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Drosophila: A Model System That Allows *in vivo* Manipulation and Study of Epithelial Cell Polarity

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1. Introduction

Epithelia are specialized tissues that emerged early in evolution to subdivide the body into distinct parts and to form barriers by lining both the outside (skin) and the inside cavities and lumen of bodies. Functions of epithelial cells include secretion, absorption, protection, transcellular transport, sensation detection and selective permeability. To achieve these different tasks, several types of epithelial tissues emerged during evolution, all of them having one feature in common that is indispensable for their function: the cells are attached to each other in order to form a layer that works as a barrier. Epithelial cells thus show a ordered morphology, they are polarized. Disruption of polarity is an important feature of epithelial cancers that accounts for more than 90% of fatal malignancies in adults, rendering the understanding of the fundamental processes needed for polarity an ongoing subject of high interest.

Epithelial cells are highly polarized and the cells are oriented so that their external, so-called apical, surfaces face the outside or a central lumen and the internal, or basolateral, surfaces are in contact with other cells and the basement membrane. These characteristics were discovered using histological and thus descriptive analyses. With the advent of molecular genetics, biochemistry and molecular cell biology our knowledge about polarization processes increased dramatically, allowing for a better understanding of the mechanisms used by epithelial cells to fulfill their specialized tasks and to act as barriers. Thus, the polarization is not only reflected in the morphology of the cells, but also in the positioning of their organelles and in the apico-basal (AB) localization of polarity protein complexes at the plasma membrane. The plasma membrane can be divided into an apical, a junctional and a basolateral domain, each domain comprising its own set of polarity proteins that are widely conserved in eukaryotes. This AB polarity is required for formation of functional epithelial tissues. The asymmetrical deployment of proteins is mediated through subcellular trafficking and the polarized localization of transcripts. Genetic studies have revealed that the polarity protein complexes function in a sequential but interdependent manner to regulate the establishment and maintenance of cellular polarity.

The proteins involved in epithelial cell polarization are largely conserved between species, as mentioned above, even between vertebrates and invertebrates. Much of our hitherto

knowledge stems from studies performed in model organisms and until now it is impossible to culture whole epithelia for a long time. Existing *in vitro* cell culture systems give important insights into epithelial cell function, but mechanisms following biological input from the living and developing organism are obviously missed in these systems. Therefore, the fruit fly, *Drosophila,* provides an excellent model system for studying columnar epithelial cells (Fig. 1), allowing their static and especially dynamic analysis in the context of a whole living organism. Furthermore, state of the art microscopy imaging techniques can be easily coupled to the extensive genetic tools available in the *Drosophila* system, allowing for *in vivo* analysis and the concomitant dissection of pathways needed for epithelial cell polarity.

In this chapter we would like to introduce *Drosophila* as a model system to study epithelial cell function, establishment and maintenance. First, we will show the similarities and differences between invertebrate and vertebrate columnar epithelial cells. Second, we will depict the current knowledge of polarity protein complex regulation, highlighting the achievements derived from work in *Drosophila*. Finally, we will provide examples that nicely show how easy *in vivo* studies on epithelial cells can be performed using *Drosophila*.

This chapter will not only give a background on epithelial cell polarity regulation, but it will highlight the importance of whole organismal studies for the understanding of epithelial tissues. Furthermore it will reveal the value of a model system like the fruit fly when deciphering mechanisms underlying biological processes.
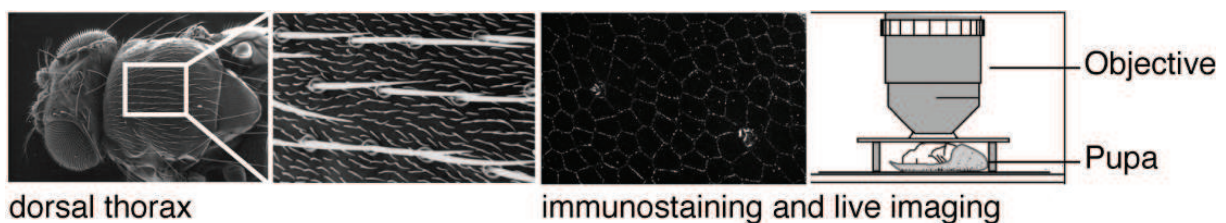

Fig. 1. Dorsal thorax of *Drosophila melanogaster*.

The dorsal thorax serves as a model system for epithelial maintenance and can easily be analyzed by immunostaining or non-invasive live imaging, using for example the dorsal thorax of the immobile, developing pupa.

