DEVELOPMENTAL DYNAMICS 236:1064–1071, 2007

Isoform-Specific Interaction of Flamingo/Starry Night With Excess Bazooka Affects Planar Cell Polarity in the *Drosophila* Wing

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Epithelia display two types of polarity, apical–basal and planar cell polarity (PCP), and both are crucial for morphogenesis and organogenesis. PCP signaling pathways comprise transmembrane proteins, such as Flamingo/Starry Night, and cytoplasmic, membrane-associated proteins such as Dishevelled. During establishment of PCP in the *Drosophila* wing, PCP proteins accumulate apically in distinct "cortical domains" on proximal and distal plasma membranes. This finding suggests that their localized function depends on prior definition of apicobasal polarity. Here, we show that overexpression of Bazooka, a PDZ-domain protein essential for apicobasal polarity in the embryo, perturbs development of PCP, but has no effect on apicobasal polarity. The PCP phenotype is associated with a failure to restrict Flamingo/Starry night to the proximal and distal plasma membranes of the wing epithelium. We further demonstrate that *flamingo* expresses two differentially spliced RNAs in wing imaginal discs, which encode two isoforms of the atypical cadherin Flamingo. The predominant Starry night–type form contains a PDZ-binding motif, which mediates binding to Bazooka in vitro. Pull-down assays support the occurrence of such an interaction in wing imaginal discs. The results suggest that interaction between the apicobasal and planar cell polarity systems has to be tightly coordinated to ensure proper morphogenesis of the wing disc epithelium. *Developmental Dynamics 236:1064–1071, 2007.* © 2007 Wiley-Liss, Inc.

Key words: cell polarity; PDZ domain; *Drosophila*

Accepted 12 January 2007

INTRODUCTION

Epithelia are characterized by a pronounced apicobasal polarity, with the apical side facing the outside or a lumen and the basal side contacting a basal lamina. In addition, many epithelia are polarized in the plane of the tissue, perpendicular to the apicobasal axis. Both apicobasal and planar polarity are crucial for morphogenesis and organogenesis of multicellular organisms. Planar cell polarity (PCP), also called tissue polarity, is under the control of the "tissue polarity" genes, some of which provide an extrinsic spatial cue for the orientation of the proximodistal axis, while others interpret this cue, thereby stabilizing the asymmetry of the cell (reviewed in Adler, 2002; Eaton, 2003; Strutt, 2003). In the adult *Drosophila* wing, PCP is manifest in the regular alignment of hairs: a single hair emerges from the distal corner of each hexagonally shaped cell and points distally. The PCP pathway comprises several core components, which can be grouped into two classes: class I provides extrinsic spatial cues, required to instruct the orientation of the axis; class II genes interpret this cue and reinforce and stabilize the asymmetry. Class II members include the

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Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: Kn250/15-3.
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DOI 10.1002/dvdy.21089
Published online 15 February 2007 in Wiley InterScience (www.interscience.wiley.com).

transmembrane proteins Frizzled and Flamingo/Starry night, and the cytoplasmic, membrane-associated protein Dishevelled. During the establishment of PCP in the epithelium of the wing discs of *Drosophila*, PCP proteins cluster in "cortical domains," which are restricted to the apicolateral regions. Before hair formation, the cortical domains become further restricted to the proximal and/or distal plasma membranes. Restriction of a given PCP protein to one or both of these domains depends on the function of the others (reviewed in Adler, 2002; Keller, 2002; Eaton, 2003; Fanto and McNeill, 2003; Strutt, 2003). The unconventional, seven-pass transmembrane cadherin Flamingo (Fmi), also known as Starry night (Stan), becomes restricted to both the proximal and distal cell boundaries, where it mediates homophilic adhesion (Chae et al., 1999; Usui et al., 1999). In addition, PCP proteins act at an earlier stage, during the development of the hexagonal shape of the cells. Here, they have been suggested to be involved in remodeling adherens junctions by recruiting Sec5, a component of the exocyst, to the sites of Fmi/Stan accumulation (Classen et al., 2005).

The observation that PCP proteins are apically localized in epithelial cells raises the question as to the control of this restriction. In *Drosophila* and vertebrates, apical–basal polarity of many epithelia is controlled by a group of evolutionarily conserved proteins, Bazooka/Par3, *Dm*Par-6/Par6, and *D*aPKC/aPKCζλ, which are often associated in a complex localized apical to the zonula adherens (Knust and Bossinger, 2002; Müller and Bossinger, 2003; Macara, 2004). In *Drosophila*, these proteins are essential for the polarization of different cell types, such as epithelial cells, neuroblasts (the precursors of the central nervous system), and oocytes (Kuchinke et al., 1998; Wodarz et al., 1999, 2000; Petronczki and Knoblich, 2001; Betschinger et al., 2003; Hutterer et al., 2004). A second evolutionarily conserved protein complex, initially identified in epithelia of *Drosophila*, comprises the transmembrane protein Crumbs, which is linked by its C-terminal amino acids (ERLI) to the membrane-associated guanylate kinase (MAGUK) Stardust (Bachmann et al., 2001; Hong et al., 2001). Stardust recruits the scaffolding proteins *D*Lin-7 and *D*PATJ into the complex (Kamberov et al., 2000; Roh et al., 2002; Bachmann et al., 2004). The proteins are localized in the subapical region, apical to the zonula adherens, a site that corresponds to that of tight junctions (which are not found in invertebrate epithelia) in vertebrate cells, and most of them are essential for the establishment and/or maintenance of epithelial polarity in the *Drosophila* embryo.

