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Strabismus is asymmetrically localised and binds to Prickle and Dishevelled during *Drosophila* planar polarity patterning

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SUMMARY

Planar polarity decisions in the wing of *Drosophila* involve the assembly of asymmetric protein complexes containing the conserved receptor Frizzled. In this study, we analyse the role of the *Van Gogh/strabismus* gene in the formation of these complexes and cell polarisation. We find that the Strabismus protein becomes asymmetrically localised to the proximal edge of cells. In the absence of *strabismus* activity, the planar polarity proteins Dishevelled and Prickle are mislocalised in the cell. We show that Strabismus binds directly to Dishevelled and Prickle and is able to recruit them to membranes. Furthermore, we demonstrate that the putative PDZ-binding motif at the C terminus of Strabismus is not required for its function. We propose a two-step model for assembly of Frizzled-containing asymmetric protein complexes at cell boundaries. First, Strabismus acts together with Frizzled and the atypical cadherin Flamingo to mediate apicolateral recruitment of planar polarity proteins including Dishevelled and Prickle. In the second phase, Dishevelled and Prickle are required for these proteins to become asymmetrically distributed on the proximodistal axis.

Key words: *Drosophila*, Planar polarity, Strabismus, Prickle, Dishevelled, Frizzled

INTRODUCTION

During animal development, almost all cell types become polarised in some way (Shulman and St Johnston, 1999). One particular example of this is the phenomenon of planar polarity in which epithelial cells become polarised in the plane of the epithelium (Eaton, 1997). Planar polarity has been extensively investigated in the cuticle of *Drosophila*, where it is manifest in a variety of different structures (Adler, 1992). These studies have led to the identification of a large number of genes required for planar polarity decisions (Adler, 2002). Central among these is the Frizzled (Fz) seven-pass transmembrane receptor (Vinson et al., 1989), which in this context acts through a non-canonical Wnt/Fz signalling pathway to control cell polarity decisions (McEwen and Peifer, 2000).

It has recently been discovered that Fz becomes asymmetrically localised to the distal edge of polarising cells of the pupal wing of *Drosophila* (Strutt, 2001). It colocalises in this location with its downstream signalling component Dishevelled (Dsh) (Axelrod, 2001; Shimada et al., 2001). At the same time, the Prickle (Pk) LIM-domain protein localises to the proximal cell edge (Gubb et al., 1999; Tree et al., 2002) and the sevenpass transmembrane atypical cadherin Flamingo (Fmi, also known as Starry Night) and the ankyrin repeat protein Diego (Dgo) localise to both proximal and distal cell edges (Feiguin et al., 2001; Usui et al., 1999). The data so far reported suggest that the activity of each of these five proteins is required for each of the others to become correctly localised. The mechanism of localisation is not fully understood, although it has been suggested that feedback loops mediated by Fz/Dsh and Pk may be important (Axelrod, 2001; Strutt, 2001; Tree et al., 2002).

A further factor that might be expected to be asymmetric in wing cells is the product of the *Van Gogh/Strabismus* (*Vang*/*Stbm*) locus. This gene was identified as being required for planar polarity throughout the adult cuticle of *Drosophila*, including the eye and wing (Taylor et al., 1998; Wolff and Rubin, 1998). Its loss-of-function phenotypes closely resemble those of other genes that produce asymmetrically localised proteins, and it shows genetic interactions with the *fz* and *pk* loci (Adler et al., 2000). Furthermore, we have recently reported that a fusion of Vang/Stbm to yellow fluorescent protein (Stbm-YFP) becomes asymmetrically localised during eye development (Strutt et al., 2002).

Vang/*Stbm* encodes a novel protein with four hydrophobic stretches that probably cross the membrane (Wolff and Rubin, 1998). The final three amino acids match the consensus for a PDZ-binding domain (PBM), suggesting that Stbm might interact with PDZ-domain proteins. Homologues are found throughout the animal kingdom, including worms, fish, frogs, mice and humans (Darken et al., 2002; Goto and Keller, 2002; Kibar et al., 2001; Park and Moon, 2002; Wolff and Rubin, 1998). As most vertebrate homologues have been named ‘Strabismus’, in this report we will refer to the *Drosophila* locus by this name.

Studies in vertebrates have demonstrated roles for *stbm* homologues in regulating polarised cell movements, in particular convergent extension during gastrulation and neural tube closure (Darken et al., 2002; Goto and Keller, 2002; Jessen et al., 2002; Kibar et al., 2001; Park and Moon, 2002). Assays of deleted forms of Stbm in zebrafish and *Xenopus* embryos suggest that the putatively intracellular C-terminal region is most likely to be important for function of the molecule (Goto and Keller, 2002; Park and Moon, 2002). Furthermore, vertebrate Stbm has been shown to bind to a vertebrate Dsh homologue through this C-terminal region (Park and Moon, 2002). Surprisingly, although binding requires the PDZ domain of Dsh, the putative PBM of Stbm was not required. Furthermore, studies in vertebrates have led to conflicting conclusions about the importance of the PBM (Darken et al., 2002; Goto and Keller, 2002; Park and Moon, 2002).

In this study, we investigate the role of the *Drosophila* Stbm protein in planar polarity patterning of the wing. Using both genetic and molecular techniques, we show that Stbm acts in a hierarchy of molecules that lead to the assembly of asymmetric protein complexes. In particular we find that Stbm binds to Pk and regulates its subcellular distribution and levels. Functional dissection shows that this binding requires the C-terminal intracellular domain of Stbm. However, genetic rescue experiments demonstrate that there is no critical role for the putative PBM in *Drosophila* planar polarity patterning.

