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Planar cell polarization requires Widerborst, a B′ regulatory subunit of protein phosphatase 2A

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SUMMARY

We have identified *widerborst* (*wdb*), a B′ regulatory subunit of PP2A, as a conserved component of planar cell polarization mechanisms in both *Drosophila* and in zebrafish. In *Drosophila*, *wdb* acts at two steps during planar polarization of wing epithelial cells. It is required to organize tissue polarity proteins into proximal and distal cortical domains, thus determining wing hair orientation. It is also needed to generate the polarized membrane outgrowth that becomes the wing hair. Widerborst activates the catalytic subunit of PP2A and localizes to the distal side of a planar microtubule web that lies at the level of apical cell junctions. This suggests that polarized PP2A activation along the planar microtubule web is important for planar polarization. In zebrafish, two *wdb* homologs are required for convergent extension during gastrulation, supporting the conjecture that *Drosophila* planar cell polarization and vertebrate gastrulation movements are regulated by similar mechanisms.

Key words: *Drosophila*, Widerborst, PP2A, Zebrafish, Planar polarity

INTRODUCTION

Coordination of planar polarity within groups of cells is crucial for organismal development, from gastrulation (Heisenberg et al., 2000; Wallingford et al., 2000; Wallingford et al., 2001) through to the differentiation of complex tissues (Eaton, 1997; Mlodzik, 2000; Adler and Lee, 2001). The alignment of hairs and bristles formed by the cuticle-secreting epithelium of insects is a particularly striking example of planar polarization that has been well studied genetically (Adler, 1992; Adler and Lee, 2001). In the *Drosophila* wing epithelium, each cell polarizes its actin and microtubule cytoskeleton within the plane of the epithelium to generate a single distally directed membrane outgrowth that becomes the wing hair (Wong and Adler, 1993; Eaton et al., 1996; Eaton, 1997; Turner and Adler, 1998). Global coordination of hair orientation is controlled by the tissue polarity proteins, including Frizzled, which localize asymmetrically to form distinct proximal and distal cortical domains at the junctional region of each cell (Strutt, 2001; Shimada, 2001; Usui, 1999; Axelrod, 2001; Feiguin, 2000). Although two of these proteins, Frizzled and Flamingo, are transmembrane proteins that could potentially recognize a ‘polarization’ ligand, no such ligand has been identified, and the signals and cell biological mechanisms that orient the polarity of the proximodistal domains are unknown.

Many genes with important roles in cell polarization may cause organismal or cell lethality when mutated, precluding their identification as tissue polarity genes in loss-of-function screens. We therefore performed an overexpression screen to identify new genes involved in tissue polarization (Feiguin et al., 2000). We address the function of *widerborst*, a B′ regulatory subunit of PP2A identified in this screen. Our data suggest that *widerborst* plays roles in both cortical polarization and hair outgrowth in the *Drosophila* wing. They further suggest that *widerborst* is part of a conserved planar polarization mechanism that also operates during vertebrate gastrulation.

MATERIALS AND METHODS

Isolation of Wdb alleles by EMS mutagenesis

Drosophila EP3559 males were treated with EMS and crossed to either *tubulinGAL4*/TM3Sb or *ApGAL4/CyO* virgin females. Emerging non-Sb progeny were examined for duplicated thoracic bristles and apparent revertants were recrossed to *tubulinGAL4*/TM3Sb or *ApGAL4/CyO*. We recovered five EP3559 chromosomes that repeatedly failed to produce a bristle duplication phenotype. Sequencing revealed point mutations within the Wdb transcript for four of these.

DNA constructs

Three different dominant negative *mts*-expressing constructs were produced by cloning N-terminally truncated subfragments generated by PCR into pUAST via *Eco*RI and *Xho*I linkers. The truncated proteins began at amino acids 165, 181 and 214. Dominant negative *wdb* was produced by cloning an N-terminally truncated PCR subfragment of *wdb* into pUAST via *Eco*RI and *Xho*I linkers. The truncated protein begins at amino acid 94.

Scanning electron microscopy

Flies were dried and stored in a desiccator under vacuum. To prepare for scanning electron microscopy, dried wings were mounted on metal knobs with double-sided adhesive tape. The knobs were placed in a sputter coated (Balzers) and coated with bold (twice for 1 minute at 0.1 Torr, 100 V and 15 Ma). Scanning electron microscopy was performed with a Zeiss Novascan-30.

Image processing

Standard image processing was performed using Adobe Photoshop 6.0 and Adobe Illustrator 9.01 (Adobe Systems) Deconvolution of images of anti-tubulin-stained wings was performed on 15 0.2 μm serial confocal sections with Huygens software.

Sequence alignment

The multiple sequence alignment of the selected PP2A B′ sequences was constructed using ClustalX with standard gap parameters and the BLOSUM-matrix series.

The phylogenetic tree was constructed with ClustalX, whereby gap positions were excluded and correction for multiple substitutions was turned on. The tree was bootstrapped with 1000 trials. The fungal sequences were used as an outgroup to root the phylogenetic tree. The branch length indicated in the upper right hand corner corresponds to 0.05 amino acid substitutions.

Antibodies

Mouse anti-Flamingo and rat anti-Dishevelled were kindly provided by Tadashi Uemura (Usui et al., 1999; Shimada et al., 2001). Guinea pig anti-Coracle was a gift from Rick Fehon (Fehon et al., 1994). Mouse anti-α and -β tubulin monoclonals were purchased from Sigma (Cat. No. T5168 and T4026). Rabbit anti-Wdb was raised to the C-terminal peptide PATTNAKIKQDKADN by Sigma-Genosys (Cambridgeshire, UK). The antibody was affinity purified using the immunizing peptide and used at a dilution of 1:300. The antibody was judged to be specific in immunofluorescence studies because its staining was elevated in cells overexpressing *wdb* and because staining disappeared in clones mutant for the *IP* allele of *wdb.* The antibody did not detect any specific bands when used on western blots.

Processing of wings for immunohistochemistry

Pupae were dissected in Graces tissue culture medium (Sigma) at room temperature on 3 cm plastic tissue culture dishes. During the dissection process, it was crucial that the wings not be damaged or stretched; we have found that stretching the wing disturbs the organization of the planar microtubule webs, on which Wdb is localized. In many cases where wings had been stressed, for example, by attempting to make an opening in the distal cuticle, microtubules collapsed to one side of the cells over large regions of the wing, along with Wdb protein. This sometimes led to apparent misorientation of Wdb staining. Distal localization of Widerborst was observed when wings were fixed with cold methanol, which efficiently preserves the structure of the planar microtubule webs, but not when they were fixed with paraformaldehyde, which does not (data not shown).

We dissected pupae as follows: for 18-30 hour pupae, the whole pupa was removed from the pupal case. The head and abdomen were then pierced, and the internal fat droplets removed. The head and abdomen were then removed gently, taking care not to exert force on the wings. Pupae were then opened along the dorsal thorax, and the cuticle covering the wing hinge region was broken, exposing the underlying epithelium at the proximal end of the wing. No attempt was made to remove the cuticle coving the wing itself. These carcasses were placed into 3 cm petri dishes containing 100% methanol precooled to 4°C and left on ice for 2 minutes. The dish containing the wings was then transferred to a metal plate at –20°C and left for a further 18 minutes. The dish was then placed on ice and the wings were removed to another 3 cm dish containing 90% methanol in water and left on ice for 3 minutes. Wings were then transferred to dishes containing 50% methanol, where they were separated from the rest of the carcass and detached from the surrounding cuticle.

