Planar polarity genes in the Drosophila wing regulate the localisation of the FH3-domain protein Multiple Wing Hairs to control the site of hair production

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The core planar polarity proteins play important roles in coordinating cell polarity, in part by adopting asymmetric subcellular localisations that are likely to serve as cues for cell polarisation by as yet uncharacterised pathways. Here we describe the role of Multiple Wing Hairs (Mwh), a novel formin homology 3 (FH3)-domain protein, which acts downstream of the core polarity proteins to restrict the production of actin-rich prehairs to distal cell edges in the Drosophila pupal wing. Mwh appears to function as a repressor of actin filament formation and, in its absence, ectopic actin bundles are seen across the entire apical surface of cells. We show that the proximally localised core polarity protein Strabismus acts via the downstream effector proteins Inturned, Fuzzy and Fritz to stabilise Mwh in apico-proximal cellular regions. In addition, the distally localised core polarity protein Frizzled positively promotes prehair initiation, suggesting that both proximal and distal cellular cues act together to ensure accurate prehair placement.

KEY WORDS: Planar polarity, Multiple Wing Hairs, Drosophila, Cell polarity, Frizzled, Actin

INTRODUCTION

The development of multicellular organisms requires the coordinated specification of both cell fates and cell polarities. One example of coordinated cell polarisation is planar polarity, whereby cells within epithelial sheets adopt uniform polarities relative to the plane of the tissue. Although widespread in nature, the mechanisms underlying the establishment of planar polarity have been most often studied in insect cuticles; nevertheless, essential roles in vertebrate development are now recognised (reviewed by Strutt, 2003; Klein and Mlodzik, 2005; Wang and Nathans, 2007).

In many contexts, a key event in the coordinated planar polarisation of epithelia is the asymmetric subcellular localisation of a group of "core" planar polarity proteins to opposite cell edges. This process is best characterised in the Drosophila pupal wing, in which the seven-pass transmembrane protein Frizzled (Fz) and the cytoplasmic proteins Dishevelled (Dsh) and Diego (Dgo) localise to distal apicolateral junctional regions, the four-pass transmembrane protein Strabismus (Sibm, also known as Van Gogh) and the cytoplasmic protein Prickle (Pk) localise proximally, and the seven-pass transmembrane cadherin Flamingo (Fmi, also known as Starry Night) localises both distally and proximally (reviewed by Klein and Mlodzik, 2005). The localisations of the core proteins define distinct distal and proximal apicolateral membrane domains, which are thought to act as cues for subsequent cell polarisation events mediated by downstream effector genes.

The best-characterised morphogenetic event regulated by the asymmetric localisation of the core polarity proteins is the production of a single, distally pointing trichome from each cell of the wing blade (Gubb and Garcia-Bellido, 1982; Wong and Adler, 1993). Trichome formation begins with increased actin bundling close to the distal cell vertex at ~32 hours of pupal life, leading to formation of a prehair that contains both F-actin and microtubules (Mitchell et al., 1983; Wong and Adler, 1993; Eaton et al., 1996; Turner and Adler, 1998). Electron microscopy has revealed that shortly prior to prehair formation, the apical cell surface is covered in electron-dense 'pimples' (Gould et al., 2005), which are believed to be precursors for microvillus formation. Prehair initiation is manifested by actin bundles sprouting from a region covering several pimples close to the distal vertex. This suggests that it is the local activation of pimples to polymerise actin that specifies the site of prehair formation.

Four genes have been identified that act downstream of the core polarity proteins to specify the site of prehair initiation, namely inturned (in), fuzzy (fy), fritz (frtz) and multiple wing hairs (mwh) (Gubb and Garcia-Bellido, 1982; Wong and Adler, 1993; Lee and Adler, 2002; Collier et al., 2005). Interestingly, whereas loss of core polarity protein function (and thus of asymmetric localisation) causes formation of a single prehair in the cell centre, loss of function of these downstream effectors leads to the formation of multiple prehairs at cell edges. This led to the suggestion that the downstream effectors repress prehair formation throughout the cell periphery, whereas the core planar polarity proteins promote prehair formation at the distal cell edge by locally counteracting the effectors (Wong and Adler, 1993).

