Drosophila Rab23 is involved in the regulation of the number and planar polarization of the adult cuticular hairs

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ABSTRACT

The planar coordination of cellular polarization is an important, yet not well understood aspect of animal development. In a screen for genes regulating planar cell polarization in *Drosophila*, we identified *Rab23*, encoding a putative vesicular trafficking protein. Mutations in the *Drosophila* *Rab23* orthologue result in abnormal trichome orientation and the formation of multiple hairs on the wing, leg and abdomen. We show that *Rab23* impairs hexagonal packing of the wing cells, and that it plays a role in cortical polarization of the polarity proteins. We found that Rab23 is able to associate with the proximally accumulated Prickle protein, although Rab23 itself does not appear to display a polarized subcellular distribution in wing cells. The absence of *Rab23* leads to increased actin accumulation in the sub-apical region of the pupal wing cells that fail to restrict prehair initiation to a single site. *Rab23* acts as a dominant enhancer of the weak multiple hair phenotype exhibited by the core polarity mutations, whereas the *Rab23* homozygous mutant phenotype is sensitive to the gene dose of the planar polarity effector genes. Together, our data suggest that Rab23 contributes to the mechanism that inhibits hair formation at positions outside of the distal vertex by activating the planar polarity effector system.
INTRODUCTION

The formation of properly differentiated organs often requires the planar coordination of cell polarization within tissues, a feature referred to as planar cell polarity (PCP) or tissue polarity. Although planar polarity is evident in many vertebrate tissues (such as fish scales, bird feathers and cochlear epithelium) and it has recently been shown that PCP regulation is highly conserved throughout the animal kingdom (FANTO and MCNEILL 2004; SEIFERT and MLODZIK 2007; SIMONS and MLODZIK 2008; STRUTT 2003), such polarization patterns are best studied in the fruitfly, Drosophila melanogaster. PCP in flies is manifest in the mirror-image arrangement of ommatidia in the eye, in the adult cuticle, which is decorated with parallel arrays of hairs and sensory bristles, and in the wing, which is covered by distally pointing hairs (or trichomes) (ADLER 2002). Wing hairs form during the pupal life when each cell produces a single microvillus-like prehair stiffened by actin and microtubules. In wild type wing cells prehairs form at the distal vertex of the cells and extend distally as they grow (WONG and ADLER 1993).

Mutations in PCP genes result in abnormal wing hair polarity patterns and wing hair number (GUBB and GARCIA-BELLIDO 1982; WONG and ADLER 1993). Based on their cellular phenotypes (i.e., prehair initiation site and number of hairs per cell), initial studies placed PCP genes into three groups: the first group (often called the core group) includes frizzled (fz), dishevelled (dsh), starry night (stan) (also known as flamingo), Van Gogh (Vang) (also known as strabismus), prickle (pk) and diego (dgo), the second group consists of inturned (in), fuzzy (fy) and fritz (frtz) (referred to as planar polarity effectors or In group), whereas the third group includes multiple wing hairs (mwh) (WONG and
Double mutant analysis demonstrated that these phenotypic groups also represent epistatic groups, and it was proposed that the PCP genes may act in a regulatory hierarchy, where the core group is on the top, whereas the In group and mwh are being downstream components (Wong and Adler 1993). Subsequent work identified several other PCP genes as well. Some of these have been placed into the Fat/Dachsous group (Adler et al. 1998; Strutt and Strutt 2002), while another group consists of cytoskeletal regulators, including Rho1 and Drok (Adler 2002; Strutt et al. 1997; Winter et al. 2001). Genetic analysis of these two groups has led to models in which the Fat/Dachsous group acts upstream of the core proteins (Ma et al. 2003; Yang et al. 2002), while Rho1 and Drok act downstream of Fz (Strutt et al. 1997; Winter et al. 2001). Although the existence of a single, linear PCP regulatory pathway is debated (Casal et al. 2006; Lawrence et al. 2007), it is clear that in the wing PCP genes regulate (1) the number of prehairs, (2) the place of prehair formation, and (3) wing hair orientation.

While the molecular mechanism that restricts prehair formation to the distal vertex of the wing cells is elusive, it has been well established that the core PCP proteins adopt an asymmetrical subcellular localization when prehairs form (Axelrod 2001; Bastock et al. 2003; Das et al. 2004; Shimada et al. 2001; Strutt 2001; Tree et al. 2002; Usui et al. 1999), which appears to be critical for proper trichome placement. In addition, it has recently been found that the In group of proteins and Mwh also display an asymmetrical pattern with accumulation at the proximal zone (Adler et al. 2004; Strutt and Warrington 2008; Yan et al. 2008). These studies concluded that the core PCP proteins are symmetrically distributed until 24 hours after prepupa formation (APF),
when they become differentially enriched until prehair formation begins at 30-32 hours APF. This transient asymmetric localization ends by 36 hours APF (ADLER 2002; MIHALY et al. 2005; STRUTT 2003). It has recently been shown that Fz and Stan containing vesicles are transported preferentially toward the distal cell cortex in the period of 24-30 hours APF (SHIMADA et al. 2006), and hence, polarized vesicular trafficking might be an important determinant of PCP protein asymmetry. Other recent studies, however, challenged the view that PCP protein polarization is limited to 24-32 hours APF. Instead, it has been suggested that at least a partial proximal-distal polarization is already evident at the end of larval life and during the prepupal stages (6 hours APF). Polarity is then largely lost at the beginning of the pupal period, but becomes evident again in several hours until hair formation begins (CLASSEN et al. 2005). Thus, molecular asymmetries are clearly revealed during wing hair formation, yet the molecular mechanisms that contribute to the establishment of these asymmetrical patterns are not well understood.

In a large-scale mosaic type of mutagenesis screen, we identified Drosophila Rab23, encoding a vesicle trafficking protein, as a PCP gene involved in the regulation of trichome orientation and number in various adult cuticular structures, including the wing, abdomen and leg. We show that Rab23 plays a role in cortical polarization of the core PCP proteins in the wing, and that Rab23 associates with at least one core protein, Pk. Additionally, we found that Rab23 contributes to the mechanism that restricts actin accumulation and thus, prehair initiation to a single site within each wing cell.

MATERIALS AND METHODS
Fly strains and genetics: Fly strains used are described in FlyBase, except for the different Rab23 alleles (see below) and the Ubx-Flp stock (kindly provided by J. Knoblich, IMP Vienna) (EMERY et al. 2005). The Rab23\textsuperscript{T69A} allele has been isolated during an F2 FRT/Flp (Xu and Rubin 1993) mosaic mutagenesis screen. FRT82B chromosomes were mutagenized with ENU (1.6mM), mutant clones were induced by Ubx-Flp, PCP phenotypes were analyzed on the wing and notum. The Rab23\textsuperscript{51} excision allele was generated by remobilization of the P(RS5)5-SZ-3123 (DrosDel) P-element insertion line by using standard techniques. The Rab23 RNAi line has been provided by the VDRC RNAi Centre (IMP-IMBA, Vienna), whereas the w; UASp-YFP-Rab23 lines were generous gifts from M. Scott (Stanford University). Flip out clones of UASp-YFP-Rab23 were generated using w,hsFLP; ActP-FRT-\textsuperscript{y+}-FRT-Gal4,arm-lacZ with a 2 hours heat shock at 37°C during the early third instar larval stage. For overexpression studies we used Act5C-Gal4 or Sal-Gal4. In line with FlyBase, we use starry night instead of flamingo, and Van Gogh instead of strabismus.

