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 ortholog result in abnormal trichome orientation and the formation of multiple hairs on the wing, leg, and abdomen. We show that Rab23 is required for hexagonal packing of the wing cells. We found that Rab23 is able to associate with the proximally accumulated Prickle protein, although Rab23 itself does not seem to display a polarized subcellular distribution in wing cells, and it appears to play a relatively subtle role in cortical polarization of the polarity proteins. The absence of Rab23 leads to increased actin accumulation in the subapical 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.

Mutations in PCP genes result in abnormal wing hair polarity patterns and wing hair number (Gubb and Garcia-Bellido 1982; Wong and Adler 1993). On the basis of 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 (stax) (also known as flamingo), Van Gogh (vang) (also known as strabismus), prickle (pk), and diego (dgo); the second group consists of interuned (in), fuzzy (fy), and friz (frtz) (referred to as planar polarity effectors or In group); whereas the third group includes multiple wing hairs (mwh) (Wong and Adler 1993). 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 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 (Strutt et al. 1997; Winter et al. 2001; Adler 2002). Genetic analysis of these two groups has led to models in which the Fat/Dachsous group acts upstream of the core proteins (Yang et al. 2002; Ma et al. 2003), while Rho1 and Drok act downstream of Fz (Strutt et al. 1997; Winter et al. 2001). Although the existence of a single,

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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 (Usui et al. 1999; Axelrod 2001; Shimada et al. 2001; Strutt 2001; Tree et al. 2002; Bastock et al. 2003; Das et al. 2004), 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 asymmetric 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 hr after prepupa formation (APF), when they become differentially enriched until prehair formation begins at 30–32 hr APF. This transient asymmetric localization ends by 36 hr APF (Adler 2002; Strutt 2003; Mihály et al. 2005). 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 hr 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 hr 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 hr APF). Polarity is then largely lost at the beginning of the pupal period, but becomes evident again in several hours until hair formation begins (Clasen 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 modest 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-Flip stock (kindly provided by J. Knoblich, IMP, Vienna) (Emery et al. 2005). The Rab23\textsuperscript{Q96A} allele has been isolated during an F2 FRT/Flip (Xu and Rubin 1993) mosaic mutagenesis screen. FRT82B chromosomes were mutagenized with ENU (1.6 mM), mutant clones were induced by Ubx-Flip, and PCP phenotypes were analyzed on the wing and notum. The Rab23\textsuperscript{Q96A} excision allele was generated by remobilization of the P(R55)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, UAS-Flip-Rab23 lines were generous gifts from M. Scott (Stanford University). Flip out clones of UAS-Flip-Rab23 were generated using w, hsFLP, ActP-FRT5+FRT-Gal4, arm-lacZ with a 2-hr heat shock at 37°C during the early third instar larval stage. For overexpression studies we used Act5C-Gal4 or Sad-Gal4. In line with FlyBase, we use starry night instead of flaminings, and Van Gogh instead of strabismus.

Cuticle preparation: Abdominal cuticles and first thoracic legs were prepared according to a protocol by 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-kb XhoI restriction fragment from BACR03P13 (BACPAC Resources) was cloned into a pcDNA3.1 vector. To clone the Rab23\textsuperscript{Q96A} transcript, total RNA was isolated from homozygous mutant adults with Trizol (Invitrogen), cDNA was synthesized with Revert Aid (Fermentas), and RT–PCR was carried out with primers specific to the first and third exon of Rab23, respectively. Primers were used as follows: Rab23Ex3 5’-CCCGGCACCAA CATCATTAG-3’ and Rab23Ex1 5’-TCGATGCTCGGA CGAG-3’. Molecular cloning of the RT–PCR product revealed that Rab23\textsuperscript{Q96A} encodes a hybrid transcript consisting of the first (noncoding) exon of Rab23, part of the first intron of Rab23, followed by 443 bp from the 5′ end of the P(R55)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 52 N-terminal amino acids, including 13 of the highly conserved and functionally essential part of the GTPase domain (Santos and Nebreda 1989). Thus, these results suggest that Rab23\textsuperscript{Q96A} encodes a functionally strongly, if not entirely, impaired protein.

