Summary of the Ph.D. thesis

The isolation of new tissue polarity genes in *Drosophila* and the genetic analysis of Rab23 involved in the regulation of the number and planar organization of the adult cuticular hairs

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Introduction and aims

Epithelial cells commonly become polarized within the plane of the cell layer during development, a property referred to as planar cell polarity (PCP) or tissue polarity. This phenomenon is evident in many vertebrate tissues (such as the ordered arrangement of scales on fish or feathers of birds, hairs of mammalian skin, the cilia of the respiratory tract or oviduct or stereocilia of the sensory epithelium in the organ of Corti). Although recent reports demonstrated that PCP regulation is highly conserved throughout the animal kingdom, such polarity patterns are perhaps best studied in *Drosophila*. PCP in flies is most evident in the wing, which is covered by uniformly polarized, distally pointing hairs, in the epidermis where sensory bristles and trichomes point to the posterior, and in the eye where PCP is manifest in a mirror symmetric arrangement of ommatidia.

Genetic and molecular studies of *Drosophila* led to identification of a set of genes, called PCP genes that are directing the establishment of PCP. Mutations in a number of genes result in abnormal wing hair polarity patterns without major effects on other aspects of tissue morphogenesis. Based on their cellular phenotypes, initial studies placed PCP genes into four groups. Mutations in the first group including *ft (fat), ds (dachsous)* and *fj (four-jointed)* (referred to as the Ft/Ds group), display wing hairs with reversed polarity derived from prehairs that form at the cell edges (Adler et al., 1998; Strutt and Strutt, 2002). The second group (core group) includes *fz (frizzled), dsh (disheveled), fmi (flamingo), stbm (strabismus), pk (prickle)* and *dgo (diego),* mutants of which impair hair orientation to a various degree, some cells display double hairs, and prehairs typically form in the center of the cells (Wong and Adler, 1993). The third group consists of *in (inturned), fy (fuzzy), frtz (fritz)* (In group) and causes the formation of multiple (mostly two) hairs per cell that are roughly equal in size, slightly misoriented, and derive from prehairs from around the cell cortex in abnormal positions. Finally the fourth group includes *mwh (multiple wing hairs)* that is characterized by the presence of four or more hairs equal in size in each cells initiated from periphery. Double mutant analysis demonstrated that these phenotypic groups also represent epistatic groups from which *mwh* is epistatic to all the other groups, the In group is epistatic to Ft/Ds group and core group and the core group is epistatic to Ft/Ds group. These data together suggest that PCP genes act in a regulatory hierarchy where the Ft/Ds group is on the top followed downstream by core, In and *mwh* groups. Although the existence of such a linear hierarchy is debated (Casal et al., 2006; Lawrence et al., 2007) it is clear that PCP genes regulate (1) wing hair orientation, (2) the place of hair formation, and (3) the number of trichomes formed.

Whereas, the molecular mechanism that restricts prehair formation to the distal vertex of the wing cells is still elusive, it has been demonstrated that the core proteins adopt an asymmetrical subcellular localization when prehair form. This localization appears to be critical for proper trichome placement (Mihály et al., 2005; Seifert and Mlodzik, 2007). The core PCP proteins are first recruited to the apical cell surface and subsequently segregate into complementary apical sub domains before the onset of hair formation. Fmi localizes to proximal and distal surfaces (Ursui et al., 1999; Shimada et al., 2001), Fz, Dsh and Dgo localizes specifically to the distal surface (Axelrod, 2001; Strutt, 2001; Das et al., 2004) while Pk and Stbm localizes on the proximal surface (Tree et al., 2002b; Bastock et al., 2003). Subsequent work revealed that, in addition to the core proteins, In protein also displays an asymmetrical pattern with accumulation to the proximal zone (Adler et al., 2004). Significantly, the absence of any of the core PCP proteins prevents the asymmetric localization of the other PCP proteins and that of In. However, in agreement with the epistatic data, asymmetric core PCP protein localization does not depend on *in* function (Adler et al., 2004). Very recent findings demonstrate that Fy and Frtz also show a proximal enrichment, and, together with In, activate Mwh that is enriched on the proximal side of the wing cell as well (Yan et al., 2008; Strutt and Warrington, 2008).

