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A single *frizzled* protein has a dual function in tissue polarity

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SUMMARY

The *Drosophila frizzled* (*fz*) gene is required for the development of normal tissue polarity in the epidermis. Genetic epistasis experiments argue that *fz* is at the top of a regulatory hierarchy that controls the subcellular site for prehair initiation within the cells of the pupal wing (Wong and Adler, 1993; *J. Cell Biol.* 123, 209-221). Genetic mosaic experiments indicate that *fz* has both cell autonomous and cell non-autonomous functions that are separately mutable (Vinson and Adler, 1987; *Nature* 329, 549-551). Two species of *fz* mRNA have been identified, raising the question as to whether the two functions are provided by a single protein or by two separate protein species. We generated transgenic flies that express each of these mRNAs under the control of an hsp70 promoter. Only one of the transgenes (*hsfzI*) showed any *fz* activity. At 29°C, the *hsfzI* transgene provided almost complete rescue of a null *fz* mutation, indicating that the protein encoded by this cDNA can fulfill both *fz* functions. Overexpression of the *hsfzI* transgene resulted in two distinct tissue polarity phenotypes depending on the time of heat shock.

Key words: *Drosophila melanogaster*, *frizzled*, *inturned*, overexpression, tissue polarity

INTRODUCTION

In *Drosophila*, adult cuticular structures typically display a common local orientation; bristle sense organs and hairs (trichomes) point posteriorly on the thorax and abdomen and distally on appendages. This alignment presumably reflects the tissue polarity of the epidermis that formed the cuticle (see Adler, 1992 for review). Six genetic loci have been studied that are required for the development of normal tissue polarity in the wing. These are *frizzled* (*fz*), *prickle*/*spinyleg* (*pk/sple*), *dishevelled* (*dsh*), *inturned* (*in*), *fuzzy* (*fy*) and *multiple wing hair* (*mwh*) (Gubb and Garcia-Bellido, 1982; Adler, 1992; Wong and Adler, 1993). Mutations in these genes cause defects in both hair polarity and number. Based on differences in the mutant phenotypes and epistasis data, Wong and Adler (1993) proposed that the tissue polarity genes are part of a genetic pathway that controls hair polarity by specifying the subcellular location for prehair initiation.

The best characterized tissue polarity gene is *fz* (Adler et al., 1987, 1990, 1994a; Vinson and Adler, 1987; Vinson et al., 1989; Park et al., 1994a,b). An extensive cDNA clone analysis revealed evidence for two classes of *fz* transcripts that differ by the use of an alternative 3′ exon (Adler et al., 1990). The more abundant transcript (which we refer to as *fz*I) is encoded by five exons and encodes an integral membrane protein (FzI) with an odd number (probably 7) of transmembrane (TM) domains (Park et al., 1994a). The less abundant transcript (*fz*II) encodes a protein (FzII) that is essentially a truncated version of the FzI protein. It is expected to contain four transmembrane domains (Adler et al., 1990). Mutations that inactivate the *fz* gene are spread over approximately 100 kb of genomic DNA (Vinson et al., 1989; Adler et al., 1990) and the large size and exon structure is conserved in the *Drosophila virilis* homologue (K. H. Jones and P. N. Adler, unpublished data). The large gene size could be required for complex spatial or temporal regulation of *fz* gene expression.

Studies in genetic mosaics demonstrated that *fz* has two functions (Vinson and Adler, 1987). In mutant mitotic clones in the wing, most *fz* alleles (including null alleles) behave cell non-autonomously. Wild-type cells distal to a clone display abnormal hair polarity. This suggests that *fz* function is required for transmission of an intercellular polarity signal along the proximodistal axis of the wing. However, some *fz* alleles behave cell autonomously in clones, suggesting that *fz* function is also required for cells to respond to the intercellular polarity signal (Vinson and Adler, 1987; Wong and Adler, 1993). These two functions of the *fz* locus (cell non-autonomous and cell autonomous) could require proteins made from both *fz* transcripts or a single protein made from either.

To evaluate the functions of the two *fz* transcripts, we generated transgenic flies that express each of the two cDNAs under the control of a heat-shock promoter (*hsfzI* and *hsfzII*). In this study, we report that ubiquitous expression of *hsfzI*, but not *hsfzII*, can rescue the *fz* mutant phenotype. This demonstrates that the FzI protein can provide both the cell non-autonomous and cell autonomous *fz* functions for tissue polarity in the epidermis. We also find that during pupal development heat-shock induced overexpression of *hsfzI*, but not *hsfzII*, results in a tissue polarity phenotype. Early heat shocks (6 hours or more before prehair initiation) result in a phenotype

that resembles *fz, pk* and *dsh* mutants (Wong and Adler, 1993). Later heat shocks (3 hours or less before prehair initiation) result in a phenotype that resembles *in* and *fy* mutants (Wong and Adler, 1993).

MATERIALS AND METHODS

Stocks

Most stocks are described in Lindsley and Zimm (1992). Several were obtained from the *Drosophila* stock centers at Indiana University and Bowling Green State University. The *fz* alleles used here have been described previously (Adler et al., 1987, 1994a). The *in* alleles (in^{HC31}, in^{IH56} and $in^{II53)}$ were isolated in this laboratory and are described elsewhere (Adler et al.,1994b). The genotypes shown in Fig. 8 are the following: fz^{HD21}/fz^{D21}, 29°C (*fz* weak); fz^{HD21}/fz^{D21}, 18°C (*fz* moderate); fz^{R52}/fz^{D21} (*fz* strong); in^{HC31}/in^{Df} (*in* weak); in^{II53}/in^{Df} (*in* moderate); in^{IH56}/in^{Df} (*in* strong).

Construction of *hsfz* transgenic flies

fz cDNAs were cloned into a modified Bluescript vector, pBHS, which contains the hsp70 promoter from pCaSpeR and an SV40 poly(A) addition sequence (Park et al., 1994a). For construction of *hsfzI*, the *fz*-coding region was amplified using PCR and cloned into pBHS (see Park et al., 1994a for details). For *hsfzII*, a *Hin*dIII-*Eco*RI fragment was subcloned into pBHS. For both constructs, a *Kpn*I-*Not*I fragment containing the hsp70-*fz* cDNA-poly(A) site cassette was subcloned into the pW8 P-element transformation vector. Germline transformation was performed according to Spradling and Rubin (1982).

Scoring of tissue polarity phenotypes

Tissue polarity phenotypes involve both abnormal hair polarity and hair number. To score the hair number phenotype, we counted the number of cells that form more than one hair (multiple hair cells) in a defined region of the wing on the dorsal surface. Abnormal hair polarity is inherently more difficult to quantify. To do this, we calculated the fractional area of the wing with grossly abnormal hair polarity. The criterion that we used for 'abnormal' was having a polarity that is 45° or greater different from wild type. To calculate the fractional area, we marked abnormal regions of individual wings on a diagram. We then scanned the diagrams (Adobe Photoshop) and measured the marked areas (NIH Image program). Slides were scored blind and statistical significance was determined using a non-parametric test (Instat program - Mann-Whitney).

Induction of *hsfz* transgenes

For 29°C inductions, eggs were collected in vials at 25°C and then moved to a 29°C air incubator within 1-5 days. For 37°C heat-shock inductions, white prepupae were transferred to fresh vials, aged at 25°C and submerged in a 37°C water bath for 1 hour.

Mounting fly wings

Flies were stored in 70% ethanol. Wings were dehydrated in 100% ethanol and mounted in euparal.

Scanning electron microscopy

Flies were dehydrated in ethanol and desiccated in a critical point dryer before sputter coating with platinum.

Mitotic clone analysis

Female flies containing a hair morphology marker *forked* (f^{36a}) (cytological map location 15F) were crossed to males containing a copy of *hsfzI* located distal to *forked* (cytological map location 10C-D) on the X chromosome. Clones were induced by gamma-irradiation at 3-4 days after egg lay (AEL). To ensure that most pupae received at least some heat induction during the sensitive period, irradiated vials of larvae and pupae were heat shocked daily from day 5-8 AEL (for 1 hour at 37°C). Heat-shocked control clones (generated in non-transgenic background) had no tissue polarity phenotype.