MATERIALS AND METHODS

Fly stocks and genetics

P[w$^+$, Act-Stbm-YFP] and *P[w$^+$, Arm-Fz-GFP]* have been described (Strutt et al., 2002; Strutt, 2001). *P[w$^+$, Act-Stbm-ΔPBM]* was made as for *P[w$^+$, Act-Stbm-YFP]*, except it expresses only the Stbm ORF with the last three amino acids removed. *stbm^6* (Wolff and Rubin, 1998), *stbm$^{Vang\text{-}A3}$* (Taylor et al., 1998), *fz^{15}*, *fz^{23}*, *fz^{25}* (Jones et al., 1996), *fmi^{E59}* (Usui et al., 1999), *pk-sple^{13}* (Gubb et al., 1999) and *dsh^3* (Wehrli and Tomlinson, 1998) have all been molecularly defined as null alleles or genetically defined as strong or amorphic alleles for function in the wing. *Df(2R)w45-30n* uncovers the *stbm* locus (Taylor et al., 1998). Loss-of-function mitotic clones were generated using the FLP/FRT system (Xu and Rubin, 1993) and marked using *Arm-lacZ* (Vincent et al., 1994). Overexpression used the UAS/GAL4 system (Brand and Perrimon, 1993) and the *ptc-GAL4* driver or *Act>>GAL4, UAS-lacZ* (Ito et al., 1997). *UAS-Stbm* was made by cloning the full-length Stbm ORF (Wolff and Rubin, 1998) into the vector pUAST. *UAS-Fz* (Adler et al., 1997), *UAS-Fmi* (Usui et al., 1999), *UAS-Pk* (Gubb et al., 1999) and *UAS-Dsh* (Neumann and Cohen, 1996) have been described. *strabismus* [*stbm* (Wolff and Rubin, 1998)] and *Van Gogh* [*Vang* – FlyBase (Taylor et al., 1998)] are the same gene, as are *flamingo* [*fmi* (Usui et al., 1999)] and *starry night* [*stan* – FlyBase (Chae et al., 1999)].

Immunostaining

Immunostaining was carried out as previously (Strutt, 2001). Primary antibodies used were mouse anti-β-gal (Promega), mouse anti-FLAG M5 (Sigma), mouse anti-Myc 9E10 (Santa Cruz), rabbit anti-Pk (Tree et al., 2002), rat anti-Dsh (Shimada et al., 2001), mouse anti-Fmi (Usui et al., 1999), rabbit anti-Dlg (Woods and Bryant, 1991), mouse anti-Arm 7A1 (Riggleman et al., 1990) (obtained from Developmental Studies Hybridoma Bank, Iowa). Anti-Stbm was raised in rabbits against a bacterially expressed peptide corresponding to amino acids 406-584, affinity purified against the same region. Secondary antibodies were conjugated to Alexa-488 or Alexa-568 (Molecular Probes) or Rhodamine-Red-X or Cy5 (Jackson). Unless otherwise stated, confocal sections are of the most apical regions of pupal wing cells, representing the average of several confocal image planes for a total image depth of about 1 μm.

Biochemistry

For transient transfection, COS-7 cells were grown in 24-well plates. FuGENE 6 (Roche) was used to transfect 200 ng of each plasmid per well, and cells were either fixed for immunostaining or lysed for immunoprecipitation 24 hours later. All proteins were expressed using CMV promoter plasmids. Full-length Stbm, FLAG-Stbm (FLAG-tagged at the N-terminus), Stbm-ΔPBM (Stbm ORF with final three amino acids deleted), CD2-Stbm-Cterm-ΔPBM (amino acids 1 to 246 of rat CD2, fused to amino acids 301 to 581 of Stbm), Dsh and Pk were cloned in pcDNA3.1 (Invitrogen). pCS-Fz and pCS-Dsh-GFP have been described previously (Tree et al., 2002). Myc-Pk was tagged with 6-Myc epitopes at the N terminus by cloning in the vector pCS2+MT.

For immunoprecipitation, 1/5 of the lysate from a single well was used for each reaction, diluted in IP buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.25% Triton X-100, Roche protease inhibitors). Immunoprecipitations were carried out overnight at 4°C using anti-FLAG M2 (Sigma) or anti-Myc 9E10 (Santa Cruz) and protein G Sepharose (Sigma). Proteins were detected on Western blots using affinity purified anti-GFP (Abcam), anti-Myc 9E10 (Santa Cruz) or anti-Rat CD2 OX34 (Serotec), and HRP-conjugated secondary antibodies (DAKO). Detection was using ECL (Amersham) or Supersignal West Dura (Pierce).

RESULTS

Stbm localises proximally in the adherens junction zone in cells of the pupal wing

We investigated the subcellular localisation of Stbm protein during wing morphogenesis using both a Stbm-YFP expressing transgene (Strutt et al., 2002) and using specific antibodies raised against Stbm. During the third instar stage, Stbm-YFP in the wing pouch localises unevenly around apicolateral cell boundaries (Fig. 1A). Based on its molecular homology as a multi-pass transmembrane protein, we assume that Stbm is present in the outer cell membrane. At 18 hours of pupal life, a similar pattern is seen, Stbm-YFP still being distributed patchily in an apicolateral ring (Fig. 1B). By 24 hours, there is preferential distribution of Stbm-YFP to proximodistal cell boundaries, which is clearly present at 28 hours and persists until at least 32 hours, which corresponds to the time of trichome initiation (Fig. 1C,D and data not shown). Although we are only able to obtain poor immunostaining of pupal wings using our antibodies against Stbm, the pattern seen confirms that Stbm-YFP is a faithful reporter of Stbm protein distribution (Fig. 1E,F).

The timecourse and distribution of Stbm broadly fits that described for other planar polarity proteins such as Fmi, Fz, Dsh and Pk-Sple (Axelrod, 2001; Shimada et al., 2001; Strutt, 2001; Tree et al., 2002; Usui et al., 1999). Consistent with this, we find good colocalisation between Stbm-YFP and other polarity proteins (Fig. 1G,H and data not shown). We confirmed the localisation of Stbm-YFP to the adherens junction zone by costaining for Armadillo (Peifer, 1993) distribution (Fig. 1J). Conversely, Stbm-YFP shows no overlap with the distribution of Discs-Large (Woods and Bryant, 1991), which is localised in the septate junction region (Fig. 1I). Mosaic analysis revealed that Stbm-YFP becomes preferentially distributed to the proximal edges of cells (arrowheads, Fig. 1J) with no appreciable accumulation at distal edges.

