a mechanism that is different from Dsh that is activated by Wg. For example, overexpressed Dsh may interact with Axin through binding to a domain that is different from the Axin domain that interacts with Wg-activated Dsh.

Conclusions: This work and other studies suggest that Dsh is a modular protein with specific domains dedicated to Wg and planar polarity signaling. How is Dsh activity regulated and how does it mediate Wg signaling? The targeting of Dsh to the cell membrane and the regulation of its phosphorylation state are correlated with Wg and planar polarity signaling. The DEP domain is necessary to localize Dsh to the cell membrane (Axelrod *et al.* 1998) and we have identified a putative SH3-binding site that may also be important for membrane localization of Dsh. It will be interesting to examine the phosphorylation state of the Dsh protein that is produced from the alleles generated in these screens and to determine if protein from alleles that mutate the potential SH3-binding site are membrane localized. CK1, CK2, and PAR-1 bind to Dsh and phosphorylate it. Moreover, CK1 and PAR-1 are positive regulators of Wg signaling that promote stabilization of β-catenin and induce the expression of Wnt target genes (Willert *et al.* 1997; Peters *et al.* 1999; Sakanaka *et al.* 1999; Sun *et al.* 2001). Thus, Dsh localization to the cell membrane may be controlled by CK1 and PAR-1, which may lead to changes in Dsh activity.

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LITERATURE CITED

Adler, P. N., C. Vinson, W. J. Park, S. Conover and L. Klein, 1990 Molecular structure of frizzled, a Drosophila tissue polarity gene. Genetics **126:** 401–416.

Ashburner, M., 1989 *Drosophila: A Laboratory Handbook.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Axelrod, J. D., J. R. Miller, J. M. Shulman, R. T. Moon and N. Perrimon, 1998 Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. Genes Dev. **12:** 2610–2622.

Bate, M., and A. Martinez Arias, 1993 *The Development of Drosophila melanogaster.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Behrens, J., B. A. Jerchow, M. Wurtele, J. Grimm, C. Asbrand *et al.*, 1998 Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3 beta. Science **280:** 596–599.

Bhanot, P., M. Brink, C. Harryman Samos, J. C. Hsieh, Y. S. Wang *et al.*, 1996 A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature **382:** 225–230.

Bhanot, P., M. Fish, J. A. Jemison, R. Nusse, J. Nathans *et al.*, 1999 Frizzled and Dfrizzled-2 function as redundant receptors

for Wingless during Drosophila embryonic development. Development **126:** 4175–4186.

Blair, S. S., 1992 Engrailed expression in the anterior lineage compartment of the developing wing blade of Drosophila. Development **115:** 21–33.

Boutros, M., and M. Mlodzik, 1999 Dishevelled: at the crossroads of divergent intracellular signaling pathways. Mech. Dev. **83:** 27–37.

Boutros, M., N. Paricio, D. I. Strutt and M. Mlodzik, 1998 Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell **94:** 109–118.

Boutros, M., J. Mihaly, T. Bouwmeester and M. Mlodzik, 2000 Signaling specificity by Frizzled receptors in Drosophila. Science **288:** 1825–1828.

Brand, A. H., and N. Perrimon, 1993 Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:** 401–415.

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

Cadigan, K., and R. Nusse, 1997 Wnt signaling: a common theme in animal development. Genes Dev. **11:** 3286–3305.

Chen, C. M., and G. Struhl, 1999 Wingless transduction by the Frizzled and Frizzled2 proteins of Drosophila. Development **126:** 5441–5452.

Cox, D. N., B. Lu, T. Q. Sun, L. T. Williams and Y. N. Jan, 2001 Drosophila par-1 is required for oocyte differentiation and microtubule organization. Curr. Biol. **11:** 75–87.

Diaz-Benjumea, F. J., and S. M. Cohen, 1995 Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. Development **121:** 4215–4225.

Guo, S., and K. J. Kemphues, 1995 par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell **81:** 611–620.

Hamada, F., Y. Tomoyasu, Y. Takatsu, M. Nakamura, S. Nagai *et al.*, 1999 Negative regulation of Wingless signaling by D-axin, a Drosophila homolog of axin. Science **283:** 1739–1742.