The best-studied effectors are in, fy and frtz, which all act cell-autonomously upstream of mwh (Gubb and Garcia-Bellido, 1982; Wong and Adler, 1993; Park et al., 1996; Collier and Gubb, 1997; Collier et al., 2005) and encode, respectively, a putative two-pass transmembrane protein, a putative four-pass transmembrane protein and a WD40-repeat cytoplasmic protein. Interestingly, the ectopic trichome phenotype of null alleles of all three loci is enhanced at lower temperatures, leading to the suggestion that these proteins act in a microtubule-dependent process (Adler et al., 1994).

An important recent observation is that the In protein localises at the proximal apicolateral edges of wing cells under control of the core polarity proteins, shortly prior to prehair formation (Adler et al., 2004). This localisation also requires the activity of fy and frtz, but not mwh. Two alternative models have been put forward to...
explain the requirement of proximally localised In for distal prehair initiation: In might promote local formation of a repressor of prehair initiation, thus restricting initiation to distal regions; alternatively, In could act positively to promote polarised transport of a factor required for prehair initiation to the distal cell edge (Adler et al., 2004). Both models challenged the existing assumption that distally localised Fz/Dsh act as the primary determinants for prehair initiation, and instead suggest that proximally localised Stbm/Pk might be the crucial cue.

The phenotype of mwh mutants is stronger than that of in, fy or frtz, displaying a greater number of inappropriate prehair initiations per cell (Wong and Adler, 1993). By genetic criteria, mwh acts downstream of the other effectors and is therefore the factor most likely to interact directly with the actin cytoskeleton, perhaps by acting as a repressor of pimple activation. However, thus far, the molecular identity of the gene product of mwh is unknown.

The widespread requirements of core planar polarity protein activity in invertebrate and vertebrate morphogenesis, and the associated observation of asymmetric core protein subcellular localisation, suggest that it will be important to understand how the ‘distal’ Fz/Dsh cue and/or the ‘proximal’ Stbm/Pk cue control cell localisation, suggest that it will be important to understand how the associated observation of asymmetric core protein subcellular development.

**RESULTS**

**Roles of the ‘proximal’ and ‘distal’ cues in specifying the site of prehair initiation**

To assess the requirements for potential prehair initiation cues provided by localisation of the core polarity proteins Fz/Dsh/Dgo distally or Stbm/Pk proximally, we generated cells that contained only one of these cues at a known cell edge. In cells lacking fz, Dsh/Dgo are no longer recruited to the junctions (Axelrod, 2001; Shimada et al., 2001; Das et al., 2004), but, at cell edges touching non-mutant cells, Stbm/Pk are strongly recruited (Tree et al., 2002; Bastock et al., 2003), giving rise to a localised ‘proximal’ cue in a cell lacking a ‘distal’ cue. Strikingly, in such cells containing just a proximal cue, prehair initiation is still seen to occur at the opposite cell edge (Fig. 1B). However, in fz cells away from the clone edge, prehair initiation occurs in the cell centre as previously reported (Wong and Adler, 1993), but is noticeably delayed.

Patches of cells lacking stbm activity present the opposite situation, in which no proximal cue is present, but, in cells touching non-mutant cells, a distal cue containing Fz/Dsh assemblies (Strutt, 2001; Bastock et al., 2003). Cells containing only a localised distal cue were also seen to initiate a prehair at the site of this cue, despite the lack of a proximal cue at the opposite cell edge (Fig. 1C). Notably, stbm mutant cells within the clone showed a shorter delay in prehair initiation than observed with fz tissue. In addition, prehair initiation was only consistently seen in the cell centre in the fourth row of cells away from the clone edge. In the third row of cells, about half produced a prehair that was positioned towards the cell edge nearest to the clone boundary, whereas in the second row of mutant cells, almost all prehairs initiated closer to the cell edge nearest the clone boundary.

To compare further the effects on prehair initiation of cells only having a distal or proximal cue, we generated wings containing clones of fz mutant cells abutting clones of stbm mutant cells (fz; stbm twin clones) (Strutt and Strutt, 2007). At the boundary between the fz and stbm clones, the core polarity proteins showed normal asymmetric localisation, but the neighbouring cells were all mutant and failed to asymmetrically localise core polarity proteins. Hence, we can rule out any potential influence of asymmetric core protein
localisation in neighbouring cells on the site of prehair initiation, and focus on the effects of the asymmetric localisation straddling a single cell–cell boundary. Consistent with the effects observed around isolated fz and stbm clones, cells containing a distal cue initiated a prehair at this cell edge, whereas the neighbours containing a proximal cue assembled a prehair towards the opposite cell edge (Fig. 1D). We again saw a greater delay in prehair initiation in fz mutant cells versus stbm mutant cells, and also found that the second row of stbm mutant cells had a site of prehair initiation that was influenced by their polarised neighbours on the clone edge.