For transfection experiments we used the following constructs: pAWV-Rab23, pHWH-Rab23, pPWW-Rab23, BLMRT-FRP-Rab23, pAWV-Rab23\textsuperscript{Q96A}, pPWW-Rab23\textsuperscript{Q96A}, pPHW-Rab23\textsuperscript{Q96A}, BLMRT-Rab23\textsuperscript{Q96A}-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, pAct5C-Gal4 was cotransfected to drive expression from UAS promoters. Expression from the BLMRT vectors was induced by 1 mM CuSO\textsubscript{4} for 4 hr or 500 μM CuSO\textsubscript{4} overnight.

Drosophila S2 cells were transfected with Effectene (Qiagen) for 4 hr or 500 μM CuSO\textsubscript{4} overnight. For transfection experiments we used the following constructs: pAWV-Rab23, pHWH-Rab23, pPWW-Rab23, BLMRT-FRP-Rab23, pAWV-Rab23\textsuperscript{Q96A}, pPWW-Rab23\textsuperscript{Q96A}, BLMRT-Rab23\textsuperscript{Q96A}-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, pAct5C-Gal4 was cotransfected to drive expression from UAS promoters. Expression from the BLMRT vectors was induced by 1 mM CuSO\textsubscript{4} for 4 hr or 500 μM CuSO\textsubscript{4} overnight.

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 prepupae 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 (Feiguin 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-FA 1:400 (Roche), rabbit anti-HA (Sigma), mouse anti-Fe 1:10 (DSHB), and mouse anti-Stat 1:100 (DSHB). Actin was stained with Rhodamine-Phalloidin 1:100 (Molecular Probes). Bright-field images were collected using Zeiss Axiocam MOT2. Confocal images were collected with an Olympus FV1000 LSM microscope. Images were edited with Adobe Photoshop 7.0 CE and Olympus Fluoview.

**Immunoprecipitation and Western blot analysis:** For immunoprecipitation experiments 100 wild-type or Rab23T69A homozygous mutant pupae (28–30 hr 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 hr 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 hr at RT to deplete nonspecifically binding proteins. For immunoprecipitation 60 μl of CL-4B Sepharose beads were incubated with 30 μl anti-Rab23 antibody for 2 hr 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 at 4°C. SDS-PAGE and Western blot analysis were carried out according to standard protocols.