2. Establishment of epithelial cell polarization in metazoans

Epithelial cells have an adhesive belt that encircles the cells apically, the zonula adherens (ZA). It assembles from the aggregation of spot adherens junctions. Basally, integrin-based focal contacts connect the epithelial cells to the basement membrane. Vertebrate epithelial cells develop a tight junction (TJ) apical to the ZA, which impedes intercellular diffusion and forms a region of close membrane contacts. In *Drosophila*, the functional equivalent to TJs is the septate junction (SJ) that lies basal to the ZA. A domain with similar protein composition as found in TJs is located apically of the ZA in the subapical region (SAR). Only a single junction, the *C. elegans* apical junction (CeAJ), has been identified in *C. elegans*, which resembles the ZA of *Drosophila* and vertebrates (Knust & Bossinger, 2002; Tepass et al., 2001) (Fig. 2). Thus, the overall structure of epithelial cells is highly conserved between species. The polarity protein complex located close to the ZA, the junctional Par complex, has been shown to play an essential role in the establishment of epithelial polarity.
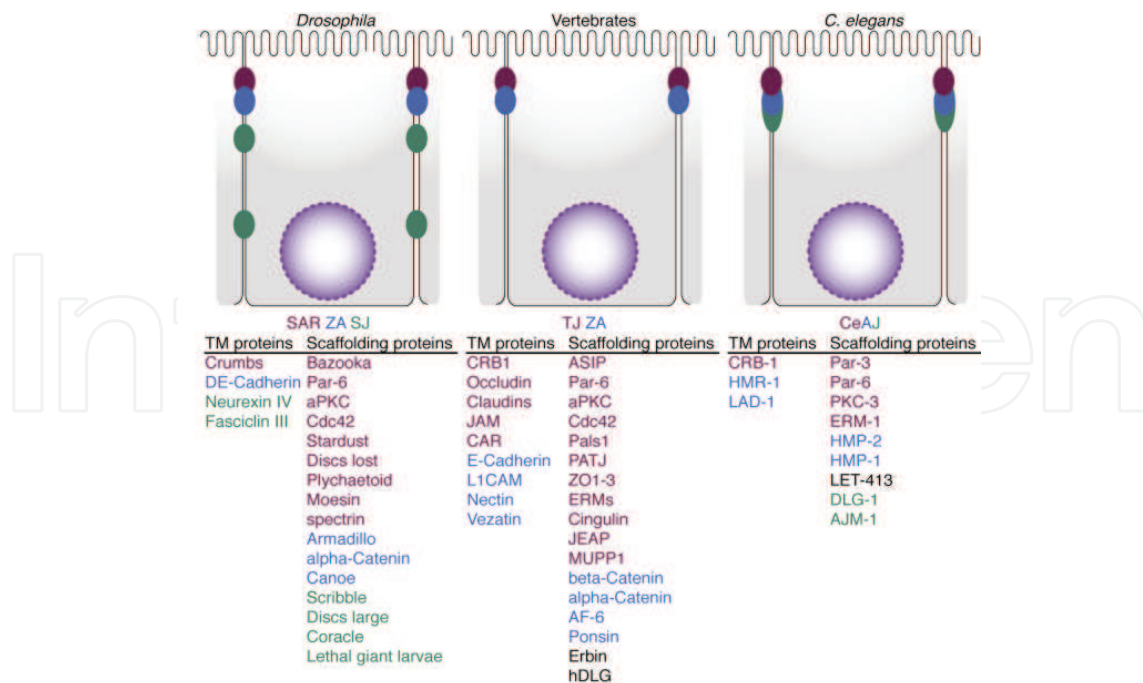
Drosophila		Vertebrates		C. elegans	
SAR ZA SJ		TJ ZA		CeAJ	
TM proteins	Scaffolding proteins	TM proteins	Scaffolding proteins	TM proteins	Scaffolding proteins
Crumbs	Bazooka	CRB1	ASIP	CRB-1	Par-3
DE-Cadherin	Par-6	Occludin	Par-6	HMR-1	Par-6
Neurexin IV	aPKC	Claudins	aPKC	LAD-1	PKC-3
Fasciclin III	Cdc42	JAM	Cdc42		ERM-1
	Stardust	CAR	Pals1		HMP-2
	Discs lost	E-Cadherin	PATJ		HMP-1
	Plychaetoid	L1CAM	ZO1-3		LET-413
	Moesin	Nectin	ERMs		DLG-1
	spectrin	Vezatin	Cingulin		AJM-1
	Armadillo		JEAP		
	alpha-Catenin		MUPP1		
	Canoe		beta-Catenin		
	Scribble		alpha-Catenin		
	Discs large		AF-6		
	Coracle		Ponsin		
	Lethal giant larvae		Erbin		
			hDLG		

Fig. 2. (adapted from Knust & Bossinger, 2002). Epithelial cell characteristics and typical polarity protein composition for *Drosophila*, vertebrate and *C. elegans* epithelial cells.