Taken together, these results suggest that asymmetric localisation of the core polarity proteins across cell junctions provides both a distal cue for prehair initiation, which promotes initiation at the same cell edge, and a proximal cue that promotes initiation towards the opposite cell edge. It should be noted, that a distal cue is always associated with a proximal cue in the next cell, and vice versa, by virtue of the mutually dependent localisations of the core polarity proteins. Thus, it is formally possible that only one of these cues has a direct effect on the site of prehair initiation, and that the apparent effect of the other cue is in fact the result of a signal from the adjacent cell.

One way in which an adjacent cell might influence prehair formation in a neighbour would be if assembly of a prehair produced a physical cue that influences the cytoskeleton of the neighbouring cell and induces prehair formation at the opposite edge. To test this, we generated mutant cells containing only a proximal or distal polarity cue, juxtaposed to cells that are unable to form a prehair by virtue of being mutant for shavenoid (sha) (Ren et al., 2006). Interestingly, cells on the edges of a fz clone, adjacent to sha mutant cells, formed prehairs at the expected cell edge despite the lack of prehairs in neighbouring cells (Fig. 1E). Similarly, cells containing only a distal cue also formed prehairs close to the cell edge (Fig. 1F). Hence, prehair formation in neighbouring cells is not necessary to enable cells with just a proximal or distal cue to position prehairs correctly, although cell–cell communication of another form cannot be excluded.

Additionally, we investigated the increased delay in prehair initiation in fz mutant cells versus stbm mutant cells. In theory, Fz and Stbm could be required simply to localise a prehair-promoting cue and a prehair-repressing cue, respectively, and/or could also be required for the activity of the cue. Hence, cells mutant for both fz and stbm might contain neither cue, or could contain one or both cues uniformly distributed.

Interestingly, stbm clones in a uniformly fz mutant background show no difference in the time of prehair initiation between single- and double-mutant tissue (Fig. 1G). This is consistent with Stbm acting to modulate the distribution of a cue but not altering its overall activity, such that the cue is equally active in cells with and without Stbm. Conversely, fz clones in a stbm mutant background show
prehairs initiating sooner in stbm mutant tissue than in stbm; fz double-mutant tissue (Fig. 1H). This suggests that Fz is able to promote prehair initiation positively and that in the absence of Fz this promoting activity is lost. Together, our results suggest that prehair initiation is controlled by an inhibitory cue that is normally localised proximally in a Stbm-dependent manner, but is not strictly dependent upon Stbm for its activity, and by a Fz-dependent cue that positively promotes prehair formation.

**Fy, Frtz and In are putative effectors of the proximal prehair initiation cue**

Loss of activity of fy, frtz or in results in more than one prehair initiating in ectopic positions in the cell, consistent with inappropriate activation of a prehair-promoting cue or the loss of a prehair-repressing cue (Gubb and García-Bellido, 1982; Wong and Adler, 1993; Collier et al., 2005). Notably, In localises proximally with Stbm, in a Stbm-dependent manner (Adler et al., 2004), suggesting that In mediates the proximal cue. Proximal localisation of In also depends upon fy and frtz activity (Adler et al., 2004), consistent with the three gene products acting together to regulate prehair initiation.

We raised an antibody against Frtz and found that Frtz also localises to proximal junctions (Fig. 2A), and that this localisation depends upon in and fy activity (Fig. 2B,C), but not on the downstream-acting gene mwh (Fig. 2D). Frtz junctional localisation also required stbm activity (Fig. 2E), but was only reduced in pk mutants (Fig. 2F), consistent with the effects of loss of pk on the distribution of Stbm (Bastock et al., 2003). Similarly, in fz mutant cells away from clone borders, significant junctional localisation was retained (Fig. 2G). Fluorescent intensities of Frtz immunolabelling in the junctional regions of mutant tissue were quantitated. Interestingly, within in and fy tissue, levels were the same as within frtz tissue, indicating that junctional Frtz protein is undetectable. However, in fz tissue, fluorescent intensities were ~50% of wild-type levels, and, in stbm tissue, fluorescent intensities still achieved 25% of wild-type levels. This suggests that although Frtz is not noticeably localised to junctions in stbm tissue, the protein is nevertheless still present within the cell, supporting the contention that Stbm acts to localise Frtz activity but not necessarily to control its levels. (As our Frtz antibodies did not work in immunoblotting, we were unable to more directly assess these protein levels.)