**RESULTS**

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 ortholog. 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 Witteman 2001). This allele, designated Rab23T69A, 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 fz or dsh, they are exhibited in every 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 data not shown), clearly indicating a requirement in the regulation of hair orientation. Because no other Rab23 alleles were available, we generated independent Rab23 alleles by remobilizing the P(RS5)5Sc-3123 Pelement insertion sitting in the first intron of Rab23 (Figure 1B). One of these alleles, Rab23mut1, is homozygous viable and displays wing hair phenotypes identical to that of Rab23T69A (Figure 2G). Rab23T69A is viable over Rab23mut1 and similarly, both mutations are viable over deficiency chromosomes uncovering Rab23. Each of these mutant combinations display a strong multiple hair phenotype and weak hair orientation defects (Figure 2, H and I; supporting information, Figure S3). As the severity of the wing phenotypes is identical in the Rab23 transheterozygous, homozygous, and hemizygous mutants, by genetic criteria, both 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 three residue (equivalent to that of T69 in Rab23) in other small GTPases leads to a strong loss of function (Spooerner et al. 2001). Moreover, we revealed that Rab23mut1 encodes a hybrid transcript that cannot be translated into a functional protein (see details in MATERIALS AND METHODS) suggesting that Rab23mut1 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 data 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 on the wing. First, we examined prehair initiation in *Rab23*51 homozygous mutant pupal wings and *Rab23*T69A mutant clones at 31 hr 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*T69A 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 ~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 ( Claussen et al. 2005). It has also been shown that the core PCP mutations partly interfere with normal cellular packing ( Claussen 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 ~30–32 hr 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 nonhexagonally shaped cells is ~11%, whereas in Rab23<sup>F<sup>51</sup></sup> homozygous mutant wings this ratio is increased to 27% (Figure 5C). As a comparison, we measured the packing defects in two core PCP mutants, Vang<sup>6</sup> and dsh<sup>1</sup>, where the ratio of the nonhexagonal 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 whether 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 nonhexagonally 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 data 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 weakly impairs late cortical polarization of the PCP proteins:** Given that the absence of asymmetrically localized core PCP proteins alters hexagonal packing of the wing cells ( Claussen 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 mutant wings (Figure S1). However, by 24–30 hr APF, although the core PCP proteins still accumulate into apicolateral complexes, they fail to polarize properly in a Rab23 mutant tissue (Figure 6, A–C’). Most notably, the PCP proteins are often not as completely removed from the anterior–posterior (A–P) cell boundaries as in wild type (Figure 6B). Also sometimes even if intracellular PCP polarity is obvious, the angle of polarity is not well correlated from cell to cell (Figure 6A). Interestingly, we found that in Rab23 mutant clones that exhibited a delay in actin accumulation, mislocalization was always evident (n = 26) in most parts of the mutant tissue (Figure 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 have 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 hr 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 whether the abnormal-looking PCP protein localization is not simply the consequence of irregular cell packing. 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 a 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 dsh1 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 much 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 MATERIALS AND METHODS), we expressed a YFP::Rab23 fusion protein to assess localization in wing cells. Note that YFP::Rab23

![Image](https://example.com/image.png)
fully rescues the Rab23 mutant phenotypes (Figure 2L), indicating that it is a functional protein. Between 24 and 32 hr 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 subapical cytoplasmic domain with occasional accumulations in punctate structures (Figure 8, A–A’). A very similar localization pattern was found before 24 hr APF (data not shown), and at 36 hr 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 proximodistally polarized distribution from the onset of pupal development until 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 (data 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 Rab23T69A (data 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

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**Figure 4.—** Rab23 mutations impair prehair initiation. (A–A’): Prehair initiation in wild-type pupal wings at 31 hr 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 Rab23373084 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–E’, and in white in A’, B’, C’, D’, and E’. Bar, 10 μm.
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 whether PCP protein localization was altered by Rab23 in Fz/Dsh and Vang/Pk cotransfected 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 (data not shown), it was not possible to test whether 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, co-immunoprecipitation 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 preimmune serum (Figure 9G). The same was true for the purified His-tagged protein (data not shown), therefore the anti-Rab23 serum appeared suitable for biochemical experiments. Indeed, anti-Rab23 co-immunoprecipitated Pk from wild-type but not from Rab23° mutant pupal protein extracts (28–30 hr 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 cannot 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 other 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) (Krasnow 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 (fz21, Vang6, phk^{Np6-13}), and the In group (frtz1 and inn1) and mwh1. The double mutants with fz, Vang, and phk^{Np6-13} displayed the type of hair orientation defects found in the corresponding single core PCP mutants (Figure 10, E and G, and data 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, and R), phk^{Np6-13} having the strongest effect (Figure 10R). In the phk^{Np6-13}; Rab23 combination we noted a partial suppression of...
the very strong and stereotyped hair orientation defects typically exhibited by the \(pk^{lo}\) 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 \(pk^{lo}\), \(Rab23\) double mutant exhibited a very high number of multiple hairs (Figure 10, J and K). Overall, the phenotype was almost identical to that of \(fzt\) or \(in\) (Figure 10K and Figure S3). The \(fzt\); \(Rab23^{+/+}\) and \(in\), \(Rab23^{+/+}\) combinations displayed essentially identical PCP defects as the \(fzt\) or \(in\) 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 \(in\), \(fzt\), and \(mwh\) are epistatic to \(Rab23\), and therefore they are likely to act downstream of, or later than that of \(Rab23\). 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 \(Rab23\), 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.