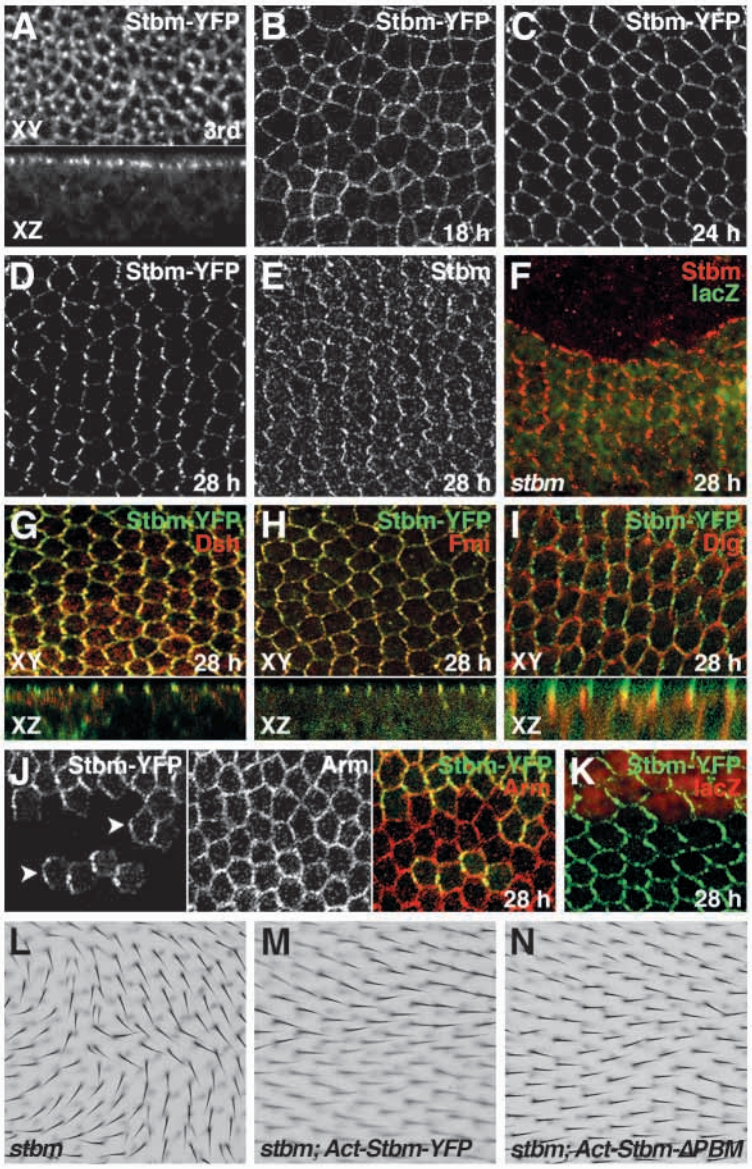

Fig. 1. Localisation of Stbm in the developing wing. Confocal images of third instar wing disc (A) or pupal wings (B-K), or images of dorsal surface of adult wings between vein 3 and vein 4 (L-N). Distal is rightwards and anterior is upwards. (A-D) Stbm-YFP is apicolateral and progressively becomes distributed to proximodistal cell boundaries. (E) Staining with an antibody against Stbm shows the protein to be apicolateral and at proximodistal cell boundaries. (F) No Stbm staining (red) is seen in a *stbm*^{6} mutant clone (labelled by absence of green *lacZ* staining). Note that Stbm is ectopically localised in cells on edge of clone, owing to the non-autonomous phenotype of *stbm* clones in the wing (Taylor et al., 1998). (G-I) Stbm-YFP (green) colocalises with Dsh (red, G) and Fmi (red, H) but not Dlg (red, I). (J) Mosaic expression of Stbm-YFP (white in left panel and green in right panel) reveals it to be preferentially localised to proximal cell boundaries (arrowheads), where it is colocalised with the adherens junction marker Armadillo (white in middle panel and red in right panel). Note in this experiment that all cells have endogenous *stbm* activity and patterning is normal. A subset of cells also express Stbm-YFP. At the boundaries between cells that express Stbm-YFP and those that do not, it is possible to see at which cell boundary Stbm-YFP preferentially localises. (K) Stbm-YFP (green) localises normally in a *stbm*^{6} mutant clone (labelled by absence of red *lacZ* staining). (L) *stbm*^{6}*/Df(2R)45-30n.* (M) *stbm*^{6}*/Df(2R)45-30n; P[w*$^{+}$*; Act-Stbm-YFP]/+*. (N) *stbm*^{6} *P[w*$^{+}$*; Act-Stbm-ΔPBM]/stbm*$^{Vang\text{-}A3}$.

We carried out two further controls for the use of the *Stbm-YFP* transgene. First, we showed that Stbm-YFP localises normally to proximodistal boundaries in the absence of endogenous *stbm* function (Fig. 1K); and second we found that ubiquitous *Stbm-YFP* expression is able to rescue the *stbm* polarity defect in the wing, eye and leg (Fig. 1M and data not shown).

We note that fusion of YFP to the C terminus of Stbm would be expected to mask the putative PBM. Therefore our rescue of *stbm* phenotypes by *Stbm-YFP* expression suggests that this motif is not essential for gene function. We confirmed this by expressing a form of Stbm lacking the PBM and found that this also rescues the *stbm* polarity phenotype in the wing (Fig. 1N).

Stbm, Fmi and Fz promote the apicolateral localisation of planar polarity proteins

Of the planar polarity proteins so far studied that exhibit asymmetric apicolateral localisation, in each case tested this localisation partly depends upon the function of those other planar polarity genes with asymmetrically distributed gene products (Axelrod, 2001; Shimada et al., 2001; Strutt, 2001; Tree et al., 2002; Usui et al., 1999).

We have previously reported that *stbm* function is required for normal Fz asymmetric localisation (Strutt, 2001). Loss of *stbm* leads to a reduction in apicolateral Fz-GFP, with the remaining protein showing a hazy distribution with no proximodistal modulation (Fig. 2A). Loss of *fz* also disrupts Stbm-YFP localisation, a reduction in apicolateral levels being observed and no proximodistal modulation being evident (Fig. 2B). It is known that *fmi* activity is required for apicolateral localisation of Fz (Strutt, 2001) and Dsh (Shimada et al., 2001). We find that loss of *fmi* also greatly reduces Stbm-YFP apicolateral localisation (Fig. 2D) and loss of *stbm* somewhat reduces apicolateral localisation of Fmi (Fig. 2C). However, loss of *fz* has only a negligible effect on apicolateral localisation of Fmi (Strutt, 2001; Usui et al., 1999).