Cuticle preparation: Abdominal cuticles and first thoracic legs were prepared according to a protocol by Ian Duncan (DUNCAN 1982).

DNA techniques and tissue culture: DNA constructs for transgenic flies and transfection experiments were created by standard cloning techniques. Detailed cloning procedures can be obtained from the authors upon request. To create a Rab23 genomic rescue construct, a 12.3 kbs XbaI restriction fragment from BACR03P13 (BACPAC Resources) was cloned into a pCaSpeR4 vector. To clone the Rab23\textsuperscript{51} transcript, total RNA was isolated from homozygous mutant adults with Trizol reagent (Invitrogen),
cDNA was synthesized with Revert Aid (Fermentas), RT PCR was carried out with primers specific to the first and third exon of Rab23, respectively. Primers used were as follows: Rab23Ex3 5’-CCCCGCCACAACATCATTAG-3’, Rab23Ex1 5’-TCAAATTTCTGATCGCGACGAG-3’. Molecular cloning of the RT PCR product revealed that Rab2351 encodes a hybrid transcript consisting of the first (non-coding) exon of Rab23, part of the first intron of Rab23, followed by 443 basepairs from the 5’ end of the P(RS5)5-Sz-3123 insertion precisely until the end of ORF0 (O’HARE and RUBIN 1983) that is fused to the third exon of Rab23 (Figure 1B). If translation from this fusion transcript begins with the ATG of ORF0, the predicted fusion protein is entirely devoid of Rab23 sequences because ORF0 and the third exon of Rab23 are not in the same phase. If a downstream ATG were used, M53 of Rab23 is the next starting point. However, translational initiation from M53 would result in a mutant Rab23 protein that is lacking of 52 N-terminal aminoacids, including 15 of the highly conserved and functionally essential part of the GTPase domain (SANTOS and NEBREDA 1989). Thus, these results suggest that Rab2351 encodes a functionally strongly, if not entirely, impaired protein.

For transfection experiments we used the following constructs: pAVW-Rab23, pHW-Rab23, pPVW-Rab23, BLMT-RFP-Rab23, pAVW-Rab23Q96A, pPVW-Rab23Q96A, pHW-Rab23T69A, BLMT-Rab23Q96A-EGFP, pAC5.1-Fz-Myc, pAC5.1-Dsh-EGFP, pAC5.1-HA-Dgo, pAC5.1-6xMyc-Pk, pRME-Vang-HA (a kind gift from T. Wolff). When necessary, pActin5c-Gal4 was cotransfected to drive expression from UAS promoters. Expression from the BLMT vectors was induced by 1 mM CuSO4 for 4 hours or 500 μM CuSO4 overnight.
Drosophila S2 cells were transfected with Effectene (Qiagen), and incubated in Drosophila Schneider’s medium (Lonza) for 24 hours before fixation.

**Antibody production:** Rab23 antibody was raised in mouse injected with a bacterially produced, His-tagged, full length Rab23 protein. After four boosts, the crude serum was tested for immunostainings and Western-blot analysis. As a strong background staining was evident in our immunostainings, the serum subsequently was only used for biochemical experiments.

**Immunohistochemistry:** For pupal wing analysis white pre-pupae were collected, aged, and dissected at the desired time and fixed in 4% formaldehyde in PBS. Stainings were done according to standard procedures (Wong and Adler 1993). Drosophila S2 cells were fixed as described in (Matusek et al. 2008).

For immunostainings we used the following primary antibodies: mouse anti-Rab23 1:100, mouse anti-β-gal 1:1000 (Promega), rabbit anti-β-gal 1:1000 (Molecular Probes), mouse anti-GFP 1:200 (DSHB), rabbit anti-GFP 1:1000 (Santa-Cruz Biotechnology), rabbit anti-Vang 1:500 (Rawls and Wolff 2003), rabbit anti-Dgo 1:200 (Feiguen et al. 2001), mouse anti-In 1:1000 (Adler et al. 2004), rabbit anti-Pk 1:2000 (Tree et al. 2002), mouse anti-Myc 1:400 (Roche), mouse anti-HA 1:400 (Roche), rabbit anti-HA (Sigma), mouse anti-Fz 1:10 (DSHB), mouse anti-Stan 1:10 (DSHB). Actin was stained with Rhodamine-Phalloidine 1:100 (Molecular Probes). Brightfield images were collected using Zeiss Axiocam MOT2. Confocal images were collected with Olympus FV1000 LSM microscope. Images were edited with Adobe Photoshop 7.0CE and Olympus Fluoview.
**Immunoprecipitation and Western-blot analysis:** For immunoprecipitation experiments 100 wild type or Rab2351 homozygous mutant pupae (28-30 hours APF) were lysed in 1 ml Lysis buffer (0,1% SDS, 0,2% NaDoc, 0,5% NP-40, 150 mM NaCl, 50 mM TrisHCl, pH=8.0) for 1 hour at 4°C. Insoluble materials were pelleted with a centrifugation step and the clear supernatant was used for further studies. A portion of the cleared lysate was used as Western-blot control. The remaining lysate was then preincubated with 100 µl IgG free Protein-A Sepharose (CL-4B, Pharmacia) beads for 1 hour at RT to deplete non-specifically binding proteins. For immunoprecipitation 60 µl of CL-4B Sepharose beads were incubated with 30 µl anti-Rab23 antibody for 2 hours at RT in 1 ml final volume in Lysis buffer (precomplex). A portion of precomplex was kept as bead control. Immunoprecipitation was carried out overnight 4°C. SDS-PAGE and Western-blot analysis were carried out according to standard protocols.