**Drosophila Rab23 is not required for Hedgehog signaling:** It was previously shown that the mouse \(Rab23\) ortholog is an essential negative regulator of \(Sonic hedgehog\) (\(Shh\)) signaling during neural patterning of the mouse embryo (Eggenschwiler et al. 2001). Contrasting to that, we found that Drosophila \(Rab23\) is not expressed in the embryonic CNS (data not shown), and it follows that CNS defects were not detected in \(Rab23\) mutant embryos, therefore \(Rab23\) is unlikely to play a role in neural development in flies. Nevertheless, Drosophila \(hedgehog\) (\(hh\)) is required for the proper development of many different tissues from the embryonic stages to adulthood (Ingham and McMahon 2001). Thus, to determine further whether Drosophila \(Rab23\) plays a role in \(Hh\) signaling, we analyzed \(Rab23\) homozygous mutant embryos and adult tissues. We found no evidence for a \(Rab23\) requirement in \(Hh\) signaling, as for example, the embryonic cuticle pattern or anterior–posterior patterning of the wing remained normal in \(Rab23\) mutants (data not shown). Moreover, if \(Rab23\) were a negative regulator of \(Hh\) signal transduction in flies, the loss of \(Rab23\) should activate the \(Hh\) pathway (such as the loss of \(patched\)) 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 (Struhl et al. 1997; Lawrence et al. 1999), 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 subapical 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 subcellular structure (i.e., the cuticular hair) in every body region we examined. However, it does not appear to play any role in the planar orientation of multicellular 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, requirement, or, such as mutations of the core group, affect the polarization of every tissue and structure, regardless of whether they are hairs, bristles, or unit eyes. Focusing on the wing, loss of Rab23 results

**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′ and B′), whereas Vang and Pk display a partial overlap with that of Rab23 (C′ and 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 nontransfected 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 preimmune serum. The predicted molecular weight of wild-type Rab23 is 30 kDa, whereas HA-Rab23 is ~40 kDa. (H) Immunoprecipitations from lysates of wild-type and Rab23 homozygous mutant 30 hr 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 Rab23 mutants. Bar, 5 μm.
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 mwh (strong orientation defects and multiple hairs in almost every cell). As compared to Rho1 and Drok, Rab23 displays a similar adult wing hair phenotype in mutant clones with
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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 subapical 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\(^{\text{se}}\); 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 pk\(^{\text{se}}\); 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.

Respect to multiple hairs, while the orientation defects are less clear in Rho1 and Drok mutants (Strutt et al. 1997; Winter et al. 2001) than in Rab23. Moreover, a significant difference exists at the molecular level, because, unlike Rab23, Rho1 and Drok do not play a role in cortical polarization of the core PCP proteins (C. Pataki and J. Mihály, unpublished results). Given that our Rab23 alleles genetically behave as strong LOF or null alleles, 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 a critical cue for cell polarization. Thus, the Rab23-induced weak alterations in wing hair polarity are best explained by the similarly modest effect on PCP protein asymmetries. Because Rab23 is able to associate with Pk, it follows that Rab23 is likely to play a role in the proximal accumulation of 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.
defects within the core PCP group (Clasen 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 moh. 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<sup>e</sup>; Rab23 double mutant wings do not show stronger packing defects than a pk<sup>e</sup> single mutant (data 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 orthologs, 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 (Evans et al. 2003; Eggenschwiler et al. 2006; Wang et al. 2004). 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 (Eggenschwiler et al. 2001; Wallingford et al. 2002; Takeuchi et al. 2003; Veeman et al. 2003; Li et al. 2007; Cooper et al. 2008). Moreover, it is also known that blocking of Rab23 or Pk function in vertebrate embryos can both lead to a spina bifida phenotype (Eggenschwiler et al. 2001; Wallingford et al. 2002; Takeuchi et al. 2003; Li et al. 2007). 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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LITERATURE CITED


