Twinstar, the *Drosophila* homolog of cofillin/ADF, is required for planar cell polarity patterning

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Planar cell polarity (PCP) is a level of tissue organization in which cells adopt a uniform orientation within the plane of an epithelium. The process of tissue polarization is likely to be initiated by an extracellular gradient. Thus, determining how cells decode and convert this graded information into subcellular asymmetries is key to determining how cells direct the reorganization of the cytoskeleton to produce uniformly oriented structures. Twinstar (Tsr), the *Drosophila* homolog of Cofilin/ADF (actin depolymerization factor), is a component of the cytoskeleton that regulates actin dynamics. We show here that various alleles of *tsr* produce PCP defects in the wing, eye and several other epithelia. In wings mutant for *tsr*, Frizzled (Fz) and Flamingo (Fmi) proteins do not properly localize to the proximodistal boundaries of cells. The correct asymmetric localization of these proteins instructs the actin cytoskeleton to produce one actin-rich wing hair at the distal-most vertex of each cell. These results argue that actin remodeling is not only required in the manufacture of wing hairs, but also in the PCP read-out that directs where a wing hair will be secreted.

**KEY WORDS: ADF/cofilin, Twinstar, Planar cell polarity, Lim Kinase, Frizzled, Flamingo, *Drosophila***

**INTRODUCTION**

Remodeling of the cytoskeleton is crucially required for various forms of cellular locomotion, such as chemotaxis and axon growth cone guidance. In these examples, the cellular architecture becomes differentially polarized in response to extracellular gradients of signaling molecules. How extracellular information is decoded by cells and used to direct the appropriate cytoskeletal polarization is currently the focus of intense study. A similar polarization mechanism occurs during the establishment of planar cell polarity (PCP), in which cells adopt a uniform orientation within the plane of an epithelium (Adler, 2002; Gubb and Garcia-Bellido, 1982; Uemura and Shimada, 2003). PCP is found throughout the metazoa, but is made particularly obvious in the fly wing as the outgrowth of single hairs from the distal-most vertices of cells, all collectively pointing towards the distal end of the cell (Wong and Adler, 1993). The wing is thus decorated with a multitude of hairs with the same orientation. PCP is evident in many other tissues of the fly, including the eye, where it is manifest not from the projections from single cells, but rather in the arrangement of a group of cells, the ommatidium (Wehrli and Tomlinson, 1995; Zheng et al., 1995) (Fig. 1A).

The PCP signal has been suggested to be a gradient of one or more extracellular ligands that is read by Frizzled (Fz), a serpentine receptor (Krasnow and Adler, 1994; Vinson and Adler, 1987; Vinson et al., 1989), which then regulates the cellular redistribution of itself and other core group PCP proteins that include: Disheveled (Dsh) (Axelrod, 2001; Krasnow et al., 1995), a cytoplasmic protein; Flamingo (Fmi), a cadherin also known as Starry Night (Stan) (Chae et al., 1999; Usui et al., 1999); Prickle (Pk), a LIM domain protein (Tree, 2002); and Van Gogh (Vang), also called Strabismus (Stbm) (Taylor et al., 1998; Wolff and Rubin, 1998). However, there is no direct evidence for a graded extracellular signal controlling PCP in *Drosophila*. Some experiments suggest instead that a presently undefined cell-to-cell mechanism propagates the PCP signal (Lawrence et al., 2004; Ma et al., 2003; Matakatsu and Blair, 2004; Strutt, 2002; Yang et al., 2002). The redistribution of the PCP core group proteins in the wing is not well understood and several mechanisms can be envisioned, but the result of the polarization signal is that Fz and the other core-group proteins are selectively redistributed to one or both sides of the cell. Fz and Dsh relocalize to the distal side of the cell (Axelrod, 2001; Strutt, 2001), Pk relocalizes to the proximal side, and Fmi is relocalized to both the proximal and distal sides of the cell (Tree, 2002; Usui et al., 1999).

Redistribution of these molecules to the proximodistal (PD) boundaries creates a characteristic zig-zag pattern (Fig. 1B). In animals mutant for any of the core group proteins, the appropriate asymmetric relocalization of the remaining PCP proteins does not occur, and the wing hairs are aberrantly positioned and oriented (Fig. 1D). This loss of polarity is probably a direct effect of mislocalization of the core group proteins; however, there is evidence that is at odds with this model (Lawrence et al., 2004).