The transmembrane (TM) and scaffolding proteins are color-coded depending on their localization. SAR: subapical region; ZA: zonula adherens; TJ: tight junction; CeAJ: *C. elegans* apical junction.

2.1 The junctional Par complex

About 20 years ago, Kemphues and colleagues identified six Par (partitioning defective) proteins, Par-1 to Par-6, and an atypical protein kinase C (aPKC, known as PKC-3 in *C. elegans* and homolog to human PKCζ) in a screen for partition defective cell division in the one cell stage embryo in *C. elegans*, resulting all in loss of the anterior-posterior axis of the embryo when mutated (Cheng et al., 1995; Kemphues et al., 1988; Kirby et al., 1990). Five of the Par proteins - all but Par-2 - as well as aPKC are highly conserved throughout the animal kingdom and are needed for cell polarization. Par-1, Par-4 (also known as LKB1) and aPKC are kinases, Par-3 and Par-6 are PDZ (PSD95, DlgA, ZO-1)-domain containing proteins and Par-5 is a 14-3-3 protein. Par-3, Par-6 and aPKC form a complex localized at the anterior cell cortex of the one cell stage *C. elegans* embryo, while Par-1 and Par-2 remain at the posterior cortex and Par-4 and Par-5 localize uniformly at the cortex. The polarized localization of these proteins is triggered upon fertilization by sperm entry, which enriches the RacGAP CYK-4 (Cytokinesis defect-4) at the posterior pole to give a spatial cue for polarity. CYK-4 functions as a GTPase activating protein (GAP) for small GTPases like Rho, Cdc42 or Rac. Thus its localized activity leads to a gradient of acto-myosin via the inactivation of small GTPases, which distributes the Par proteins in a polarized manner (Jenkins et al., 2006) (Fig. 3). One of the essential functions of the Par complex comprised of Par-3, Par-6 and aPKC, is to set-up epithelial polarity in metazoans in response to the formation of initial cell-cell contact or discrete membrane domains, whereas the basolateral Par complex (Par-1) functions to promote the expansion of the lateral membrane. In general, Par proteins have been found to regulate cell polarization in many different contexts in diverse animals: in epithelia, in directed cell migration, in polarized

cells like neurons or in self-renewing cells; suggesting that they form part of an ancient and fundamental mechanism of cell polarization.
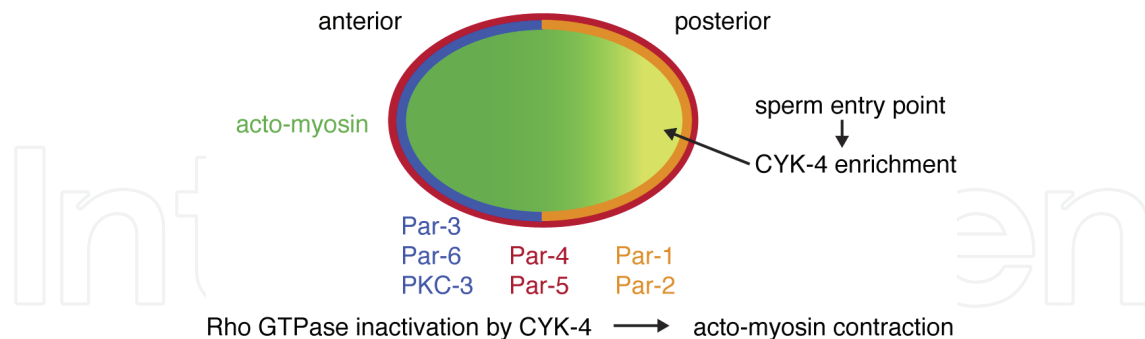

Fig. 3. Par protein distribution in the one cell stage *C. elegans* embryo.

Sperm entry marks the posterior pole and leads to an enrichment in CYK-4 protein, which functions as a GAP for Cdc42, Rho and Rac, thus leading to a gradient of acto-myosin and to the spatial restriction of the different Par complexes.