**RESULTS**

In order to identify new planar polarity genes, we employed the FRT/Flp mosaic system to induce homozygous mutant clones on the wing and notum of mutagenized flies by Ubx-Flp (EMERY et al. 2005). Genetic mapping of one of the new PCP mutants, displaying multiple hairs in mutant clones, showed that the mutation affected the *Drosophila Rab23* orthologue. Rab23 belongs to the Rab family of small GTPases known to play a role in vesicular membrane transport (ZERIAL and McBRIDE 2001). Sequence analysis of the new allele revealed a point mutation (T69A) in the Switch I region of the GTPase domain of Rab23, affecting an amino acid residue that is invariant in the whole
small GTPase superfamily (Figure 1A) (VETTER and WITTINGHOFER 2001). This allele, designated \( \text{Rab23}^{T69A} \), is semilethal, but homozygous mutant animals occasionally survive till adulthood and display a strong multiple wing hair phenotype as well as hair orientation defects (Figure 2, C, D and F). Although these trichome orientation defects are relatively mild compared to \( f_{z} \) or \( dsh \), they are exhibited in every \( \text{Rab23} \) mutant wing. Whereas in wing sectors B and C hair orientation is mildly affected, the deflection from wild type orientation is obvious in sectors A, D and E (Figure 2, A-F, and not shown), clearly indicating a requirement in the regulation of hair orientation. Because no other \( \text{Rab23} \) alleles were available, we generated independent \( \text{Rab23} \) alleles by remobilizing the \( P(RS5)5-Sz-3123 \) P-element insertion sitting in the first intron of \( \text{Rab23} \) (Figure 1B). One of these alleles, \( \text{Rab23}^{51} \), is homozygous viable and displays wing hair phenotypes identical to that of \( \text{Rab23}^{T69A} \) (Figure 2G). \( \text{Rab23}^{T69A} \) is viable over \( \text{Rab23}^{51} \) and similarly, both mutations are viable over deficiency chromosomes uncovering \( \text{Rab23} \). Each of these mutant combinations displays a strong multiple hair phenotype, and weak hair orientation defects (Figure 2, H and I, Figure S2). As the severity of the wing phenotypes is identical in the \( \text{Rab23} \) trans-heterozygous, homozygous and hemizygous mutants, by genetic criteria, both \( \text{Rab23} \) alleles behave as strong loss-of-function (LOF) or null alleles. In agreement with this, it has already been reported that impairment of the Threonine residue (equivalent to that of T69 in Rab23) in other small GTPases leads to a strong loss of function (SPOERNER et al. 2001). Moreover, we revealed that \( \text{Rab23}^{51} \) encodes a hybrid transcript that can not be translated into a functional protein (see details in Materials and Methods) suggesting that \( \text{Rab23}^{51} \) is a null allele.
The wing hair phenotypes of both Rab23 alleles can be fully rescued by providing a single wild type copy of the Rab23 gene (Figure 2K, and data not shown). Hence, the LOF analysis and the rescue experiments together indicate that Rab23 in Drosophila is not essential for viability, however, it is involved in the regulation of wing hair number, and to a lesser degree, wing hair orientation. Consistently, silencing of Rab23 by RNAi using ubiquitously expressed, as well as wing specific drivers, resulted in a moderately strong multiple hair phenotype (Figure 2J), without affecting viability (data not shown).

**Rab23 impairs hair polarity and number on the adult cuticle**

Because PCP is also manifest in tissues other than the wing, we examined the eyes, notum, legs and abdomen of Rab23 mutant flies. We found that ommatidial polarity and the orientation of the epidermal bristles on the notum, legs and abdomen are unaffected by Rab23, and these mutants also lack the duplicated tarsal joint phenotype typical of the core PCP mutants (Figure 3, and not shown). Conversely, Rab23 affects the orientation and number of trichomes covering the legs and abdomen (Figure 3). The orientation defects are equally evident on the legs, tergites, sternites (Figure 3) and pleural regions of the abdominal cuticle (not shown). Interestingly, hair polarity looks randomized all over the tergites (Figure 3F), that is different from other PCP mutations exhibiting polarity reversions or orientation defects affecting only certain areas along the anterior-posterior axes of the tergites (Casal et al. 2002; Lawrence et al. 2004). Additionally, the formation of multiple hairs is also obvious on the leg and the abdominal cuticle (Figure 3). Together, these data suggest that Rab23 identifies a unique class of PCP genes that is specifically required for the regulation of trichome orientation and
number in all body regions examined without affecting the planar polarization of multicellular structures such as ommatidia or sensory bristles.

**Rab23 impairs prehair initiation, and hexagonal packing of pupal wing cells**

To investigate *Rab23* function at the cellular level, we subsequently focused our analysis to the wing. First, we examined prehair initiation in *Rab23<sup>51</sup>* homozygous mutant pupal wings and *Rab23<sup>T69A</sup>* mutant clones at 31 hours APF. In the absence of *Rab23* apical actin accumulation is not restricted to the distal vertex of the cells. Instead, we detected large amounts of diffusely organized actin filaments in the apical region of the wing cells (Figure 4, A-C”). Presumably as a consequence of the failure to restrict the site of actin accumulation, many cells developed more than one prehair that, similarly to mutations of the In group, formed in abnormal positions around the cell periphery (Figure 4, B and D). Additionally, we noted that prehair initiation is somewhat delayed in *Rab23<sup>T69A</sup>* mutant clones (Figure 4, D-D”). This delay is particularly obvious in clones induced in the proximal half of wing sector B and C (in 85.7% of the clones, n=23), conversely, the delay is less frequent in clones from distal B and C sectors (25%, n=20). Clones with delayed prehair initiation are more randomly distributed in wing sectors D and E in about 50% of clones examined (n=36). Beyond these effects, the clonal analysis also revealed that *Rab23* acts in a cell autonomous manner, as the presence of multiple hairs was always restricted to the mutant cells (Figure 4, E-E”).

Recent studies uncovered that the wing epithelial cells are irregularly shaped throughout larval and early pupal stages, but most of them become hexagonally packed shortly before prehair formation (CLASSEN *et al.* 2005). It has also been shown that the
core PCP mutations partly interfere with normal cellular packing (CLASSEN et al. 2005). While analyzing Rab23 mutant pupal wings, we noticed that some of the Rab23 mutant wing cells also fail to adopt a hexagonal shape at around 30-32 hours APF (Figure 5B). We quantified this effect in the D region of the wing distal to the posterior cross vein (Figure 2A), and found that in wild type wings the ratio of the non-hexagonally shaped cells is about 11%, whereas in Rab23T69A homozygous mutant wings this ratio is increased to 27% (Figure 5C). As a comparison, we measured the packing defects in two core PCP mutants, Vang6 and dsh1, where the ratio of the non-hexagonal cells was 30% and 28%, respectively (Figure 5C). Because the strength of the Rab23 induced packing defects is comparable to the effect of the core PCP mutations, these data suggest that Rab23 might also be an important determinant of cellular packing.

Next we addressed if the packing defects revealed in Rab23 mutant wings correlate with the hair orientation defects and/or the formation of multiple hairs, also exhibited upon loss of Rab23. However, we found no obvious correlation with the hair orientation defects (not shown). Similarly, when we compared the average prehair number of hexagonally and non-hexagonally packed Rab23 mutant wing cells, we failed to reveal any correlation between the presence of multiple hairs and the cellular packing defects (Figure 5D). To extend this analysis, we investigated the packing defects of other mutations causing multiple hairs, such as in, frtz and mwh. These mutants exhibit somewhat weaker packing defects than Rab23 (Figure 5C, and not shown), but as for Rab23, the formation of multiple hairs does not appear to correlate with irregular cell shape (Figure 5D). Taken together, these results demonstrate that cell packing has no direct effect on the number of prehair initiation sites in the wing epithelial cells.
**Rab23 is required for cortical polarization of the PCP proteins**