The PCP pathway is not a simple linear biochemical cascade as there is clear evidence for multiple branches. For example, after Fz relocalizes to the distal end of the cell, it regulates the non-muscle myosin regulatory light chain protein (MRLC; Mlc2 – FlyBase) and Myosin II through the small GTPase RhoA and Rho kinase (Rok) (Winter et al., 2001) (Fig. 1C). RhoA and Rok mutants show defects only in the number of wing hairs per cell, and not hair polarity errors. By comparison, mutants for the PCP core group show both wing hair number defects and inappropriate hair orientation (Fig. 1D). The current view is that Fz lies near the top of the PCP intracellular signaling and controls a branching biochemical pathway that organizes a polarized cell to project one distally oriented hair.
Fig. 1. Aspects of planar cell polarity. (A) In a wild-type eye, ommatidia are rotated uniformly above and below the dorsoventral equator. (B) Localization of the polarity cue. In wild-type wing cells, Fz is localized to the distal sides of cells, whereas Fmi is localized to both the proximal and distal sides of cells. (C) The basic PCP pathway that determines wing hair polarity. (D) Classes of PCP defects in the wing epithelium. In wild-type, a single hair is secreted from the distal-most vertex of each cell. Mutations of the PCP core group genes (fz, fmi, stan, dsh, pk, and vang/stmb) cause hairs to project from central locations and to have a non-distal orientation. RhoA and Rok mutants show multiple wing hairs projected from the distal side of a cell. The tsr mutants show a single wing hair that is projected in a non-distal orientation and is not centered through the distal-most vertex of a cell.

TsR, the *Drosophila* homolog of coflin (CFL1)/ADF, belongs to a family of small actin-binding proteins (Bamburg, 1999). Coflin/ADF binds filamentous actin, inducing a twist in the actin filament that enhances the rate of actin depolymerization from the pointed end (Carlier et al., 1997; Lappalainen and Drubin, 1997). Coflin/ADF also severs F-actin, thereby increasing the number of actin filament barbed ends and increasing the rate of actin polymerization. Coflin/ADF can therefore enhance both actin polymerization and actin polymerization. The activity of Coflin/ADF is regulated through phosphorylation (Arber et al., 1998; Yang et al., 1998). Coflin/ADF is inactivated by Lim kinase 1, and reactivated by the phosphatases Slingshot (Niwa et al., 2002) and Chronophin (Gohla, 2005). As one of the main regulators of actin cytoskeleton remodeling, coflin/ADF is required for many different processes in the cell, including: cell motility (Chen et al., 1997); cell polarity during migration (Dawe et al., 2003); endocytosis in yeast (Lappalainen and Drubin, 1997); axon guidance (Kuhn et al., 2000); and cytokinesis (Gunsalus et al., 1995). In this report, we show that TsR is also required for the establishment of PCP and for the redistribution of the PCP core proteins Fz and Fmi to the PD boundary of cells during establishment of PCP in the wing. These data argue that actin remodeling is intimately involved in the polarization and protein redistribution mechanism.

**MATERIALS AND METHODS**

**Molecular cloning and mutagenesis**

Temperature sensitive *tsr* mutations were created by site-specific mutagenesis in the context of a 6.4 kb *tsr* genomic fragment. The oligonucleotides were: AATGTAAAAGATCTGATAGCGATCTT for *tsr*^272^ and CGGGAAAGCCTGCTGACGCGAAGCGCCACCGA-CCGC for *tsr*^139^. Mutations were amplified using the QuickChange kit (Stratagene), then subcloned in the context of the *tsr* 6.4 kb genomic rescue fragment (Chen et al., 2001) into the pB[4cat] vector (Thummel et al., 1988). p[WHHTG] is an abbreviation for P[w^m, hsp70-3,tsp^ns] in which the *tsr* protein-coding region is fused to the hsp70 heat shock promoter and cloned into the pW8 vector. The *tsr* genomic fragment, p[WHHTG], is 2548 bp, starting 101 bp upstream of the translation start site ATG and continuing to the *P* element site that is 385 bp downstream of the TAA stop codon.

The Limk cDNA LD15137 was obtained from the Berkeley *Drosophila* Genome Project and subcloned into the pUAST transformation vector (Brand and Perrimon, 1993). Disruption of the second Lim domain in human Lim kinase has previously been shown to increase its in vivo activity significantly (Arbers and Gilks, 1999). Therefore, a mutated form of Lim (called Limk^NgoMV1^), was made by modifying p[UAST-Limk^NgoMV1^] by restriction digestion with NgoMV1, creating an in-frame deletion of amino acids A109 to A175 that removes a sizeable part of the second Lim domain.