To obtain prepupal wings, we held on to the anterior tip of the prepupa with number 5 forceps and gently severed the pupal case approximately one third of distance from the anterior end. Wings usually remained attached to the head region and they were transferred to cold methanol (as described above) without being directly touched.

Wings were then accumulated in PBS + 0.1% Triton X-100 (PBT) in one well of a 24-well tissue culture plate. After rinsing twice with PBT, the wings were blocked for 10 minutes in PBT + 0.5% FCS, then incubated overnight with relevant antibodies.

In situ hybridization and RNA/morpholino oligonucleotide injection (zebrafish)

In situ hybridization with digoxigenin-incorporated antisense RNA probes was as described (Heisenberg et al., 1996). RNA and morpholino antisense oligonucleotides against zebrafish *wdb1* and *wdb2* were injected as described (Heisenberg et al., 2001). Control-morpholino oligonucleotides (Gene Tools) and morpholinos against various other genes (*axin1*, *sqt*, *cyc*) were injected to determine the specificity of the obtained phenotypes.

RESULTS

To identify new genes involved in planar polarization, we used an EP modular misexpression screen (Rorth, 1996; Feiguin et al., 2000). We induced expression of 2500 different EP lines in the developing wing and thorax, and first selected those lines that induced ectopic or apolar bristles in the thorax. We chose for further study those lines that also produced defects in hair polarity or number. Expression from EP3559 caused bristle duplication in the thorax and induced many cells throughout the wing to form multiple rather than single hairs (compare Fig. 1A with 1B), suggesting that the gene activated by EP3559 might regulate planar polarization.

To identify the gene overexpressed by EP3559, we cloned and sequenced the adjacent DNA and identified corresponding ESTs in the BDGP (Berkeley Drosophila Genome Project) database; these were derived from gene CG5643 in the GadFly database. Sequencing revealed no differences in the coding potential of the cDNAs, but suggested that alternative splicing gives rise to transcripts differing in their 5′ untranslated sequence (submitted to the GadFly genome annotation database). Three different cDNAs were placed under the control of a GAL4UAS promoter and used to generate transgenic flies. After inducing expression in the wing, we observed that only the cDNA with the longest 5′ untranslated region, LD2456, caused multiple wing hair formation (Fig. 1C). This shows that CG5643 indeed represents the gene causing the multiple wing hair phenotype, and suggests that the 5′ untranslated region may play a part in its post-transcriptional regulation.

We named the gene from which LD2456 is derived *widerborst*, which, in German, means something stubborn or recalcitrant (derived from wider, meaning against, and borst, meaning bristle). It encodes a B′ regulatory subunit of protein phosphatase 2A (PP2A), an enzyme that is conserved from yeast to mammals. PP2A is a holoenzyme that consists of a catalytic (C) subunit, an A regulatory subunit and one of a large family of B, B′ or B″ subunits. The latter subunits are thought to regulate the activity of the C subunit and provide substrate