Strikingly, many core PCP components are localized apically in epithelia. However, despite a considerable knowledge on the regulation of each of the two processes, nearly nothing is known about the connection between these two axes of polarity. A link between the two axes of polarity has been suggested to occur in the eye (Djiane et al., 2005), where the scaffold protein *D*PATJ, a component of the apical Crumbs complex, recruits both the serpentine receptor Fz1 and the atypical protein kinase C (*D*aPKC), which inactivates the former. Increased levels of Bazooka or loss of *D*PATJ in the eye interferes with the differential activation of Fz and leads to a PCP phenotype.

Data presented here show for the first time that the PCP gene *fmi/stan* expresses both of the two predicted isoforms in the wing. In addition, they demonstrate that the Stan isoform, which contains a PDZ-binding motif, specifically interacts with Bazooka and that its restricted localization is perturbed upon overexpression of Bazooka, resulting in a PCP phenotype. These results point to a fine-tuned balance between components controlling apicobasal and planar cell polarity in the wing epithelium.

RESULTS

fmi/stan Expresses Two Isoforms in the Wing Imaginal Discs, One of Which Binds to Bazooka In Vitro

fmi/stan, a gene that is essential for the correct development of PCP, encodes an unconventional cadherin. We noticed that the amino acid sequences annotated as Stan (Chae et al., 1999) and Fmi (Usui et al., 1999)—both encoded by the *fmi* gene—diverge at their C-terminal ends. Closer inspection of the predicted gene structure (www.flybase.org) suggested that an alternatively spliced 7-bp exon accounts for this difference (Fig. 1A). If this exon is included in the transcript, the protein product terminates in the Fmi sequence -DSEAEY. Omission of the exon results in the Stan-type C-terminal sequence -ERNIDDDETTV, which ends in a type I PDZ domain-binding motif (-S/T-X-V; Harris and Lim, 2001). To evaluate the expression of the two isoforms, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) on mRNA from wing imaginal discs of third-instar larvae, using primers designed to amplify both isoforms. Sequence analysis of subcloned PCR fragments indicated that the *stan* transcript predominates, because 11 of 11 clones examined were of that type. However, *fmi*-type transcripts are also present in the wing disc, as verified by using an alternative primer specific for the 7-bp exon.

Because Fmi/Stan is expressed apically and one isoform terminates with a PDZ-binding motif, we set out to analyze whether Bazooka, an apical scaffold protein with three PDZ (**P**SD-95, **D**iscs large, **Z**O-1) domains (Kuchinke et al., 1998), might interact with the C-terminus of Fmi/Stan. PDZ domains are protein–protein interaction motifs, which often bind to C-terminal sequences of transmembrane proteins (Harris et al., 2001). So we analyzed whether either isoform could bind the PDZ domains of Bazooka by performing GST pull-down assays. A fusion protein comprising all three PDZ domains of Bazooka (Baz_{PDZ1-3}) linked to GST was found to pull down a protein of the size predicted for Fmi/Stan (300 kDa; Usui et al., 1999) from lysates of wild-type wing discs (Fig. 1B). The anti-Fmi antibody used to detect this protein is directed against the extracellular domain (Usui et al., 1999), and should recognize both isoforms. We, therefore, tested Stan- and Fmi-type proteins (fused to GST) separately for direct interaction with the Bazooka protein containing the three PDZ domains (translated in vitro). Only the Stan-type fusion protein with its PDZ-binding motif pulled down Bazooka (Fig. 1C). Further experiments showed that the first PDZ