**Drosophila strains**

To express thermosensitive forms of TsR from transgenes in a background that lacks endogenous TsR activity, the following crosses were conducted, using a transgene located on the X-chromosome or 3rd chromosome. y w^+/tsr*^272*t; Fz^+/+. was crossed to y w^+; p[C4cat-tsr*^272*t]^{1C}; tsr*^272^ and was created on the 3rd chromosome by crossing. y w^+/; tsr*^272^; p[C4cat-tsr*^272*t]^{1C}; tsr*^272^ and was observed with the line w, p[WHHTG]; tsr*^272^/TSTL. Fz localization was examine by crossing in the Fz-GFP transgene (Strutt, 2001). Limk was overexpressed in the dorsal wing blade with: y w^+; P[UAST-Limk^NgoMV1^] (three independent lines) and the GAL4 driver: y w; P[GawB]ap[2A]/CyO[y^+] (Calla et al., 1996).

**Microscopy**

Confocal

Fluorescent microscopy was performed at the CNSI Advanced Light Microscopy/Spectroscopy Shared Facility at UCLA. Images were acquired using Leica Confocal Software (Leica Microsystems, Heidelberg GmbH) and were analyzed using NIH ImageJ. Confocal images were adjusted to best show protein localization and F-actin structures. Owing to the variability of fluorescence with each fluorophore within a sample and genotype, the relative intensities of expression are not intended be compared between wild type and the tsr*^272*tsr*^139^ mutant.

Bright field

Adult wings were rinsed in 100% ethanol, transferred to PBS, then transferred into Hoyer’s medium for mounting. Slides were baked overnight at 65°C and examined on a Zeiss Axioskop.

Scanning electron microscopy

Adult cuticles were examined with a Hitachi S-2460N scanning electron microscope. Images were acquired using the Quartz PCI version 3 imaging management system.

Staging mutant wing development

The cross to obtain y w^+/tsr*^272*t/; w^+/tsr*^139^, tsr*^272^/tsr*^139^ flies (Fig. 2B) was performed at 18°C. The homozygous *tsr* mutants are weak and develop at a variable and slower rate than their heterozygous siblings. Therefore, in addition to staging the wings by measuring time after puparium formation
**Antibodies and immunohistology**

Mutant pupae were staged at 18°C. Pupal cases were cut across the top of the operculum, and then cut laterally two-thirds of the way down the ventral side. Pupae were then fixed for 1 hour or 24 hours in 5% formaldehyde in PBS at 4°C. The pupal case was gently torn off at the cut exposing the pupa, but leaving the ends of the wings restrained by the remaining pupal case. The membrane encasing the wing was torn near the wing hinge, and the wing was gently pulled out. Pupal wings were rinsed three times in PBT (1X PBS, 1% Tween 20); blocked in PBT (+2% BSA) for at least 30 minutes; incubated overnight in a 1/10 dilution of primary Fmu monoclonal antibody at 4°C; rinsed three times in PBT for 10 minutes; blocked in PBT (+2% BSA) for at least 30 minutes; and incubated in 1/1000 dilution of secondary antibody [goat anti-mouse conjugated Alexa Fluor 594 (Molecular Probes)]. An α-GFP antibody conjugated with Alexa-Fluor 488 (Molecular Probes) was used a 1/200 dilution to enhance visualization of Fz-GFP. The α-Arm monoclonal antibody (Riggleman et al., 1990) was used at a 1/10 dilution (Developmental Studies Hybridoma Bank, product N2 7A1).

**RESULTS**

**tsr conditional alleles have a planar cell polarity mutant phenotype**

Tsr is required for cytokinesis, reducing the usefulness of mosaic analysis experiments to determine its role during development. We therefore engineered and tested two different forms of tsr mutants that are sensitive to temperature: one type being thermo-labile proteins, the other a heat-shock inducible gene. We have previously shown that a transformed wild-type tsr genomic fragment (P[minim-wr; tsr1]) can rescue the lethality of homozygous tsr96 null mutants (Chen et al., 2001). Two thermolabile mutations, tsrV27Q and tsr139, were independently introduced into the tsr-coding region of P[minim-wr; tsr1].