It has been shown that the core PCP proteins are found in symmetric complexes until 24 hours APF, when they relocalize and become asymmetrically enriched until prehair formation begins at 30-32 hours APF, but then, by 35 hours APF, they again fail to display an asymmetrical pattern.

How exactly these elements act together to polarize tissues properly, is still an unsolved problem. A model or working hypothesis that is consistent with most of the experimental data is as follows. First a directional clue that is provided by the Fj, Ds and Ft proteins determines the axis of polarization. This somehow initiates the asymmetric relocalization of the core PCP proteins that is enlarged by feedback mechanisms between the core elements themselves. This will lead to high Fz signaling activity on the distal side of the cell, which will subsequently activate (an as yet unidentified) effector proteins in the distal vertex where they initiate prehair formation. Additionally, it is believed that the In complex prevents hair formation on the proximal side of the cell. Although this model explains many observations, the mechanistic details on protein localization are largely missing or poorly understood, the link between Fz signaling and asymmetric localization is not understood and it remains a mystery how upstream elements are coupled to the asymmetric enrichment of PCP proteins.

Furthermore the tissue specific downstream components or their way of action is still an open problem.

The aim of this work was to answer a part of these questions by isolating new PCP genes, and by studying their role in PCP signaling and development.

Materials and methods

- *Drosophila* genetics
    - mutagenesis screen (EMS, ENU)
    - P-element excision
    - determination of lethal phase
    - phenotypic analysis of wings, notum, eye, abdomen and legs
    - generation of transgenic lines
- DNA techniques:
    - PCR
    - RT-PCR
    - cloning
- Biochemistry:
    - Rab23 antibody production
    - Western- blot
    - immunoprecipitation
- Microscopy:
    - Nomarski images: Zeiss Axiocam MOT2
    - confocal images: Olympus FV1000 LSM
- Immunohistochemistry:
    - dissections
    - immunostainings
- Statistical analysis

Results and discussion

In order to isolate new PCP genes, we employed the FRT/Flp mosaic system to induce homozygous mutant clones on the wing and notum by Ubx-Flp. After chemical mutagenesis of the 2L, 2R and 3R chromosomal arms, we have isolated 33 new PCP mutants, 3 on 2L, 3 on 2R and 27 on 3R. Complementation analysis and genetic mapping of our mutants revealed

that one of the new complementation groups (consisting of five members) identifies *Kuzbanian-like* (*Kul*). This gene behaves like a core polarity gene because it affects PCP on the notum, wing and eye. The new alleles carry amino acid changes in the metalloprotease domain of the Kul protein, four of them in the C481, and one of them in the V603 position. Deeper analysis of these mutants is subject of future investigations.

Genetic mapping of another new PCP mutant from 3R, has led to the conclusion that this allele carries a point mutation in the Switch 1 region of the GTPase domain of the *Rab23* gene. The mutation affects an aminoacid residue (T69, corresponding to T35 of Ras) that is conserved in the whole small GTPase superfamily. This point mutant (named $Rab23^{T69A}$) is semilethal and displays a strong multiple wing hair phenotype, and mild hair orientation defects.

Because no other *Rab23* alleles were available, we generated independent alleles by P-element remobilization. One of these alleles, $Rab23^{51}$ is homozygous viable and displays a strong multiple wing hair phenotype. $Rab23^{T69A}$ and $Rab23^{51}$ maintain their phenotype over deficiency chromosomes uncovering the gene, behaving as strong LOF or functionally null mutants. We were able to fully rescue the phenotype of these mutants by providing one copy of the wild type *Rab23* gene. Silencing of *Rab23* by *RNAi* using ubiquitously expressed drivers resulted in a moderately strong multiple hair phenotype but neither viability nor the morphogenesis of tissues other then the wing were affected. The *Rab23* mutants exhibit trichome orientation defects and multiple hairs on the abdomen and legs as well, without affecting bristle polarity. Together these data suggest that *Rab23* is a peculiar PCP gene that has a role in determination of trichome orientation and number without affecting the polarity pattern multicellular structures, such as bristles or unit eyes.