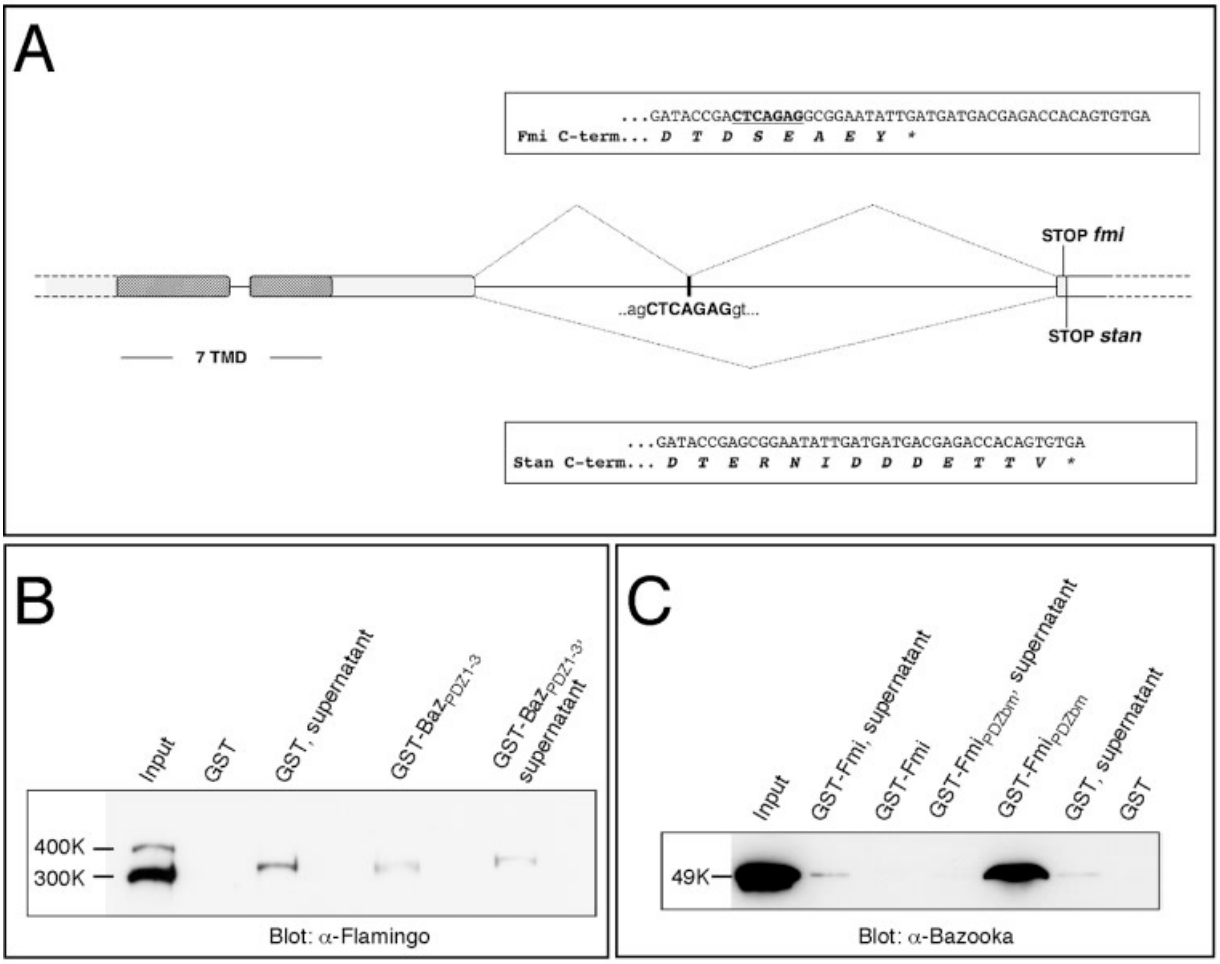

Fig. 1. The Stan isoform of Flamingo interacts with Bazooka. **A:** Alternative splicing of a 7-bp exon accounts for carboxy-terminal diversification of the protein products of the *fmi/ stan* gene. Part of the exon–intron structure of *fmi* is depicted (according to FBgn0024836 at www.flybase.org). Boxes, exons; striped sections, the seven transmembrane-domain region (7TMD). *fmi*- and *stan*-specific modes of splicing and the resulting C-termini of the two different gene products are shown above and below the gene region map, respectively. The 7-bp exon in the *fmi* gene product is underlined. Only the Stan-type isoform contains a terminal-binding motif for PDZ domains (TTV). **B:** GST pull-down assays. Protein extracts from wild-type wing imaginal discs were incubated with the three PDZ domains of Bazooka fused to GST (GST-Baz_{PDZ1-3}) or with GST alone. Only GST-Baz_{PDZ1-3} pulls down a protein of approximately 300 K. The larger band of approximately 400 K in the input is likely to represent the unprocessed protein (Usui et al., 1999). **C:** A GST fusion protein containing the C-terminal sequence of the Stan-type isoform with the PDZ-binding motif (GST-Fmi_{PDZbm}) or the Fmi-type isoform lacking this motif (GST-Fmi) was incubated with an in vitro translated portion of Bazooka containing the three PDZ domains. Only the C-terminus of the Stan-type isoform containing the PDZ-binding motif pulls down the three PDZ domains of Bazooka.

domain of Bazooka is required, but not sufficient, for strong binding, while the third PDZ domain is dispensable (data not shown).

Overexpression of Bazooka Causes Defects in PCP During Wing Development

To further demonstrate the interaction between Bazooka and Fmi/Stan, we analyzed cell clones either lacking or overexpressing Bazooka in the wing epithelium. Loss of *bazooka* in the embryo leads to defects in apicobasal polarity in epithelia and neuroblasts (Müller and Wieschaus, 1996; Kuchinke et al., 1998; Wodarz et al., 1999). In the forming blastoderm, the first epithelium in the *Drosophila* embryo to develop, Bazooka acts as an early apical cue required for positioning of the zonula adherens (ZA; Harris and Peifer, 2004, 2005). Similarly as in eye imaginal discs (Hong et al., 2003), however, cell clones in the wing imaginal discs homozygous mutant for the *bazooka* null allele baz^{Xi106} do not show any defect in apicobasal polarity (Fig. 2A,B), as revealed by the proper localization of apical components, such as *D*aPKC (Fig. 2A,A′) or markers of the ZA (Fig. 2B,B′). Similarly, lack of Bazooka does not perturb PCP (Fig. 2C,C′).