Western blot analysis and immunostaining

Western analysis of Fz protein was done as previously described (Park et al., 1994a). Briefly, to obtain an adequate signal-to-noise ratio, it is essential to do a partial membrane purification of the sample: tissues were dissected in PBS, transferred to hypotonic buffer (0.1× BSS = 1.0 mM Tricine base, 5.5 mM NaCl, 4.0 mM KCl, 0.7 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5.0 mM sucrose, pH 6.95; Wilcox, 1986) and homogenized in a microcentrifuge tube. The homogenate was centrifuged for 1 minute at 1,000 ***g*** in a microfuge to remove large pieces of debris. The supernatant was then recentrifuged for 10 minutes at 13,000 ***g***. The resulting pellet is composed of small vesicular material and contains essentially all of the Fz protein remaining in the sample after removal of poorly homogenized material in the first pellet. This second pellet was solubilized in SDS sample buffer and run on SDS-PAGE without boiling the sample. Fz protein was detected with a 1:10 dilution of 1C11 monoclonal antibody (Park et al., 1994a) and the ECL detection system (Amersham). In some experiments, the blot was washed and reprobed with an anti-actin monoclonal antibody, to control for variations in loading. Due to our use of prestained molecular weight markers in these experiments, the sizes reported here are only approximations. For quantitation of westerns, we scanned the films (Adobe Photoshop) and measured the average density of the bands (the NIH image program). Immunostaining of pupal wings was done as described in Park et al. (1994a).

Actin staining

Pupal wings were stained with rhodamine-labelled phalloidin, as previously described (Wong and Adler, 1993).

RESULTS

We generated transgenic flies in which each of the two classes of *fz* cDNAs is controlled by the *hsp70* promoter (*hsfzI* and *hsfzII*) (see Materials and Methods for a description of the constructions). We obtained five *hsfzI* lines and two *hsfzII* lines. All the transgenic lines were homozygous viable and phenotypically wild type at 25°C. Whenever tested, all lines of a given type behaved similarly, with only slight quantitative variation. Therefore, we will refer to all lines of each type as either *hsfzI* or *hsfzII*.

hsfz transgenes direct expression of Fz proteins in the pupal wing

Endogenous Fz protein is expressed uniformly on the surface of pupal wing cells before and during hair development (Park et al., 1994a, b). To confirm that the proteins from both transgenic lines were expressed normally, we immunostained pupal wings from heat-shocked *hsfzI* and *hsfzII* lines. The pattern of staining at the cell periphery was similar to the pattern of the endogenous protein (Fig. 1D), indicating that Fz proteins expressed from both *hsfz* lines accumulated in or near the plasma membrane after heat induction. The level of staining in both lines (Fig. 1A,B) was greatly increased compared to wild-type controls (Fig. 1C). Cytoplasmic staining was also elevated in the transgenic lines, possibly due to a buildup of overexpressed Fz proteins in the Golgi or a basal endosome.

To verify that both *hsfz* lines expressed stable Fz protein of

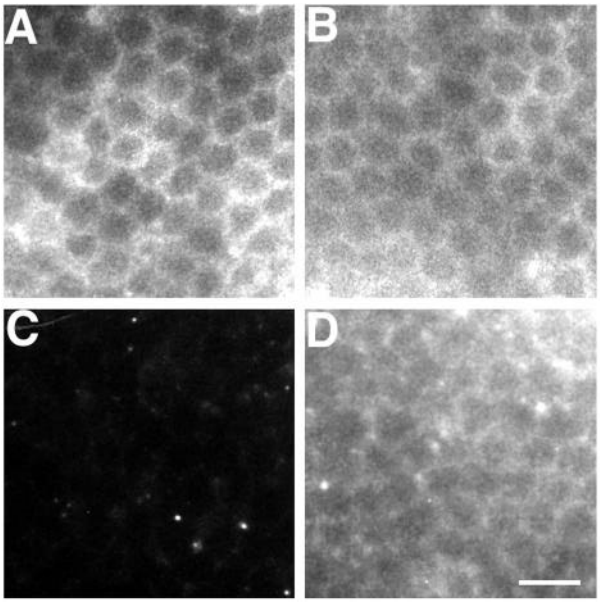

Fig. 1. Immunostaining of hsfzI, hsfzII and wild-type Fz proteins in the pupal wing. Pupae were heat shocked at 37°C for 1 hour and allowed to recover for 1 hour at 25°C before immunostaining with anti-Fz antibody. To compare the relative intensity of staining, the preparations shown in A-C were photographed at the same exposure and all subsequent processing of the images was identical. (A) *hsfzI* (B) *hsfzII* (C) wild type (D) The same wing as in C photographed with a longer exposure to show the honeycomb pattern of membrane staining of the endogenous protein (see also W. Park et al., 1993). Note the higher intensity of the staining in A and B compared to C and the similarity of the pattern in A, B and D. The endogenous Fz staining is typically quite faint due to the rarity of the protein. Bar, 10 μM.

the predicted size, we used western blot analysis of protein derived from whole pupae. As expected, *hsfzI* lines (Fig. 2A) expressed a Fz protein (FzI) that migrated as a broad band at approximately 60×10^3 M_r, consistent with its open reading frame of 581 amino acids (Vinson et al., 1989). The 60×10^3 M_r band comigrated with endogenous Fz protein extracted from wild-type controls (Fig. 2C) and remained elevated for greater than 36 hours after heat shock. In some experiments, the 60×10^3 M_r band resolved into a doublet, as is also seen with the endogenous protein (Park et al., 1994a). We also detected a much larger form of FzI protein; the apparent size of this form, ~125×10^3 M_r, suggests that it could be a dimeric form of the FzI protein (see below). We note that this form of the FzI protein appears to accumulate, relative to the monomeric form of the protein, over time.

hsfzII lines (Fig. 2B) expressed a smaller protein (FzII) that migrated as a doublet at ~42×10^3 M_r, consistent with its smaller open reading frame of 415 amino acids. No endogenous Fz protein of this size was detected. The 42×10^3 M_r *hsfzII* protein was detectable for about 5 hours after heat shock. Surprisingly, most of the heat-inducible *hsfzII* protein migrated at an apparent relative molecular mass of ~86×10^3 and an additional minor species migrated at ~168×10^3 M_r. The apparent sizes of the larger protein species are very close to calculated sizes of dimeric and tetrameric forms of the FzII protein (84 and 168×10^3 M_r). As noted above, we also detected a form of FzI protein that is close to the expected size of an FzI dimer. These observations raised the possibility that we were detecting Fz multimers. To test this idea, we examined extracts from pupae that overexpressed both transgenes. In these pupae, we detected a new band at the expected size of an FzI:FzII heterodimer (~100×10^3 M_r) (not shown), consistent with the idea that overexpressed Fz proteins may multimerize under certain conditions. As we have seen no genetic evidence of Fz dimerization (such as dominant negative alleles), or evidence of Fz dimers in extracts from wild-type pupae, the significance of these putative multimers remains unclear. Another example of SDS-resistant multimers of a membrane protein has recently been reported (Weisz et al., 1993).

hsfzI rescues *fz* mutant phenotypes

To assess the ability of the individual transgenes to rescue a *fz* mutant phenotype, we crossed the transgenes into a *fz* mutant background and induced expression by a variety of treatments. Rescue of the adult mutant phenotype was judged by