First, we changed amino acids 139 through 143 from the sequence EEKLR to AAALA and called the corresponding allele tsrV27Q. This mutation mimics the temperature-sensitive cof1-22 mutation of yeast Coflin (Lappalainen et al., 1997) (Fig. 2A). Second, we changed amino acid 27 from valine to glutamine and called the corresponding allele tsr139, which created an additional PCP-associated phenotype. Male flies of genotype P[WHTG]/Y; tsrV27Q/+ showed extensive PCP defects (Fig. 3C-E); leg bristles often showed non-distal orientations (Fig. 3E); and bracts, the small hair-like structures at the base of the bristle sockets, showed opposite orientations (Fig. 3I). Such defective tarsal segmentation phenotypes, although not an obvious PCP defect, also occur in a number of polarity mutants such as fz, dsh and prickle (Held et al., 1986) and are regarded as a PCP-associated phenotype. Male flies of genotype P[WHTG]/Y; tsr139/+ showed a relatively healthy appearance, over 90% showed clear PCP defects of the wing. An adult wild-type wing grown at 18°C shows the normal pattern of uniformly distally pointing hairs (Fig. 3A). The tsrV27Q/H9004 wing shows an abnormal pattern of non-distally pointing hairs (Fig. 3B). When grown at temperatures above 18°C, there was an increase in lethality and other non-PCP-related defects; hence, for this analysis tsrV27Q/H9004 flies were grown at 18°C. Tsr is probably required for many different processes during development. The observation that tsrV27Q/H9004 flies grown at 18°C were relatively healthy, but had a high incidence of PCP defects indicates that the PCP defect is more sensitive to a reduction of Tsr activity than to other defects caused by these tsr mutations.

P[WHTG] is a P-element insertion on the X chromosome capable (albeit at a low frequency) of rescuing the lethality of the tsr96-null allele without heat-shock treatment. The rescue was more efficient when flies are raised at a high temperature, such as 28°C. Rescued flies of genotypes P[WHTG]/P[WHTG]; tsrV27Q/H9004 and P[WHTG]/Y; tsrV27Q/H9004 showed extensive PCP defects (Fig. 3C-I): in adult wings, hairs did not uniformly point distally (Fig. 3C); in the abdomen, fine hairs and bristles frequently had non-posterior orientations (Fig. 3E); leg bristles often showed non-distal orientations and displayed aberrant tarsal joints and joint duplications (Fig. 3F-H); and bracts, the small hair-like structures at the base of the bristle sockets, showed opposite orientations (Fig. 3I). Such defective tarsal segmentation phenotypes, although not an obvious PCP defect, also occur in a number of polarity mutants such as fz, dsh and prickle (Held et al., 1986) and are regarded as a PCP-associated phenotype. Male flies of genotype P[WHTG]/Y; tsr139/+ showed limited PCP defects. Male flies of genotype P[WHTG]/Y; tsrV27Q showed limited rescue of the lethality caused by the tsr96 null allele; however, when put in transheterozygous combination: tsrV27Q/H9004; tsr139/H9004 (hereafter abbreviated as tsrV27Q/tsr139; Fig. 2B) there was a complete rescue of lethality when grown at 18°C. When grown at 25°C, only a few rare escapers of this genotype survived, which was indicative of the temperature-sensitive nature of these alleles (see Table 1). Interestingly, although the tsrV27Q/tsr139 flies grown at 18°C had a relatively healthy appearance, over 90% showed clear PCP defects of the wing. A wild-type wing grown at 18°C shows the normal pattern of uniformly distally pointing hairs (Fig. 3A). The tsrV27Q/H9004 wing shows an abnormal pattern of non-distally pointing hairs (Fig. 3B). When grown at temperatures above 18°C, there was an increase in lethality and other non-PCP-related defects; hence, for this analysis tsrV27Q/tsr139 flies were grown at 18°C. Tsr is probably required for many different processes during development. The observation that tsrV27Q/tsr139 flies grown at 18°C were relatively healthy, but had a high incidence of PCP defects indicates that the PCP defect is more sensitive to a reduction of Tsr activity than to other defects caused by these tsr mutations.