To further evaluate the role for Bazooka during epithelial development, we conducted overexpression experiments. Targeted expression of Bazooka in the posterior compartment of wing imaginal discs, driven by *en*-Gal4, has no obvious effect on the apicobasal axis, as revealed by the proper localization of *D*aPKC (Fig. 2D,D′), *D*PATJ, or ZA markers (data not shown). Although there was no obvious defect in apicobasal polarity upon overexpression of Bazooka in the posterior compartment of the wing epithelium, two mutant phenotypes were observed in the adult wing: the posterior compartment of the wing becomes

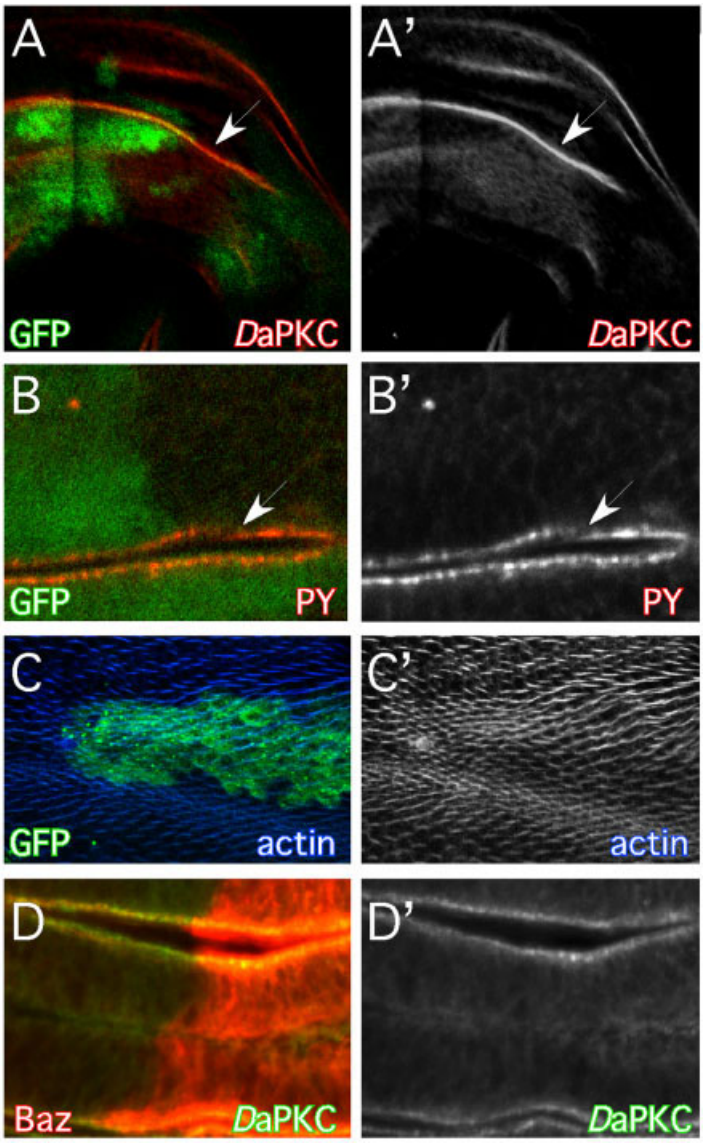

Fig. 2. Loss of Bazooka does not affect apicobasal or planar polarity in the wing epithelium. **A,A′,B,B′:** *baz*^{Xi10} mutant cell clones in wing imaginal discs of third instar larvae (marked by the loss of green fluorescent protein [GFP], green) still show correct localization of apical markers, such as *D*aPKC (A,A′) and Tyr-phosphorylated epitopes, which mark the zonula adherens (B,B′, arrows). **C,C′:** Cell clones in the wing imaginal discs of third-instar larvae homozygous mutant for the *bazooka* allele *baz*$^{815\text{-}8}$ (marked by GFP, green) exhibit a wild-type orientation of hairs, as revealed by actin staining (blue). **D,D′:** Overexpression of Bazooka (red) in the posterior compartment of the wing does not affect apical localization of *D*aPKC (green). Apical localization is nicely visible at the folds of the discs.

broader and shorter along the proximodistal axis (Fig. 3A,B), and the wing hairs no longer align proximodistally. Instead, groups of hairs oriented in parallel form whorls and often point anteriorly or posteriorly rather than distally (Fig. 3A′,B′).

Wing hairs are actin-rich structures formed during pupal development. In wild-type wing discs at 32 hr after puparium formation (APF), a single prehair emerges at the distal vertex of each hexagonal cell. By 33 hr, APF prehairs have extended further and are now aligned in parallel (Fig. 4A′). In discs that overexpress Bazooka in the posterior compartment, Bazooka protein is strongly enriched at the apicolateral plasma membrane (compare the anterior and posterior compartments in Fig. 4B). In these cells, mispositioning and misalignment of the hairs is clearly visible at 33 hr APF (Fig. 4B′,B″). Only occasionally more than one hair is formed per cell. Strikingly, in Bazooka-overexpressing cells that are juxtaposed to wild-type cells of an anterior compartment, hair localization and orientation are normal (Fig. 4B,B″). To rule out the possibility that the latter phenotype is due to the particular situation at the anterior–posterior compartment boundaries, cell clones overexpressing Bazooka were induced. In these cases, defects in PCP were only observed at the center of a clone; cells at the margin were not affected, suggesting that adjacent wild-type cells suppress the mutant phenotype in cells at the margin of the clone (Fig. 4C,C″).

Defects in the positioning and orientation of wing hairs point to errors in the establishment of PCP, possibly affecting the subcellular localization of Fmi/Stan. Before 30 hr APF in wild-type wing discs, Fmi/Stan is distributed around the apical cortex of the cells (not shown; Usui et al., 1999). Between 30 and 36 hr APF, before initiation of the prehairs, Fmi/Stan accumulates at the distal and proximal membranes of the cells (Fig. 5A, white arrowheads). By this time, all cells have adopted a hexagonal shape and are more tightly packed (Fig. 5A; Classen et al., 2005). In contrast, cells residing within zones of Bazooka overexpression driven by *en*Gal4 (1) fail to concentrate Fmi/Stan at their distal and proximal edges, exhibiting instead a patchy accumulation of Fmi/Stan on all apical membranes; and (2) remain irregularly packed (Fig. 5A–C, lower halves). Hence, overexpression of Bazooka is associated with disruption of Fmi/Stan localization and failure to undergo the normal change in cell shape. Overexpression of Fmi itself, on the other hand, results in a regular pattern of single trichomes pointing toward the anterior–posterior compartment boundary, due to a reorientation of the axis of polarity caused by ectopic localization of Fmi to the anterior and posterior membranes (Usui et al., 1999). In contrast, *en-Gal4/UAS-baz* provokes a randomization of hair orientation (and occasional multiple wing hairs); this phenotype is likely to be linked to a patchy localization of Fmi, with some protein remaining at the proximal and distal cell membranes (see Fig. 5A).