2.2 The apical Crumbs complex

The apical region of polarized epithelial cells harbors Crumbs (Crb), a transmembrane protein with 30 EGF-like repeats in the extracellular domain that binds with its intracellular domain to the MAGUK (membrane-associated guanylate kinase) protein Pals1 (protein associated with Lin7; also known as Stardust in *Drosophila*). Crb also recruits PATJ (Pals1-associated tight junction protein) into the most-apical complex (Tepass & Knust, 1993). Pals1 and PATJ are cytoplasmic scaffolding proteins with several protein-protein interaction domains including L27 domains, SH3 (Src homology 3) domains, guanylate kinase (GUK) domains and PDZ domain. Overexpression or siRNA-mediated down-regulation of any of the three components in mammalian epithelial cells leads to defects in AB polarity formation suggesting the requirement of the complex as a whole for the proper development of cell polarity (Roh et al., 2003; Shin et al., 2005; Straight et al., 2004).

2.3 Basolateral domain: Lethal giant larvae, Discs large and Scribble

The basolaterally localized proteins Lethal giant larvae (Lgl), Discs large (Dlg) and Scribble (Scrib) cooperatively regulate cell polarity, junction formation and cell growth in epithelial cells. All three genes were identified as tumor suppressors in *Drosophila*. Lgl has at least four WD40 repeats, forms homo-oligomers and associates with the cytoskeleton by binding to non-muscle myosin II. Dlg contains three PDZ domains, a SH3 domain and a GUK domain. Scrib is a LAP (leucine-rich repeats and PDZ domain) protein, containing 16 leucine-rich-repeats (LRRs) and 4 PDZ domains (Yamanaka & Ohno, 2008).

2.4 Adherens junctions proteins

The adherens junctions harbor the E-Cadherin/β-Catenin/α-Catenin (E-Cad/β-Cat/α-Cat) complex that is indispensable for establishing and maintaining cell-cell adhesion. Cadherins are a large family of transmembrane glycoproteins that form homophilic, calcium-dependent interactions with neighboring cells (Gumbiner et al., 1988). E-Cadherin is the predominant epithelial isoform of cadherin. Its extracellular domain is composed of five

ectodomain modules (EC1-EC5), with the most membrane-distal module (EC1) mediating binding with the E-Cad on the adjacent cell. Calcium ions bind between the EC domains to promote a rod-like conformation required for trans-interactions (Fig. 4). The cytoplasmic tail binds to β-Cat (known as Armadillo, Arm in *Drosophila*) in order to link E-Cad to α-Cat in metazoans (Pacquelet & Rørth, 2005). Arm is furthermore linked to the Par complex in *Drosophila* by binding to Bazooka (Baz), the *Drosophila* Par-3 homolog (Capaldo & Macara, 2007; Oda et al., 1994).