The effects of loss of *stbm* on Dsh and Pk localisation were also tested. In this case, Dsh localisation is strongly reduced (Fig. 2E). However, the effect of loss of *stbm* on Pk localisation was unexpected. In *fz*, *dsh* or *fmi* mutations, Pk exhibits reduced apicolateral and/or proximodistal location (Tree et al., 2002), but in *stbm* clones Pk shows greatly increased levels in the cytoplasm as well as some localisation at the apicolateral cell cortex (Fig. 2F). This suggests that Stbm normally plays a role in either destabilising or otherwise reducing cellular levels of Pk protein, or in regulating transcription or stability of *pk* mRNA.

Overall, we conclude that Stbm, Fmi and Fz each promote the stable apicolateral localisation of at least a subset of other polarity proteins to the cell cortex (see Discussion). Notably, we find that overexpression of Fmi, Fz or Stbm does not significantly promote apicolateral accumulation of other polarity

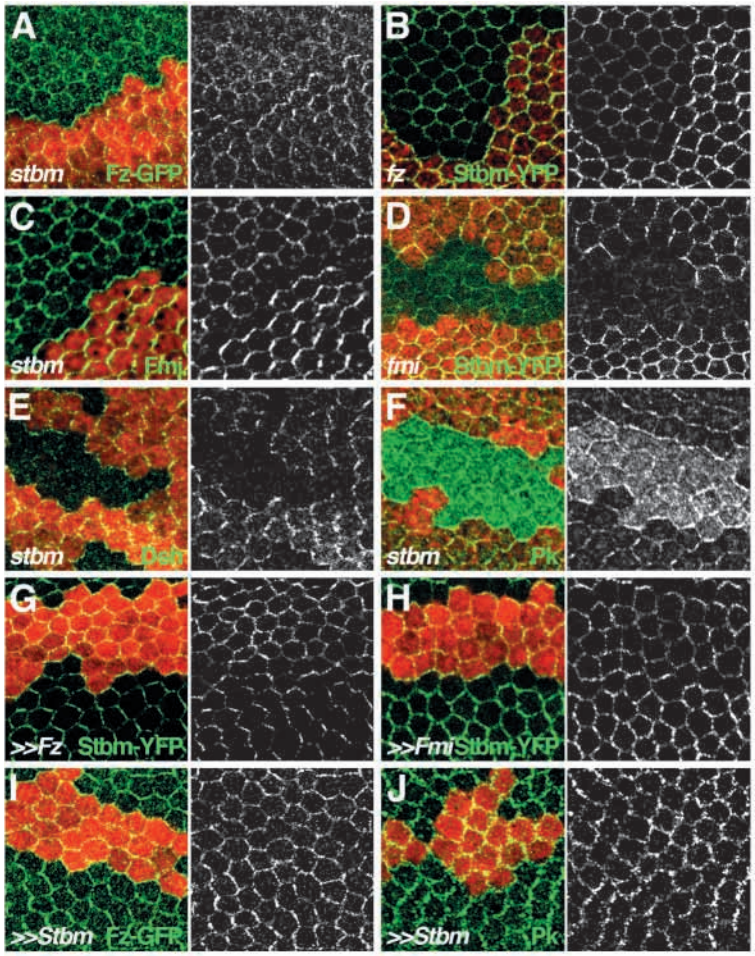

Fig. 2. *stbm*, *fz* and *fmi* are required for apicolateral asymmetric localisation of polarity proteins. Confocal images of pupal wings at about 28 hours. Distal is right and anterior is upwards. (A-F) Loss-of-function clones; mutant tissue indicated by absence of *lacZ* staining (red, left panels). (G-J) Clones of overexpression indicated by *lacZ* staining (red, left panels). Note that in all genotypes except *fmi*, the clones also have significant non-autonomous effects on planar polarity that leads to ectopic localisation of polarity proteins in cells surrounding the clone. (A) Fz-GFP in $stbm^{Vang\text{-}A3}$. (B) Stbm-YFP in fz^{25}. (C) Fmi in $stbm^{Vang\text{-}A3}$. (D) Stbm-YFP in fmi^{E59}. (E) Dsh in $stbm^{6}$. (F) Pk in $stbm^{6}$. (G) Stbm-YFP in *Act-GAL4/UAS-Fz* overexpression clone. (H) Stbm-YFP in *Act-GAL4/UAS-Fmi* overexpression clone. (I) Fz-GFP in *Act-GAL4/UAS-Stbm* overexpresssion clone. (J) Pk in *Act-GAL4/UAS-Stbm* overexpression clone.

proteins (Fig. 2G-J), although it does disrupt proximodistal localisation. We interpret this to mean that these factors have specific roles in the apicolateral recruitment of a polarity protein complex, but cannot promote aggregation above normal levels.

Pk and Dsh promote asymmetric proximodistal localisation and accumulation of polarity proteins

Removal of *dsh* or *pk-sple* function results in similar phenotypes, as regards the subcellular localisation of Fz, Fmi and Stbm [Fig. 3A-E; see also (Shimada et al., 2001; Strutt, 2001)]. In both genotypes, levels of apicolaterally localised polarity proteins are close to normal (although do sometimes appear slightly reduced) and the proteins are tightly associated with the cell boundaries, but nevertheless no asymmetric localisation is evident on the proximodistal axis. Thus, unlike Fz, Fmi and Stbm, neither of these proteins appears to play a major role in apicolateral recruitment of other proteins.

As Pk and Stbm both localise proximally in cells, we were interested in whether they might act together. Simultaneous removal of both *pk-sple* and *stbm* results in a strong reduction in apicolateral Fmi (Fig. 3F). As loss of *stbm* also causes a significant reduction in apicolateral Fmi (Fig. 2C), this suggests that Stbm alone plays an important role in apicolateral localisation of Fmi, acting upstream of Pk, but that Stbm and Pk may also cooperate in this process.

As previously reported (Tree et al., 2002), increased Pk levels lead to higher levels of Fz, Dsh and Fmi in apicolateral complexes (Fig. 3G). High Pk also promotes increased apicolateral accumulation of Stbm-YFP (Fig. 3H). We also find that elevated Dsh also leads to increased levels of Stbm-YFP in apicolateral complexes (Fig. 3I), the phenotype being very similar to that caused by overexpression of Pk.