Given that the absence of asymmetrically localized core PCP proteins alters hexagonal packing of the wing cells (Clasen et al. 2005), and packing was altered in Rab23 mutant wings, we asked whether Rab23 affects PCP protein localization. To this end, we examined the localization pattern of several core PCP proteins in Rab23 mutant pupal wings. These experiments showed that the initial polarization pattern of Stan in prepupal wings is essentially identical in wild type and Rab23<sup>51</sup> mutant wings (Figure S1). However, by 24-30 hours APF the core PCP proteins fail to polarize properly in a Rab23 mutant tissue (Figure 6, A-C’), although they still accumulate into apicolateral complexes (Figure 6, A-C’). Interestingly, we found that in Rab23 mutant clones that exhibited a delay in actin accumulation mislocalization was always evident (n=26) in most part of the mutant tissue (Fig. 4D’) independent of the wing region the clone was from. In clones that did not exhibit a delay in actin accumulation, localization was strongly affected in the distal C region in every case (n=7), whereas in other wing sectors the effect was restricted to a smaller region within the clone (n=24). Thus, instead of a regional specificity, it appears that altered PCP protein localization correlates with the delay in actin accumulation, i.e. localization defects are more evident in delayed clones than in others. In agreement with this, we’ve been unable to find a clear regional specificity in Rab23 homozygous mutant wings. Instead, smaller or larger areas in which the “zigzag” pattern was altered were evident in all wing regions (n=25). Consistent with previous findings that In localization depends on core PCP protein localization (Adler et al. 2004), we found that the lack of Rab23 impairs In localization as well (Figure 6, D
and D’). Together, these results suggest that Rab23 contributes to the late (24-30 hours APF) apical, cortical polarization of the PCP proteins.

As we pointed out above, Rab23 is also required for hexagonal packing of the wing cells, therefore it was necessary to clarify if the abnormal looking PCP protein localization is not simply the consequence of irregular cell packing. In order to address this issue, we carefully examined Pk localization in wild type and Rab23 mutant wings in such hexagonal cells whose six neighbors were hexagonally shaped as well and therefore packing defects could not interfere with localization (Figure 7). This analysis revealed that a Rab23 mutant hexagonal cell has significantly higher chance of displaying impaired PCP protein localization than a wild type cell (Figure 7, A-C). Because impaired PCP protein localization is also associated with cellular packing defects in the core PCP mutants, we also quantified Pk localization in dsh\(^1\) mutant hexagonal wing cells. In agreement with previous work that provided a general assessment of Pk localization in dsh mutants (TREE et al. 2002), we found that dsh strongly disrupts the asymmetric accumulation of Pk in the hexagonal cells (Figure 7C). Hence, it appears that the core PCP mutations have a stronger effect on PCP protein polarization than that of Rab23.

**The subcellular distribution of Rab23 in pupal wing cells**

To gain insight into the mechanism whereby Rab23 contributes to core PCP protein localization, we examined the subcellular localization of Rab23 in developing pupal wings. Given that the anti-Rab23 serum we raised does not appear to be suitable for immunohistochemical analysis (see Methods), we expressed a YFP::Rab23 fusion protein
to assess localization in wing cells. Note that YFP::Rab23 fully rescues the Rab23 mutant phenotypes (Figure 2L), indicating that it is a functional protein. Between 24 and 32 hours APF, when the core PCP proteins repolarize, the YFP::Rab23 protein expressed uniformly in the wing exhibits a strong plasma membrane association in the junctional zone, and also in the basolateral zone (Figure 8, A-B”). Additionally, we detected an elevated YFP::Rab23 level in the sub-apical cytoplasmic domain with occasional accumulations in punctate structures (Figure 8, A-A”). A very similar localization pattern was found before 24 hours APF (not shown), and at 36 hours APF. The expression of the YFP::Rab23 fusion protein in flip-out clones also led to similar observations, and, importantly, it confirmed the lack of any apparent proximal-distal polarization (Figure 8, C-D”). Thus, Rab23 itself does not display a proximo-distally polarized distribution from the onset of pupal development till shortly after trichome placement. As a comparison, we examined the localization pattern of YFP::Rab23Q96L, a constitutively active and YFP::Rab23S51N, a dominant negative (DN) form of Rab23. The overall patterns were similar to wild type YFP::Rab23, but the activated form displayed stronger membrane association, whereas the DN exhibited higher accumulation in the cytoplasm than wild type (Figure S2). Because this is in full agreement with the membrane cycle model of Rab GTPases (Behnia and Munro 2005), we conclude that although our localization studies are based on overexpression, they are likely to reflect the normal localization of Rab23 to a great extent.

Next, we addressed the question whether increased amounts of Rab23 would affect wing development and PCP protein localization. However, overexpression of YFP::Rab23 had no effect on wing development, and the protein did not impair PCP
protein localization (not shown). The overexpression of the activated form had no effect on pupal wing development either, while the DN induced a weak multiple hair phenotype that was however much weaker than that of Rab23\(^{31} \) (not shown) and therefore this allele was not used for further studies.

**Rab23 associates with the Pk protein**

As *Rab23* seems to be required for PCP protein polarization, we wondered whether core protein(s) might be directly regulated by, or associated with Rab23. Because YFP::Rab23 displays a strong, uniform membrane association when expressed in pupal wing cells, and the core PCP proteins are also enriched in membrane complexes, our initial colocalization studies in pupal wings were not informative with regard to the identification of potential Rab23 partners. To get around this problem, we used cultured S2 cells in which Rab23 and the core PCP proteins are normally not expressed, or only expressed at a moderate level (BHANOT *et al.* 1996; and our unpublished results). We compared the subcellular distribution of the core proteins in cultured S2 cells transfected with the appropriately tagged version of the Fz, Dsh, Pk, Dgo and Vang proteins in the presence and in the absence of Rab23. Additionally, we examined if PCP protein localization was altered by Rab23 in Fz/Dsh and Vang/Pk co-transfected cells. Together, these studies led to the conclusion that the presence of Rab23 does not modify the subcellular distribution of the core proteins, including the membrane localization of Fz and Fz/Dsh. However, we noticed that while Fz, Dsh and Dgo do not colocalize with Rab23 (Figure 9, A-B”, and not shown) (for Fz 0 % of the cells exhibited colocalization, n=29), Vang and Pk partially colocalize with Rab23 (Figure 9, C-D”) (partial
colocalization for Pk was evident in 96% of the cells, n=54). Because the Vang and Pk proteins did not show cell membrane localization when expressed alone or together (not shown), it was not possible to test if Rab23 dependent vesicle trafficking affects the subcellular distribution of Vang and Pk. Nonetheless, these results indicate that Rab23 might affect core protein localization by regulating Vang and/or Pk distribution. Consistent with this possibility, the activated form of Rab23 (Rab23Q96A) displayed a strong colocalization with Pk (Figure 9, E-E”), whereas the T69A mutant version exhibited a very low level of colocalization (in 1 out of 26 cells) (Figure 9, F-F”).