Rab23 Regulates Hair Number and Polarization


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Drosophila Rab23 Is Involved in the Regulation of the Number and Planar Polarization of the Adult Cuticular Hairs

Csilla Pataki, Tamás Matusek, Éva Kurucz, István Andó, Andreas Jenny and József Mihály

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10.1534/genetics.109.112060
Figure S1.—Rab23 is not required for early cortical polarization of the PCP proteins. (A–B’). Early cortical polarization of Stan at 6 hours APF is not affected by Rab23. Wild type (A) and Rab23<sup>51</sup> (B) pupal wing cells display a similar level of polarization of Stan at 6 hours APF, schematized in panels A’ and B’, respectively (black lines are drawn between cells with coherent Stan polarity). Scale bar: 10 μm.
Figure S2.—The subcellular distribution of DN and CA YFP::Rab23 in pupal wing cells. (A) In the junctional zone the wild type YFP::Rab23 protein (in green) is enriched in the plasma membrane of pupal wing cells and displays a weak diffused staining in the cytoplasm as well. (B) The dominant negative (DN) form of YFP::Rab23 (in green) is partly membrane associated, but displays a stronger accumulation in the cytoplasm than the wild type protein. (C) The constitutively active (CA) form of YFP::Rab23 (in green) is enriched in the plasma membrane, whereas the cytoplasmic staining is somewhat weaker than that of the wild type protein. A-C displays apical sections, while Z axis reconstructions (made along the white lines indicated on A-C) are shown below these panels. Scale bar: 10 μm.
FIGURE S3.—Genetic interaction studies and double mutant analysis with Rab23 and in. (A) A wild type wing exhibiting distally pointing trichomes. (B) A $\text{Rab}23^{7004}$ and a $\text{Rab}23^{7004}/\text{Rab}23^{51}$ mutant wing exhibiting multiple hairs and moderately strong hair orientation defects. (D) $\text{in}^1$, (E) $\text{in}^1$, $\text{Rab}23^{7004}$ and (F) $\text{in}^1$, $\text{Rab}23^{7004}/\text{Rab}23^{51}$ mutant wings. $\text{in}^1$ dominantly enhances the number of multiple hairs exhibited by $\text{Rab}23^{7004}/\text{Rab}23^{51}$ (compare C to F), whereas the PCP defects in regard of wing hair number and orientation are identical in $\text{in}^1$ and $\text{in}^1$, $\text{Rab}23^{7004}$ (compare D to E). Photomicrographs were taken from the D region of the wing and were positioned the same way as the ones in Fig. 2. Quantification of wing hair numbers is presented in Fig. 10, P-R.
**FIGURE S4.**—*Rab23* increases the number multiple hairs induced by hs-Fz expression. (A) Schematized wing, multiple wing hairs were counted in the proximal half of the A region on the dorsal side of the wing (a square with dashed lines indicates the area). Quantification of wing hair numbers in wings that express hs-Fz is shown on the right. Note that both *Rab23* alleles increase the number of multiple hairs produced. We examined 12-17 wings for each genotype. To test for significance we applied the *t*-test as a statistical method, *P* indicates probability.
Figure S5.—Quantification of the average number of trichomes per cell in Rab23, in and frtz mutant combinations. (A) Schematized wing, trichomes were counted in the C region (area bordered by dashed lines) immediately distal to the anterior cross vein (acv) until the line of the posterior cross vein (pcv). (B) Rab23\textsuperscript{51}, (C) frtz\textsuperscript{1} and (D) frtz\textsuperscript{1}; Rab23\textsuperscript{51} mutant wings. Wing cells with more than two hairs are indicated with red circles, note that the number of such cells is higher in the double homozygous mutant combination than in the single mutants. (E) Quantification of the average number of trichomes per cell in Rab23 and In group mutant combinations. In double mutant wings the average number of trichomes per cell is higher that in the appropriate single mutants.

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