It has been suggested that elevated levels of Pk result in increased Fz signalling and that this accounts for higher protein levels in apicolateral complexes (Tree et al., 2002). However, we find that overexpressing Pk in a *fz* mutant background still results in higher levels of apicolateral proteins (Fig. 3J), indicating that such accumulations are not a result of increased Fz signalling. Furthermore, overexpression of Fz is known to activate Fz signalling (Krasnow et al., 1995), but does not result in similar increased apicolateral accumulation of polarity proteins (Fig. 2G). It is interesting to note that the elevated levels of Pk in *stbm* clones do not result in increased apicolateral levels of polarity proteins.

Overall, our results suggests that whereas Dsh and Pk do not play a major role in apicolateral recruitment of polarity proteins, they are crucially important for their asymmetric distribution on the proximodistal axis and they also promote increased aggregation or stability of polarity proteins at the cell cortex.

Stbm interacts directly with Dsh and Pk

Our understanding of the respective roles of different planar polarity proteins in cell polarisation is limited by our lack of knowledge about their biochemical interactions and functions. We have therefore investigated the properties of Stbm using in vitro assays.

It has recently been reported that vertebrate homologues of Stbm and Dsh associate both in vivo and in vitro (Park and Moon, 2002). As during the later stages of cell polarisation in the wing, Dsh is localised distally (Axelrod, 2001) and Stbm is localised proximally (this work), the significance of these findings for the *Drosophila* system are unclear. We therefore tested whether *Drosophila* Stbm could associate with *Drosophila* Dsh. We found that Stbm and Dsh associate using two different assays, when expressed in COS7 tissue culture cells. First, the proteins co-immunoprecipitate (Fig. 4A) and secondly Stbm is able to qualitatively recruit Dsh from cytoplasmic vesicles to Stbm containing membranes (Fig. 5C,

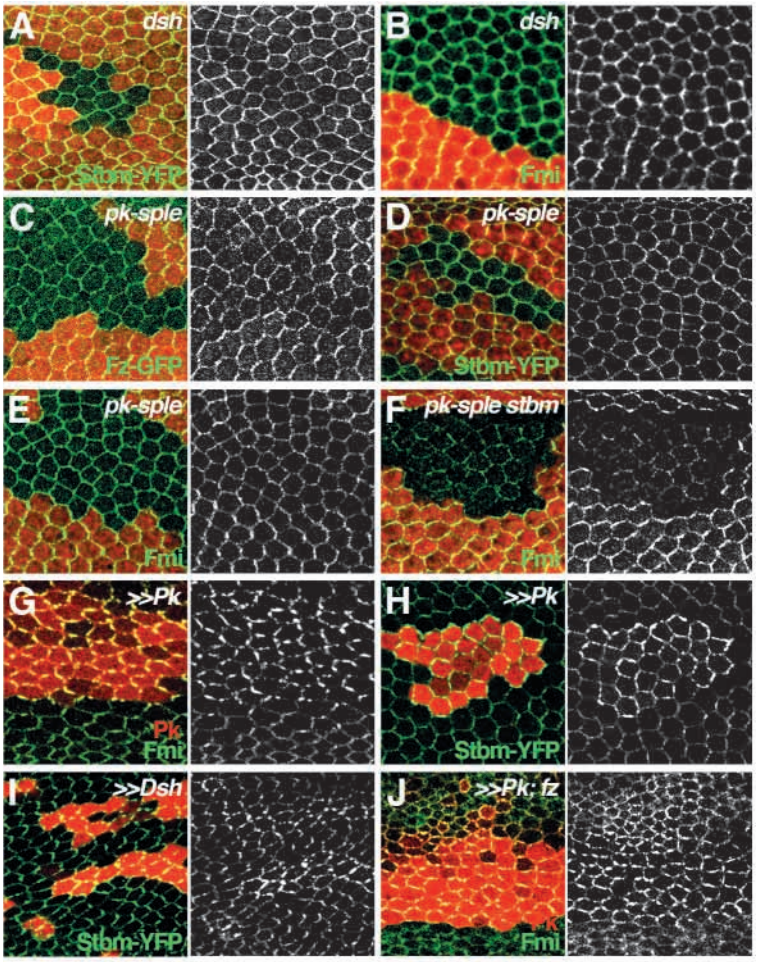

Fig. 3. *pk* and *dsh* are required for asymmetric localisation and accumulation of polarity proteins. Confocal images of pupal wings at about 28 hours. Distal is rightwards and anterior is upwards. (A-F) Loss-of-function clones, mutant tissue indicated by absence of *lacZ* staining (red, left panels). (G-J) Clones of overexpression indicated by *lacZ* staining (red, left panels, H,I) or Pk staining (red, left panels, G,J). (A) Stbm-YFP in dsh^3. (B) Fmi in dsh^3. (C) Fz-GFP in $pk\text{-}sple^{13}$. (D) Stbm-YFP in $pk\text{-}sple^{13}$. (E) Fmi in $pk\text{-}sple^{13}$. (F) Fmi in $pk\text{-}sple^{13}$ $stbm^6$ double mutant clone. Note that Fmi is ectopically localised in cells surrounding clone, owing to the non-autonomous phenotype of *stbm* clones in the wing (Taylor et al., 1998). (G) Fmi in *Ptc-GAL4/UAS-Pk* wing at compartment boundary. (H) Stbm-YFP in *Act-GAL4/UAS-Pk* overexpression clone. (I) Stbm-YFP in *Act-GAL4/UAS-Dsh* overexpression clone. (J) Fmi in *Ptc-GAL4/UAS-Pk;* fz^{15}/fz^{23} wing at compartment boundary.

note that under these expression conditions, Stbm is largely associated with the Golgi).