To support the relevance of the observations made in S2 cells, and to verify whether Rab23 associates with Vang and Pk in vivo, coimmunoprecipitation experiments were carried out. Western-blot analysis of S2 cells transfected with HA-tagged Rab23 demonstrated that HA-Rab23 is specifically recognized by our anti-Rab23 serum, but not by the pre-immune serum (Figure 9G). The same was true for the purified His-tagged protein (not shown), therefore the anti-Rab23 serum appeared suitable for biochemical experiments. Indeed, anti-Rab23 co-immunoprecipitated Pk from wild type but not from Rab23sl mutant pupal protein extracts (28-30 hours APF) (Figure 9H). In parallel, we were unable to detect Vang or Stan in the Rab23 complex (Figure 9H), despite the fact that Vang is known to bind Pk (BASTOCK et al. 2003; JENNY et al. 2003). Thus, our data suggest that Rab23 interacts with Pk that could explain the localization problems of Pk and, indirectly, the other core PCP proteins. However, because the PCP complexes might be sensitive to biochemical manipulations or might undergo very dynamic changes, we can not exclude that Rab23 directly regulates the distribution of Vang or some of the other PCP proteins as well.
**Rab23 cooperates with the core PCP and In group of genes to regulate wing hair number**

If the sole function of *Rab23* were to modulate the cortical polarization of *Pk*, and possibly some other core PCP proteins, it would be expected that *Rab23* mutants exhibit similar phenotypic defects as *pk* mutants or mutations of the core group. Indeed, even if not as strongly as typical for the core PCP mutants, *Rab23* impairs wing hair orientation. However, beyond that effect, the strong multiple hair phenotype of *Rab23* is different from the defects exhibited by the core PCP mutants. Thus, *Rab23* appears to have two distinct activities during the establishment of tissue polarity in the wing. The first is a role in late PCP protein polarization, while the second is to restrict actin accumulation and prehair initiation to a single site. Consistent with a specific role in the restriction of prehair formation, we found that *Rab23* dominantly enhances the weak multiple hair phenotype of the core mutations (without affecting the hair orientation defects) (Figure 10, C, D, H, I and P), while the *Rab23* homozygous mutant phenotype is sensitive to the gene dose of the In group and *mwh* (Figure 10, L and Q, and Figure S3) known to play a role in the regulation of wing hair number. In contrast, *Rho1* and *Drok* mutations, affecting two cytoskeletal regulators of trichome placement, do not exhibit dominant genetic interaction with *Rab23* (Figure 10Q). Similar to *in*, *Rab23* enhances the multiple hair phenotype induced by late hs-Fz overexpression (Figure S4) (Krashnow and Adler 1994; Lee and Adler 2002), whereas *Rho1* and *Drok* suppressed it (Strutt et al. 1997; Winter et al. 2001). Hence, the dominant interaction studies suggest that *Rab23*
cooperates with the core PCP and In group of genes, but not the Rho pathway, during the regulation of wing hair number.

To further probe the relationship between *Rab23* and the PCP genes, we examined double mutant combinations of *Rab23* and mutations of the core group (*fz*<sup>21</sup>, *Vang*<sup>6</sup>, *pk<sup>pk-sple13</sup>*, *pk<sup>pk30</sup>*), the In group (*frtz*<sup>1</sup>, *in*<sup>1</sup>) and *mwh*<sup>1</sup>. The double mutants with *fz*, *Vang* and *pk<sup>pk-sple</sup>* displayed the type of hair orientation defects found in the corresponding single core PCP mutants (Figure 10, E and G, and not shown). However, they exhibited a synergistic interaction with regard to their multiple hair phenotype, which is stronger than the sum of the individual mutants (Figure 10, B, C, E-G, R), *pk<sup>pk-sple</sup>* having the strongest effect (Figure 10R). In the *pk<sup>pk30</sup>*; *Rab23* combination we noted a partial suppression of the very strong and stereotyped hair orientation defects typically exhibited by the *pk<sup>pk30</sup>* single mutant (Figure 10, H and J). Additionally, with respect to wing hair number, we observed an even stronger synergistic effect than with *fz* and *Vang*, since the *pk<sup>pk30</sup>*; *Rab23* double mutant exhibited a very high number of multiple hairs (Figure 10, J and R). Overall, the phenotype was almost identical to that of *frtz*<sup>1</sup> or *in*<sup>1</sup> (Figure 10K, and Figure S2). The *frtz*<sup>1</sup>; *Rab23<sup>51</sup>* and *in*<sup>1</sup>, *Rab23<sup>T69A</sup>* combinations displayed essentially identical PCP defects as the *frtz*<sup>1</sup> or *in*<sup>1</sup> single mutants (Figure 10M, and Figure S3), except that the average trichome number per cell was somewhat higher in the double mutants than in the corresponding single mutant (Figure S5). Finally, the *mwh*, *Rab23* double mutants displayed an identical PCP phenotype to that of *mwh* single mutants (Figure 10, N and O).

With respect to the determination of prehair initiation site, the double mutant analysis revealed that *in*, *frtz* and *mwh* are epistatic to *Rab23*, and therefore they are
likely to act downstream of, or later than that of \textit{Rab23}. However, the core PCP proteins appear to cooperate with Rab23 in a more complex manner. In the double mutant assay they exhibit a synergistic interaction in regard of wing hair number, suggesting that they act in parallel and/or redundant signaling pathways. Yet, the core PCP mutations not only exhibit a dominant genetic interaction with that of \textit{Rab23}, but Rab23 seems to bind Pk and appears to play a role in cortical polarization of the PCP proteins, which makes it unlikely that they only act through completely independent pathways.

\textit{Drosophila Rab23 is not required for Hedgehog signaling}

It was previously shown that the mouse \textit{Rab23} orthologue is an essential negative regulator of \textit{Sonic hedgehog} (\textit{Shh}) signaling during neural patterning of the mouse embryo (EGGENSCHWILER \textit{et al.} 2001). Contrasting to that, we found that \textit{Drosophila Rab23} is not expressed in the embryonic CNS (data not shown), and it follows that CNS defects were not detected in \textit{Rab23} mutant embryos, therefore \textit{Rab23} is unlikely to play a role in neural development in flies. Nevertheless, \textit{Drosophila hedgehog} (\textit{hh}) is required for the proper development of many different tissues from the embryonic stages to the adulthood (INGHAM and McMHAON 2001). Thus, to determine further if \textit{Drosophila Rab23} plays a role in Hh signaling, we analyzed \textit{Rab23} homozygous mutant embryos and adult tissues. We found no evidence for a \textit{Rab23} requirement in Hh signaling, as for example, the embryonic cuticle pattern or anterior-posterior patterning of the wing remained normal in \textit{Rab23} mutants (data not shown). Moreover, if \textit{Rab23} were a negative regulator of Hh signal transduction in flies, the loss of \textit{Rab23} should activate the Hh pathway (such as the loss of \textit{patched}) and would be expected to lead to phenotypic
effects similar to the overexpression of Hh. However, in the case of the abdominal cuticle, Hh overexpression induces reversed hair polarity (Lawrence et al. 1999; Struhl et al. 1997), that is clearly distinct from the effect of Rab23, which induces randomized polarity and multiple hairs (Figure 3). Together, these findings indicate that although the Rab23 protein appears to be highly conserved throughout evolution (Guo et al. 2006), its role in Hh signaling is likely to be restricted only to vertebrates.

**DISCUSSION**

Here we have shown that *Drosophila* Rab23 is required for the planar organization of the adult cuticular hairs covering the epidermis of the wing, leg and abdomen. Rab23 appears to regulate two main aspects of trichome development that are hair orientation and hair number. In pupal wing cells, the absence of Rab23 leads to increased actin accumulation in the sub-apical region and the formation of multiple hairs. In addition, Rab23 mutations impair hexagonal packing of the wing cells, and to a lesser degree, affect cortical polarization of the PCP proteins. Although, Rab23 does not appear to exhibit a polarized distribution in wing cells, we found that it associates with Pk that normally accumulates in the proximal cortical domain.