Stable epithelial adhesions require the F-actin network (Cavey et al., 2008) and the E-Cad/β-Cat/α-Cat complex is linked to actin via α-Cat, vinculin and α-actinin. It is a matter of debate whether the α-Cat-actin interaction indeed stabilizes the AJ complex since it has been shown in mammalian cells that α-Cat binds only in its homodimeric state to actin, whereas its binding to β-Cat is restricted to the monomeric form. Thus, the interaction of the E-Cad/β-Cat/α-Cat complex with the actin cytoskeleton is dynamic by way of association and dissociation of α-Cat with the complex and with actin filaments. Homodimeric α-Cat directly regulates actin-filament organization by suppressing Arp2/3 mediated actin polymerization, most likely by competing with the Arp2/3 complex for binding to actin filaments (Drees et al., 2005; Yamada et al., 2005). The observations made by Drees, Yamada and colleagues thus suggests a 'dynamic stabilization' of the AJ complex through actin-α-Cat interactions. In the light of a developing epithelial tissue, where a modulation of the cell-cell contacts needs to take place in order to allow for cell rearrangements and growth without compromising epithelial integrity and barrier properties (Classen et al., 2005), such a dynamic interaction between the stabilizing actin cytoskeleton and the AJ complex seems to be indispensable. Thus, this suggests the existence of adjustable mechanisms stabilizing the adherens junctions proteins at the junctions allowing for plasticity of the epithelial tissue; mechanisms that needed to be further elucidated *in vivo* to completely understand epithelial development and maintenance and associated E-Cad transport and turnover.
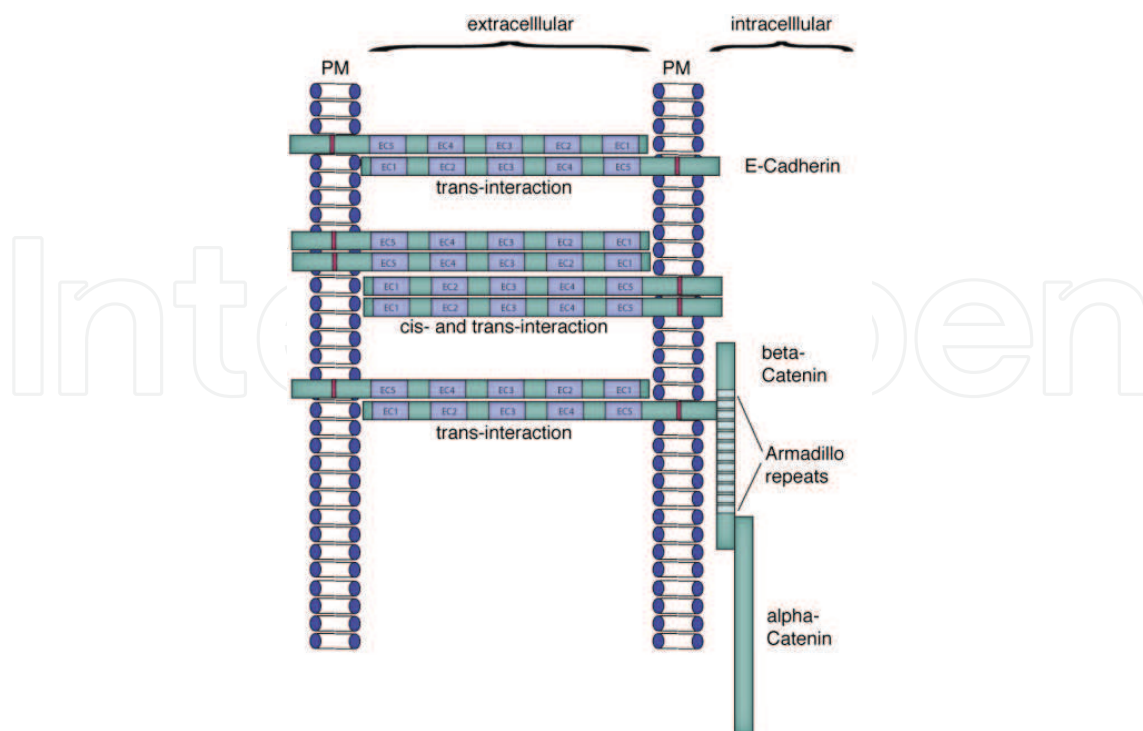

Fig. 4. E-Cadherin/β-Catenin/α-Catenin interaction.

β-Catenin binds to the cytoplasmic tail of E-Cadherin. α-Catenin binds to the N-terminal region of β-Catenin. E-Cadherin molecules can interact with each other in the extracellular space in cis and in trans, both interactions need Ca^{2+}. PM: plasma membrane.

3. Establishment of epithelial cell polarization in *Drosophila*

In *Drosophila*, the syncytial blastoderm gives rise to the first epithelium during cellularization. Membrane invaginations form, the so-called furrow canals, which already display discrete domains apical and lateral to the nuclei (Foe & Alberts, 1983; Mavrakis et al., 2009; Schejter & Wieschaus, 1993). Baz localizes below aPKC and Par-6 as the epithelium forms and its positioning is independent of aPKC and Par-6 but dependent on cytoskeletal cues given by the apical scaffold and dynein-mediated basal-to-apical transport as well as by cues that still need to be elucidated. Baz recruits Par-6 and aPKC, and subsequently Baz and Par-6 recruit Crb and PATJ respectively. aPKC in turn stabilizes apical Crb. Par-6 positioning is dependent on aPKC and on activated Cdc42 (Harris & Peifer, 2005). A transient basal adherens junction made up of ZA proteins E-Cad, Arm, α-Cat and PATJ forms and its assembly is coupled to correct Baz positioning (Hunter & Wieschaus, 2000). Once cellularization is complete, the lateral dispersed spot-like adherens junctions coalesce apically to form the belt-like ZA. In the SAR, Crb binds to Stardust (Sdt, Pals1 homolog of *Drosophila*) via its C-terminus and recruits PATJ into the complex. Crb also provides a link to the apical membrane cytoskeleton, which might reinforce the ZA, by binding to *Drosophila* Moesin (membrane-organizing extension spike protein) via its FERM (4.1, Ezrin, Radixin, Moesin) binding site and to spectrin (Médina et al., 2002). Spectrin is a tetrameric actin crosslinking protein. The spectrin cytoskeleton composition differs in the apical and basolateral domain of epithelial cells, thus giving spatial cues. The basolateral localization of the proteins Lgl, Dlg and Scrib depends on the presence of each of the three proteins and a failure of localization leads to the expansion of the apical Crb complex into more lateral domains. Consequently, the ZA does not form correctly, leading to multilayering of cells. Dlg and Scrib localize to the basolateral domain and the septate junctions just below the ZA, whereas Lgl co-localizes only partially with them below the septate junctions. Lgl is also found in the cytoplasm (Bilder & Perrimon, 2000; Bilder et al., 2000; Woods & Bryant, 1991).