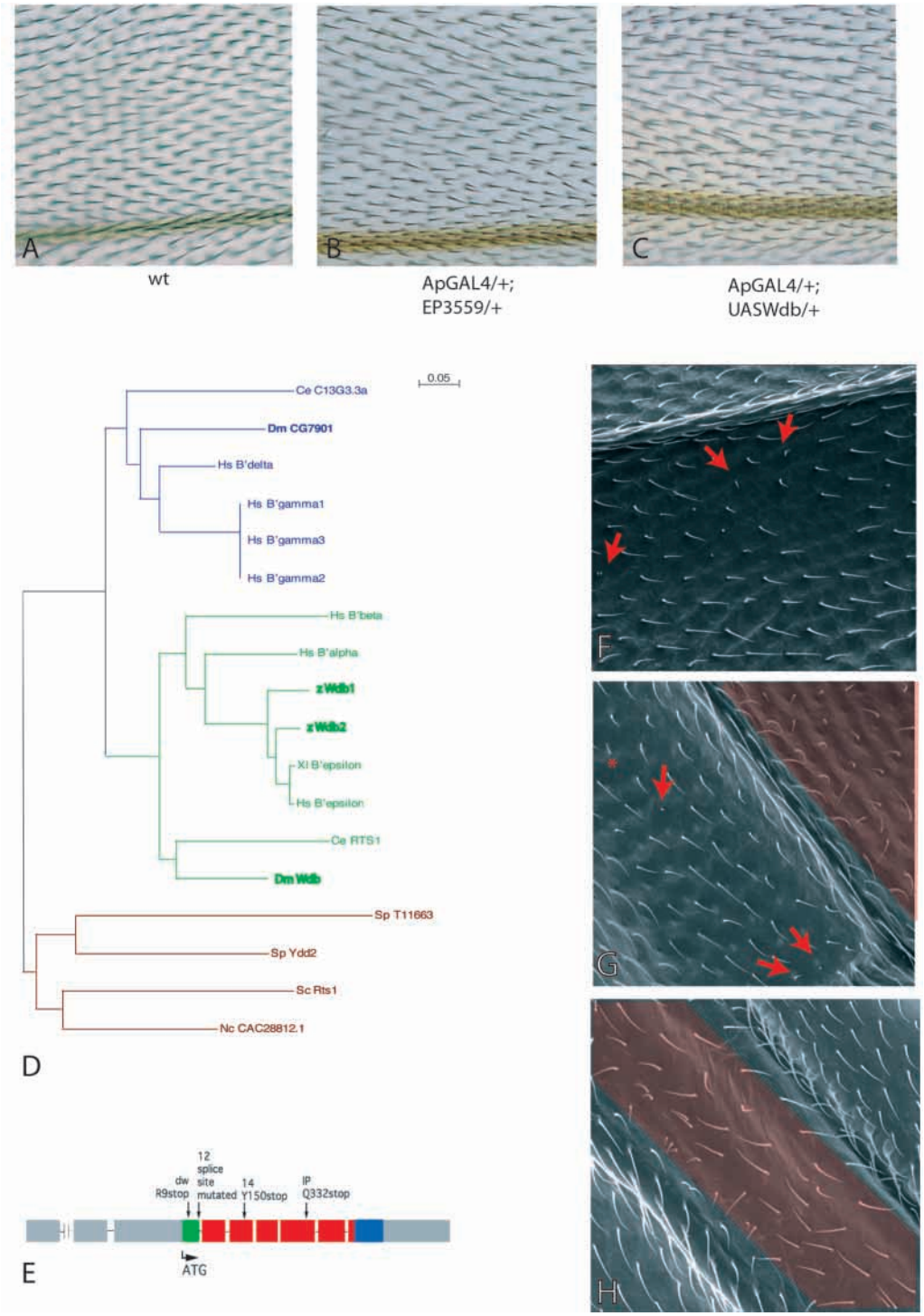

Fig. 1. Widerborst is a B′ subunit of PP2A required for planar polarization. (A) Wild-type wing epithelial cells each make a single hair. (B) Driving expression from EP3559 on the dorsal wing blade with Apterous GAL4 causes multiple wing hairs to form. (C) Expression of a cDNA (LD3434) corresponding to CG5643 (the locus downstream of EP3559) produces a similar phenotype. (D) Dendritic tree depicting the relationships between different PP2A B′ subunits from fungi (Sp T11663, Sp Ydd2, Sc Rts.1, Nc CAC28812.1), *C. elegans* (Ce C13G3.3a, Ce RTS.1), *Drosophila* (Dm CG7901, Dm Wdb), *Xenopus* (Xl B′ ε), zebrafish (zWdb1, zWdb2) and *Homo sapiens* (Hs B′ α, β, γ, δ and ε). The bar in the upper right -hand corner depicts the branch length corresponding to 0.05 amino acid substitutions per residue. (E) Location of EMS mutations within the Wdb transcript, and structure of the Wdb dominant-negative construct. Grey regions are non-coding. The red region is strongly conserved between all members of the B′ subfamily. Green indicates sequences conserved only between Widerborst and the α and ε subfamily members. Blue indicates divergent sequence unique to Widerborst. Arrows indicate the positions at which, wdb^{dw}, wdb^{12}, wdb^{14}, and wdb^{IP} are mutated and the resulting sequence changes. wdb^{dw} and wdb^{12} probably cause short N-terminal truncations (see Materials and Methods) because they make protein that is detectable with an antibody against the C terminus. (F) The ventral wing blade of a wdb^{dw}/wdb^{IP} transheterozygote shows regions where hair formation fails or is compromised. Stunted hairs are often misoriented (arrows). (G) The ventral wing blade of a fly that expressed dominant-negative Wdb along the anteroposterior compartment boundary, under the control of PatchedGAL4. The expressing region is indicated in red. Expressing cells form hairs that are stunted and misoriented. Occasionally, hairs fail to form. Posterior to the expression domain, hair formation and orientation are affected non-autonomously (asterisk and arrows). (H) The ventral wing blade of a fly that expressed dominant negative Wdb along the anteroposterior compartment boundary (red). Even hairs of normal morphology display orientation defects.

specificity. In metazoans, the B′ subunits have diverged into two related subclasses (Fig. 1D). The central regions of these proteins are strongly conserved, but they differ at their N and C termini (Fig. 1E). The protein encoded by *widerborst* is more closely related to the human α, β and ε subunits (62-66% identity) than to the β or γ subunits (52-59% identity). Its sequence suggests that *wdb* might influence tissue polarization by regulating PP2A activity with respect to specific targets.

To ask whether Wdb was necessary for tissue polarization, we sought loss-of-function *wdb* mutations. After treating

EP3559 males with EMS, we crossed them to flies containing either *tubulinGAL4* or *apterousGAL4* and screened for chromosomes that could no longer produce the bristle phenotypes characteristic of *wdb* over-expression. Five different 'revertant' chromosomes were isolated in this fashion; four of the five (wdb^{12}, wdb^{14}, wdb^{IP}, and wdb^{dw}) fell into one complementation group. Each mutation was homozygous lethal, and either lethal or semi-lethal with other *wdb* alleles.

We analyzed these possible *wdb* mutants in three different ways. First, we looked for mutations in the *wdb* transcribed region. Second, we quantified the extent to which overexpression of the different alleles could cause multiple wing hair formation. Finally, using an antibody to the Wdb C terminus, we measured Wdb protein accumulation caused by *patchedGAL4*-driven expression from the EP element. These data are summarized in Table 1 and Fig. 1E.

From these analyses, we concluded that wdb^{14} is closest to a null; it contains a nonsense mutation at codon 150 that removes two-thirds of the protein, including most of the highly conserved region. Consistent with this, the C-terminal antibody fails to detect overexpressed Wdb14 protein, and the mutation completely suppresses the formation of multiple wing hairs caused by overexpression.

Wdb^{IP} harbors a stop codon at position 332 and encodes a C-terminally truncated protein that is significantly longer than Wdb^{14} and contains most of the central, conserved region. Wdb^{IP} may retain some function, because wdb^{dw}/wdb^{IP} escapers have weaker wing phenotypes than wdb^{dw}/wdb^{14} escapers (see below). As expected, the truncated protein encoded by wdb^{IP} is not recognized by the C-terminal antibody (Table 1).

Wdb^{dw} suffered a nonsense mutation at position 9, and thus might potentially encode the shortest protein. However, expression of wdb^{dw} from the EP insertion produced protein that could still be detected with the C-terminal antibody. This suggests that translational reinitiation probably occurs at a downstream methionine, resulting in an N-terminally truncated protein. Despite its inability to produce duplicated bristles, wdb^{dw} expression caused almost as many multiple wing hairs as did the wild type gene, suggesting that the mutation is mild with respect to tissue polarity.

Wdb^{12} is mutated in the donor splice site that follows the first coding exon. As the C-terminal antibody still detects protein expressed from wdb^{12}, albeit at reduced levels, we presume either that splicing still occurs to some extent or that an alternative translational initiation site is used. Its mild suppression of the multiple wing hair phenotype confirms that wdb^{12} is a weak allele.

Table 1. Effect of *wdb* mutations on protein accumulation and overexpression phenotype

Allele	Protein (% of wild type)	Number of cells giving multiple hairs in the fifth cell
wdb12	17±10 (*n*=12)	4±2 (*n*=12)
wdbdw	35±15 (*n*=14)	n.c.
wdbIP	0*	0 (*n*=15)
wdb14	0*	0 (*n*=15)
EP3559 (WT)	100±16 (*n*=11)	49±20 (*n*=17)

Flies with EP3559 chromosomes containing the indicated mutations were crossed to Apterous GAL4, and the average number of cells giving wing hair duplications in the fifth cell was calculated. (Suppression was comparable in other regions of the wing.) The combination of Apterous GAL4 with EP3559 wdb^{dw} was semi-lethal, and the multiple wing hair phenotype could not be assessed. Expression from EP3559 wdb^{dw} using other GAL4 drivers produced wing patterning defects, suggesting that this allele has dominant negative properties.

To assess protein accumulation, the expression of either wild-type or mutant *wdb* alleles was driven by EP3559 along the AP compartment boundary under the control of Patched GAL4. After staining with an antibody to the C terminus of Wdb, brightness was quantified using the plot profile function of NIH image by subtracting the signal in surrounding tissue from that within the overexpression stripe. The measurements for each mutant were normalized to that of wild type.

*No overexpression stripe was observed for either *wdb14* or *wdbIP*, therefore protein expression was not quantified.

Widerborst is a multifunctional protein with an important role in hair formation

As the *wdb* mutations were homozygous lethal, we first examined their effects on hair formation by placing them on FRT chromosomes and generating homozygous clones by mitotic recombination. We were unable to recover clones of the null mutation, wdb^{14}, suggesting that cells that have no Wdb activity do not survive. We did recover clones of the partial loss-of-function mutations wdb^{dw}, wdb^{IP} and wdb^{12}, but weaker alleles had no effect on hair formation or polarity (data not shown). Wdb^{IP} clones, however, caused outgrowths in the wing and eye, suggestive of ectopic Wingless signaling (M. H., unpublished).

Because clonal analysis failed to resolve whether Widerborst was involved in polarization or hair formation, we examined hairs in wings from wdb^{dw}/wdb^{14} and wdb^{dw}/wdb^{IP} flies. Although most flies of these genotypes die before emerging, we were able to analyze wings from the occasional escapers. These wings contained bald patches on the wing blade where hair formation had either failed, or was compromised (Fig. 1F). The stunted hairs observed in these regions were sometimes of altered polarity (arrows). These defects occurred most often on the ventral wing blade between veins 3 and 4, a region in which hairs are normally somewhat shorter and finer than in the rest of the wing. This part of the wing may be more sensitive to conditions that discourage hair formation. Clones of wdb^{IP} or wdb^{dw} that fell outside of this small region would not be expected to have any effect. Loss of hairs was more severe in wdb^{dw}/wdb^{14} than in wdb^{dw}/wdb^{IP} wings. Overall, the phenotypes given by *wdb* transheterozygotes suggest that endogenous Wdb positively regulates hair formation and may impinge on hair polarity.