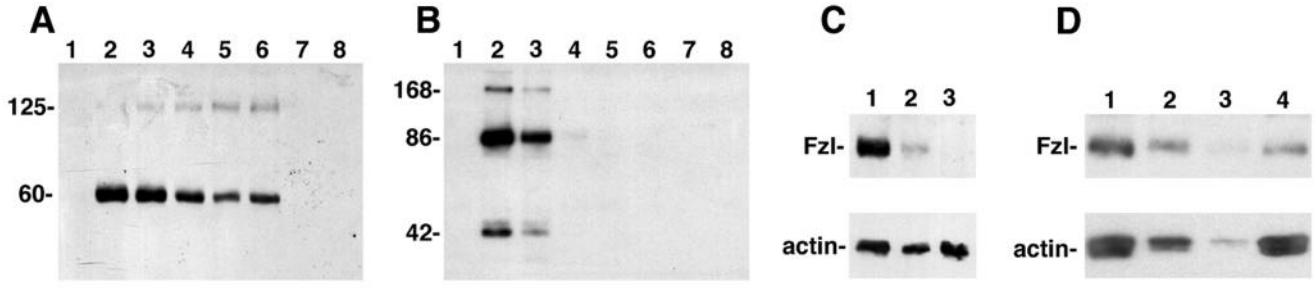

Fig. 2. Western analysis of FzI and FzII protein stability and size. (A,B) Time course of decay of the Fz proteins expressed after a 1 hour heat induction of *hsfz* transgenes. (A) *hsfzI* (B) *hsfzII.* Lanes in A and B are as follows: (1) no heat shock (2) 1 hour (3) 5 hours (4) 10 hours (5) 24 hours and (6) 36 hours after heat shock. (C) Fz protein (above) from pupae raised at 29°C. Actin protein (below) was used as a control for sample loading. Each lane contains protein from 10 pupal wings. Lane (1) wild type (2) *hsfzI; fz.* Note the amount of Fz protein produced under rescue conditions is significantly less than wild type. (3) *fz*. In these experiments, fz^{K21}/fz^{3} was used instead of fz^{K21}/fz^{D21} as a negative control. This is also a protein null genotype. (D) The relative amount Fz protein in a line with multiple copies of *hsfzI* compared to the endogenous level. Actin controls for loading are shown below. (1-3) A dilution series of Fz protein extracts from *hsfzI* pupal wings. The number of wing equivalents in each extract are as follows: lane 1, 10 *hsfzI* wings; lane 2, 5 *hsfzI* wings; lane 3, 2.5 *hsfzI* wings; lane 4, 10 wild-type wings. Note that 10 wild-type wings have roughly the same amount of Fz protein as 5 *hsfzI* wings. By densitometry, we estimate that the Fz protein in this multi-copy *hsfzI* line is approximately 2.3× the endogenous level.

comparing the polarity of thoracic bristles and wing hairs to both wild-type and mutant controls. We found that heat-shock inductions (37°C for 1 hour) of *hsfzI*, but not *hsfzII*, during early pupal development resulted in dramatic improvement of the *fz* null phenotype (fz^{-}). We obtained the best rescue, of both the wing (Fig. 3) and thoracic (Fig. 4) phenotypes, when we raised *hsfzI;* fz^{-} flies at 29°C throughout development (see Figs 3D,4D). We postulate that growth at 29°C mimics wild-type expression, by providing low-level expression of the transgene over an extended time.

To quantify the degree of rescue, we scored two distinct aspects of the *fz* phenotype: (1) the area of the adult wing with abnormal hair polarity (Fig. 5) and (2) the number of multiple hair cells (although compared to other tissue polarity mutants, *fz* wings have relatively few multiple hair cells, this number is a good measure of the severity of the *fz* phenotype. [see Fig. 8 and Adler et al., 1987]). The data showed that *hsfzI* provided an average of 94% rescue of both phenotypes in the presence of a null allele for the endogenous *fz* gene (Fig. 6A,B) and, in most cases, 100% rescue of the weak fz^{R53} allele (average 99% rescue of the polarity defect and 96% rescue of the multiple hair cell phenotype) (Fig. 6C,D). Although the 29°C treatment results in nearly perfect rescue of the null wing phenotype, most wings had a slight residual phenotype that was usually less severe than our weakest allele fz^{R53} (Fig. 5B, E). We believe that this slight phenotype represents a quantitative rather than a qualitative deficit, as some wings showed complete rescue. Because *fz* mutations are recessive, we expected that the amount of Fz protein in *hsfzI;* fz^{-} flies reared at 29°C would be less than half of the wild-type level. Western blot analysis of pupal wing extracts confirmed this prediction (Fig. 2C). We estimate from densitometry that under rescue conditions the pupal wings contain approximately 20% of the wild-type levels of Fz protein. This result indicates that *hsfzI* activity is comparable to that of the endogenous gene; i.e., overexpression of *hsfzI* is not necessary to bypass a requirement for a second gene product (e.g., the FzII protein).

In addition to thoracic and wing phenotypes, *fz* mutants have rough eyes and misoriented leg and abdominal bristles. We did not attempt to quantify rescue of these phenotypes. Nevertheless, it was clear that *hsfzI* (but not *hsfzII*) significantly rescued these phenotypes as well.

In contrast to the dramatic rescue activity that we observed with *hsfzI*, induction of *hsfzII*, by any heat-shock regimen that we tested, had no effect on either the null (Figs 3C, 5C, 6A,B) or weak *fz* phenotypes (Figs 5F, 6C,D).

Overexpression of *hsfzI* results in tissue polarity phenotypes

To determine whether *fz* overexpression affects tissue polarity development, we used lines that carried either *hsfzI* or *hsfzII* transgenes in a wild-type genetic background (fz^{+}) and heat shocked at various times during late larval and pupal development. We found that heat-shock inductions of *hsfzI* pupae between 0 and 36 hours after pupariation (AP) resulted in abnormal wing hair polarity and hair number phenotypes similar to those of tissue polarity mutants. Heat shocks of *hsfzI* pupae also resulted in weak thoracic bristle (Fig. 4B) and roughened eye (not shown) phenotypes, like those of other tissue polarity mutants, but we did not attempt to study these further. In contrast, heat-shock inductions of *hsfzII* pupae had no detectable effect.

Developmental timing determines the quality of the *fz* overexpression phenotype

The sensitive period for *hsfzI* overexpression in the wing (0-36 hours AP) corresponds to the period before prehair initation in the pupal wing (Wong and Adler, 1993). After the start of prehair initiation (approximately 36 hours AP), heat-shock induction of *hsfzI* had no effect (compare Fig. 7E,F). Thus, wing hair differentiation is sensitive to *hsfzI* overexpression only before prehair initiation.

The quality of the *hsfzI* overexpression phenotypes depended on the time of heat shock. Early heat shocks (0-30 hours AP, i.e., greater than 6 hours before prehair initiation) resulted in major disruptions of hair polarity and occasional multiple hair cells. These phenotypes resembled *fz*, *dsh* and *pk* mutants (compare Fig. 7A,B). In contrast, late heat shocks (33-36 hours AP, i.e., less than 3 hours before prehair initiation) resulted in numerous multiple hair cells as well as abnormal hair polarity. These phenotypes closely resembled *in* and *fy* mutants (compare Fig. 7C,D). We will refer to these two types of phenotypes as the early (or *fz*-like) and late (or *in*-like) *hsfzI* overexpression phenotypes respectively.