Thus, epithelial polarity establishment has been extensively studied in *Drosophila*, which is due to the fact that the syncytial blastoderm-to-epithelium transition can be easily studied in wild-type and mutant conditions. Freshly laid eggs are collected from the flies and subsequently staged to observe cellularization (defects) at different timepoints.

4. Establishment of epithelial cell polarization in vertebrates

The TJs of vertebrate epithelial cells separate apical and basolateral membrane domains and harbor the transmembrane proteins occludin, claudin family members and junctional adhesion molecules (JAMs) (Fig. 2). All TJ proteins interact directly with cytoplasmic, PDZ-domain containing proteins like ZO-1, ZO-2, ZO-3 (Zonula occludens 1-3), Par-3 and Pals1. These cytoplasmic proteins recruit other cytoskeletal (F-actin) or signaling molecules (Mertens et al., 2005).

Many results explaining epithelial polarity establishment in vertebrates rely on a cell-based system: In MDCK (Madin-Darby canine kidney) cells, ZO-1 binds to the C-terminus of claudins and JAM via two of its PDZ domains and Par-3 directly associates with the C-terminus of JAM. Subsequently, and similar to what has been shown in *Drosophila*, Par-6, aPKC and Cdc42 are recruited to Par-3 and form a complex that is needed for the development of normal tight junctions (Afonso & Henrique, 2006; Chen & Macara, 2005; Joberty et al., 2000). Claudins play a central role in polarity establishment, since they also interact with the Crb complex: PATJ, whose expression depends on Pals1, interacts with claudin and ZO-3 via two of its PDZ domains and with Pals1 via its N-terminus. Pals1 interacts with its PDZ domain with the cytoplasmic tail of Crb, which reinforces the association of Crb with the Par complex (Lemmers et al., 2003; Straight et al., 2004).

5. Regulation of cell polarity in the epithelium

5.1 Regulation of the junctional Par complex

As described above, Par-3/Baz recruits Par-6 and aPKC in vertebrates and *Drosophila* and this has been confirmed by ectopic expression of Par-3, which leads to ectopic Par-6 and aPKC recruitment in mammalian cells (Joberty et al., 2000), indicating a general mechanism for this recruitment. Par-6 also plays an important role in Par complex localization and activation since it interacts with Cdc42 via its PDZ and semi-CRIB domain, with Par-3/Baz via its PDZ domain and with aPKC through its PB1 (Phox and Bem1) domain. Concomitant binding to aPKC and Cdc42 causes a conformational change in Cdc42-GTP, leading to aPKC activation (Hutterer et al., 2004; Joberty et al., 2000). Activated aPKC in turn is needed for correct Par-3 localization in mammalian cells and has been shown to spatially regulate the basolateral protein complex in *Drosophila*. aPKC phosphorylates Par-3 which in turn dissociates from the Par complex, possibly allowing the regulative interaction of aPKC with other proteins and thus leading to correct cell-cell contact formation and epithelial polarization (Joberty et al., 2000; Nagai-Tamai et al., 2002). At the basolateral side, Par-6 localization is excluded by Lgl in *Drosophila*, however the exclusion of the Par complex in mammalian cells has been shown to be regulated by Par-1 kinase activity: Par-1 phosphorylates Par-3, which leads to the binding of Par-5 (14-3-3) to Par-3 and thus inhibits the formation of the Par complex by blocking Par-3 oligomerization and binding to aPKC (Benton & St Johnston, 2003). Par-1 activation is regulated by Par-4 through phosphorylation of the activation loop of the Par-1 kinase domain (Suzuki & Ohno, 2006) and it is inactivated by phosphorylation by aPKC, which causes binding to Par-5 (14-3-3) and inhibition of plasma membrane binding in the apical domain (Hurov et al., 2004) (Fig. 5).