Careful comparison of the Rab23 mutant phenotype with that of the other PCP mutations reveals that the phenotypic effect of Rab23 differs from all of the known PCP genes. Most notably, Rab23 has a specific requirement in the development of one particular type of sub-cellular structure (i.e. the cuticular hair) in every body regions we examined. However, it does not appear to play any role in the planar orientation of multi-
cellular units such as ommatidia in the eye or the sensory bristles of the adult epidermis. In contrast to this, other PCP genes typically exhibit a tissue specific, but not structure specific, requirements, or, such as mutations of the core group, affect the polarization of every tissue and structure, regardless whether they are hairs, bristles or unit eyes. Focusing on the wing, loss of \textit{Rab23} results in weak trichome orientation defects and a relatively strong multiple hair phenotype (mostly double hairs). This is clearly different from the core PCP phenotypes (strong hair orientation defects and few multiple hairs), or the phenotypes of the In group and \textit{mwh} (strong orientation defects and multiple hairs in almost every cells). As compared to \textit{Rho1} and \textit{Drok}, \textit{Rab23} displays a similar adult wing hair phenotype in mutant clones in respect of multiple hairs, while the orientation defects are less clear in \textit{Rho1} and \textit{Drok} mutants (STRUTT \textit{et al.} 1997; WINTER \textit{et al.} 2001) than in \textit{Rab23}. Moreover, a significant difference exists at the molecular level, because, unlike \textit{Rab23}, \textit{Rho1} and \textit{Drok} do not play a role in cortical polarization of the core PCP proteins (C.P. and J. M. unpublished results). Given that our \textit{Rab23} alleles genetically behave as strong LOF or null alleles, \textit{Rab23} identifies a unique class of PCP genes dedicated to the regulation of trichome planar polarization.

Although some recent data suggested that the establishment of properly polarized cortical domains is not an absolute requirement for correct trichome polarity in the wing (STRUTT and STRUTT 2007), asymmetric accumulation of the PCP proteins is thought to serve as critical cue for cell polarization. Thus, the \textit{Rab23} induced weak alterations in wing hair polarity are best explained by the similarly modest effect on PCP protein asymmetries. Because \textit{Rab23} is able to associate with \textit{Pk}, it follows that \textit{Rab23} is likely to play a role in the proximal accumulation of \textit{Pk}. Given that the Rab family of proteins is
known to control membrane trafficking, our results provide further support for models suggesting that polarized membrane transport is an important mechanism for the asymmetric accumulation of the PCP proteins (SHIMADA et al. 2006). Although Rab23 showed a specific interaction with Pk, technical limitations might have prevented the detection of interactions with other core PCP proteins, and hence it is possible that the mechanism whereby Rab23 contributes to cortical polarization is not limited to Pk regulation. One additional candidate is the transmembrane protein Vang that partly colocalizes with Rab23 in S2 cells (this work) and has been shown to bind Pk (BASTOCK et al. 2003; JENNY et al. 2003). Thus, through binding to Pk, Rab23 might affect Vang localization or signaling capacity. Irrespective of whether Rab23 directly affects the localization of only one or more PCP proteins, in the wing Rab23 has a relatively modest effect on protein localization, and, as a consequence, on hair orientation, indicating that Rab23 has a minor or largely redundant role in this tissue. Interestingly, however, Rab23 induces much stronger trichome orientation defects on the abdominal cuticle. Although it is not proven formally, genetic analysis suggests that asymmetric PCP protein accumulation (or at least polarized activation) is likely to occur in the abdominal histoblast cells as well. Hence, with respect to protein polarization Rab23 may act in a tissue specific manner playing a largely dispensable role in the wing, but having a critical role in the abdominal epidermis.

Correct trichome placement at a single distally located site is clearly a crucial step in planar polarization of the wing cells. Current models suggest that prehair initiation is controlled by an inhibitory cue localized proximally in a Vang-dependent manner, and by a Fz-dependent cue that positively regulates hair formation at the distal vertex (STRUTT
and WARRINGTON 2008). Whereas it is not clear how the distal cues work, with regard to the proximal cues it is known that Vang and Pk colocalize with the effector proteins In, Fy and Frtz that control the localization and activity of Mwh, which is thought to regulate prehair initiation directly by interfering with actin bundling in the sub-apical region of cells (STRUTT and WARRINGTON 2008; YAN et al. 2008). We found that Rab23 severely impairs trichome placement in the wing leading to the formation of multiple hairs, which indicates a role in the repression of ectopic hair initiation. Where does Rab23 fit into the regulatory hierarchy of trichome placement? Our double mutant analysis suggests that Rab23 is upstream of the In group and mwh, and acts at the same level as the core PCP genes. The synergistic genetic interaction between Rab23 and the core PCP mutations indicates that they function in parallel pathways during the restriction of prehair initiation. Remarkably, the pk; Rab23 double mutants exhibit an almost identical phenotype to mutations of the In group, suggesting that, unless we assume the existence of an In independent restriction system, Pk and Rab23 together are both necessary and sufficient to fully activate the In complex. In pk single mutants the proximal accumulation of In is severely impaired, yet multiple hairs rarely develop, indicating that proper In localization plays only a minor role in the restriction mechanism. Conversely, in Rab23 single mutants In localization is weakly affected, but multiple hairs often form, suggesting that the major function of Rab23 is related to In activation. Thus, it appears that the proximally restricted activation of In on the one hand is ensured by Pk, that mainly plays a role in proper In localization, and on the other hand by Rab23, that seems to be required for In activation. At present, the molecular function of the In system is unknown, and it is therefore also unclear how Rab23 might contribute to the activation of
the In complex. Nevertheless, because Rab23 has a weaker multiple hair phenotype than
in, but the pkpk; Rab23 double mutant is nearly as strong as in, it is conceivable that In
activation is not exclusively Rab23 dependent but, beyond a role in protein localization,
Pk has a partial requirement as well.

The regulation of cellular packing is an interesting, yet only lately appreciated
aspect of wing development. It has been reported by Classen et al. (2005) that the wing
epithelium is irregularly packed throughout larval and prepupal stages, but shortly before
hair formation it becomes a quasihexagonal array of cells. Hexagonal repacking depends
on the activity of the core PCP proteins (CLASSEN et al. 2005). However, defects in
packing geometry do not appear to directly perturb hair polarity in core PCP mutant wing
cells. The possible exception to this rule is pk that exhibits very strong hair orientation
defects and induces the strongest packing defects within the core PCP group (CLASSEN et
al. 2005; LIN and GUBB 2009). Additionally, another study revealed that irregularities in
cell geometry are associated with polarity defects in the case of fat mutant clones (MA et
al. 2008). Thus, cell geometry is not the direct determinant of cell polarity, but in some
instances cell packing seems to impact on PCP signaling and hair orientation. Here we
have shown that in the wing Rab23 is predominantly involved in the regulation of wing
hair number, and it is also required for hexagonal packing of the wing epithelium. Do
these packing defects correlate with the severity of the multiple hair phenotype? Our data
argue against this idea for the case of Rab23, and also for the cases of other strong
multiple hair mutants, such as in, frtz and mwh. Therefore, cell shape has no direct effect
on the regulation of the number of prehair initiation sites, and Rab23 appears to regulate
hexagonal packing and hair number independently.
As Rab23 and Pk are both required for cellular packing, and Rab23 associates with Pk, it is possible that they cooperate during the regulation of packing. This is in agreement with the observation that \( pk^{pk}; \) Rab23 double mutant wings do not show stronger packing defects than a \( pk^{pk} \) single mutant (not shown). However, other interpretations are also possible, hence further investigations will be required to understand how Rab23 and Pk regulates cellular packing, and to clarify the impact of packing geometry on PCP establishment in the wing.