Using the same techniques, we also find a similar interaction between Stbm and Pk (Fig. 4B, Fig. 5F). We next asked whether Stbm preferentially associated with Dsh or Pk. Co-expressing all three proteins, we found that the presence of either Dsh or Pk does not significantly inhibit the interactions between Stbm and Pk or Stbm and Dsh, respectively (Fig. 4C,D). Indeed, all three proteins colocalise (Fig. 5G). Transfecting up to five times more of either the Dsh or Pk expression plasmid failed to disrupt these interactions (data not shown). It has recently been reported that Dsh and Pk specifically interact with each other (Tree et al., 2002). In our assay, in our best experiments we were also able to see this interaction (Fig. 4E), but it was much weaker than the interactions between Stbm and Pk and between Stbm and Dsh. Furthermore, co-expression of Dsh and Pk resulted in only negligible colocalisation in COS7 cells (Fig. 5H).

Based on a similar heterologous tissue culture assay, it has been reported that Pk is capable of antagonising the well-characterised interaction between Fz and Dsh at the cell cortex (Tree et al., 2002). Consistent with our failure to observe an appreciable interaction between Dsh and Pk, we also found no effect of Pk co-expression on recruitment of Dsh to the cortex by Fz (Fig. 5J). Indeed, transfection of a four times excess of the Pk expression plasmid relative to the Dsh expression plasmid still had no effect. We carried out this assay both in COS7 cells, where Fz is able to efficiently recruit Dsh (Fig. 5I) and in U-2 OS cells as used in the previous study (data not shown). Currently we are unable to explain the discrepancy between our results and the previous study. However, we note that overexpression of Pk in vivo does not reduce membrane recruitment of Dsh (Tree et al., 2002).

Vertebrate studies have indicated that the PBM of vertebrate Stbm is not necessary for binding to Dsh (Park and Moon, 2002), consistent with our own findings that the PBM of *Drosophila* Stbm is not absolutely required for its function. We now find that the PBM is not necessary for binding to *Drosophila* Dsh or Pk (Fig. 5L,M,O,Q). However, the binding activity is located within the C-terminal putative intracellular tail (Fig. 5O,Q), which can efficiently recruit either protein in the absence of the PBM when tethered to the outer cell membrane by the heterologous transmembrane domain of rat CD2. Furthermore, the same CD2-StbmCterm-ΔPBM fusion protein co-immunoprecipitates with Pk (Fig. 4F).

As already noted, it is perhaps surprising that Stbm binds to Dsh, as ultimately these molecules become localised to the opposite sides of cells (or cell-cell boundaries) in the developing wing. However, we find that these proteins can colocalise from much earlier in wing development. For example, in the third instar wing pouch where polarity proteins are not visibly asymmetrically localised, Stbm-EYFP and Dsh colocalise in apicolateral regions of the cell (Fig. 5R). This is consistent with Stbm and Dsh directly associating during the symmetric phase of apicolateral polarity protein localisation. However, it is also possible that this colocalisation is due to assembly of randomly orientated asymmetric complexes across cell-cell boundaries (see Discussion and Fig. 6).

DISCUSSION

The phenomenon of asymmetric localisation of planar polarity proteins during cell polarisation is now well established (reviewed by Strutt, 2002). However, much remains unknown about the mechanisms underlying this asymmetric localisation.

Studies of protein localisation and genetic dissection suggests that the process of asymmetric localisation of planar polarity proteins in the wing can be divided into two parts: first, a phase in which proteins are localised apicolaterally to the adherens

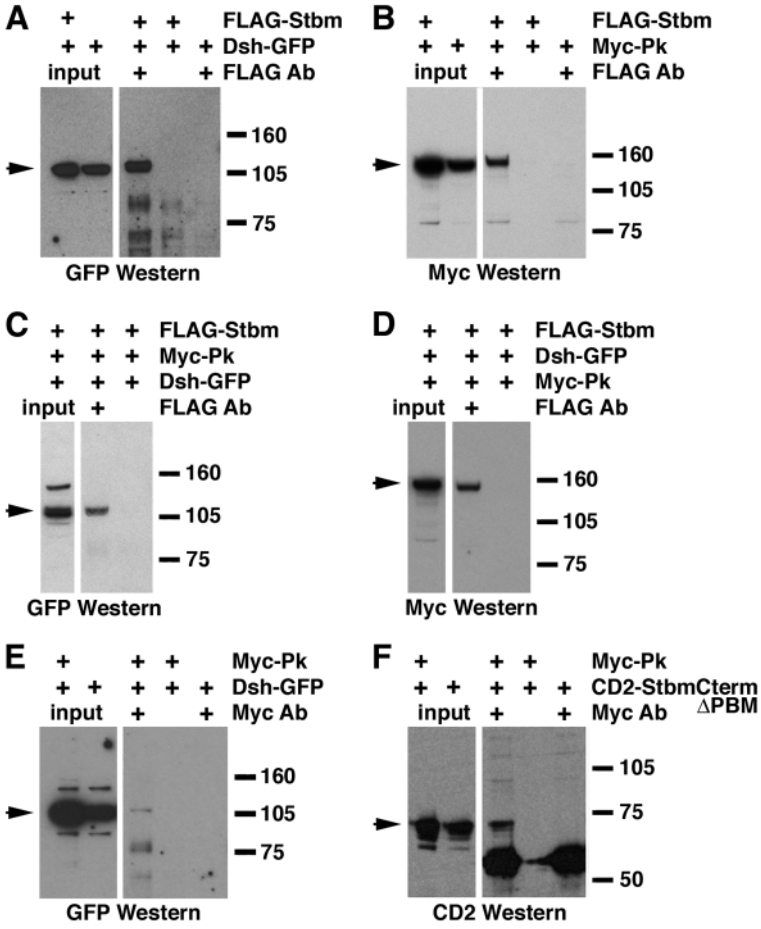

Fig. 4. Stbm co-immunoprecipitates Dsh and Pk. Immunoprecipitations from COS7 cells transfected with the plasmids indicated. Proteins in the lysate prior to immunoprecipitation are shown on the left (input), and either five times (B-D) or 10 times (A,E,F) equivalent immunoprecipitated material is on the right. (A) Dsh-GFP co-immunoprecipitated with FLAG-Stbm. (B) Myc-Pk co-immunoprecipitated with FLAG-Stbm. (C,D) Lysate containing FLAG-Stbm, Dsh-GFP and Myc-Pk immunoprecipitated with anti-FLAG co-immunoprecipitates both Dsh-GFP (C) and Myc-Pk (D). (E) Weak co-immunoprecipitation of Dsh-GFP by Myc-Pk. (F) Myc-Pk co-immunoprecipates a fusion of CD2 extracellular and transmembrane regions to Stbm C-terminal intracellular domain lacking the putative PBM. Arrows indicate specific bands of expected molecular weights detected by western blotting for co-immunoprecipitated proteins.

junction zone; and then a second stage in which molecules become asymmetrically distributed on the proximodistal axis (Strutt, 2002). Taking our new data with that already published (Axelrod, 2001; Feiguin et al., 2001; Shimada et al., 2001; Strutt, 2001; Tree et al., 2002; Usui et al., 1999), we draw a number of conclusions about the mechanisms responsible.