Although incomplete loss of function interfered with hair formation in some regions of the wing, we suspected that the null phenotype of Wdb might be more severe. As cell lethality prevented us from analyzing hairs formed by cells totally missing Wdb activity, we designed a dominant-negative version of the protein analogous to one described for a vertebrate B′ γ1 regulatory subunit. N-terminal truncation of this γ subunit has been shown to dominantly inhibit dephosphorylation of paxillin by PP2A (Ito et al., 2000). We hoped that a similar truncation of Wdb might dominantly inhibit dephosphorylation of Wdb targets. We expressed a Wdb construct from which the first 93 amino acids had been deleted along the AP compartment boundary under the control of *ptcGAL4*, and examined the resulting wings by scanning electron microscopy. Hairs that formed in the region of the wing expressing N-terminally truncated Wdb were often

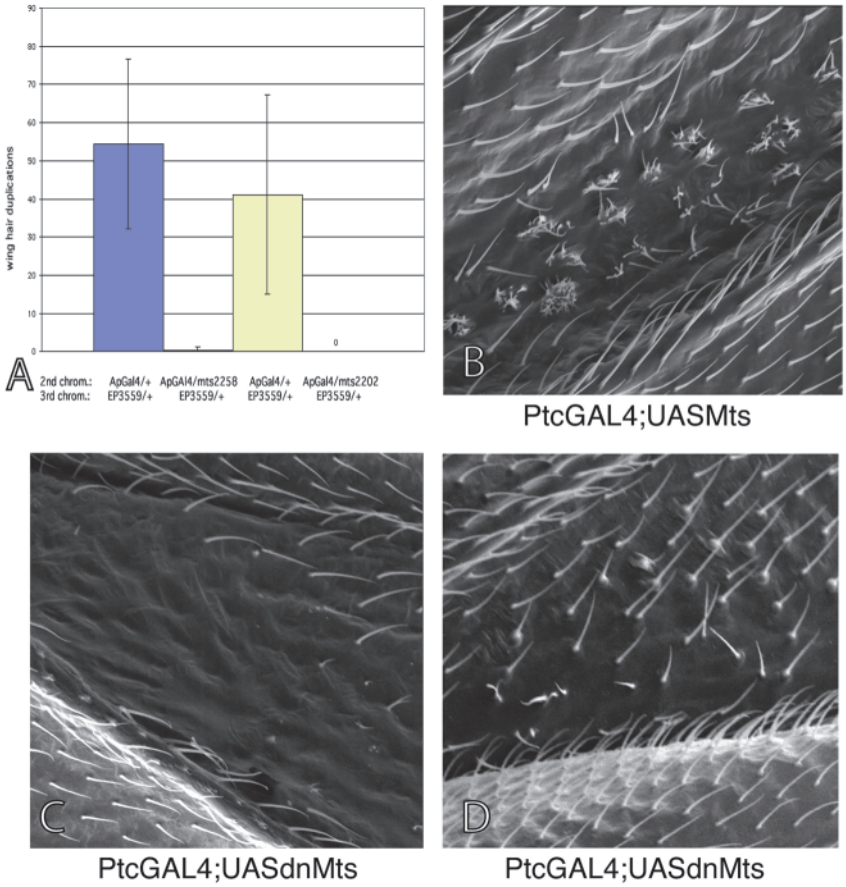

Fig. 2. Widerborst activates the catalytic subunit of PP2A, which promotes hair formation. (A) The multiple wing hair phenotype caused by Widerborst overexpression is suppressed by removing one copy of *mts*. Each bar represents the average number of wing hair duplications in the region between veins 4 and 5, distal to the posterior crossvein. At least 12 wings were averaged. (B) Wing from a fly that expressed the wild-type catalytic subunit of PP2A along the AP compartment boundary under the control of PatchedGAL4. Each overexpressing cell makes prodigious numbers of hairs that point in all directions. (C) Wing from a fly that expressed a dominant negative catalytic subunit along the AP compartment boundary. Wing hairs are stunted or fail to form. (D) Dominant-negative catalytic subunit expression wing showing a milder phenotype than in C; hairs display stunting and polarity defects.

stunted and sometimes absent, as might be expected from the phenotype observed for partial loss of Wdb function (cells expressing the dominant negative are shaded red in Fig. 1G,H). Furthermore, the stunted hairs produced by Wdb dn-expressing cells were often of abnormal or even reversed polarity, similar to those occasionally produced by Wdb transheterozygotes. The polarity defects are probably not a secondary consequence of faulty hair outgrowth, as cells expressing dnWdb also exhibited polarity alterations when hair morphology was otherwise normal (Fig. 1H). The effects of dominant-negative expression also resembled those of mild loss of Wdb function in that they were more severe on the ventral side of the wing. Hairs in the patched expression domain on the dorsal side of the wing were most often wild type in appearance, although no obvious differences in expression level were observed. These results show that dnWdb expression produces defects qualitatively similar to, through stronger than, those produced by mild *wdb* loss of function.

To confirm that the defects produced by dominant-negative Wdb were due to inhibition of Wdb rather than another activity, we asked whether co-expressed wild-type Wdb could reduce their severity. Co-expression of the wild-type protein strongly suppressed the defects in hair orientation and length (data not shown), indicating that the molecular functions disrupted by dominant negative Wdb are ones that can actually be performed by Wdb itself. These results do not rule out the possibility that dnWdb also interferes with a second protein that acts redundantly with Wdb.

Interestingly, hairs near the Wdb dominant negative expression domain were occasionally stunted or missing (Fig. 1G, arrows), suggesting that dnWdb expression can non-autonomously disrupt hair formation.

Widerborst promotes hair formation by activating the PP2A catalytic subunit microtubule star

B′ regulatory subunits are thought to act by modulating the activity of the catalytic subunit of PP2A with respect to specific protein targets. To address the effect of Widerborst on the activity of the catalytic subunit, we tested whether reducing the level of the C subunit could modify the *wdb* overexpression phenotype. Fig. 2A shows that transheterozygosity for two different strong alleles of *microtubule star* (*mts*), the gene that encodes the *Drosophila* PP2A C subunit, greatly reduces the number of cells forming multiple hairs in *wdb*-overexpressing wings. These data suggest that Wdb overexpression exerts its effects by positively regulating the enzymatic activity of the catalytic subunit of PP2A.

To confirm that PP2A activity could regulate hair formation, we examined the effect of overexpressing the catalytic subunit. Fig. 2B shows a wing in which *mts* was expressed along the compartment boundary under the control of the patched promoter. Strikingly, cells that overexpress *mts* can construct as many as 20 wing hairs that cover their apical surface and point in all directions. In those cells that make fewer wing hairs, it is also clear that hair polarity is disturbed. These data indicate that unregulated activity of the catalytic subunit enlarges the region of the cell capable of hair formation and interferes with normal polarization.