Hart, M. J., R. de los Santos, I. N. Albert, B. Rubinfeld and P. Polakis, 1998 Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Curr. Biol. **8:** 573–581.

Hino, S., S. Kishida, T. Michiue, A. Fukui, I. Sakamoto *et al.*, 2001 Inhibition of the wnt signaling pathway by idax, a novel dvl-binding protein. Mol. Cell. Biol. **21:** 330–342.

Huynh, J. R., J. M. Shulman, R. Benton and D. St Johnston, 2001 PAR-1 is required for the maintenance of oocyte fate in Drosophila. Development **128:** 1201–1209.

Ikeda, S., S. Kishida, H. Yamamoto, H. Murai, S. Koyama *et al.*, 1998 Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. EMBO J. **17:** 1371–1384.

Kay, B. K., M. P. Williamson and M. Sudol, 2000 The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. FASEB J. **14:** 231–241.

Kishida, S., H. Yamamoto, S. Hino, S. Ikeda, M. Kishida *et al.*, 1999 DIX domains of dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability. Mol. Cell. Biol. **19:** 4414–4422.

Klingensmith, J., R. Nusse and N. Perrimon, 1994 The Drosophila segment polarity gene dishevelled encodes a novel protein required for response to the wingless signal. Genes Dev. **8:** 118–130.

Kloss, B., J. L. Price, L. Saez, J. Blau, A. Rothenfluh *et al.*, 1998 The Drosophila clock gene double-time encodes a protein closely related to human casein kinase Iepsilon. Cell **94:** 97–107.

Krasnow, R. E., L. L. Wong and P. N. Adler, 1995 dishevelled is a component of the frizzled signaling pathway in Drosophila. Development **121:** 4095–4102.

Li, L., H. Yuan, C. D. Weaver, J. Mao, G. H. Farr, III *et al.*, 1999a Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. EMBO J. **18:** 4233–4240.

Li, L., H. Yuan, W. Xie, J. Mao, A. M. Caruso *et al.*, 1999b Dishevelled proteins lead to two signaling pathways. Regulation of LEF- 1 and c-Jun N-terminal kinase in mammalian cells. J. Biol. Chem. **274:** 129–134.

Mayer, B. J., 2001 SH3 domains: complexity in moderation. J. Cell Sci. **114:** 1253–1263.

Moses, K., and G. M. Rubin, 1991 Glass encodes a site-specific DNA-binding protein that is regulated in response to positional signals in the developing Drosophila eye. Genes Dev. **5:** 583–593.

Noordermeer, J., P. Johnston, F. Rijsewijk, R. Nusse and P. A. Lawrence, 1992 The consequences of ubiquitous expression of the wingless gene in the Drosophila embryo. Development **116:** 711–719.

Noordermeer, J., J. Klingensmith, N. Perrimon and R. Nusse, 1994 *dishevelled* and *armadillo* act in the *wingless* signalling pathway in *Drosophila*. Nature **367:** 80–83.

Pastink, A., C. Vreeken, E. W. Vogel and J. C. Eeken, 1990 Mutations induced at the white and vermilion loci in Drosophila melanogaster. Mutat. Res. **231:** 63–71.

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

Perrimon, N., and A. P. Mahowald, 1987 Multiple functions of segment polarity genes in Drosophila. Dev. Biol. **119:** 587–600.

Peters, J. M., R. M. McKay, J. P. McKay and J. M. Graff, 1999 Casein kinase I transduces Wnt signals. Nature **401:** 345–350.

Rulifson, E. J., C. H. Wu and R. Nusse, 2000 Pathway specificity by the bifunctional receptor frizzled is determined by affinity for wingless. Mol. Cell **6:** 117–126.

Sakanaka, C., J. B. Weiss and L. T. Williams, 1998 Bridging of beta-catenin and glycogen synthase kinase-3beta by Axin and inhibition of beta-catenin-mediated transcription. Proc. Natl. Acad. Sci. USA **95:** 3020–3023.

Sakanaka, C., P. Leong, L. Xu, S. D. Harrison and L. T. Williams, 1999 Casein kinase iepsilon in the wnt pathway: regulation of beta-catenin function. Proc. Natl. Acad. Sci. USA **96:** 12548–12552.