Unlike the vertebrate orthologues, *Drosophila* Rab23 is not an essential gene and does not appear to regulate Hedgehog signaling. Given that Rab GTPases are thought to regulate vesicular transport and that mouse Rab23 localizes to endosomes (Evans et al. 2003), it was expected that Rab23 regulates the trafficking of vesicle associated Hedgehog signaling components. However, in the mammalian systems no clear link between endocytosis, Rab23 and the subcellular localization of Hedgehog signaling elements has been identified (Eggenswiler et al. 2006; Evans et al. 2003; Wang et al. 2006). Our finding that Rab23 associates with Pk suggests that Rab23 might be directly involved in the regulation of Pk trafficking, and therefore Pk could be the first known direct target of Rab23. Interestingly, there is a significant overlap reported in the embryonic expression domains of the vertebrate Pk and Rab23 genes in the region of the dorsal neural ectoderm, the somites and the limb buds (Cooper et al. 2008; Eggenswiler et al. 2001; Li et al. 2007; Takeuchi et al. 2003; Veeman et al. 2003; Wallingford et al. 2002). Moreover, it is also known that blocking of Rab23 or Pk function in vertebrate embryos can both lead to a *spina bifida* phenotype (Eggenswiler et al. 2001; Li et al. 2007; Takeuchi et al. 2003; Wallingford et al. 2002).
2002). These observations raise the possibility that, unlike the Rab23 involvement in Hedgehog signaling, the Rab23-Pk regulatory connection is evolutionarily conserved.

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FIGURE LEGENDS

Figure 1. Conservation of the Rab GTPases and the structure of the Drosophila Rab23 gene. (A) Multiple alignment of small GTPase family members; the highly conserved GTPase domain is indicated with a black line above the alignment. The Rab23T69A mutation affects a threonine residue that is invariant in the small GTPase superfamily. (B) The Rab23 genomic region is indicated on top with the position of the P(RS5)5-Sz-3123 insertion used to generate the Rab2351 allele. The structure of the
RH23273 EST clone, corresponding to a full length Rab23 cDNA, is shown below (black boxes indicate coding region, white ones untranslated regions). Further below we indicate the genomic structure of the Rab2351 allele, note the 1.7 kb foreign DNA from the P-element insertion line. The possible transcript of the Rab2351 allele is shown at the bottom of the figure. Translation from this transcript cannot result in a functional Rab23 protein because of premature termination.

Figure 2. Rab23 mutant wings display PCP defects. (A) A schematized wing indicating the main wing blade regions (A-E) and the two cross-veins (acv: anterior cross vein, pcv: posterior cross vein). Distal is on the right and anterior is on top on all panels. Wild type wing cells exhibit a single, distally pointing hair (B, E), whereas Rab23T69A (C, D, F) and Rab2351 (G) mutant wings exhibit multiple hairs and orientation defects in every wing regions. Photomicrographs were taken from the A region (B, C), the C region (D) and the D region (E-L) of the wing (dashed areas in panel A). Wings of Rab23T69A/Df(3R)BSC47 (H), Rab2351/Df(3R)BSC47 (I), and wings in which Rab23 is silenced by RNAi (J), also display multiple hairs and hair orientation defects. (K-L) The wing PCP phenotypes induced by Rab23 can be fully rescued by a single copy of the Rab23 genomic rescue construct (Rab23-GR) (K), and by Act-Gal4 driven expression of UAS-YFP::Rab23 (L).

Figure 3. Rab23 affects hair polarity on the leg and the abdominal cuticle. (A-B) Photomicrographs of the femur (male first leg), the third sternite (female) (C-D) and the fourth tergite (female) (E-F). Proximal is on left, distal is on right on A-B, anterior is on
top, posterior is on bottom on C-F. (A) A wild type leg exhibiting distally pointing hairs and bristles. (B) A Rab23 mutant leg is covered by distally pointing bristles, however, trichome orientation defects, and occasionally, the formation of multiple hairs are evident. (C) Hairs and bristles of a wild type sternite and (E) tergite point posteriorly. In Rab23 mutants sternite (D) and tergite (F) hairs display largely randomized orientations and the appearance of multiple hairs is also evident, whereas, bristle orientation is not significantly altered.

Figure 4. Rab23 mutations impair prehair initiation (A-A”) Prehair initiation in wild type pupal wings at 31 hours APF. Note that each wing cell develops an actin-rich prehair at its distal vertex. (B-B”) Apical actin level is increased in Rab2351 mutant pupal wing cells, many of which fail to restrict prehair initiation to a single site. (C-C”) Z axis projections of the wing shown in panel B, projection is shown along the white line indicated on B-B”. Note that Stan and actin are accumulated near the apical cell surface. (D-D”) Prehair initiation is delayed in a Rab23T69A mutant clone (derived from the proximal half of wing sector C, and marked by the absence of β-gal staining, in blue) as prehairs are shorter in the mutant tissue than in the surrounding wild type tissue. Note that some cells initiate multiple hairs that form around the cell periphery. Moreover, the P-D accumulation of Stan is partly impaired within the mutant tissue. (E-E”) Multiple hairs are not seen outside of Rab23T69A mutant clones indicating that Rab23 acts cell-autonomously. In all panels, cell borders are labeled with Stan staining (green in A,A’,B,B’,C,C’,D,D’,E and E’), actin is labeled in red in A,B,C,D,E and E’, and in white in A”,B”,C”,D” and E”. Scale bar: 10 µm.
Figure 5. *Rab23* impairs hexagonal packing of the pupal wing cells. (A) DE-cadherin staining in wild type and (B) *Rab23* \(^{51}\) mutant pupal wings at 30h APF. In wild type wings by this stage of development most cells acquire a hexagonal shape, whereas *Rab23* wings display an increased ratio of non hexagonal cells (indicated with white dots). (C) Quantification of the ratio of the non-hexagonally shaped cells in wild type, *Rab23* mutant and other PCP mutant wings. We examined 10-25 wings for each genotype and counted at least 100 cells per wing. To test for significance we applied the \(t\)-test as a statistical method, \(P\) indicates probability. (D) Quantification of the ratio of cells that exhibit multiple hairs in hexagonal (white columns) and in non hexagonal (gray columns) cells in *Rab23* \(^{51}\), \(in^1\) and *frtz* \(^{1}\) mutants. Scale bars: 5 \(\mu\)m.