Proteins that have so far not been linked to epithelial polarity can of course also affect the functionality of the junctional Par complex, and in the upcoming years new regulators will for sure be identified. One example for such a regulation is protein phosphatase-2A (PP2A), a heterotrimeric serine/threonine phosphatase with broad substrate specificity and diverse cellular functions like cell growth and regulation of the cytoskeleton. PP2A inhibits aPKC function and dephosphorylates TJ components thereby triggering junction disassembly in mammalian cells (Nunbhakdi-Craig et al., 2002) (Fig. 5).
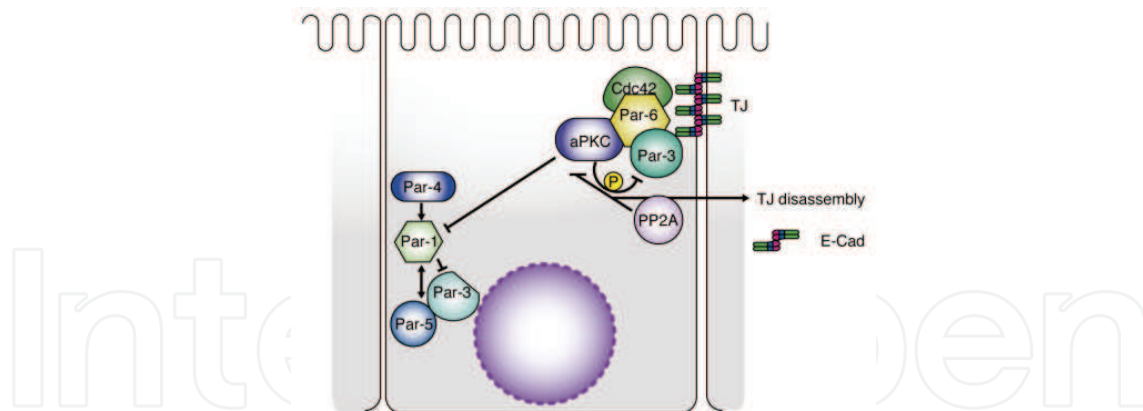
Fig. 5. Regulation of the junctional Par complex in mammalian cells.

aPKC inhibits the function of Par-1, which inactivates Par-3 in the basolateral domain. Par-4 activates Par-1. Cdc42 binds to Par-6 and thereby activates the Par complex. aPKC phosphorylates Par-3, which then dissociates from the Par complex. PP2A blocks aPKC function and leads thus to TJ disassembly. TJ: tight junction; PP2A: protein phosphatase-2A.

5.2 Regulation of the junctional E-Cad/β-Cat/α-Cat complex

The junctional E-Cadherin/β-Catenin/α-Catenin complex is needed for cell-cell adhesion in epithelial cells and its localization and maintenance must thus be strictly regulated. In mammalian cells, disassembly of the apical junctional complex is driven by reorganization of apical F-actin involving cofilin-1-dependent depolymerization and Arp2/3-assisted repolymerization as well as myosin II mediated contraction (Ivanov et al., 2004). Actin organization, myosin II phosphorylation and therefore localization and regulation of gene transcription and E-Cad localization is affected by Rho, which counteracts Cdc42 and Rac activity and thus inhibits AJ formation (Sturge et al., 2006). Cdc42, Rac and Rho are indispensable for epithelial polarity regulation at the junctional domain. Cdc42 promotes Par complex formation in vertebrates and *Drosophila* as depicted above and it promotes TJ development by activation of Rac in mammalian cells. Rac is also activated at the junctional domain through the GEF Tiam1 (T-cell lymphoma invasion and metastasis-1), which also binds to Par-3, providing a link between Rac and the junctional polarity protein complex. This activation is needed to counteract Rho activity, since Rho favors TJ disassembly through Rho kinase (ROCK) mediated myosin II phosphorylation (van Leeuwen et al., 1999). In *Drosophila*, Cdc42/Par-6/aPKC furthermore regulate E-Cad endocytosis, by recruiting and interacting with the actin and dynamin machinery needed for vesicle scission (Georgiou et al., 2008, Harris & Tepass, 2008, Leibfried et al., 2008) (Fig. 6).