Salic, A., E. Lee, L. Mayer and M. W. Kirschner, 2000 Control of beta-catenin stability: reconstitution of the cytoplasmic steps of the wnt pathway in Xenopus egg extracts. Mol. Cell **5:** 523–532.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shulman, J. M., R. Benton and D. St Johnston, 2000 The Drosophila homolog of C. elegans PAR-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. Cell **101:** 377–388.

Siegfried, E., T. B. Chou and N. Perrimon, 1992 wingless signaling acts through zeste-white 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. Cell **71:** 1167–1179.

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

Smalley, M. J., E. Sara, H. Paterson, S. Naylor, D. Cook *et al.*, 1999 Interaction of axin and Dvl-2 proteins regulates Dvl-2-stimulated TCF-dependent transcription. EMBO J. **18:** 2823–2835.

Spradling, A. C., 1986 P element-mediated transformation, pp. 175–197 in *Drosophila: A Practical Approach*, edited by D. B. Roberts. IRL Press, Oxford.

Strapps, W. R., and A. Tomlinson, 2001 Transducing properties of Drosophila Frizzled proteins. Development **128:** 4829–4835.

Strovel, E. T., D. Wu and D. J. Sussman, 2000 Protein phosphatase 2Calpha dephosphorylates axin and activates LEF-1-dependent transcription. J. Biol. Chem. **275:** 2399–2403.

Strutt, D. I., U. Weber and M. Mlodzik, 1997 The role of RhoA in tissue polarity and Frizzled signalling. Nature **387:** 292–295.

Sun, T. Q., B. Lu, J. J. Feng, C. Reinhard, Y. N. Jan *et al.*, 2001 PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. Nat. Cell Biol. **3:** 628–636.

Tamai, K., M. Semenov, Y. Kato, R. Spokony, C. Liu *et al.*, 2000 LDL-receptor-related proteins in Wnt signal transduction. Nature **407:** 530–535.

Theisen, H., J. Purcell, M. Bennett, D. Kansagara, A. Syed *et al.*, 1994 dishevelled is required during wingless signaling to establish both cell polarity and cell identity. Development **120:** 347–360.

Tomancak, P., F. Piano, V. Riechmann, K. C. Gunsalus, K. J. Kemphues *et al.*, 2000 A Drosophila melanogaster homologue of Caenorhabditis elegans par-1 acts at an early step in embryonic-axis formation. Nat. Cell Biol. **2:** 458–460.

van de Wetering, M., R. Cavallo, D. Dooijes, M. van Beest, J. van Es *et al.*, 1997 Armadillo co-activates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell **88:** 789–799.

Wehrli, M., S. T. Dougan, K. Caldwell, L. O'Keefe, S. Schwartz *et al.*, 2000 arrow encodes an LDL-receptor-related protein essential for Wingless signalling. Nature **407:** 527–530.

Wieschaus, E., C. Nüsslein-Volhard and G. Jürgens, 1984 Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. Roux's Arch. Dev. Biol. **193:** 296–307.

Willert, K., M. Brink, A. Wodarz, H. Varmus and R. Nusse, 1997 Casein kinase 2 associates with and phosphorylates dishevelled. EMBO J. **16:** 3089–3096.

Willert, K., C. Y. Logan, A. Arora, M. Fish and R. Nusse, 1999 A Drosophila Axin homolog, Daxin, inhibits Wnt signaling. Development **126:** 4165–4173.

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

Wodarz, A., U. Hinz, M. Engelbert and E. Knust, 1995 Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. Cell **82:** 67–76.

Wong, H. C., J. Mao, J. T. Nguyen, S. Srinivas, W. Zhang *et al.*, 2000 Structural basis of the recognition of the dishevelled DEP domain in the wnt signaling pathway. Nat. Struct. Biol. **7:** 1178–1184.

Yamaguchi, T. P., 2001 Heads or tails: Wnts and anterior-posterior patterning. Curr. Biol. **11:** R713–R724.

Yamamoto, H., S. Kishida, M. Kishida, S. Ikeda, S. Takada *et al.*, 1999 Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability. J. Biol. Chem. **274:** 10681–10684.

Yanagawa, S., F. Van Leeuwen, A. Wodarz, J. Klingensmith and R. Nusse, 1995 The Dishevelled protein is modified by Wingless signaling in Drosophila. Genes Dev. **9:** 1087–1097.

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