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![Fig. 2. Temperature-sensitive tsr mutations. (A) Sequence comparisons of yeast, human and Drosophila cofilin sequences (Lappalainen et al., 1997). The cof1-22 mutation that gave a temperature sensitive phenotype in yeast is in red and denoted above the sequence. This mutation was used to predict and engineer a similar temperature-sensitive mutation in Drosophila, tsr139, that is in red and denoted below the sequence. The position of the tsrV27Q mutation is also indicated. (B) The cross generating tsrV27Q/tsr139, tsr96 progeny. The conditional alleles P[w+], tsr96 and P[w+], tsrV27Q together rescued the lethality caused by the tsr96 mutation at the permissive temperature and allowed analysis of PCP defects in different tissues.](image-url)
Table 1. Characteristics of the temperature sensitive tsr\(^{139}\) and tsr\(^{V27Q}\) mutations

<table>
<thead>
<tr>
<th>Temperature</th>
<th>tsr(^{139})</th>
<th>P[w(^{+}), tsr(^{139})](^{1C})</th>
<th>tsr(^{V27Q})</th>
<th>P[w(^{+}), tsr(^{V27Q})](^{1C})</th>
<th>tsr(^{139})</th>
<th>P[w(^{+}), tsr(^{139})](^{1C})</th>
<th>tsr(^{V27Q})</th>
<th>P[w(^{+}), tsr(^{V27Q})](^{1C})</th>
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<tr>
<td>16°C</td>
<td>Lethal 1st instar</td>
<td>Pupal lethal adult escapers (0/73)</td>
<td>Pupal lethal adult escapers (0/66)</td>
<td>Adult progeny fertile females males escapers (25/231)</td>
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<td></td>
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<tr>
<td>18°C</td>
<td>Lethal 1st instar</td>
<td>Pupal lethal adult escapers (5/913) two males</td>
<td>Pupal lethal adult escapers (1/1153) one male</td>
<td>Adult progeny fertile females males escapers (129/1481) two males</td>
<td></td>
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<tr>
<td>25°C</td>
<td>Lethal 1st instar</td>
<td>Few 2nd instar larvae</td>
<td>Few 2nd instar larvae</td>
<td>Few pupae</td>
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<tr>
<td>29°C</td>
<td>Lethal 1st instar</td>
<td>Lethal 1st instar</td>
<td>Lethal 1st instar</td>
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Transheterozygous adults for the mutations tsr\(^{139}\) and tsr\(^{V27Q}\) survive and are relatively healthy at 16°C and 18°C, but few animals survive to the late pupal stage at 25°C.

Ts1\(^{396}\)/CyO, y\(^{+}\) appeared wild type; therefore, the phenotypes seen with P[WHTG]-rescued tsr\(^{396}\) homozygotes were not caused by the P-element insertion on the X chromosome, but rather resulted from a reduction in Tsr activity.

Polarity defects were also observed with the fine hairs of the notum. These hairs point to the posterior on wild-type nota, whereas many hairs adopted non-posterior orientations in a tsr\(^{V27Q}/tsr^{139}\) mutant (Fig. 3I). Occasionally, multiple hairs are observed (Fig. 3I, arrow); these abnormalities also occur in \(fz\) and dsh mutant animals (Krasnow et al., 1995; Wong and Adler, 1993). The tsr\(^{V27Q}/tsr^{139}\) genotype described above carried tsr\(^{V27Q}\) and tsr\(^{139}\) transgenes on the X chromosome; nearly identical phenotypes were observed with P[WHTG]-rescued tsr\(^{396}\) homozygotes (data not shown), indicating that the mutant phenotypes were not caused by the P-element but rather to reduced Tsr activity. This finding that compromised Tsr activity results in multiple defects that mimic those of mutants in the PCP pathway suggests that Tsr is a necessary component of this pathway.

Ts1 is required for the distal orientation of wing hairs

In wild type, a single wing hair emerges from the distal vertex of each wing cell. Wing hairs in PCP mutants lose this distal orientation and initiate from the central region of a cell. To determine the location of tsr\(^{V27Q}/tsr^{139}\) prehair emergence, pupal wings were treated with phalloidin to visualize filamentous-actin-based structures. Distinct differences between wild-type and mutant wing cells were observed. Wild-type prehairs (~64 hours after puparium formation (APF) at 18°C) were first observed as F-actin accumulations at the distal site of cells (Fig. 4A). By contrast, the site of prehair initiation in tsr\(^{V27Q}/tsr^{139}\) wings was variable. In moderately to severely affected wings, prehairs were first observed as F-actin accumulations near cell centers (Fig. 4B, dot), or as long F-actin fibers that span from near cell centers to the distal side of cells (Fig. 4B, asterisk). During prehair emergence (over 64 hours APF at 18°C), wild-type prehairs were oriented along the PD axis and centered through the distalmost vertex of each cell (Fig. 4C). By contrast, tsr\(^{V27Q}/tsr^{139}\) prehairs were not oriented along the PD axis and had emerged from either side adjacent to the distal-most vertex.
of a cell (Fig. 4D). Although not completely penetrant, this phenotype was seen in the vast majority (>90%) of tsrV27Q/tsr139 wings grown at 18°C. In addition to abnormal prehair emergence in tsrV27Q/hsa139 mutant wings, the F-actin accumulation on apical cell surfaces was increased and variable when compared with the F-actin accumulation in wild-type wing cells (data not shown). As Tsr functions to depolymerize F-actin, an increase in F-actin accumulation in wild-type wing cells (data not shown). As Tsr appears to play a role in the PCP pathway, we asked whether Tsr is required for PCP patterning in Drosophila. Therefore, developmental stages were also determined by examining structural features of the pupal wing.