To determine whether endogenous PP2A activity was required for either hair outgrowth or polarization, we wanted to examine the effect of loss of PP2A function. As null alleles of *mts* are cell lethal (Wassarman et al., 1996), we approached the problem by expressing dominant negative versions of the protein. Truncations of the catalytic subunit that remove the active site have been shown behave as dominant negatives based on their ability to interact non-productively with A and B subunits (Evans et al., 1999). We constructed similar truncations of *mts* and expressed them under the control of the

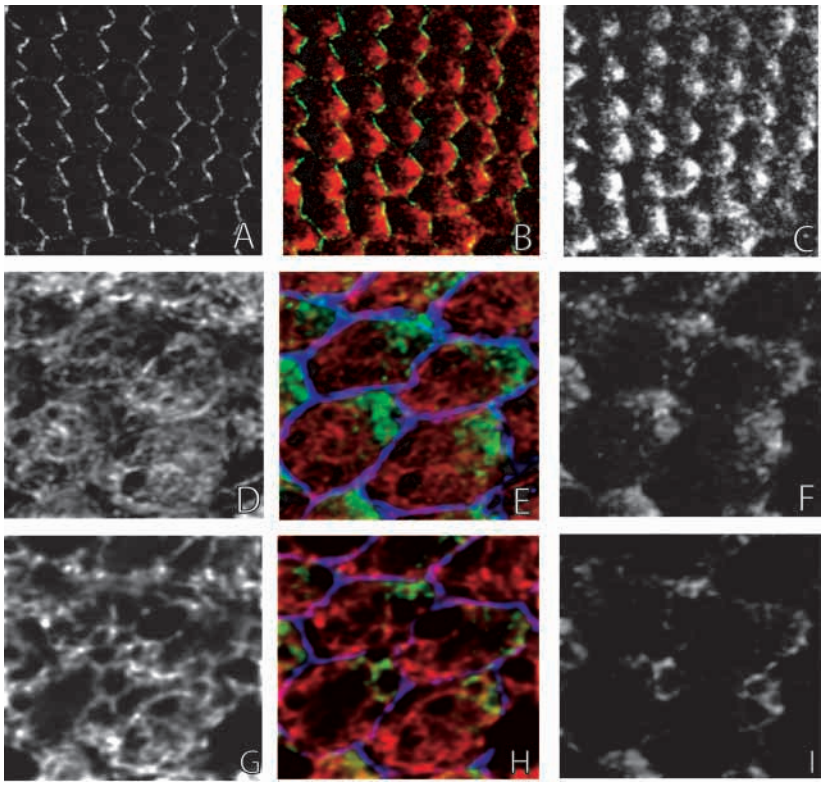

Fig. 3. Localization of Widerborst. (A-C) Widerborst (red), Flamingo (green) localization at 26 hours after puparium formation. Widerborst (B,C) is present on cell-internal spots and forms a distoproximal concentration gradient, unlike Flamingo (A,B), which is present on the proximal and distal cortex. (D-F) Nine optical sections separated by 0.25 μm were deconvolved and projected to give these images. Widerborst (D, green) is concentrated distally in the region of planar microtubules (red, F). (G-I) A single section from the projection shown in D-F. Widerborst (green) colocalizes exactly with microtubules (red). Coracle (blue) indicates cell boundaries. In all panels, distal is towards the right.

patched promoter during wing development. Wings derived from these flies are devoid of hairs over a significant region of the AP compartment boundary (Fig. 2C). In regions where hairs could be seen, they were often of abnormal orientation (Fig. 2D). Taken together, these data show that PP2A activity is necessary both for hair formation and hair orientation.

Widerborst localizes to distal microtubules before hair formation

Because regulation of the PP2A catalytic subunit by Widerborst affects hair formation, we wondered how activation might normally be restricted to the distal side of the cell. To address this question, we examined the subcellular localization of Wdb shortly before and during hair formation. Between 26 and 28 hours after puparium formation (apf) the tissue polarity genes Fz, Dsh, Fmi and Dgo are expressed at high levels at the proximodistal cortex (Usui et al., 1999; Feiguin et al., 2000; Axelrod, 2001; Shimada et al., 2001; Strutt, 2001) (Fig. 3A,B), but hairs will not form for another 3 to 5 hours. At this stage, Wdb protein is distributed on predominantly cytoplasmic punctate structures that are most abundant distally (Fig. 3B,C). Co-staining with antibodies to tubulin revealed that these spots of Wdb at least partially overlap with a subset of microtubules. Many microtubules in wing epithelial cells are organized in a meshwork lying parallel to the epithelial plane at the level of apical junctions (Eaton et al., 1996). Fig. 3D shows a projection of the microtubules comprising this ‘planar web’. Widerborst is enriched on microtubule bundles on the distal side of this web; colocalization is seen most clearly in single sections (Fig. 3G-I). During hair formation, Wdb polarity is lost, although it still associates with microtubules in general, including those in the outgrowing hair (data not shown). The enrichment of Wdb on distal microtubules suggests that PP2A is locally activated there and that microtubule associated proteins may be dephosphorylated in a polarized fashion before hair formation.

To identify the time at which the Wdb polarization mechanism is active, we examined Wdb localization at earlier stages of wing development. In third instar wing discs, and in early pre-pupal discs, Wdb was not polarized (data not shown). By 8 hours app, after the everted wing pouch had flattened significantly, Wdb became enriched on the microtubules to one side of each cell; surprisingly, its orientation at this stage was predominantly proximal with a slight anterior slant, rather than distal (Fig. 4A-C). By 18-20 hours apf, Wdb had relocalized distally (Fig. 4D,E). Flamingo, by contrast, was polarized only slightly, if at all (Fig. 4A). These results show that epithelial cells develop a polarized planar axis, reflected by Wdb localization, in pre-pupal wings. The polarity of the axis is initially proximal, but reverses direction sometime between 8 and 18 hours after puparium formation. The timing of distal Wdb reorientation suggests that it either precedes or is coincident with the onset of cortical domain polarization.

Wdb polarization is independent of Frizzled signaling

The subcellular localization of Wdb suggests that it does not polarize by binding directly to components of the cortical tissue polarity complex. To examine whether Wdb localization depends on the cortical domains at all, we stained *dsh*^{1} mutant wings for Wdb. In *dsh* mutant cells, both Frizzled and Flamingo fail to polarize along the proximal distal axis (Usui et al., 1999; Strutt, 2001). Strikingly, Wdb is well polarized in these wings (Fig. 5A). This observation suggests that polarized proximodistal cortical domains are not necessary for Wdb polarization.

To further examine the role of the cortical domains, we examined Wdb polarization in wings mutant for *starry night/flamingo*. In Flamingo mutant clones, both Frizzled and Diego not only fail to polarize, but do not localize the cortex at all (Feiguin et al., 2000; Strutt, 2001). Nevertheless, Wdb is normally polarized in *stan*^{3} mutant wings (Fig. 5B). Taken together, these data suggest that Wdb polarization does not require formation of polarized cortical domains.

To ask whether Wdb would respond to dominant reorientation of proximodistal cortical domains, we examined its localization in wings that expressed Frizzled under the

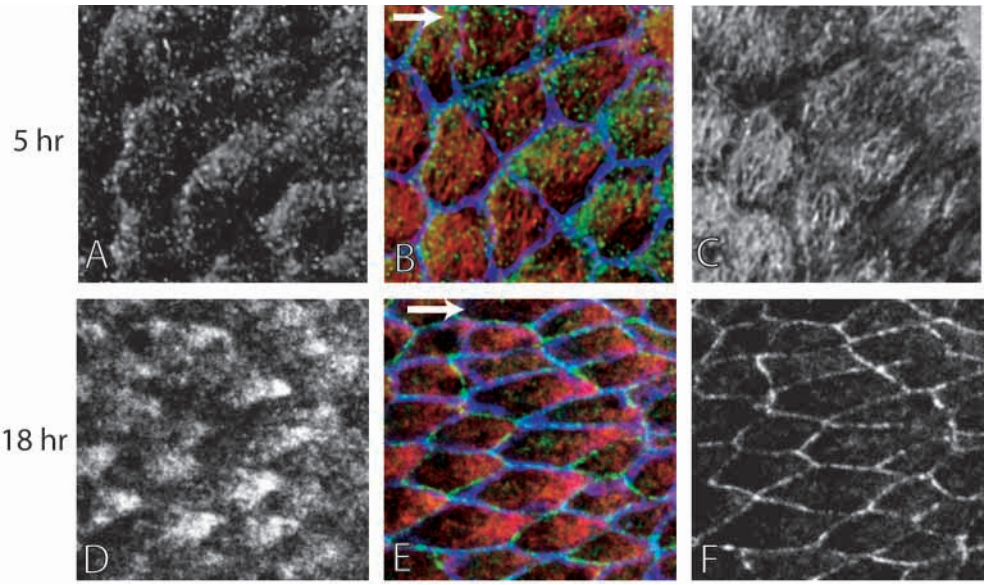

Fig. 4. Widerborst polarizes by 18 hours after puparium formation. (A-C) Widerborst (A; green in B), microtubules (C; red in B) and coracle (blue) in wings from pupae aged for 5 hours at 29°C. Widerborst is localized to the proximal side of the cell. The arrow in B points distally, and anterior is upwards. (D-F) Widerborst (D; red in E) is polarized distally by 18 hours after puparium formation (pupae aged at 25°C). Flamingo (F; green in E) is only slightly polarized at this stage, compared with 24 hours (see Fig. 3A). Coracle (blue) outlines cell boundaries. The arrow in E points distally.