Figure 6. *Rab23* is required for cortical polarization of the PCP proteins. (A-D') *Rab23* \(^{T69A}\) mutant clones at 30 hours APF, marked by the absence of \(\beta\)-gal staining (blue). The proximal-distal polarization of Pk (in green) (A, A'), Vang (in green) (B, B'), Stan (in green) (C, C') and In (in green) (D, D') is impaired in many of the mutant cells. Photomicrographs were taken from the C region (B, C) and the D region (A, D) of the wing. A', B', C' and D': single images of the green channel of A, B, C and D. Scale bar: 10 \(\mu\)m.

Figure 7. Pk is mislocalized in hexagonally packed *Rab23* and *dsh* mutant wing cells. (A-B) Pk staining on wild type (A) and *Rab23* \(^{51}\) mutant wings (B). Pk localization was examined in those hexagonally shaped cells whose all six neighbors were hexagonal as
well, white dots represent cells with impaired Pk localization. Note that Rab23$^{5/}$ mutant wings exhibit a higher ratio of cells with localization defects than wild type. Mislocalization is assigned to those cells where Pk localization is not restricted to the P-D cell boundaries but appears at the anterior-posterior cell boundaries as well. Proximal is on left, anterior is up on A and B. (C) Quantification of hexagonal cells with impaired Pk localization in wild type, Rab23$^{5/}$ mutant and dsh$^{1}$ mutant wings. 6-15 wings were analyzed for each genotype. To test for significance we applied the t-test as a statistical method, P indicates probability. Scale bars 5 $\mu$m.

**Figure 8. The subcellular distribution of YFP::Rab23 in pupal wing cells.** (A-A”) In the junctional zone the YFP::Rab23 protein (in green) is enriched in the plasma membrane of pupal wing cells and displays a diffuse staining in the cytoplasm. In the basolateral zone (B-B”) YFP::Rab23 is also enriched in the plasma membrane, but staining in the cytoplasm is much weaker than in junctional zone. Pupal wings depicted on A-B” were stained 39 hours APF at 22°C (corresponding to 31 hours APF at 25°C), actin is labeled in red in these panels. A and B are two different optical planes of the same image, the distance between the two sections is 7 $\mu$m. (C-D””) The subcellular localization of YFP::Rab23 (green) expressed in flip-out clones. DE-cadherin (red) labels the junctional zone. Note the lack of any obvious proximal-distal polarization (C-C”), and the strong co-localization with DE-cadherin in the junctional zone (D-D”). D-D” display a Z section of the wing along the white line indicated on C-C”. Proximal is on left, distal is on right on each panels. Scale bars: 10 $\mu$m in A-B and 20 $\mu$m in C.
**Figure 9. Rab23 associates with Pk.** (A-F”’) S2 cells cotransfected with Rab23 and Fz (A-A”), Rab23 and Dsh (B-B”), Rab23 and Vang (C-C”), Rab23 and Pk (D-D”), Rab23Q96A and Pk (E-E”) and Rab23T69A and Pk (F-F”). Rab23 is labeled in green, core PCP proteins are labeled in red. Fz and Dsh do not show a significant colocalization with Rab23 (A”, B”), whereas Vang and Pk display a partial overlap with that of Rab23 (C”, D”). Rab23Q96A, the activated form of Rab23, exhibits a strong colocalization with Pk (E-E”), while the Rab23T69A mutation reduces colocalization with Pk (F-F”). (G) Western-blot analysis of non-transfected S2 cells, and S2 cells transfected with HA-tagged Rab23. Rab23-HA is specifically recognized by anti-Rab23 and anti-HA, but not by the pre-immune serum. The predicted molecular weight of wild type Rab23 is 30kDa, whereas HA-Rab23 is about 40kDa. (H) Immunoprecipitations from lysates of wild type and Rab2351 homozygous mutant 30 hours pupae using anti-Rab23 and probed with anti-Rab23 (upper left), anti-Pk (upper right), anti-Vang (lower left) and anti-Stan (lower right). Rab23 co-immunoprecipitates Pk but not Vang and Stan from wild type pupae, whereas Rab23 and Pk could not be precipitated from Rab2351 mutants. Scale bar: 5 µm.

**Figure 10. Genetic interaction studies and double mutant analysis with Rab23 and other PCP mutations.** (A) Wild type wing with single distally pointing hairs. (B) Rab2351 mutant wing exhibiting multiple hairs and modest hair orientation defects. (C) fz21, (F) Vang6 and (H) pk6k30 wings displaying very few if any multiple hairs, and strong hair orientation defects. Rab2351 significantly increases the number of multiple hairs in the fz21, Rab2351/fz21 (D), and pk6k30; Rab2351/+ (I) mutant combinations. (L) frtz1/+; Rab2351 mutant wings display a higher number of multiple hairs than a Rab2351
homozygous mutant wing (compare L to B), indicating that \textit{frtz} is a dominant enhancer of \textit{Rab23}. The double mutant combinations \textit{fz}, \textit{Rab23} \textit{E}, \textit{Vang} \textit{G}, \textit{Rab23} \textit{M} and \textit{pk}; \textit{Rab23} \textit{J} display very high number of multiple hairs and strong hair orientation defects. The orientation defects in \textit{pk}; \textit{Rab23} seems to be weaker than in \textit{pk} \textit{J} (compare J to H). \textit{frtz} \textit{K} mutant wings display a very high number of multiple hairs and strong orientation defects, such as the \textit{frtz}; \textit{Rab23} \textit{M} double mutants. Note that the \textit{pk}; \textit{Rab23} double mutant exhibits an identical PCP phenotype as \textit{frtz} (compare J to K). The \textit{mwh} single mutant wing hair phenotype (N) is essentially identical to the one of a \textit{mwh}, \textit{Rab23} \textit{O} double mutant. Photomicrographs were taken from the D region of the wing and were positioned the same way as the ones in Fig. 2. (P) Quantification of wing hair numbers in core PCP and \textit{Rab23} mutant combinations. \textit{Rab23} dominantly enhances the number of multiple wing hairs exhibited by core PCP mutations. (Q) Quantification of wing hair numbers in mutant combinations that were homozygous for \textit{Rab23} and heterozygous for an In group mutation or \textit{mwh} or a mutation of the Rho pathway. \textit{frtz}, \textit{fy} and \textit{mwh} dominantly enhance the number of multiple wing hairs exhibited by \textit{Rab23} whereas \textit{Drok} and \textit{RhoA} had no significant effect. The \textit{in} allele also had no effect in this wing region but in other wing regions it significantly enhanced the number of multiple hairs exhibited by \textit{Rab23}/\textit{Rab23} \textit{T69A} (see also Fig. S3). (R) Quantification of wing hair numbers in double homozygous mutant combinations of \textit{Rab23} and core PCP mutations. The numbers indicate a synergistic genetic interaction. During the quantitative analysis hairs were counted in the proximal half of wing sector A, we examined 10-22 wings for each genotype. To test for significance we applied the \textit{t}-test as a statistical method, P indicates probability.


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<tr>
<th>Genotype</th>
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**Western-blot**

- **M**: 40kDa, 30kDa
- **S2**: 55kDa, 30kDa, 66kDa
- **HA-Rab23**: 92kDa, 55kDa
- **α-HA**: 300kDa

**Immunoprecipitation**

- **M**: lysate, IP
- **lysate beads**: α-Vang, α-Stan