The three putative multipass transmembrane proteins Fmi, Fz and Stbm all play important roles in the first step of localising planar polarity proteins to the apicolateral adherens junction zone (Fig. 6A). We believe that Fmi acts at the top of the hierarchy in this process, as, in its absence, negligible amounts of any planar polarity proteins become apicolaterally localised (Feiguin et al., 2001; Shimada et al., 2001; Strutt, 2001; Tree et al., 2002) (this work). Stbm is also key, because, in its absence, both Fz (Strutt, 2001) and Fmi recruitment are reduced (this work). Additionally, Stbm is also required for Dsh apicolateral recruitment and for efficient localisation of Pk to membranes. Fz is not significantly required for apicolateral recruitment of Fmi (Strutt, 2001), but is partly needed for apicolateral localisation of Stbm and is absolutely required for apicolateral localisation of Dsh (Axelrod, 2001; Shimada et al., 2001). Hence, in the absence of Fmi, Fz or Stbm, one or more planar polarity proteins do not become apicolaterally localised and the process of asymmetric localisation on the proximodistal axis does not occur.

An important question is which of these factors are directly binding together, in the process of apicolateral recruitment. So far no direct protein interactions have been reported for Fmi, although it is tempting to speculate that Fmi might bind directly to Fz and Stbm in the process of apicolateral recruitment. However, Fz is able to recruit Dsh to membranes in a heterologous cell type (Axelrod et al., 1998), suggesting that these factors directly interact. In addition, vertebrate Stbm and Dsh homologues have been shown to directly interact (Park and Moon, 2002). We now show direct interactions between *Drosophila* Stbm and Dsh, and Stbm and Pk. This suggests a model in which Dsh and Pk both become apicolaterally localised as a result of direct interactions with Fz and Stbm. Notably, in the absence of Stbm, Pk accumulates in the cytoplasm, suggesting that its interaction with Stbm is important for regulating its level in the cell in addition to its subcellular localisation.

At the stage when the planar polarity proteins are apicolaterally localised, but prior to the stage when they are asymmetrically localised on the proximodistal axis of the wing, it is possible that they are present in either ‘symmetric’ or ‘asymmetric’ complexes assembled across cell-cell boundaries (Fig. 6B). If the complexes were symmetric, then Fmi, Fz, Stbm, Pk and Dsh would all be present in a complex together on the same side of the cell-cell boundary. Such symmetric complexes would then subsequently evolve into asymmetric complexes, with Fz/Dsh at distal cell edges and Stbm/Pk at proximal cell edges and Fmi on both sides. Alternatively, the initial apicolateral complexes formed could be asymmetric, with Fz/Dsh always on the opposite side of the cell-cell boundary from Stbm/Pk. These asymmetric complexes would initially be randomly orientated relative to the axes of the wing, but would gradually become aligned to the proximodistal axis. We favour the possibility that planar polarity protein complexes are initially symmetric, as Stbm directly interacts with Dsh and these molecules colocalise during earlier stages of wing development. However, it has been reported that Pk and Dsh-GFP do not precisely colocalise in early pupal wings (Tree et al., 2002), which supports the early presence of asymmetric complexes.

After the apicolateral recruitment of planar polarity proteins, over a number of hours their localisation alters such that they become asymmetrically distributed on the proximodistal axis of the wing. Although Dsh and Pk play negligible roles in the