control of the patched promoter (Fig. 5C-F). In these wings, Flamingo, Diego and Dsh (Usui et al., 1999; Feiguin et al., 2000; Axelrod, 2001; Shimada et al., 2001) (Fig. 6D) relocalize perpendicular to the normal proximodistal axis. The polarization of Wdb, however, is unchanged (Fig. 5C,E,F). These data show that the proximodistal domains formed by tissue polarity proteins are neither necessary nor sufficient to specify the axis of Wdb polarization.

Polarization of Fmi and Dsh depends on Wdb

To ask whether cortical polarization might depend on Widerborst, we examined the localization of Fmi and Dsh in cells expressing dominant-negative Wdb along the AP compartment boundary. Fig. 5G-L shows dnWdb-expressing wings that have been stained with an antibody to Widerborst, and either Fmi (Fig. 5H,I) or Dsh (Fig. 5K,L). The cells that express dnWdb are identifiable by their elevated staining with the Widerborst antibody (Fig. 5G,H,J,K). In these wings, it is clear that expression of dnWdb causes both Fmi and Dsh to accumulate uniformly around the cortex at high levels (Fig. 5H,I,K,L). By contrast, Dsh and Fmi are normally polarized in most of the cells that do not express dominant negative Wdb. These data indicate that Wdb activity is required for normal cortical polarity.

Interestingly, the expression of dnWdb appears to inhibit the distal accumulation of endogenous wild type Wdb in the cells adjacent to the dnWdb-expressing domain (Fig. 5G,J). This observation is consistent with the non-autonomous effects on hair formation observed in the adult wings of these flies (Fig. 1G). Furthermore, Dsh and Fmi are often depolarized in cells up to five cell diameters away from the dnWdb-expression domain (Fig. 5H,K). This might either be due directly to lower levels of endogenous Widerborst, or to non-autonomous propagation of cortical depolarization in the dnWdb-expression domain.

To ask whether Widerborst was required for the cytoskeletal integrity of wing epithelial cells, we examined the effect of dominant negative expression on actin, microtubules and Coracle. Cortical organization of both filamentous actin and Coracle appears essentially normal in dnWdb-expressing cells (Fig. 6A,B,C,F), suggesting that failure to polarize Flamingo and Dishevelled distribution does not result from gross defects in the subcortical actin cytoskeleton. By contrast, the organization of the planar microtubule web was perturbed by expression of dominant negative Wdb. Although dnWdb-expressing cells accumulate microtubules to at least normal levels, their ordered, web-like structure is not maintained (Fig. 6D-F). This suggests that Wdb normally directs the dephosphorylation of a protein that is important for microtubule organization and that the structure of the planar web may contribute to the development of cortical polarity.

Widerborst is needed for convergent extension movements during zebrafish gastrulation

The PP2AB′ regulatory subunits of the α/ε family are highly conserved and homologous genes are present from worms to humans. To ask whether the function of *widerborst* has been conserved in different cellular processes that require cell polarization, we examined whether it played a role in the regulation of gastrulation movements in zebrafish. One of the main cellular rearrangements during gastrulation in zebrafish and *Xenopus* is convergent extension. In convergent extension, cells move to the dorsal side of the gastrula to redistribute there along the forming anteroposterior body axis. Convergent extension movements are driven by mediolateral cell intercalations that require prior mediolateral polarization of cells. In recent studies, it has been shown that convergent extension movements depend on some of the same proteins that are responsible for organizing planar polarity in *Drosophila*, including zebrafish homologs of *dishevelled* and *strabismus/Van Gogh* (Heisenberg et al., 2000; Wallingford et al., 2000; Park and Moon, 2001).

We identified two zebrafish *widerborst* homologs (*wdb1* and *wdb2*), that both clearly fell into the α/ε subclass of B′ regulatory subunits (Fig. 1D); the proteins they encode are 63 and 64% identical to Dm and Wdb, respectively. Zebrafish *wdb1* and *wdb2* are maternally provided and expressed during