The biochemical data raise the possibility that the PCP phenotype induced upon Bazooka overexpression is

the result of a direct interaction between Bazooka and Stan in the pupal wing. This idea is strongly supported by the observation that the PCP phenotype is completely suppressed by removing one copy of *fmi/stan* (using fmi^{192} or fmi^{E59}; Fig. 6A,B, and data not shown). This finding can be explained by assuming that halving the dose of Fmi may reduce the amount of ectopic Fmi protein. In line with this view, deletion of one copy of *strabismus*, which encodes a Fmi-stabilizing transmembrane protein (Bastock et al., 2003), was also found to suppresses the PCP phenotype (Fig. 6C). In contrast, halving the copy number of *dishevelled* (*dsh*), which functions in signal transduction downstream of Fz during positioning of the hairs, has no effect on the Bazooka-induced PCP phenotype (Fig. 6D).

It is well established that interactions between PDZ domains and their ligands can be somewhat promiscuous. For example, the PDZ domain of vertebrate Par-6 binds to the C-terminus of CRB3, the N-type Ca^{2+} channel, and neurexin (Bezprozvanny and Maximov, 2001; Lemmers et al., 2004). We, therefore, tested the effects of overexpressing *D*Lin-7 and Discs large, two scaffold proteins that contain one and three type I PDZ domains, respectively (Woods and Bryant, 1991; Bachmann et al., 2004), in the wing disc. Neither of them produced a *baz*-like PCP phenotype upon overexpression (Fig. 3C,C′, and data not shown). Given the observation, that induction of a PCP phenotype could only be induced by overexpression of *bazooka*, the Bazooka–Stan interaction seems to be specifically required for induction of the PCP phenotype.

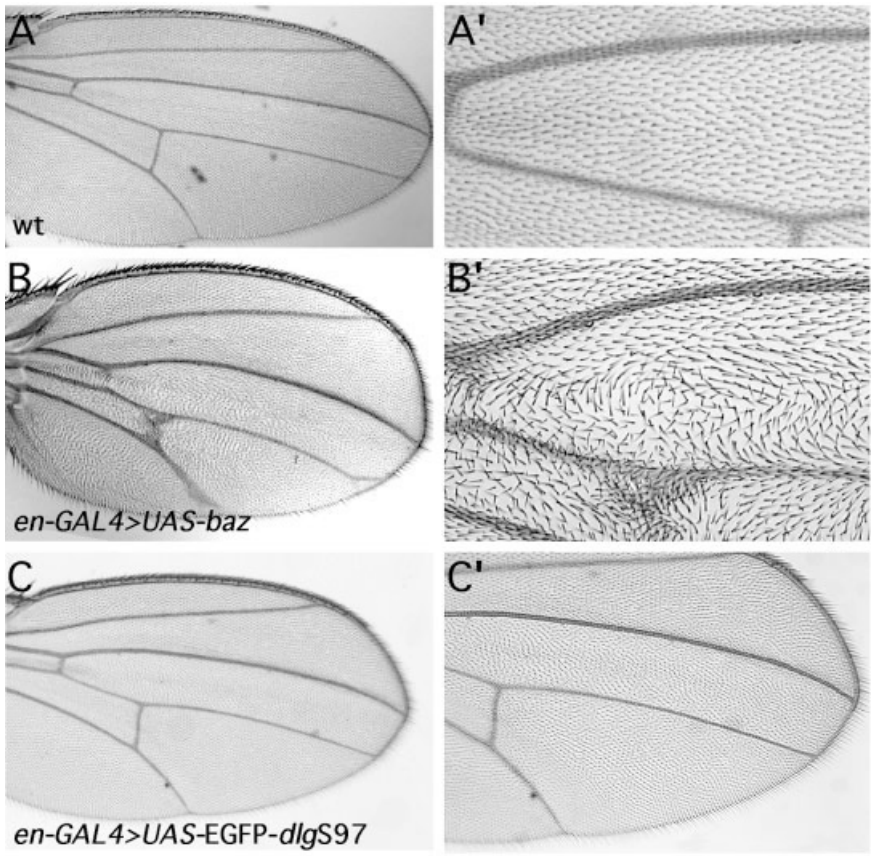

Fig. 3. Overexpression of Bazooka induces defects in planar cell polarity in the wing. **A,A′:** Wild-type wing. Hairs are aligned parallel and point distally. **B,B′:** *UAS-baz/en-Gal4* wing, overexpressing Bazooka in the posterior compartment. The posterior compartment is shorter and broader and the hairs are irregularly orientated. **C,C′:** *UAS-EGFP-dlg/en-Gal4* does not induce a planar polarity phenotype upon overexpression.