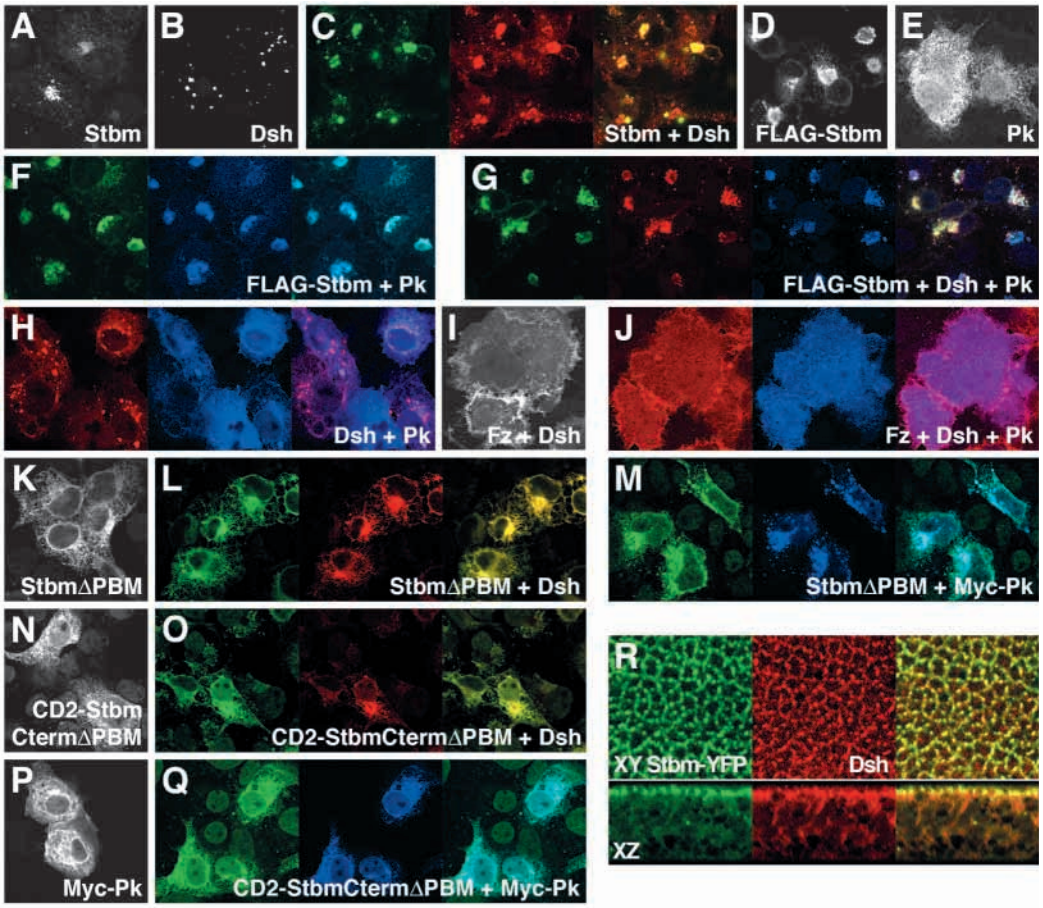

Fig. 5. Stbm recruits Dsh and Pk to membranes. Confocal images of COS7 cells transfected with the plasmids indicated (A-Q), or third instar wing disc expressing Stbm-YFP (R). Distribution of proteins expressed singly is shown in white. Stbm (detected with anti-Stbm), FLAG-Stbm (detected with anti-FLAG) and Stbm-YFP (detected by YFP fluorescence) is always shown in green in multiply labelled panels. Dsh (detected with anti-Dsh) is always shown in red in multiply labelled panels. Pk (detected with anti-Pk) and Myc-Pk (detected with anti-Myc) are always shown in blue in multiply labelled panels. Stbm-ΔPBM (K-M) lacks the last three amino acids of the ORF. CD2-Stbm-Cterm-ΔPBM (N-Q) consists of the extracellular and transmembrane regions of rat CD2, fused to the C-terminal intracellular tail of Stbm with the last three amino acids deleted. (A-C) Stbm (white in A, green in C) can recruit Dsh (white in B, red in C) from cytoplasmic vesicles to Stbm-expressing membranes (largely the Golgi membranes but partly the outer cell membrane). (D-F) FLAG-Stbm (white in D, green in F) can recruit Pk (white in E, blue in F) to Stbm-expressing membranes. (G) FLAG-Stbm (green), Dsh (red) and Pk (blue) colocalise to Stbm expressing membranes. (H) Dsh (red) and Pk (blue) co-expressed together do not colocalise. (I,J) Recruitment of Dsh (white in I, red in J) to the outer membrane by Fz (I) is not disrupted by co-expression of Pk (blue in J). (K-Q) Stbm-ΔPBM (white in K, green in L,M) or CD2-Stbm-Cterm-ΔPBM (white in N, green in O,Q) can recruit Dsh (red in L,O) and Pk (white in P, blue in M,Q). (R) Stbm-YFP and Dsh colocalise apicolaterally in the wing pouch of a third instar wing disc.

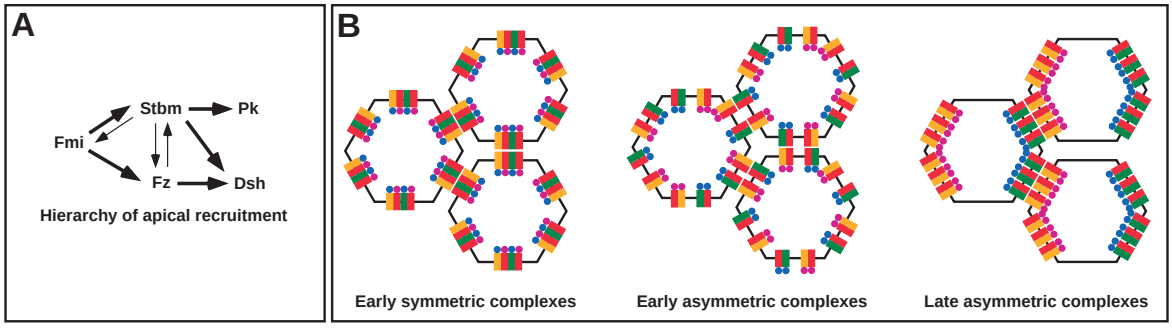

Fig. 6. Models for asymmetric localisation of planar polarity proteins. (A) We propose that initial apicolateral recruitment of planar polarity proteins occurs according to the hierarchy shown (see text for details). (B) During the early stages of asymmetric complex formation, planar polarity proteins are apicolaterally localised but not obviously asymmetrically distributed on the proximodistal axis. At this stage, they may either be in symmetric complexes in which the same proteins are present on both sides of the cell-cell boundaries (left) or asymmetric complexes that are randomly orientated relative to the proximodistal axis of the wing (middle). Ultimately, the pattern resolves to that shown (right). Proteins represented are Fmi (red), Fz (green), Dsh (blue circles), Stbm (yellow) and Pk (purple circles).

apicolateral recruitment of proteins, both are required for this subsequent proximodistal redistribution. As overexpression of both factors leads to the accumulation of polarity proteins at apicolateral cell boundaries, we suggest that they both function to promote the assembly and/or stabilisation of protein complexes. We note that removal of the function of the planar polarity gene *dgo* also blocks asymmetric proximodistal localisation but not apicolateral localisation of other polarity

proteins (Feiguin et al., 2001). Furthermore, overexpression of Dgo causes a similar accumulation of other polarity proteins at cell boundaries to that seen when Dsh and Pk are overexpressed. Therefore, we propose that Dsh and Pk act together with Dgo in the assembly of asymmetric complexes.

Recently, it has been proposed that the function of Pk in asymmetric complex assembly is to antagonise Dsh localisation to membranes (Tree et al., 2002). This model is mechanistically attractive, in providing an explanation for the formation of asymmetric complexes in which Dsh and Pk are found on opposite sides of cell-cell boundaries. However, we find that in the presence of Stbm, Dsh and Pk will colocalise at the same membranes. Furthermore, we were unable to show an effect of overexpressing Pk on the association of Fz and Dsh at membranes. In addition, high level Pk expression in vivo does not cause Dsh to lose its membrane localisation but instead appears to increase levels of Dsh at the membrane (Tree et al., 2002). Resolution of these issues will require a more detailed understanding of the composition and properties of the protein complexes involved.

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