Fz and Fmi do not correctly localize in tsr mutant wings
As Tsr appears to play a role in the PCP pathway, we asked whether the PCP core group members Fz and Fmi are properly localized in the tsrV27Q/hsa139 mutant background. If correctly localized, this would suggest that Tsr acts downstream of these proteins. If incorrectly localized, Tsr would be required for the localization of these proteins. The temperature-sensitive tsrV27Q/hsa139 mutat was grown at 18°C for this analysis. The rate of wing development in the tsrV27Q/hsa139 mutants was variable, preventing us from using pupal age as the only accurate indication of the progress of wing development. Therefore, developmental stages were also determined by examining structural features of the pupal wing.

Fz and Fmi subcellular localizations were examined in wild type and in tsrV27Q/hsa139 mutants by using the Fz-GFP transgene (Strutt, 2001) in conjunction with an anti-Fmi antibody, and an anti-Fmi antibody. In wild type, after 48-hours APF (18°C), Fz and Fmi redistribute asymmetrically to the proximodistal boundaries of cells, forming the characteristic zigzag pattern (Strutt, 2001) (Fig. 5A-C). A typical tsrV27Q/hsa139 mutant wing (Fig. 5D-F) shows a Fz-GFP and Fmi zigzag pattern that is markedly uneven compared with wild type, with gaps in the localization pattern at cell vertices (Fig. 5D), and larger gaps in which either both proteins were missing from an entire side of a cell (Fig. 5D, asterisk). In a tsrV27Q/hsa139 wing with a more severe phenotype, Fz-GFP was almost completely delocalized and Fmi appeared clustered in aggregates at cell boundaries (Fig. 5G-I). These data show that Tsr is required for the proper localization of Fz-GFP and Fmi. Although the localization of these proteins was not totally abolised, it is likely that the incomplete penetrance of this phenotype is the result of the partial rescue of the tsrV27Q null allele by the hypomorphic alleles tsrV27Q and tsr139. We anticipate that Fz-GFP and Fmi localization would be completely abolished in a stronger tsr mutant background; however, we are unable to verify this expectation because a further reduction of Tsr activity, such as raising tsrV27Q/hsa139 flies at a temperature above 18°C, results in non-PCP defects that prevent data interpretation. These defects include abnormal cell size and shape, and abnormal accumulations of F-actin on apical cell surfaces (Table 1).

In wild type, at ~64 hours APF (18°C), Fz-GFP and Fmi distributions were enriched at the PD boundaries, and prehairs had emerged at the distal-most cell vertices (Fig. 6A-D). At a similar developmental stage, in tsrV27Q/hsa139 wing cells of a moderately affected wing, both the Fz-GFP and Fmi protein distributions were
in an uneven and gapped pattern, or were missing from a cell side, and prehairs had initiated from non-distal locations (Fig. 6E-H). In a more severely affected wing, Fz-GFP was almost completely delocalized; Fmi was not localized in the characteristic zigzag pattern; and prehairs had initiated aberrantly from central locations (arrowhead) (Fig. 6I-L, respectively). The loss of Fz-GFP localization from the PD boundary and the appearance of centralized sites of prehair initiation suggests that Tsr activity is required for the asymmetric distribution of Fz.