DISCUSSION

The data presented here provide strong evidence that excess Bazooka interferes with proper PCP by means of direct interaction with Stan, the major isoform of Fmi/Stan expressed in wing imaginal discs. The existence of different carboxy-termini, which is deducible from the original molecular analyses of Fmi/Stan (Chae et al., 1999; Usui et al., 1999), has so far not been considered as a means for differential functions. Our observations, however, suggest that the difference between both isoforms may be of functional importance, because only the Stan-type isoform binds to the PDZ domains of Bazooka. This assumption is supported by the fact that the 7-bp exon, together with its flanking intron sequences, is highly conserved among various *Drosophila* species. Because we were unable to coimmunprecipitate Bazooka and Stan from wild-type imaginal discs, we cannot completely rule out the possibility that the Stan-type isoform and Bazooka only interact if the latter is overexpressed, thereby preventing the polarized distribution of Stan. However, results from GST pull-down assays using lysates of wild-type imaginal discs support a direct interaction between these two proteins. Further experiments will demonstrate whether a portion of the Bazooka protein containing only the first and the second PDZ domain is sufficient to provoke the PCP phenotype. The nonautonomous effect of wild-type cells on cells overexpressing Bazooka could be explained by assuming that Fmi/Stan-dependent PCP signalling relies on homophilic cell-to-cell interactions, which have been demonstrated in S2-cell culture (Usui et al., 1999). Ectopic Fmi/Stan on anterior and posterior membranes that are adjacent to wild-type cells would, therefore, remain inactive.

Several observations point to a more complex relationship between these two proteins. In wild-type wing imaginal discs, Bazooka is localized apically on all cell membranes throughout pupal development, while Fmi/Stan becomes restricted to the proximal–distal membranes before prehair initiation. This timing may be explained by assuming that modification of Bazooka and/or interactions with other, yet unknown, proteins may alter the binding activity of Bazooka to Fmi/Stan at anterior–posterior or proximal–distal membranes,

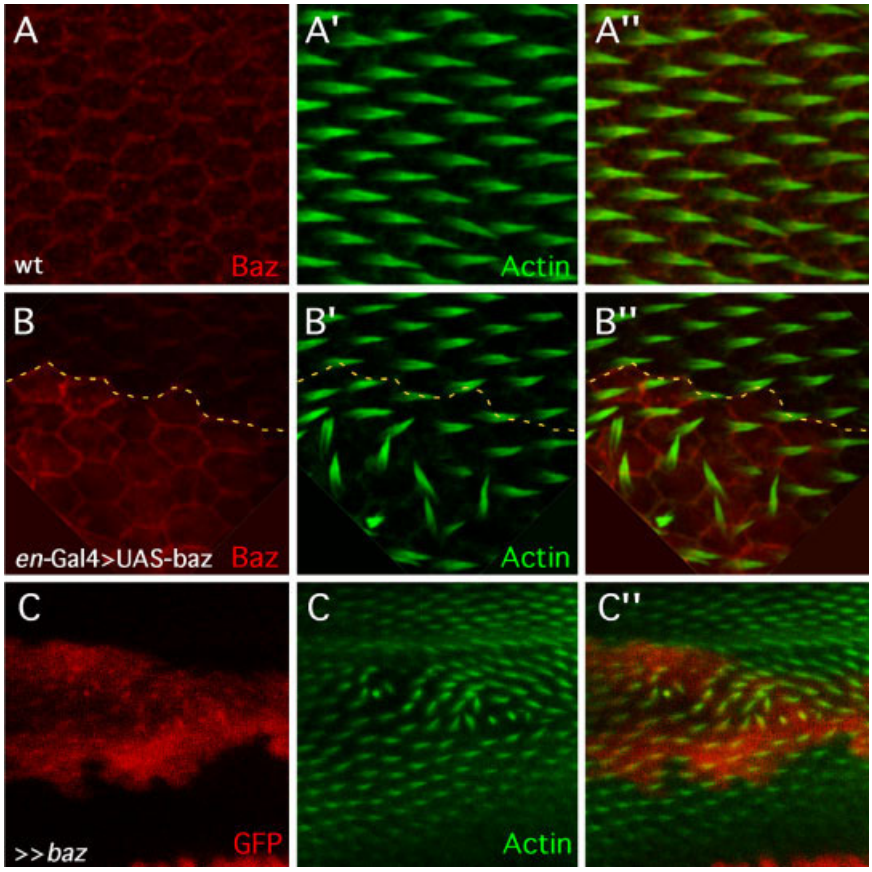

Fig. 4.

Fig. 4. Overexpression of Bazooka induces defects in planar cell polarity (PCP) during wing development. **A,A′,A″:** Wild-type pupal wing at 33 hr after puparium formation (APF). Bazooka, red; actin, green. Hairs are localized in the distal vertices of the cells and point distally. Bazooka is evenly distributed apically (the apparent higher accumulation of Bazooka at the anterior–posterior membranes is due to cross-talk from the green channel). **B,B′,B″:** Pupal wing at 33 hr APF, overexpressing Bazooka (red) in the posterior compartment. The compartment boundary is marked by a yellow dashed line. **C,C′,C″:** Cells within a clone overexpressing Bazooka (marked by GFP, red) exhibit a PCP phenotype. Proximal is left, anterior is up.