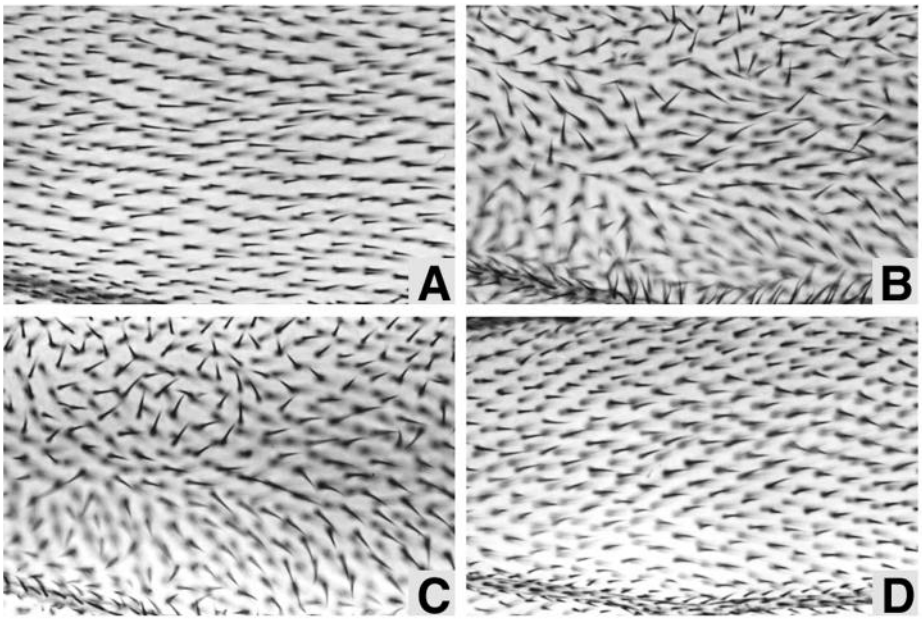

Fig. 3. Rescue of *fz* adult wing phenotype. Flies of each genotype were raised at 29°C. The region shown is the dorsal surface of the central C region (between the third and fourth longitudinal wing vein, just anterior to the posterior crossvein) of the adult wing. In all wings, distal is to the right. (A) Wild type (B) fz^{K21}/fz^{D21} (C) *hsfzII*/+; fz^{K21}/fz^{D21} (D) *hsfzI*/+; fz^{K21}/fz^{D21}. Note that *hsfzI* but not *hsfzII* rescues the *fz* phenotype.

Quantitative distinction between the *fz*-like and *in*-like phenotypes

To aid in our analysis of the overexpression phenotypes, we devised a method to evaluate quantitatively tissue polarity phenotypes of varying severity. Previous studies showed that null (or near null) mutations of the tissue polarity genes all cause abnormal hair polarity; but they differ dramatically in the frequency of multiple hair cells in the wing (Wong and Adler, 1993). For example, *fz* and *in* mutants both display abnormal hair polarity over most of the wing; however, *fz* mutants have very few multiple hair cells (~2.2%) whereas *in* mutants have many (~70%). To determine if these differences would also be seen for hypomorphic alleles, we scored an allelic series of *in* and *fz* mutations for both the number of multiple hair cells and the fraction of the wing showing abnormal polarity. We found that, at equivalent fractions of the wing showing abnormal polarity, *in* wings had many fold more multiple hair cells than *fz* wings (Fig. 8). This confirms the qualitative distinction between these mutants that is apparent to a trained observer on inspection of such wings.

Scoring of the *hsfzI* overexpression phenotypes (Fig. 8) revealed that early heat shocks (25-30 hours AP) of varying severity produced phenotypes that resembled an allelic series of *fz* mutations, whereas late heat shocks (34 hours AP) of varying severity produced phenotypes that resembled an allelic series of *in* mutations. The severity of the phenotypes depended on the time and temperature of the heat shock and on the number of copies of the transgene. For example, a strong induction (e.g., 37°C for one hour) at 28-30 hours AP gave a strong early *hsfzI* overexpression phenotype, whereas earlier (e.g., 25 hours AP) or milder (e.g., 31°C) inductions produced correspondingly weaker phenotypes. Similarly, strong induction (37°C for one hour) at 34 hours AP gave a strong late *hsfzI* overexpression phenotype, whereas earlier (e.g., 33 hours AP) or milder (e.g., 31°C) treatments gave weaker phenotypes.

To determine whether the similarity between the early and late *hsfzI* overexpression phenotypes and those of *fz* and *in* mutants extended to the subcellular level, we compared their F-actin staining patterns. In a previous study, Wong and Adler (1993) used F-actin staining of pupal wing cells to visualize developing prehairs and found a correlation between wing hair polarity and the subcellular site for prehair initiation. For example, prehairs in the wild-type pupal wing initiate from the distal vertex of polygonally shaped cells and have a uniform distal polarity (Fig. 9A). In tissue polarity mutants, both the number of prehairs formed and their site of initiation within the cell is altered. As expected from the adult wing phenotype, the F-actin staining pattern of *hsfzI* wings that were heat shocked early (Fig. 9B) was similar to that of a *fz* mutant (Fig. 9C): Prehairs initiated near the center of the cell or from an alternative location (rather than the distal vertex) along the cell periphery. In both cases, there was a lack of uniform hair polarity (compare to the wild-type pattern, Fig. 9A) and only occasional cells formed multiple prehairs. Likewise, the F-actin staining pattern of *hsfzI* wings that were heat shocked late (Fig. 9D) was similar to that of an *in* mutant (Fig. 9E). Prehairs initiated from a variety of locations along the cell periphery and many cells formed multiple prehairs.

An *hsfzI* overexpression phenotype does not require a vast excess of Fz protein or endogenous *fz* activity

To estimate the minimum level of *hsfzI* overexpression that could cause an overexpression phenotype, we constructed

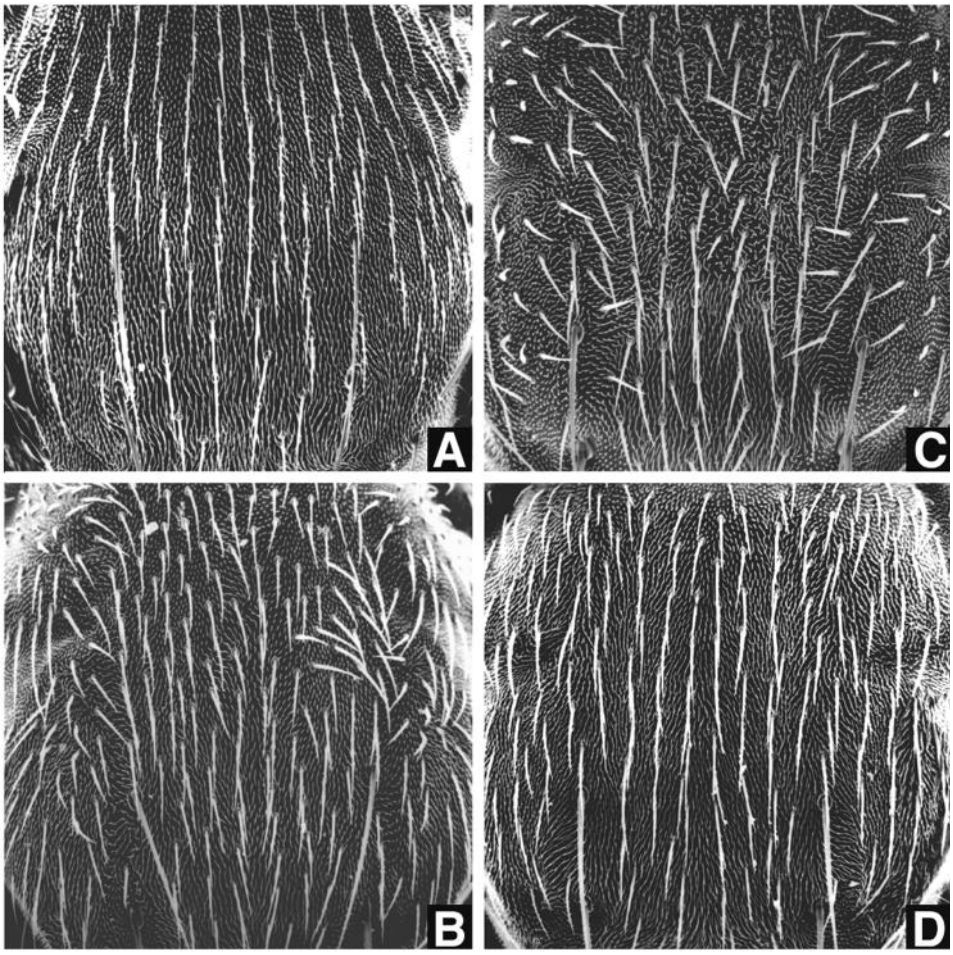

Fig. 4. Thoracic bristle phenotypes from increased and reduced *fz* function. Scanning electron micrographs of the dorsal thorax of adult flies are shown. Flies in A,C and D were raised at 29°C to induce low level expression of the transgenes. (A) Wild type: thoracic bristles are well aligned and point posteriorly. (B) *hsfzI; +*: this fly was heat shocked at 37°C at 34 hours AP to induce an overexpression phenotype. Note the abnormal polarity of some of the lateral bristles. (C) fz^{K21}/fz^{D21} showing a strong bristle phenotype. (D) *hsfzI*/+; fz^{K21}/fz^{D21}. The *fz* bristle phenotype is almost entirely rescued.