It has been shown that the mouse *Rab23* orthologue is an essential negative regulator of the Shh (Sonic-hedgehog) pathway during neuronal patterning of the embryo (Eggenschwiler et al., 2001). Therefore we analyzed *Rab23* homozygous mutant embryos and adult tissues, but we failed to find any evidence for a *Rab23* involvement in *Drosophila* Hedgehog signaling. Rab23 is not expressed in the *Drosophila* embryonic CNS and no CNS defects were detected in *Rab23* mutant embryos either, therefore most likely Rab23 has no role in neuronal development in flies indicating that the role of *Rab23* gene in Shh signaling is restricted to the vertebrates. In agreement with this, the embryonic cuticle hair pattern of *Rab23* mutants shows no sign of Rab23's involvement in Hh pathway in *Drosophila.*

To investigate *Rab23* function at the cellular level, we examined prehair initiation in $Rab23^{51}$ homozygous mutant pupal wing cells, and in $Rab23^{T69A}$ mutant clones. We found that

in the absence of *Rab23* apical actin accumulation is not restricted to the distal vertex, instead, the actin network was diffused and many cells developed multiple hairs in abnormal positions at the cell periphery. Additionally, prehair initiation is somewhat delayed in the *Rab23* mutant tissue. Since the multiple hairs were always restricted to the mutant area, *Rab23* acts cell autonomously.

Recent studies revealed 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). We noticed that some of the *Rab23* mutant wing cells fail to adopt a hexagonal shape at around 30-32 hours APF. We quantified this effect and found that in the $Rab23^{T69A}$ point mutant the ratio of the non-hexagonally shaped cells is significantly higher compared to wild type wing cells. As a comparison we measured the packing defects in two core PCP mutants, $stbm^{6}$ and dsh^{1} too. Because the strength of the *Rab23* induced packing defects is comparable to the effect of the core mutations, this 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 hair orientation defects and /or the formation of multiple hairs also exhibited upon loss of *Rab23*. However, we found that Rab23 regulates cellular packing, and hair orientation and number, independently. To extend this analysis, we investigated the packing defects of other mutations causing multiple hairs, like *in*, *frtz* and *mwh*. These mutants exhibit somewhat weaker packing defects than *Rab23*, but in the same way, the formation of multiple hairs does not appear to correlate with irregular cell shape in these cases either. These results demonstrate that cell packing has no direct effect on the number of prehair initiation sites in the wing epithelial cells. In regard of hair orientation our data support the same conclusion, that is similar to the findings of Classen et al., 2005 for the case of the core PCP mutations, but opposite to the case of fat (ft) mutant clones (Ma et al., 2008).

It has been recently shown that asymmetric accumulation of the core PCP proteins depends on hexagonal packing, and because in our mutants packing was altered, we were curious if Rab23 has any effect on core PCP protein localization in different stages of development. To address this issue, first we examined the localization pattern of Fmi in early, 6 hours APF, $Rab23^{51}$ mutant wings, but we failed to detect any localization defects in this stage of development. Next, we examined the localization pattern of the PCP proteins in later stages (24-32h APF) in $Rab23^{51}$ homozygous wings and $Rab23^{T69A}$ clones. We found that at this stage the core proteins and In fail to polarize properly in a *Rab23* mutant tissue, although

they still accumulate into apico-lateral complexes. From these experiments we concluded that *Rab23* plays a role only during the late phase of PCP protein localization.

To understand the mechanism whereby Rab23 contributes to core PCP protein localization, we examined the subcellular distribution of YFP::Rab23 in developing pupal wings. We found that at 24-32 hours APF the Rab23 protein is predominantly enriched in the cell membrane in the apico-lateral zone. This pattern was detected before and after this developmental stage, and it seems that Rab23 has no polarized distribution during pupal development. These observations were strengthened with results from examining flies expressing the YFP::Rab23 in flip-out clones or the constitutively active form of Rab23.