thus allowing polarized localization of Fmi/Stan. One likely candidate to modify Bazooka or any other component associated with it is *Drosophila* aPKC, which can bind Bazooka (Wodarz et al., 2000). Preliminary data suggest that, in wild-type wing imaginal discs, *D*aPKC accumulates preferentially at the proximal and/or distal membranes during prehair initiation (I. Wasserscheid and E. Knust, unpublished observations), but it remains to be analyzed whether this spatial restriction plays any role in PCP. According to a model put forward recently for the development of PCP in the eye (Djiane et al., 2005), *D*aPKC inhibits Fz1 by phosphorylation of its cytoplasmic tail, and higher levels of Bazooka in photoreceptors R3 and R4 prevent this inhibition. Unlike in the eye, neither loss of Bazooka nor reduction of *D*PATJ, which has been suggested to recruit *D*aPKC in the

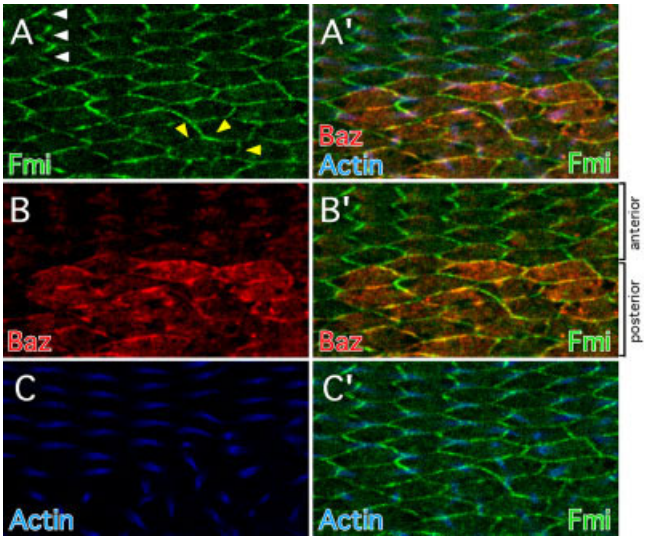

Fig. 5. Overexpression of Bazooka prevents polarized accumulation of Fmi/Stan. Wing discs at 32–33 hr after puparium formation (APF), overexpressing Bazooka in the posterior compartment (*en-Gal4/UAS-baz*). Fmi, green; Baz, red; actin, blue. **A,A′:** Cells in the anterior compartment are hexagonal and accumulate Fmi at the proximal and distal plasma membranes (white arrowheads in A). **B,B′:** Cells with high levels of Baz (B) are irregular in shape and show a patchy, apical accumulation of Fmi on all membranes (see also yellow arrowheads in A), that partially colocalize with Baz (B′). **C,C′:** Orientation of actin is perturbed in cells overexpressing Bazooka. Proximal is left, anterior up.

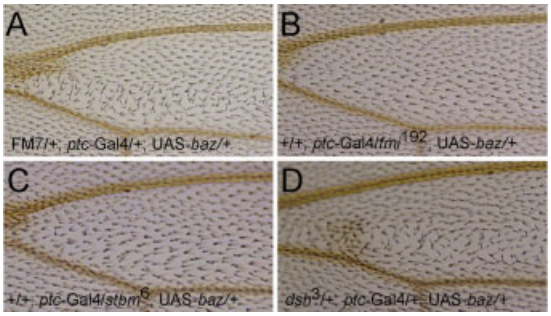

Fig. 6. *fmi*, but not *stbm* or *dsh*, suppresses the Bazooka-induced planar cell polarity (PCP) phenotype. **A:** Wing overexpressing Bazooka at the anterior/posterior compartment boundary under the control of *ptc*-Gal4 exhibits a PCP phenotype. **B:** The Baz-dependent PCP phenotype is nearly completely suppressed by removing one copy of *fmi*. **C,D:** Removal of one copy of *stbm* (C) or *dsh* (D) influences the PCP phenotype induced by overexpression of Bazooka weakly or not at all, respectively. Proximal is left, anterior up.

eye, has any effect on PCP development in the wing (this work and M. Richard and E. Knust, unpublished observations). The lack of any defect in apicobasal polarity in *bazooka* mutant cell clones in the wing or the eye imaginal disc (this work; Hong et al., 2003) is striking, given that it is absolutely required for the establishment of cell polarity in the embryo. In this respect, it does not differ from the behavior of *crumbs* or *stardust* mutant clones, which also develop normal apicobasal polarity in imaginal discs (Johnson et al., 2002; I. Wasserscheid, unpublished work). One possibility to explain this result is to assume that these genes act redundantly and that only the concomitant removal of two (or more) of them would result in a polarity phenotype. Alternatively, epithelia of the imaginal discs may differ essentially in the mechanisms that control their polarity. A difference is also obvious when Bazooka is overexpressed: although it is no longer restricted apically when overexpressed in embryonic epithelia (Wodarz et al., 2000), excess Bazooka is still localized apically in wing disc epithelia (see Fig. 2).

Several possibilities can be considered of how an interaction between Stan and overexpressed Bazooka may perturb PCP. Excess Bazooka could interfere with the lateral mobility of the Stan isoform in the plasma membrane and, thus, prevents the formation of proximodistal localization before wing hair formation. Alternatively and in a way described for other PDZ domain proteins (Standley et al., 2000), Bazooka might promote surface expression of its binding partner, thus mimicking overexpression of Stan and Fmi. Finally, overexpression of Bazooka may affect the preferential orientation of microtubuli along the proximodistal axis. Recent data suggest that polarized transport of Fz- and Fmi-containing vesicles occurs along polarized microtubule arrays and that their disruption by colchicin interferes with distal localization of prehairs (Shimada et al., 2006). It is tempting to speculate, that *bazooka*, which is required for spindle orientation in *Drosophila* neuroblasts (Kuchinke et al., 1998; Wodarz et al., 1999, 2000), provides a link between the cortex and the spindle pole in these cells. Similarly, Bazooka overexpression in wing epithelial cells may affect microtubule orientation and consequently redirect polarized transport of Fmi-containing vesicles.