In wild type at greater than 66 hours APF (18°C), prehairs have emerged and the asymmetric distribution of Fz-GFP and Fmi is less apparent, becoming more symmetrically arrayed around the cell circumferences (Fig. 7A-C). At a similar developmental stage in tsrV27Q/tsr139 wing cells, both the Fz-GFP and Fmi distributions were uneven and discontinuous, with regions of highly elevated protein concentration alternating with areas devoid of either protein (Fig. 7E-F). There was frequent colocalization between the Fz-GFP and Fmi proteins, suggesting that these proteins are closely associated (Fig. 7H). tsrV27Q/tsr139 prehairs frequently passed through the region of highest Fz-GFP/Fmi accumulation in 68% of cells counted (Fig. 7G, H, I'1; data not shown), suggesting that these accumulations present an orienting cue for the prehair outgrowth. Further, these data suggest that the tsr PCP wing defect is the result of a mislocalization of the Fz/Fmi signal and not due to an inability of the prehair to orient towards that signal.

**Fig. 6. Prehairs do not initiate from the correct location in tsr mutant wings.** Fz-GFP (A,E,I); Fmi (B,F,J); F-actin (C,G,K); and merged images of Fz-GFP in green and phalloidin-stained F-actin in red (D,H,L). (A-D) A wild-type wing during prehair initiation aged ~64 hours APF at 18°C. Fz-GFP and Fmi show the characteristic asymmetrical distribution at the PD boundaries. F-actin accumulations show a single prehair centered at the distal-most vertex of each cell (C). The merged image shows the overlay of Fz-GFP (green) and F-actin (red) localization. (E-H) A moderately affected tsr139/tsrV27Q wing of a similar age shows a Fz-GFP distribution that was interrupted at the distal cell boundaries. (E) Fz-GFP accumulated unevenly and was missing from some cell boundaries. (F) Similarly, Fmi shows an uneven distribution at PD cell boundaries. (G) The F-actin accumulation shows prehairs were not centered through the distal vertices; few were extended. (H) The merged image. (I-L) tsr139/tsrV27Q, a severely affected wing shows that Fz-GFP is not enriched at PD boundaries and the Fmi distribution at PD boundaries is uneven showing puncti of strong staining alternating with gaps in the staining pattern. Phalloidin-stained F-actin (K) shows prehairs abnormally formed near cell centers (arrowhead). The merged image shows the extent of Fz-GFP delocalization.

**Armadillo correctly localizes in tsr mutant wings**

As a control, we investigated the localization pattern of Armadillo (Arm) in tsr139/tsrV27Q pupal wings. Mutations in arm have no direct effect on PCP signaling (Axelrod et al., 1998; Boutros et al., 1998); however, Arm is linked at the adherens junction to the cytoskeleton and is therefore a marker for the general organization of the actin cytoskeleton. Pupal wings were double stained for Arm and Fmi localization. There were no obvious differences between wild type and the tsr mutant prior to the asymmetric relocalization of the core group proteins to the PD boundary of cells (data not shown). Once Fmi was redistributed to form the zigzag pattern in wild type, the Arm distribution remained in the characteristic honeycomb pattern (see Fig. S1A,B in the supplementary material). In tsr mutant wings of similar age, the Fmi distribution appeared disrupted, but the Arm distribution remained in the characteristic honeycomb pattern (see Fig. S1C,D in the supplementary material). Later during development, after the wild-type Fmi zigzag pattern was no longer prominent, the Arm protein had disappeared; however, in tsrV27Q/tsr139 wings of similar age, the Arm protein and pattern persisted (data not shown). This difference occurred well after wing hair polarity had been established. Therefore, the tsr PCP phenotype is not the result of a general disorganization of the actin cytoskeleton; rather, our findings are consistent with a direct role for tsr in PCP specification.
Overexpressing Lim kinase causes defects in planar cell polarity