These results indicate that Frtz, like In, localises proximally in a Stbm-dependent manner. We also attempted to determine the localisation of Fy using an EGFP-tagged form. We again saw preferential localisation to the apicolateral junctions, with enrichment at proximal cell edges (Fig. 2H). These results are consistent with In, Fy and Frtz all colocalising proximally and (at least in the case of In and Frtz) each requiring the activity of the other two to become localised.

**mwh encodes a novel FH3-domain protein that is more strongly localised proximally in cells**

The mwh locus has been mapped genetically to the cytological position 61E-F. As the most downstream known effector of core polarity gene function, we hypothesised that mwh might encode a protein that interacts directly with the cytoskeleton. Searching by gene ontology in FlyBase for ‘cytoskeletal protein binding’ revealed a single uncharacterised candidate gene, CG13913, in this region. A strain carrying a transgene that expresses an inducible RNAi hairpin against the CG13913 transcript (Dietzl et al., 2007) closely phenocopied the mwh phenotype (Fig. 3B). Importantly, transgenic flies expressing the CG13913 gene product fused to EGFP also showed complete rescue of the mwh phenotype (Fig. 3C). In addition, we isolated the predicted coding region of CG13913 from mwh flies by PCR and determined that the locus has been subject to a rearrangement in which an inversion breaks the coding sequence after amino acid 367 (Fig. 3D). Taken together, this is good evidence that mwh corresponds to CG13913 and also suggests that the mwh allele is likely to correspond to a null allele (see also Materials and methods).
Analysis of the CG13913 coding sequence for known protein domains (Labarga et al., 2007) revealed the presence of the Interpro GBD/FH3 (IPR014768) and FH3 (IPR010472) domains, and the point at which the inversion in mwh1 breaks the coding sequence (367, arrow). (E-F*) mwh1 clone in a 32-hour wing, labelled for Mwh (magenta), clonal marker (lacZ, green) and Fmi (blue). (F-F*) mwh1 clone in a 32.5-hour wing, labelled for Mwh (green) and actin (Ph, magenta). Arrowheads indicate trichomes in which Mwh does not colocalise strongly with actin bundles. (G-G*) mwh1 clone in 32.5-hour wing, labelled for Mwh (green) and α-tubulin (magenta). Arrowheads indicate trichomes in which Mwh does not colocalise with α-tubulin. Scale bars: 50 μm in A; 10 μm in G*.

**Mwh localisation and phosphorylation are regulated by frtz**

Next we asked whether Mwh levels and distribution were regulated by in, fy or frtz. Interestingly, in all three genotypes, the apical punctate labelling of Mwh was dramatically reduced (Fig. 4A-C). By contrast, in fz and stbm clones, the proximal enrichment of Mwh was lost (consistent with loss of proximal Frtz localisation), but the levels of apical punctate labelling were not greatly altered (Fig. 4D,E; see Fig. S1C-F in the supplementary material).

To further investigate the effects of frtz on Mwh, we used our Mwh antibody for immunoblotting. In extracts from wild-type flies, the antibody detected a number of bands in the molecular weight range expected for Mwh (~91 kDa). Only a broad band migrating at ~110 kDa was lost in mwh mutants or CG13913-RNAi extracts, consistent with this representing the Mwh protein (Fig. 5A). Expression of EGFP-Mwh gave rise to bands ~20-30 kDa larger, reflecting the expected shift in molecular weight due to the tag. Extracts from fz and stbm flies showed negligible changes in Mwh levels, consistent with the immunolabelling results (Fig. 5A). However, loss of frtz activity resulted in a slight increase in Mwh gel-mobility, with the loss of a higher-molecular-weight form (Fig. 5A,C). We surmise that the higher-molecular-weight form constitutes the apical and proximally enriched punctate populations of Mwh seen by immunolabelling, as both were reduced in frtz cells.

A plausible explanation for the presence of the higher-molecular-weight form of Mwh is that Frtz promotes post-translational modification of Mwh. When wild-type protein extracts were treated with phosphatase (Fig. 5B), the slower-migrating form of Mwh was
lost and an increase in gel-mobility similar to that seen upon loss of frtz was observed, consistent with Frtz promoting Mwh phosphorylation.