Since Rab23 is localized mainly to the plasma membrane in pupal wings and core PCP protein asymmetric localization is also linked to the membrane, it was not informative to check the colocalization between Rab23 and core elements in this system. However, we were curious to know which core protein(s) might be directly regulated by Rab23, therefore we compared the subcellular distribution of different core proteins (Fz, Dsh, Dgo, Stbm, Pk) with Rab23 in S2 cells. We observed that Pk and Stbm show a partial colocalization with Rab23. Because Pk or Stbm did not show membrane localization when expressed alone or together in S2 cells, it was not possible to test directly if Rab23 affects Pk or Stbm endocytosis. Nonetheless these results indicated that Rab23 might affect core protein distribution by regulating Pk or Stbm. This hypothesis was strengthened by the observation that in cells transfected with Rab23T69A (corresponding to the point mutant) the degree of colocalization between Rab23T69A and Pk was much lower. Unfortunately, a similar experiment was not possible in the wing because of the low quality of the available Rab23 antibody but we were able to show that Rab23 and Pk can be coimmunoprecipitated from pupal wing protein extracts, indicating that they may act in the same protein complex.

If the sole function of Rab23 would be to promote the cortical polarization of Pk, and may be some other core proteins, it would be expected that *Rab23* mutants have similar phenotype as *pk* or mutations of the core group. However, the strong multiple hair phenotype of *Rab23* is different from the orientation defects shown by core group mutants. Thus, it seems that *Rab23* has two distinct, albeit not necessarily independent, roles during the establishment of tissue polarity in wing. The first role is in PCP protein polarization, the second is to restrict actin accumulation to a single site. Consistent with the second role, we found that *Rab23* dominantly enhances the weak multiple hair phenotype of the core mutants without affecting their hair orientation defects, while the *Rab23* homozygous mutant

phenotype is sensitive to the gene dose of the In group that has role in the regulation of wing hair number.

Further, we were wondering what is the position of *Rab23* in the hierarchy of known PCP genes, therefore we initiated double mutant analysis of *Rab23* with fz^{21}, dsh^{1}, pk^{pk30}, $stbm^{6}$, in^{1}, $frtz^{1}$ and mwh^{1}. Our results have shown that in respect of the regulation of hair orientation, *Rab23* has minor or no function there, which is entirely consistent with the single mutant phenotype of *Rab23*. In respect of the determination of the prehair initiation site, the In group and *mwh* clearly appears to function downstream of or later than that of *Rab23*. Conversely, the core PCP proteins and Rab23 function in parallel pathways during the restriction of trichome placement.

Taken the Rab23 multiple wing hair phenotype and the effect on PCP protein localization together with the dominant genetic interactions and double mutant analysis, these results suggest that Rab23 links cortical PCP polarization to a mechanism that is more directly involved in the repression of hair initiation at ectopic sites. In the simplest model, Rab23 would contribute to the proximal accumulation of Pk, and would also play a role in linking In activation to the proximal cell domain.

List of publications

1. Mihály, J., Matusek, T., **Pataki, C**. (2005). Diego and friends play again. *FEBS Lett* 272, 3241-3252. **IF= 3,3**

2. Rus, F., Kurucz, É., Márkus, R., Sinenko, S. A., Laurinyecz, B., **Pataki, C**., Gausz, J., Hegedüs, Z., Udvardy, A., Hultmark, D., Andó, I. (2006). Expression pattern of Filamin-240 in Drosophila blood cells. *Gene Expr. Patterns* 6, 928-934. **IF= 2,1**

3. **Pataki, C.** (2006). Isolation of new planar polarity genes in Drosophila melanogaster. *Acta Biol Szegediensis* 50, 3-4.

4. Weber, U., **Pataki, C**., Mihály, J., Mlodzik, M. (2008). Combinatorial signaling by the Frizzled/PCP and Egfr pathways during planar cell polarity establishment in the Drosophila eye. *Dev Biol* 316, 110-123. **IF= 5,234**

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6. **Pataki, C., Matusek, T.,** Kurucz, É., Andó, I., Jenny, A., Mihály, J. Drosophila Rab23 is involved in the regulation of the number and planar polarization of the adult cuticular hairs. Published ahead of print on February 1. 2010 in Genetics **IF= 4**