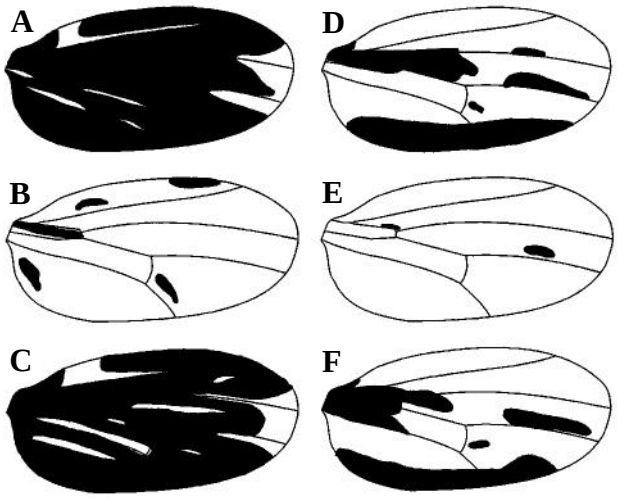

Fig. 5. Area of the adult wing with abnormal polarity. Blackened regions of the wing diagrams represent the area of the wing with abnormal polarity (>45° different from wild type, see Materials and Methods for details of scoring). Black lines represent the wing margin and vein pattern, which is normal in all cases. Four wings were scored for each genotype. Individual wings from *fz* mutant genotypes with *hsfzI* or *hsfzII* transgenes are shown. (A-C) Null genotypes (fz^{K21}/fz^{D21}) raised at 29°C. (E-F) Weak genotypes (fz^{R53}) raised at 25°C. All except E are average wings. E is the only 1 of 4 wings scored that had any abnormal regions. (A) $fz^{K21}fz^{D21}$ (B) *hsfzI;* fz^{K21}/fz^{D21} (C) *hsfzII;* fz^{K21}/fz^{D21} (D) fz^{R53} (E) *hsfzI;* fz^{R53} (F) *hsfzII;* fz^{R53}.

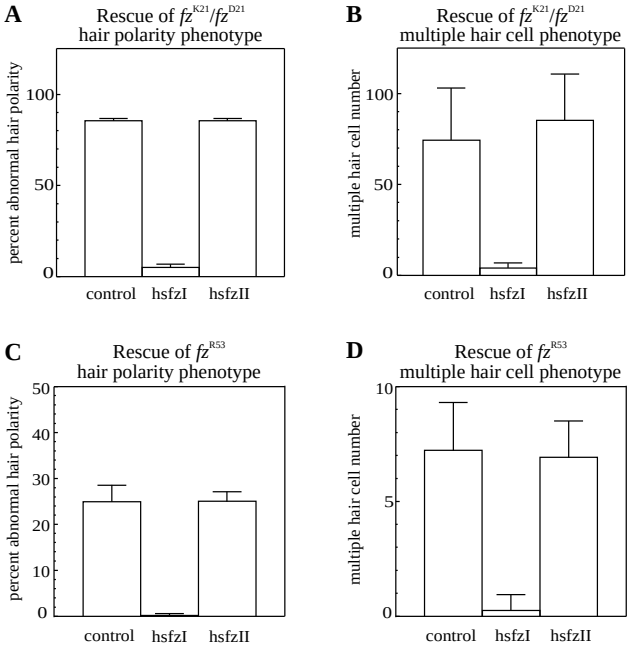

Fig. 6. Quantitation of rescue. Individual wings were scored for both abnormal hair polarity (n=4) and multiple hair cell number (n=5-9). Mean values are shown for each genotype. We scored abnormal hair polarity for the entire dorsal surface of the wing and number of multiple hair cells for the dorsal C region. (A,B) *hsfzI* rescue of a *fz* null phenotype (fz^{K21}/fz^{D21}) at 29°C. (C,D) *hsfzI* rescue of a weak *fz* phenotype (fz^{R53}) at 25°C. Three out of four of the *hsfzI;* fz^{R53} wings had no regions of abnormal polarity and no multiple hair cells. *hsfzII* had no effect on either the weak or strong *fz* phenotypes. Note differences in scale.

stocks that carried multiple copies of *hsfzI* transgenes. At 25°C wings from these flies showed small regions with altered hair polarity and occasional multiple hair cells. This phenotype presumably represents a very weak early *hsfzI* overexpression phenotype and is weaker than our weakest loss-of-function *fz* mutation (fz^{R53}). Western blot analysis (Fig. 2D) showed that pupal wings from these flies had about 2.3× the wild-type amount of Fz protein. Thus, even a modest overexpression of *hsfzI* can disrupt the normal tissue polarity system.

To determine if a functional endogenous *fz* gene is required for either of the *hsfzI* overexpression phenotypes, we examined *hsfzI*; fz^{-} flies. When these flies were heat shocked early, we found that hair polarity was often dramatically abnormal in the distal tip of the wing (not shown), which is a region that is barely affected in *fz* null mutants (see Fig. 5A). When these flies were heat shocked late, we found that multiple hair cell number was highly elevated compared to *fz* null mutants (not shown). This shows that an active endogenous *fz* gene is not essential for either the early or the late *hsfzI* overexpression phenotypes.

hsfzI generates an altered polarity signal

Previous experiments argued that *fz* either generates or transmits an intercellular polarity signal to neighboring cells (signalling function) and that it also functions in the intracellular reception or transduction of polarity information (response function) (Vinson and Adler, 1987). Disruption of either or both of these functions could account for the *hsfz* overexpression phenotypes. In this study, we used mosaic analysis to determine whether *hsfzI* overexpression disrupts the intercellular signalling function. We generated 'wild-type' clones (lacking the *hsfzI* transgene) in an *hsfzI*/+ genetic background using *forked* (f^{36a}) as a gratuitous cell marker for recombination; overexpression of *hsfzI* was induced by heat shocking during pupal development. Due to the necessity to examine large numbers of pupae, it was impractical to administer timed heat shocks in these experiments (see Materials and Methods for details). Therefore, we do not know the exact time of heat shock for pupae that produced clones.

We found that clones that lacked the *hsfzI* gene had altered hair polarity and often contained multiple hair cells (Fig. 10C-F). Vinson and Adler (1987) also observed multiple hair cells in the distal neighbors of *fz* mutant clones, indicating that an altered polarity signal can cause a multiple hair cell phenotype in cells with a normal *fz* gene. Neither altered hair polarity nor multiple hair cells was observed in control clones (Fig. 10A,B) produced in a non-*hsfzI* genetic background. The cell nonautonomy of *hsfzI* clones indicates that overexpression of *hsfzI* disrupts the intercellular polarity signal. This result has implications for the normal function of *fz* in the transmission of a polarity signal (see Discussion).

DISCUSSION

Wong and Adler (1993) have proposed that *fz* and the other tissue polarity genes are components of a genetic pathway that

functions to restrict prehair initiation to the distal vertex of pupal wing cells. They showed that *fz, dsh* and *pk* mutants share a similar subcellular phenotype in which pupal wing prehairs initiate from random locations within the cell. *in* and *fy* mutants share a distinct phenotype in which prehairs initiate from the cell periphery and a high frequency of cells form multiple prehairs. Thus, these two genes act formally as inhibitors of prehair initiation. Based on epistasis analysis of double mutants, they argued that *fz, dsh* and *pk* function upstream of *in* and *fy* in a genetic hierarchy, and that *fz* and *dsh* act to antagonize *in*/*fy* activity locally at the distal vertex of the pupal wing cell. This would restrict permissive conditions for prehair initiation to this unique subcellular location. In this study, we present evidence that is consistent with this view and that helps to define further the molecular basis for *fz* function.