Interestingly, the frtz, in and fy null phenotypes increase in strength at lower temperatures (Adler et al., 1994; Collier et al., 2005). This could be explained if Mwh stability was temperature dependent. On immunoblots, we observed a dramatic reduction in Mwh levels in wings from animals raised at 18°C, as compared with 29°C (Fig. 5C). This was additive to the effects of frtz on Mwh gel-mobility, consistent with the cold-sensitivity of in, fy and frtz nulls being a result of reduced stability of Mwh.

**mwh mutant cells show ectopic actin bundles across their apical surface**

The earliest reported manifestation of the mwh phenotype is ectopic prehair initiation at the cell edge (Wong and Adler, 1993). Using fixation conditions optimised for preservation of F-actin structures, we re-examined the earliest stages of the mwh phenotype, looking in wings from animals raised at 18°C, 25°C and 29°C. A similar phenotype was seen at each temperature (Fig. 6A-D, and data not shown). We observed that prior to and during the appearance of prehair structures at the cell periphery, cells showed excess actin bundling across the apical surface of the cells, sometimes in a ‘starburst’ pattern, with actin bundles radiating from the cell centre (Fig. 6A,B). Subsequently, prehair structures were seen at cell edges, as previously reported. Interestingly, the excess actin bundles often extended at least 1 μm basally into the cytoplasm from the apical surface, particularly once prehair initiation was underway (Fig. 6C,D). These results suggest that excess actin polymerisation across the apical surface of the cell is the primary defect in mwh, and that ectopic prehairs forming at cell edges might be a secondary consequence.

**Mwh affects actin structures in cultured cells**

From the molecular homology of Mwh with other proteins of known function, one can suggest that it could directly interact with the cytoskeleton or cytoskeletal modulators via its GBD/FH3 domain. However, the lack of other functional domains seen in formins might indicate that Mwh negatively influences actin filament formation, which would explain the unrestricted actin bundling seen across the apical surface of cells in its absence.

To gather more evidence for Mwh repressing actin filament formation, we transfected Mwh into cultured *Drosophila* S2 cells and assayed the effect on their actin cytoskeleton and behaviour. Intriguingly, cells expressing high levels of EGFP-Mwh showed an altered morphology compared with their contacting neighbours, characterised by a less rounded shape, the appearance of slender projections at the cell periphery and a reduction in F-actin bundles visible at the cell periphery (Fig. 6E). When we quantitated the reduction in fluorescent labelling intensity at the edges of transfected cells we found, on average, a 63% reduction. Furthermore, we observed that isolated transfected cells progressively developed a more dramatically altered morphology, with radially projecting slender extensions (Fig. 6F, present in 84% of transfected cells, compared with only 6% of control cells). This phenotype is reminiscent of the effects of reducing the activity of a number of proteins involved in actin dynamics, including the Arp2/3 complex, which is required for nucleation of actin filaments (Kiger et al., 2003; Kunda et al., 2003). Together, these results suggest that EGFP-Mwh overexpression can inhibit actin filament formation.

**DISCUSSION**

**The site of prehair initiation is influenced by both proximal and distal cellular cues**

Activity of the core planar polarity proteins is required in cells of the *Drosophila* pupal wing to specify prehair initiation at the distal vertex (Wong and Adler, 1993). Here we present evidence that core polarity protein localisation at both proximal and distal cell edges provides redundant cues for specifying distal prehair initiation.

Regarding the mechanistic basis of the proximal cue, this and previous work provide evidence for a plausible model (Fig. 7). The downstream effectors Frtz, In and Frtz all colocalise at the proximal cell edge with Sbtm and in a Sbtm-dependent manner. Activity of In, Frtz and Frtz is required for Mwh phosphorylation and its sub-apical subcellular localisation, which is thus concentrated towards the proximal side of the cell. Genetic studies have shown that loss of *fy*, *in*, *frtz* or *mwh* activity leads to excess prehair initiation (Wong and Adler, 1993; Collier et al., 2005), and we find that the initial defect in mwh mutant cells is excess actin bundling across the entire apical face of cells. Thus, proximal restriction of Mwh activity in the cell results in actin bundling and prehair initiation specifically in distal regions.