EXPERIMENTAL PROCEDURES

Fly Stocks and Mosaic Analysis

The following fly stocks were used: wild-type (Oregon R), *baz^{Xi106} FRT 9-2* (Wieschaus et al., 1984), *baz$^{815-8}$ FRT19A* (McKim et al., 1996), *UAS-baz6.3* (Kuchinke et al., 1998), *en*-Gal4 (Han and Manley, 1993), *omb*-Gal4 (Bloomington *Drosophila* Stock Centre), *ptc*-Gal4 (Hinz et al., 1994), *fmi^{192}*, *fmi^{frz3}* (Rawls and Wolff, 2003), *UAS-fmi* (Usui et al., 1999; kindly provided by T. Uemura), UAS-*dlgS9*, UAS-*DLin-7* (Bachmann et al., 2004), *P{ry$^{+t7.2}$ =hsFLP}1, y^{1} w^{1118}; Dr^{Mio} /TM3, ry* Sb^{1}* (Bloomington *Drosophila* Stock Centre).

Flip out clones were induced by a 2-hr heat shock (38°C) at 48–72 hr and 72–96 hr of development in *baz^{Xi106} FRT 9-2/GFP FRT9-2; hsFLP/+* females. Green fluorescent protein (GFP) -marked MARCM clones (Lee and Luo, 2001) were induced by applying the same heat-shock protocol in the offspring of *baz$^{815-8}$ FRT19A/FM7* females crossed to *hsFLP, tubG80 FRT19A/Y; Act-Gal4 UAS-CD8::GFP/CyO* males (the latter were kindly provided by T. Klein).

Immunohistochemical Analysis of Pupal Imaginal Wing Discs and Dissection of Adult Wings

Pupal wings were dissected from staged pupae and fixed for at least 30 min in 4% paraformaldehyde/phosphate buffered saline (PBS). After washing in PBS, they were incubated with Alexa-coupled phalloidin 660/488 (1:40; Molecular Probes) for 1 hr at room temperature, followed by two washes in PBS/0.1% Triton and incubation with the primary antibody in PBT/10% horse serum overnight at 4°C. Wings were washed in PBS/0.1% Triton, incubated with the secondary antibody overnight in PBS/0.1% Triton with 10% horse serum, and mounted in glycerol-propylgallate. The following antibodies were used for staining: rabbit antiBaz N-term (1:1,000; Wodarz et al., 1999), mouse monoclonal anti-Fmi antibody (1:10; Usui et al., 1999; kindly provided by T. Uemura), Cy2- and Cy3-conjugated secondary antibodies (1:200; Jackson Immunoresearch). Imaginal discs were examined with a Leica TCS NT confocal microscope, and images were processed and mounted using Photoshop 7.1 (Adobe) and Canvas 9.0 (Deneba). Wings of adult flies were kept at least overnight in isopropanol, dissected, and mounted in Canada balsam.

Generation of Expression Constructs

An 829-bp fragment encoding the C-terminal 265 amino acids of Flamingo [Thr^{3310} to Tyr^{3574}, terminal EAEY version] (see also Fig. 3A) was obtained by PCR of the embryonic cDNA library LD (www.BDGP.org) using the primers flm-cyto.s: 5′-GAATTCACG-GACACCAGTTACC and flm-STOP.a: 5′-GTCGACTCACACTGTGGTCTCG-TCATC. The resulting product was subcloned into pCR2.1-TOPO-TA (Invitrogen) and subjected to an inverse PCR to delete the 7-bp exon sequence (CTCAGAG) specific to the EAEY version, using 5′-phosphorylated primers flanking the heptanucleotide (flm-del7.s: P-5′-GCGGAATATTGATGATG-ACGA and flm-del7.a: P-5′-TCGGTA-TCCGTGATGCTTGTC). Subsequent self-ligation of the PCR product and transformation yielded the desired construct encoding Thr^{3310} to Val^{3579} of the terminal ETTV version. Primer-derived *Eco*RI and *Sal*I linkers were used to insert the fragments into the pGEX-5X1 vector (Pharmacia) to allow for IPTG-induced expression of GST-fmi fusion proteins. Primer-derived *Eco*RI and *Sal*I linkers were used for subcloning the fragment encoding the three PDZ-domains of Bazooka (von Stein et al., 2000) into pGEX-5X1 (for production of GST-fusion protein) and pGBKT7 (for in vitro transcription/translation). All PCR-derived clones were verified by sequencing.

Pull-Down Assays and Western Blot Analysis

Pull-down assays and Western blots were essentially done as described before (Bachmann et al., 2004). To

detect the high-molecular-weight Fmi polypeptides, a modified polyacrylamide gel electrophoresis protocol was used (Bolt and Mahoney, 1997).

ACKNOWLEDGMENTS

We thank Andreas Wodarz for Bazooka antibodies, Tadashi Uemura for Flamingo antibody and flies, André Bachmann and Susann Özüyaman for advice in biochemical methods, Hendrik Strumpf for help with cloning Fmi-PCR products, and David Strutt for helpful discussions. E. Knust was funded by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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