A single Fz protein has a dual function in tissue polarity

Genetic mosaic studies demonstrated that *fz* has two separately mutable functions in tissue polarity (Vinson and Adler, 1987). The directional cell non-autonomy of most *fz* alleles argued that *fz* is required for the transmission of an intercellular polarity signal along the proximal distal axis of the wing. The cell-autonomy of certain rare alleles indicated that *fz* function is also required for the intracellular response to polarity information. The identification of two transcripts (that could encode two different proteins) from the *fz* locus (Adler et al., 1990) raised the possibility that each protein would fulfill one of the two functions of the gene. We show here that *hsfzI* alone can rescue the mutant phenotype. This demonstrates that the *hsfzI* transcript is necessary and sufficient for both *fz* functions. It is not yet clear whether a single form of the protein is bifunctional, or whether the FzI protein is post-translationally modified to produce two functionally distinct forms of the protein.

The ability of *hsfzI* to rescue the *fz* null phenotype also demonstrates that the large gene structure of the *fz* locus (Adler et al., 1990) is not required for tissue polarity function in the epidermis. We had initially suspected that the large gene size (100 kb) might reflect complex regulatory information. Other loci of this size, such as those of the homeotic gene complexes, have elaborate regulatory regions (e.g., Kuziora and McGinnis, 1988; Mann and Hogness, 1990) and null mutants cannot be rescued by a heat-shock promoter-cDNA construct. It remains possible that *fz* could have an additional or redundant function which has been overlooked due to the more obvious tissue polarity function (Park et al., 1994b). Large gene size could be important for this function. This could also explain why *fz* RNA and protein are expressed in tissues and developmental stages (e.g., embryonic and neural tissues) that have no obvious connection to the development of normal tissue polarity in the epidermis (Adler et al., 1990; Park et al., 1994b).

Role of the *fzII* transcript

Our results also indicate that the *fz*II transcript is neither necessary nor sufficient for *fz* function in tissue polarity in the epidermis. In addition, we found that overexpression of the FzII protein during pupal development has no effect. The inactivity of the transgene is unlikely to be due to insufficient expression of the protein; as western analysis and immunostaining demonstrate, expression of FzII protein in pupal wing cell membranes of heat-shocked *hsfzII* animals is far greater than that of endogenous Fz protein. Genetic evidence also argues that the *fz*II transcript is not sufficient for *fz* function. As noted previously (Adler et al. 1990), the inversion breakpoint of a strong *fz* allele (*In(3L)fz*^{C21}) maps more than 15 kb downstream of the 3′ end of the *fz*II transcript. Thus, a mutation

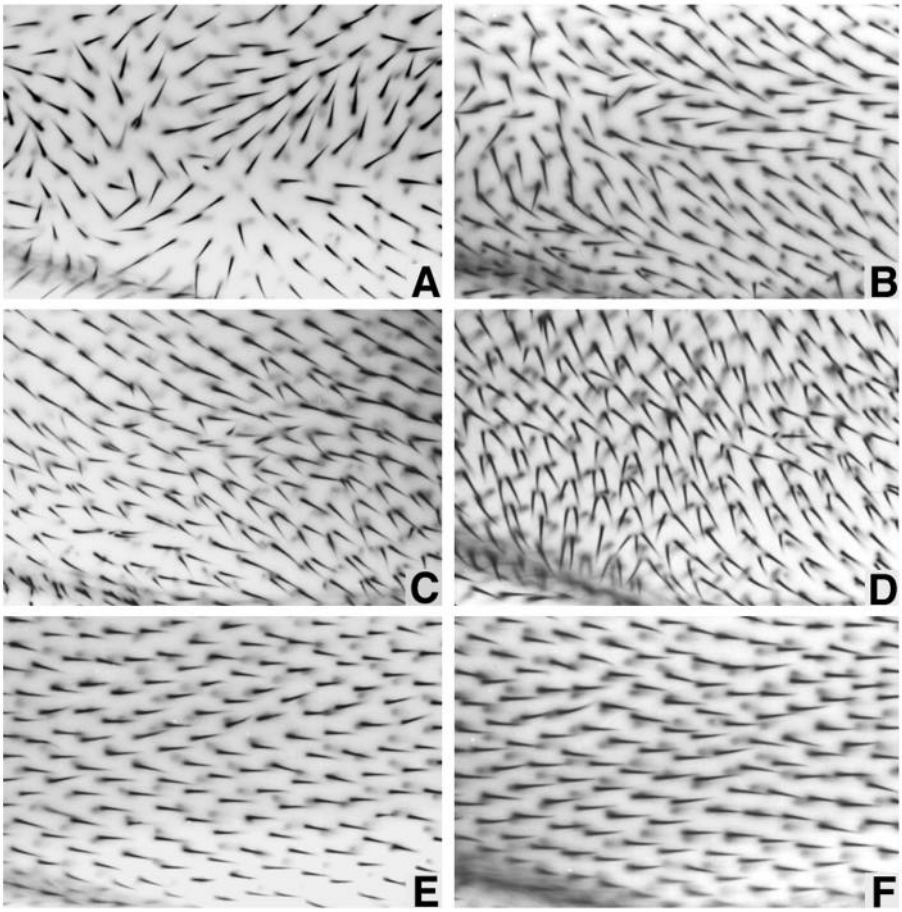

Fig. 7. *hsfzI* overexpression phenotypes resemble tissue polarity mutants. Adult wings from *hsfzI* overexpression at different times during pupal development are shown on the left. Wild type and tissue polarity mutants are shown on the right for comparison. (A) Early *(fz*-like) *hsfzI* overexpression phenotype. Pupae were heat shocked at 27 hours AP. (B) A moderate *fz* phenotype. (C) Late *in*-like *hsfz*I overexpression phenotype. Pupae were heat shocked at 34 hours AP. (D) A moderate *in* phenotype. (E) *hsfzI* pupae heat shocked at 36 hours AP. Induction of *hsfzI* at or beyond 36 hours AP had no effect. This corresponds to the time of prehair initiation (Wong and Adler, 1993). (F) Wild type.

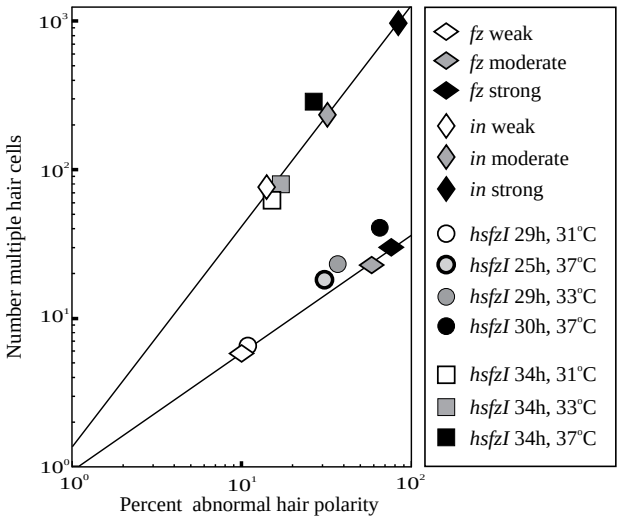

Fig. 8. *hsfzI* phenotypes resemble a hypomorphic series of either *fz* or *in* alleles, depending on the time of heat shock. We scored the C region (between the third and forth wing veins) of the wing distal to the anterior cross vein on the dorsal surface (see Materials and Methods for details). Data points represent the mean value of 5 wings scored for both phenotypes. There is a linear relationship between alleles of differing strength for each class of mutants (note double log scales). The different slopes of these lines permit a quantitative distinction between *in* and *fz* phenotypes. *hsfzI* pupae heat shocked at 30 hours AP or earlier resemble *fz* mutants, whereas *hsfzI* pupae heat shocked at 34 hours AP resemble weak to moderate *in* mutants. Note that a mild heat shock (31°C) at 34 hours AP resembles a weak *in* phenotype, as opposed to a strong *fz* phenotype.