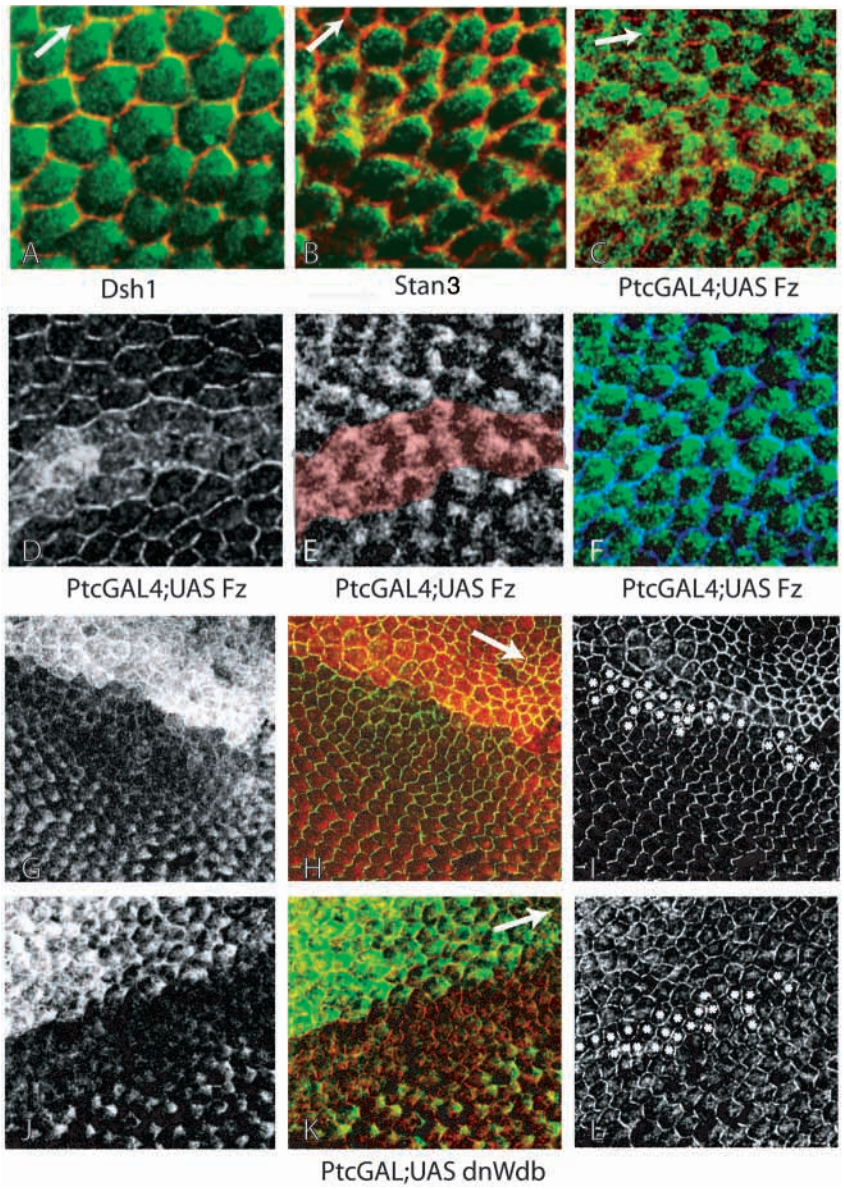

Fig. 5. Widerborst polarizes independently of Dsh and Fmi and is needed for polarization of cortical domains. Arrows point distally. (A) Widerborst (green) and Coracle (red) in a *dsh*^{1} pupal wing (aged 27 hours after puparium formation). Wdb is normally polarized. (B) Widerborst (green) and coracle (red) in a *stan*^{3} pupal wing (aged 27 hours after puparium formation). Wdb is normally polarized. (C) Frizzled, Flamingo, and Wdb staining in a pupal wing expressing Frizzled under the control of PatchedGAL4. Both Frizzled and Flamingo proteins were detected with mouse monoclonal antibodies and the red channel is a composite of their two patterns. In adjacent cells, Flamingo is repolarized perpendicular to the normal proximal-distal axis, but Wdb is normally polarized. (D) Flamingo and Frizzled, from the same image shown in C. (E) Wdb, from the same image shown in C. Fz-overexpressing cells are shaded red. (F) Wdb and Coracle, from the same image shown in C. (G-I) Wdb (G; red in H) and Fmi (I; green in H) in wings expressing dominant negative Wdb under the control of PatchedGAL4. Expression of dnWdb disrupts the polarization of wild-type Wdb up to five cells away from the overexpression domain. Fmi fails to polarize in dnWdb-expressing cells (H,I). Fmi also fails to polarize in cells near the dnWdb-expressing domain (asterisks in I). (J-L) Wdb (J; red in K) and Dsh (L; green in K) in wings expressing dominant negative Wdb under the control of PatchedGAL4. Expression of dnWdb disrupts the polarization of wild type Wdb in adjacent cells. Dsh fails to polarize in dnWdb-expressing cells (K,L). Dsh also fails to polarize in cells adjacent to the dnWdb-expressing domain (asterisks in L).

all stages of gastrulation (data not shown). To address their role(s) in convergent extension, we injected *wdb1* and *wdb2* morpholino antisense oligonucleotides alone and in combination into one-cell-stage embryos and examined the resulting phenotypes at bud stage (Table 2). Embryos injected with low concentrations of *wdb1* and *wdb2* morpholinos exhibited a shortened and broadened body axis at the end of gastrulation as monitored by in situ hybridization using *notail* (notochord), *dlx3* (anterior edge of neural plate) and *hgg* (prochordal plate) as markers (Fig. 7A-D). This phenotype suggests that the embryos are defective in convergent extension movements.

Embryos injected with higher concentrations of the same morpholinos were strongly dorsalized as seen by an expanded domain of *gsc* expression in the presumptive shield anlage at the onset of gastrulation (Fig. 7E,F). These data suggest that zebrafish Wdb regulates dorsoventral axis formation, as well as convergent extension, but that convergent extension is more sensitive to the dose of Wdb.

For both phenotypes, injections of zebrafish *wdb1* and *wdb2* morpholinos alone caused similar although sometimes weaker phenotypes than injections of a combination of both. These data may suggest that, although Wdb1 and Wdb2 perform the same functions, sufficient protein levels are only attained when

Table 2. zWdb affects convergent extension and dorsalization

RNA	Concentration (μg/μl)	Number of wild type	Number with convergent extension	Number dorsalised	Total number
dPP2A-dn(cs)	0.4	76	37	0	113
wdb-dn	0.4	88	34	0	122
zwdb-1-MO	4	16	70	0	86
zwdb-2-MO	4	14	94	0	108
zwdb-1+2-MO	2+2	14	70	0	84
zwdb-1+2-MO	8+8	0	0	84	84

dn, dominant negative; *cs*, c subunit; *MO*, morpholino.

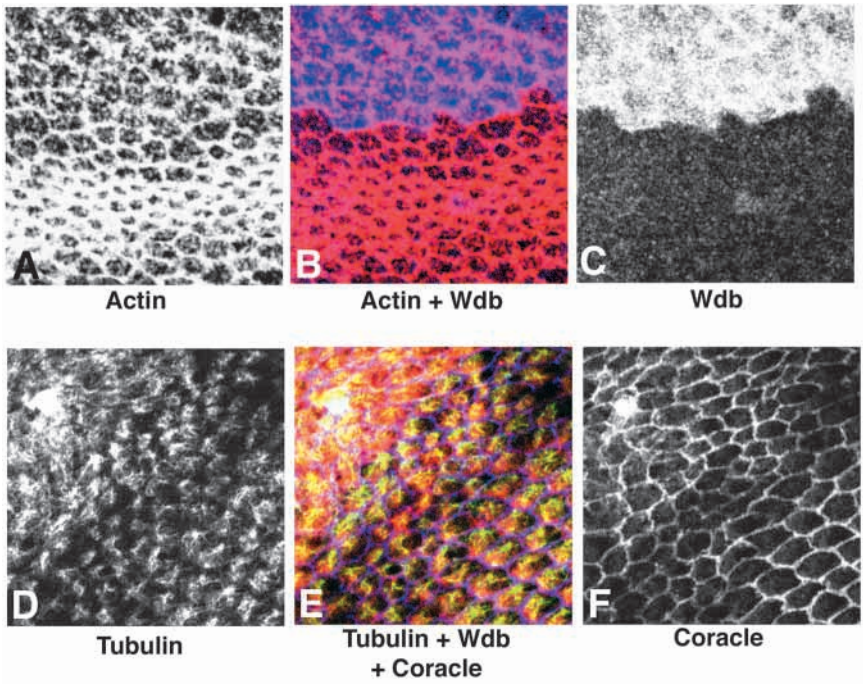

Fig. 6. Widerborst is required to organize planar microtubule webs. (A-C) dnWdb was expressed along the AP compartment boundary under the control of Ptc:GAL4. Pupal wings were fixed with paraformaldehyde and stained with phalloidin, to detect filamentous actin (A; red in B), and with anti-Wdb to detect dnWdb (C; blue in B). Cortical actin accumulates normally in dnWdb-expressing cells. The stripe of smaller cells with brighter actin staining correspond to the fourth wing vein. DnWdb-expressing cells should be compared with the larger intervien cells. Note that paraformaldehyde fixation does not preserve the polarized localization of Wdb. (D-F) dnWdb was expressed along the AP compartment boundary under the control of Ptc:GAL4. Pupal wings were fixed with methanol and stained with anti-Coracle (F; blue in E), anti-tubulin (D; green in E) and anti Wdb (red in E). The cortical organization of Coracle is normal, but microtubule structure is disturbed in dnWdb-expressing cells.

both genes are expressed. Alternatively, they may have non-redundant functions in both dorsoventral axis formation and gastrulation.

As the *Drosophila* and zebrafish Widerborst proteins show a high degree of sequence conservation, we also tested whether injection of the *Drosophila* dominant-negative *widerborst* and PP2A-C subunit constructs interfered with convergent extension movements in zebrafish. Injections of both dominant-negative constructs caused reduced convergent extension movements while dorsoventral patterning was largely unaffected, indicating that the *Drosophila* PP2A B′ α/ε subunit fulfills similar functions in zebrafish and *Drosophila* (Table 2).