Our experiments with tsr alleles suggested that regulation of the actin cytoskeleton has a crucial role in PCP and the appropriate localization of at least two of the PCP core group proteins. To verify this conclusion, we manipulated this pathway in a different manner by using Drosophila Limk, which phosphorylates ADF/cofilin thereby inhibiting its activity (Ohashi et al., 2000). Overexpression of a wild-type form of Limk showed only a weak mutant phenotype (at 23°C; data not shown). Therefore, an activated form of Limk (LimkH11032/H11032) was engineered by deleting a region of the second Lim domain (Edwards and Gils, 1999) and was expressed under Gal4-UAS transcriptional control (Brand and Perrimon, 1993). LimkH11032/H11032 was expressed in the dorsal wing blade using the apterous Gal4 (ap-Gal4) driver (Calleja et al., 1996), and the resulting phenotypes were found to be temperature sensitive. At 28°C, all the progeny died; at 25°C most animals died as pupae, but the few escapers had severe wing defects (data not shown). At 23°C, the progeny were fully viable. Approximately 10% of the progeny had wings that showed a whorled PCP pattern that is similar to the fz class of mutants (Fig. 8A), in which hairs tend to point in generally the same direction as their neighbors, while a majority of the progeny had wing hairs that frequently point in orientations independent of their neighbors (Fig. 8B). Examination of phalloidin stained UAS-LimkH11032/H11032/ap-Gal4 pupal wings showed that the PCP defects were limited to regions where the ap promoter is active, that is, on the dorsal wing blade (Fig. 8C, right panel). On the ventral wing blade, prehairs initiated as wild type from the distal-most vertices (Fig. 8D, left panel). Prehairs on the ventral wing blade had initiated aberrantly and were not centered (Fig. 8D, right panel), which is similar to the pattern of the tsrV27Q/tsr139 mutant (Fig. 8E). Animals grown at 21°C showed no defects. Thus, by compromising the actin depolymerization pathway with an independent molecular tool, we were able to phenocopy the effects on PCP seen with the tsr mutant.

DISCUSSION

Using different methods of downregulating Tsr protein activity, either by expressing thermo-labile forms of the Tsr protein or by overexpressing its inhibitor Limk, we have shown that the actin remodeling pathway is required for the correct decoding of extracellular gradients into PCP. The question to be addressed is whether PCP is disturbed indirectly because the cells are generally compromised, or whether remodeling of the actin cytoskeleton instead plays a direct and specific role in transducing positional information in the cells. The following points strongly favor the latter interpretation:

1) Reorganization of the actin cytoskeleton is required for the formation of hairs and bristles (Wong and Adler, 1993). However, the phenotypes we describe, cells produced these structures in a form that is close to wild type in appearance, yet the location and orientation of these structures are significantly altered. Morphogenesis of the eye also requires the active migration of cells, which requires the reorganization of the actin cytoskeleton. Yet, in the tsr mutant examined, the only defect in the ommatidia was of polarity.

2) PCP defects have been observed in different tissues, including the wing, thorax, leg, abdomen and eye. One might expect PCP phenotypes in one tissue or another if cells were just generally compromised, but the universality of the effect argues for a PCP-specific effect.

3) The PCP pathway directs tissue polarity in both the wing and eye, yet after PCP cues are established, the morphogenetic events that follow are very different. In the wing, PCP is manifest by the secession of a single hair from a specific location in a cell. In the eye, PCP is manifest by the inter-communication of developing photoreceptors cells to direct specific cell fates. Thus, the tsr mutations affect two distinctly different mechanisms of development. This strongly suggests tsr acts at a point of the PCP pathway common in both the wing and eye, rather than at the level of the distinct morphogenetic events that follow.

4) tsr mutants have aberrant tarsal segmentation that is characteristic of PCP mutants (Held et al., 1986). Thus, even the associated PCP effects are phenocopied in tsr mutants.

The above arguments highlight the strong genetic association between tsr and the PCP pathway, from which we infer that actin remodeling is a key step in the PCP generating mechanism. This is consistent with the work of Turner and Adler, whose data suggested that the actin cytoskeleton is important for the generation of wing hair polarity (Turner and Adler, 1998). Fz is the primary receptor for the PCP signal, and Fmi is at the top of the pathway required for the asymmetric redistribution of the PCP core proteins within the cell (Bastock et al., 2003). The molecular
mechanisms described above. Third, the clustered Fz molecules send a high-level signal resulting in the reorganization of actin filaments into the future hair or bristle.

Although our data focus on the role of cofilin/ADF, other factors required for reorganization of the actin cytoskeleton would be expected to be required for establishment of PCP. The small GTPase Rho is a known regulator of the actin cytoskeleton and is regulated by Fz signaling. During wing hair formation, a signal transduction cascade from RhoA to Rok to MRLC to Myosin II is required to limit the formation of just a single wing hair per cell (Winter et al., 2001) (Fig. 1C). Interestingly, Rho kinase is a known regulator of cofilin/ADF that acts through Link (Maekawa et al., 1999). However, the tsr mutant phenotypes described in this study are not consistent with a defect in the Rok branch of the pathway alone, as Tsr mutations affect wing hair orientation, not the number of wing hairs. In addition, Tsr is required for the proper redistribution of Fz and Fmi to the PD boundary of cells, whereas no requirement for RhoA and Rok has been demonstrated.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/9/1789/DC1

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Tsφ is required for PCP patterning in Drosophila