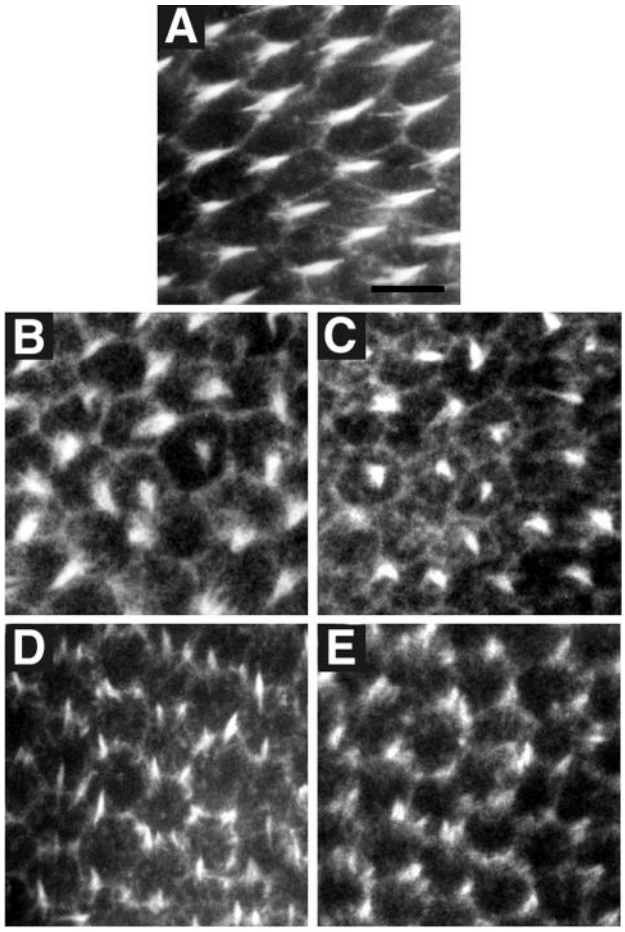

Fig. 9. Early and late *hsfzI* overexpression phenotypes resemble *fz* and *in* tissue polarity phenotypes at the subcellular level. Actin staining of wild-type, tissue polarity mutant and *hsfzI* pupal wings are shown. *hsfzI* pupae were heat shocked to produce either the early or late *hsfzI* overexpression phenotypes. (A) Wild type. Each cell forms a single prehair which initiates from a unique location at the cell periphery (the distal vertex of the cell). Prehairs from different cells have a common polarity. (B) Early (*fz*-like) *hsfzI* overexpression phenotype. Most cells form a single prehair which initiates from either the cell center or from an alternative location at the cell periphery. Prehairs from different cells lack a common polarity. (C) The *fz* phenotype. Note the similarity to B. (D) Late *in*-like *hsfzI* overexpression phenotype. Many cells form multiple prehairs which initiate from along the cell periphery. (E) The *in* phenotype. Note the similarity to D. See Wong and Adler (1993) for a detailed analysis of tissue polarity mutant phenotypes. Bar, 5 μM.

that does not directly affect the *fz*II transcript nevertheless has a strong phenotype. Taken together, the results argue strongly that the *fz*II RNA has no significant role in tissue polarity in the epidermis. It remains possible that the *fz*II RNA is required for an as yet unknown function of the *fz* locus.

fz generates/regulates a polarity signal

One of the two normal functions of *fz* in tissue polarity development is the transmission of a polarity signal. In principle, *fz* could play either a permissive (e.g., as the transporter of a diffusible morphogen) or an instructive role in this process. Our finding, that overexpression of *hsfzI* results in an altered intercellular polarity signal argues that *fz* has an instructive role in polarity signalling. Thus, we suggest that *fz* either generates or regulates the polarity signal.

How might a single Fz protein both generate/regulate a polarity signal, as well as respond to one? One way is through a branched signal transduction pathway such as in *Dictyostelium* aggregation (see Devreotes, 1989 for review). In that system, a cAMP signal activates a G-protein coupled receptor, which transduces the signal along two separate signal transduction pathways. One pathway leads to polarized cell migration (cell autonomous function) and the other to regeneration of the cAMP signal (cell non-autonomous function).

To account for *fz*'s two roles in the directional transmission of polarity information and the restriction of prehair initiation to the distal vertex of the cell, Park et al. (1994a) proposed a model adapted from one for *Dictyostelium* aggregation (see above). They suggested that a localized ligand binds to Fz on the proximal edge of a cell. This results in the polarized activation of two signal transduction pathways at the distal edge of the cell. One pathway leads to regeneration and localized release of the signal from the distal edge of the cell; the other pathway leads to assembly of the prehair initiation site at the distal vertex of the cell (Wong and Adler, 1993).

Alternatively, *fz* could function as both a receptor and as a ligand (either for itself or for another receptor). The *Drosophila* Boss protein (Kramer et al., 1991) is an example of an integral membrane protein that functions as a tethered ligand. Boss ligand functions as an inductive signal required

for cell fate specification in the eye. As previously noted (Hart et al., 1990), both Fz and Boss have seven potential TM domains and share many sequence features with the family of G-coupled receptors (which also includes the *Dictyostelium* cAMP receptor).

Fz overexpression disrupts two tissue polarity functions

We found that overexpression of FzI protein results in two qualitatively distinct phenotypes, depending on the time of heat shock. Early overexpression results in a *fz*-like phenotype whereas late overexpression results in an *in*-like phenotype. The two phenotypes could reflect disruption of two temporally distinct functions. Alternatively, they could reflect two thresholds of response to a function at a single time. According to the 'two-threshold model', *hsfzI* would affect only a late function. At that time, high FzI activity would result in an *in*-like phenotype; lower activity would result in a *fz*-like phenotype. After an early heat shock, FzI activity would decay below the threshold for producing an *in*-like phenotype, resulting in the *fz*-like phenotype. After a late heat shock, FzI activity would be above the threshold for producing the *in*-like phenotype. This model predicts that a late induction of a low level of FzI would produce an *fz*-like phenotype. However, we found that a late heat shock at low temperature (31°C) produced a weak *in*-like phenotype. This result supports the alternative model, that *hsfzI* overexpression disrupts tissue polarity functions at two distinct times in wing development. The 'early function' would likely be at 28-30 hours AP, as this is when production of the early (*fz*-like) phenotype is maximal; likewise, the 'late function' would be at 34 hours AP, when the late (*in*-like) phenotype is maximal.

Our interpretation, that *hsfzI* overexpression disrupts two temporally distinct functions raises the possibility that each phenotype represents disruption of one of the two normal functions of *fz*. For example, the early (*fz*-like) *hsfzI* overexpression phenotype could reflect disruption of the cell non-autonomous function and the late (*in*-like) *hsfzI* overexpression phenotype could reflect disruption of the cell autonomous function. However, other data argue against this idea: reducing the dose of *dsh* can dramatically suppress both types of *hsfzI* overexpression phenotypes (R.K. unpublished results) Since *dsh* functions cell-autonomously (Klingensmith et al., 1994; Theisen et al., 1994) this suggests that each phenotype has a cell-autonomous component. Our finding that *hsfzI* overexpression alters the polarity signal indicates that at least one of the phenotypes also involves a cell non-autonomous component. We propose below that the early (*fz*-like) *hsfzI* overexpression phenotype involves both cell non-autonomous and autonomous functions, and that the late (*in*-like) *hsfzI* overexpression phenotype involves only the autonomous function.