DISCUSSION

The observation that tissue polarity gene products organize proximal and distal cortical domains in the wing epithelium was the first step towards a cell biological understanding of tissue polarization. Nevertheless many aspects of tissue polarization remain mysterious. In particular, the nature of the signal that initiates polarization of the cortical domains and defines their axis is not known. Furthermore, it is clear that additional, frizzled-independent, mechanisms operate in the wing to polarize the cytoskeleton during wing hair formation and to align the axes of hair polarity between cells. Up until to now, there have been few clues as to the nature of these processes. In this work, we identify a B′ regulatory subunit of PP2A, Widerborst, that is required both for cortical polarization and for hair outgrowth.

Widerborst is unique in that it does not colocalize with other tissue polarity proteins at the cell cortex. Instead, as cortical polarization is beginning (18-24 hours apf), it is found on microtubules on the distal side of each wing epithelial cell. Furthermore, it localizes there before obvious organization of proximodistal cortical domains, and its polarization is independent of them. Strikingly, at earlier developmental stages (7-9 hours apf), Wdb polarity is not distal but proximal. These dynamic shifts in Wdb polarity and their independence

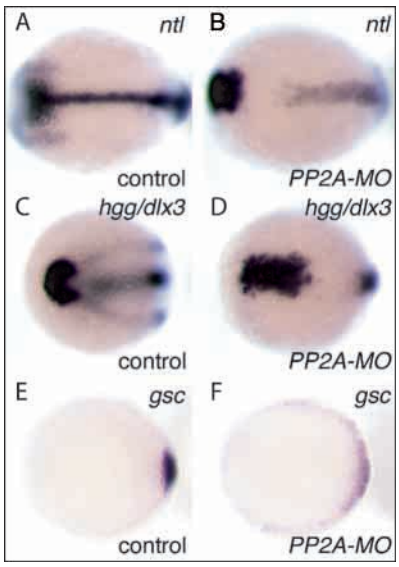

Fig. 7. Injection of morpholino antisense oligonucleotides (MO) against zebrafish *wdb 1* and *wdb2* interferes with dorsoventral patterning and convergent extension movements during gastrulation. (A,B) The notochord, outlined by expression of *ntl*, is shortened and broadened in MO (1 ng)-injected (B) versus mock-injected (A) embryos. (C,D) The shape of the prechordal plate, marked by the expression of *hgg* is elongated in MO (1 ng)-injected (D) versus mock-injected (C) embryos. (E,F) the expression of *gsc* within the shield is expanded ventrally in embryos injected with 4 ng of wdb1 and *wdb*2 MOs (F) when compared with mock-injected embryos (E) at shield stage, indicating that these embryos are dorsalized.

from previously described tissue polarity genes suggest the existence of a novel polarization mechanism.

How might Wdb operate to specify cortical polarity? When Wdb activity is reduced, components of the cortical domains like Dsh and Fmi accumulate uniformly around the cell cortex at high levels. By contrast, disruption of Frizzled signaling interferes with the accumulation of Dsh and Fmi at the cell cortex (Usui et al., 1999; Axelrod, 2001; Shimada et al., 2001). This suggests that Wdb is not required to activate Frizzled signaling, but rather is important for making it asymmetric.

Our genetic data indicate that Wdb exerts its activity by activating the catalytic subunit of PP2A with respect to specific substrates, and the localization of Wdb suggests that it does so on the distal side of the planar microtubule web. Which proteins might be targeted for dephosphorylation by Widerborst? One possibility is Dishevelled. Heterozygosity for *wdb* strongly suppressed the mwh phenotype of *dsh1* suggesting that, during tissue polarization, these two proteins act antagonistically (M. H., unpublished). Dishevelled cortical localization correlates with hyperphosphorylation (Yanagawa et al., 1995; Axelrod, 2001), and the cortical localization of Dsh is certainly expanded in Wdb dominant-negative expressing cells. Supporting this possibility, two-hybrid experiments have indicated that Dishevelled can physically interact with a *Xenopus* B′ regulatory subunit (Ratcliffe et al., 2000). If Wdb normally acted by antagonizing Dsh, then the dominant-negative might overactivate Frizzled signaling and cause defects in tissue polarity. This model is not easily reconcilable with a role for the distal localization of Wdb; one might naïvely expect an antagonist of Frizzled signaling to accumulate proximally instead of distally. Nevertheless, although the early distal localization of Wdb is suggestive, we have not proven that it is relevant to cortical polarization; for example, Wdb might have a role in transducing the Frizzled signal, for which distal localization is not required.

What might be the importance of Wdb binding to the distal microtubule web? Binding to the cytoskeleton might simply allow stable distal localization of an otherwise diffusible cytosolic molecule. More interestingly, this association raises the possibility that Widerborst directs the dephosphorylation of a microtubule-associated protein. Consistent with this idea, the structure of the planar microtubule web is disrupted by dnWdb expression. PP2A activity is important for the accumulation of stable microtubules (Gurland and Gundersen, 1993), presumably through its effects on the phosphorylation state of MAPs (Sontag et al., 1996; Gong et al., 2000a; Gong et al., 2000b). Microtubule stability can affect the binding of microtubule motor proteins (Gagnon et al., 1996; Liao and Gundersen, 1998) and can contribute to polarized protein delivery (Pous et al., 1998). In the wing, microtubules have been suggested to play important roles in hair polarity; depending on the time at which it is added, vinblastine treatment of pupal wings causes either failure of hair outgrowth or the formation of multiple wing hairs (Turner and Adler, 1998). Polarized dephosphorylation of MAPs within the planar microtubule web might bias the transport of vesicles containing components of the proximodistal cortical domains. At later stages, it might also help direct transport of components of the hair formation machinery to the distal side of the cell, or promote the stability of microtubules in the outgrowing hair. This model for Widerborst action could provide a single explanation for its effects on hair outgrowth and on cortical polarity. Identification of the relevant Widerborst substrate(s) should greatly advance our understanding of the cell biology of tissue polarization.

Our data also support other studies indicating that B′ α/ε regulatory subunits antagonize the classical Wnt signaling pathway. Experiments in *Xenopus* embryos and tissue culture cells have shown that increasing the level of a B′ α subunit inhibits Wnt signaling and causes ventralization (Li et al., 1995; Seeling et al., 1999; Ratcliffe et al., 2000). Consistent with this, our experiments show that reducing Wdb levels causes dorsalization of zebrafish embryos. Although Wdb, like Frizzled and Dishevelled, is a shared component of both planar polarization and classical Wnt signaling pathways, it probably has different functions in each; during classical Wnt signaling, the B′ α is thought to act downstream of Dishevelled, forming part of a β-catenin degradation complex (Seeling et al., 1999; Li et al., 2001) that plays no role in planar polarity signaling.

The observation that *widerborst* is needed both for distal polarization of *Drosophila* wing hairs and for convergent extension movements during zebrafish gastrulation points to a conserved role for Wdb in regulating tissue polarity in development. Furthermore, it provides additional evidence supporting the conjecture that components of the planar polarization pathway in *Drosophila* are also used to control cell polarity and movement during vertebrate gastrulation (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). To date, the evidence for this is based on analysis of various *dsh* constructs and, more recently, on the analysis of *vang/stbm* and *rhoA* during vertebrate gastrulation (Habas et al., 2001; Park and Moon, 2001). Our identification of Wdb as another shared component provides further evidence that this signaling cascade is indeed conserved between *Drosophila* and vertebrates. Additional experiments will have to address the precise function(s) of vertebrate *wdb* homologs and where *wdb* acts in the genetic pathway regulating vertebrate gastrulation movements.

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