Additional evidence for the sufficiency of a Sbtm-dependent cue for prehair initiation at opposite cell edges comes from experiments in the *Drosophila* abdomen (Lawrence et al., 2004). Here, it was reported that cells lacking fz activity, but juxtaposed to cells with fz activity, were able to produce polarised trichomes, as we also observed in the first row of cells within a fz clone in the wing.
We have less information regarding the distal cue. Its existence is based upon two pieces of evidence. First, if prehair initiation were entirely dependent on Stbm-mediated localisation of Mwh activity, then prehairs should show no bias in their site of initiation in cells lacking stbm activity. In fact, stbm mutant cells with Fz localised at one cell edge show a strong bias towards initiating prehairs at that edge. Second, if prehair initiation were controlled only by a Stbm-dependent repressive cue, then in the absence of stbm activity, Fz would have no influence over prehair initiation. Instead, in a stbm background, fz activity still weakly promotes prehair formation. Taken together, these data support the view that distally localised Fz acts as a prehair-promoting cue.

A possible mechanism of action of the distal cue is that localised Fz might be able to repress Mwh activity in distal cell regions, possibly via its known effectors RhoA and Drok (Rho1 and Rok – FlyBase) (Strutt et al., 1997; Winter et al., 2001). Alternatively, Fz might promote prehair initiation in a Mwh-independent fashion, via RhoA/Drok or other effectors.

It is notable that the absence of fz activity results in a delay in prehair formation and in a greater tendency, compared with loss of stbm, for prehairs to form in the cell centre rather than towards a cell edge. We surmise that in fz mutant cells, there is no Fz-dependent prehair-promoting cue, and the Stbm-dependent repressive cue is evenly distributed around the cell edge, resulting in delayed prehair initiation in the cell centre. Conversely, in stbm mutant cells, there is no change in the activity of the repressive cue, but the Fz-dependent prehair-promoting cue is localised to cell edges, albeit more thinly spread than in the wild-type situation. This results in approximately normally timed prehair initiation near the cell edges.

An unexplained observation is that within stbm mutant tissue, the site of prehair initiation appears to be biased towards that seen in neighbouring cells. Thus, in the first rows of cells within a clone, prehairs tend to point towards the adjacent wild-type tissue. This phenomenon is presumably independent of core protein asymmetric placement.
Fig. 7. Model for placement of prehair initiation in Drosophila. Diagram representing a z-section of apical regions of a cell, showing distal complexes (green) and proximal complexes (orange). The proximal complex recruits In, Fy and Frtz (red), which in turn recruit or activate an unknown kinase (pink) that modifies Mwh (magenta), which is consequently stabilised in apical/proximal regions. Mwh acts as an inhibitor of actin filament formation in electron-dense pimples on the surface of cells (dark grey), possibly by interfering with the activity of conventional formins. Distally in the cell, pimples become activated as a consequence of a lack of Mwh activity. The distal complex promotes pimple activation, either by repression of Mwh activity or via an alternative mechanism.

Mwh activity is regulated by phosphorylation. Treatment of cell extracts with phosphatase results in increased gel-mobility of Mwh. A similar increase in mobility is observed when frtz activity is removed, but not when stbm or fz activities are removed. Thus, at the least, Mwh phosphorylation correlates with Mwh activity and apical punctate localisation. Hence, we propose that the roles of In, Fy and Frtz might be to activate, or bring into proximity with Mwh, a kinase or kinases responsible for activating Mwh. Similarly, Fz could locally promote the dephosphorylation of Mwh to induce prehair initiation, although any such effect would have to be small, as Mwh phosphorylation is not obviously altered in the absence of Fz.

Definitive proof that phosphorylation of Mwh is important for its activity would require the identification of particular phosphorylation sites required for specific molecular functions and/or the identification of a kinase essential for Mwh activity.

How is Mwh activity regulated?
Loss of in, fy and frtz results in a similar phenotype to loss of mwh, with multiple ectopic prehairs at the cell edge preceded by excess apical actin bundling (D.S., unpublished) (Wong and Adler, 1993). As In, Fy and Frtz are all required for the apical punctate distribution of Mwh within cells, and also appear to stabilise each other (this work) (Adler et al., 2004), this suggests that In, Fy and Frtz act together to activate Mwh and promote its apical localisation. Conversely, although Stbm plays a role in localising Mwh within the cell, it is not required for its activity, as loss of stbm does not phenocopy mwh mutants in which increased apical actin bundling is observed. This role of Stbm in localising but not regulating Mwh activity is most simply explained by Stbm acting to localise, but not regulate, the activity of In, Fy and Frtz. This is supported by the observation that whereas loss of fz or stbm has a strong effect on the distribution of Frtz to the apicolateral junctions, it has a negligible effect on the apparent phosphorylation state of Mwh.