The early *hsfzI* overexpression phenotype resembles that of *fz*, *dsh and pk* mutants

The early (*fz*-like) *hsfzI* overexpression phenotype is puzzling because it resembles the *fz* loss-of-function phenotype. This phenotype is also similar to *dsh* and *pk* mutants. According to proposed models (Wong and Adler, 1993; Park et al., 1994a) Fz protein is differentially active across the cell. Thus, one possible explanation for the early (*fz*-like) overexpression

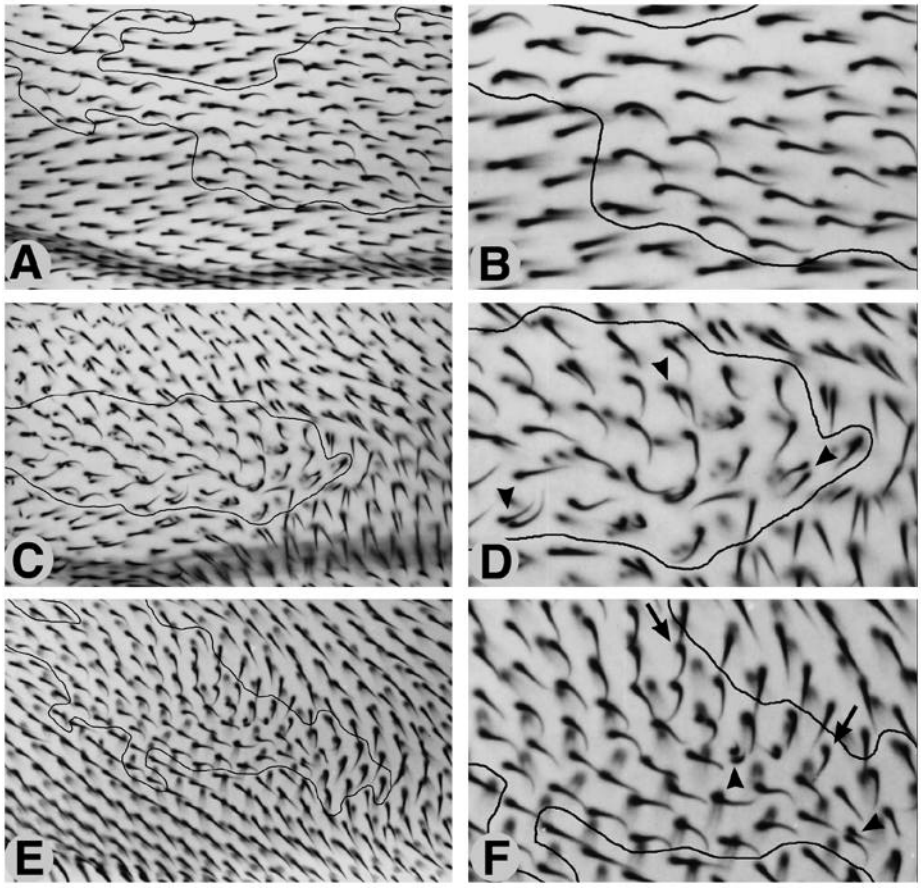

Fig. 10. Mosaic analysis of the *hsfzI* phenotype. Wings are oriented such that distal is at the right and anterior is at the top. *forked* clones were produced in wild-type or *hsfzI* genetic background and pupae were heat shocked several times to induce an overexpression phenotype. Panels on the right are higher magnification views of the panels on the left. The clone boundary is marked with a line. Arrows indicate clear examples of abnormal hair polarity. Arrowheads indicate multiple hair cells. (A,B) A control clone in a wild-type genetic background. Note the uniform distal polarity of the *forked* hairs within the clone and the absence of multiple hair cells. (C,D) An example of a clone in an *hsfzI* background. *forked* hairs near the clone boundary have an altered polarity and contain multiple hair cells. Multiple hair cells are also visible outside of the clone boundary. This demonstrates that *hsfzI* has a cell non-autonomous effect. (E,F) A second example of a clone in an *hsfzI* wing. As above, hairs near the clone boundary have an abnormal polarity and contain multiple hair cells.

phenotype is that early overexpression of FzI protein interferes with polarization of *fz* activity. This would affect both signal transmission (non-autonomous) and the selection of the prehair initiation site at the distal vertex of the cell (autonomous function). The molecular basis for the early *hsfzI* overexpression phenotype is not obvious. Overexpression of FzI might result in a failure to activate a threshold fraction of FzI protein. Alternatively, overexpression of FzI might result in ectopic activation of FzI protein throughout the cell; ubiquitous activity might be functionally equivalent to no activity. It is important to note that although they are similar, the early *hsfzI* overexpression phenotype and *fz* loss-of-function phenotypes are not identical. For example, the early *hsfzI* overexpression phenotype effects regions of the wing that are not strongly affected by *fz* null mutations (for example the distal tip of the wing).

Other cases in which overexpression and loss-of-function of a gene produce a similar phenotype, are believed to result from an altered stoichiometry of components involved in the assembly of a multimeric structure. In yeast for example, overexpression of β-tubulin prevents assembly of microtubles and produces a phenotype similar to a β-tubulin mutant. (Burke et al., 1989). In *Drosophila*, overexpression of *forked* results in a bristle phenotype similar to a *forked* mutant. (Petersen et al., 1994). *forked* is thought to encode a structural component of bristles and hairs. Although the early (*fz*-like) *hsfzI* overexpression phenotype could be related to altered stoichiometries, evidence suggests that Fz protein is not a major structural component of pupal wing cells. Its molecular structure, cell non-autonomy, time of function and relatively low level of expression (Gubb and Garcia-Bellido, 1982; Vinson and Adler, 1987; Wong and Adler, 1993; Adler et al. 1994; Park et al., 1994b) all suggest that *fz* functions as part of a signal generation transduction system that controls hair polarity by regulating the subcellular location for prehair initiation.

A better example of a gene that has an overexpression phenotype similar to its loss-of-function phenotype is the yeast *BUD2* gene (Park et al., 1993). *BUD2* encodes a GTPase activating protein (GAP) that is required for the selection of a bud initiation site at a unique location in the cell. *fz* may have an analogous role in the selection of a prehair initiation site at a unique location in the pupal wing cell (the distal vertex). Just as overexpression or deletion of *BUD2* results in a random budding pattern, overexpression or reduction of *fz* results in a random prehair initiation pattern. Unfortunately, the mechanism for *BUD2* function is not understood.

The late *hsfzI* overexpression phenotype resembles *in* and *fy* mutants

The similarity of the late *hsfzI* overexpression phenotype to that of *in* and *fy* mutants suggests to us that late FzI overexpression antagonizes *in* or *fy* function. We propose that late overexpression of FzI results (either directly or indirectly) in ectopic inhibition of *in/fy* function. Inhibition of *in*/*fy* function throughout (or in a larger than normal region of) the cell thus mimics the condition caused by reduction-of-function mutations in *in* or *fy*. This result also raises the possibility that *fz*'s putative role in inhibition of *in*/*fy* function may be temporally distinct from its role in polarity signalling.

The late (*in*-like) *hsfzI* overexpression phenotype is consistent with the proposal (Wong and Adler, 1993) that *fz* functions normally to inhibit locally *in*/*fy* activity at the distal vertex of the cell. There are several other examples in which the activity of one gene locally inhibits the activity of a downstream gene in a defined region of a tissue. For example, according to recent models for segment polarity gene function in *Drosophila* (Seigfried et al., 1994), *wingless* normally acts locally to antagonize the activity of *zeste-white 3* (*zw3*); ubiquitous expression of *wingless* via a heat-shock promoter (HS-*wg*) results in a cuticle phenotype that resembles the phenotype of *zeste-white 3* and *naked* mutants (Perrimon and Smouse, 1989; Sampedro et al., 1993; Noordermeer et al., 1994;).

It is interesting to note that *dsh* is a component of both the tissue polarity and segment polarity pathways (Perrimon and Mahowold, 1987; Theisen et al., 1994) and appears to be required for both *wg* and *fz* signalling (Klingensmith et al., 1994; Noordermeer et al, 1994; Seigfried et al., 1994; Theisen et al., 1994; R. K., unpublished results). It seem likely that the two processes also involve at least some different molecular components.

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