The regulation of Mwh activity appears to be largely post-translational, as although the subcellular distribution of Mwh changes dramatically in frtz mutant cells, total levels of Mwh are not similarly altered. Further evidence that In, Fy and Frtz regulate Mwh activity by a mechanism largely independent of Mwh protein levels comes from the observation that Mwh overexpression in the wing has no effect on the apparent phosphorylation state of Mwh.

The lack of direct vertebrate homologues of Mwh might indicate that any such effect would have to be small, as Mwh phosphorylation is not obviously altered in the absence of Fz.

Definitive proof that phosphorylation of Mwh is important for its activity would require the identification of particular phosphorylation sites required for specific molecular functions and/or the identification of a kinase essential for Mwh activity.

An alternative regulatory mechanism for Mwh, by analogy with Diaphanus family formins, would be via RhoA GTase activity (Wallar and Alberts, 2003). The FH2 domain of such formins promotes actin nucleation, an activity that is autoinhibited by interaction with the GBD. Upon interaction of the GBD with GTase-bound Rho GTases, this autoinhibition is released. Notably, genetic interaction data suggest that Fz/Dsh can activate RhoA activity (Strutt et al., 1997; Winter et al., 2001). This is consistent with a model whereby in the proximal cell, Rho GTase activity is low and Mwh inhibits prehair initiation, whereas in the distal cell, activated RhoA alleviates the inhibitory activity of Mwh.

Notwithstanding our evidence for post-translational regulation of Mwh activity in the normal context of the pupal wing, in cultured cells we do see an effect of Mwh overexpression on the actin cytoskeleton. This seems likely to be due to the much higher levels of expression that can be achieved in transfected cells as opposed to cells in vivo, and hence the result should be treated with caution, but might nonetheless suggest that S2 cells express a factor able to constitutively activate Mwh.

Our results also indicate that Mwh levels are influenced by temperature, which provides a plausible explanation for why in, fy and frtz phenotypes are stronger at 18°C than at higher temperatures (Adler et al., 1994; Collier and Gubb, 1997; Collier et al., 2005). We suggest that loss of in, fy and frtz reduces Mwh activity and that lower temperatures additively reduce Mwh levels, resulting in lower overall Mwh activity.

What is the molecular function of Mwh?
As already noted, the FH3 domain of conventional formins is thought to be involved in targeting the protein to particular cellular sites, whereas the GBD domain is involved in inhibition of the actin-nucleating function of the FH2 domain (Wallar and Alberts, 2003). A plausible model is that Mwh acts as a dominant-negative by binding via its GBD domain to other FH2-domain-containing formins involved in the nucleation of actin filaments, thereby inhibiting their activity. Notably, this dominant-negative activity of Mwh could then be inhibited distally in the cell by Fz-mediated activation of RhoA GTase activity.

Electron microscopy studies suggest that prior to prehair initiation, the apical cell surface is covered in electron-dense pimples that are normally only activated at the distal cell edge and serve as foci for actin filament formation (Guild et al., 2005). We propose that at around 32 hours of pupal development, cells receive a general signal for pimple activation that results in actin nucleation, and that Mwh activity is required to inhibit this activation at locations away from the distal cell edge.

The lack of direct vertebrate homologues of Mwh might indicate that in insects, the GBD/FH3 domain of a conventional formin has become separated from the rest of the molecule, but retained its function in inhibiting formin-mediated actin nucleation. Nevertheless, it is also plausible that the core polarity proteins would use similar regulatory mechanisms to promote local changes in
cytoskeletal structure in vertebrate cells as those employed in the Drosophila wing. Importantly, vertebrate homologues of both Fuzzy and Inturned have been shown to be involved in regulating apical actin assembly and, thus, in specifying the orientation of cilia (Park et al., 2006). By analogy with our findings, we suggest that core polarity proteins in vertebrates are likely to localise Fuzzy/Inturned activity within cells and to regulate formin activity via phosphorylation and/or Rho GTPase activation.

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