Myosin II is a motor that converts chemical energy of ATP into mechanical forces, mediating the contractility of the actin cytoskeleton. It is activated by phosphorylation of its light chain through ROCK or MLCK (myosin light chain kinase). Rho activity is therefore down-regulated at the AJs in polarized epithelial cells via the interaction of the GAP p190RhoGAP and the catenin p120-Catenin. p190RhoGAP translocation to the AJs is mediated by Rac activity, a process taking also place in *Drosophila* epithelial cells, thus suggesting a general and not organism-specific down-regulation of Rho at the adherens junctions (Magie et al., 2002; Wildenberg et al., 2006). In mammalian cells, Rac has been shown to recruit actin to sites of cell-cell contacts, where it leads to the internalization of the E-Cad/Cat complex

(Akhtar & Hotchin, 2001). Therefore, Rac seems to have a dual role in E-Cad/Cat complex maintenance by recruiting p190RhoGAP and at the same time leading to E-Cad/Cat internalization at the junctions.

Echinoid (Ed) is a component of *Drosophila* AJs that stabilizes the adhesion complex through cooperation with E-Cad and by linking the AJs to actin filaments. The C-terminal PDZ domain of Ed furthermore binds to Baz, which leads to a strong linkage between the Par complex and the AJs, since E-Cad is also bound to Baz via its interaction with Arm (Laplante & Nilson, 2006; Wei et al., 2005). Moreover, actin filaments are organized by a pathway that is regulated by Bitesize, a synaptogamin-like protein that binds to Moesin and PIP2 at the apical domain, leading to stabilization of E-Cad at the AJs (Pilot et al., 2006). These results show that the regulation of the adhesion complex depends strongly on small GTPases and the underlying acto-myosin network as well as on protein-protein interactions between adhesion and junctional polarity proteins. Thus they form part of an ancient and fundamental mechanism of cell polarization.
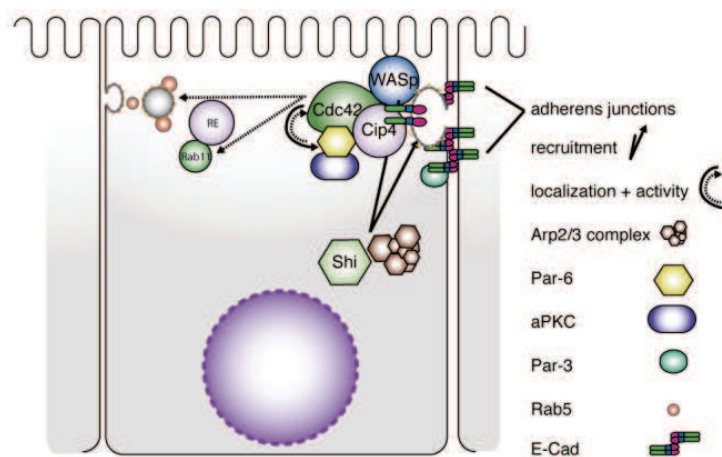

Fig. 6. Regulation of E-Cad endocytosis at the AJs in *Drosophila* epithelial cells.

Cdc42 most likely recruits together with Par-6 and aPKC the actin regulators Cip4 and WASp as well as dynamin, which is needed for endocytic vesicle scission, therefore allowing for correct endocytosis of junctional material.

5.3 Regulation of the apical Crumbs complex

The correct localization of the Crb complex depends on the Crb/Pals1/PATJ proteins themselves, on motor proteins, the Par complex and on regulation of the cytoskeleton. Most of our current knowledge is based on studies conducted on *Drosophila* epithelial cells: The apical localization of the Crb complex is highly dependent on its individual components. Crb localization depends on PATJ and on the transport of Crb protein and transcript by a cytoplasmic dynein complex. PATJ localization is in turn partially dependent on apical Crb localization, possibly resulting in a positive feedback loop between Crb and PATJ targeting (Horne-Badovinac & Bilder, 2008; Li et al., 2008; Michel et al., 2005; Tanentzapf et al., 2000). This positive regulation might be antagonized during later stages of epithelial development by the FERM protein Yurt in order to prevent an expansion of the apical domain. Yurt is localized at the basolateral domain, also in mammalian epithelial cells, but Crb recruits it to the apical membrane late during epithelial development where it counteracts Crb activity (Laprise et al.,

2006). Crb also recruits Pals1 to the apical domain and the same dynein complex used for Crb apical targeting can transport Pals1 transcript. The junctional Par complex regulates the Crb complex by phosphorylation. aPKC can interact with Crb in presence of PATJ and Pals1. As a consequence, Crb gets phosphorylated which is indispensable for correct membrane targeting of Crb and PATJ and for Crb activity (Sotillos et al., 2004). PATJ localization and ZA formation depend furthermore on Par-6 activity (Hutterer et al., 2004).

The apical and basolateral complex regulate each other and loss of the basolateral complex leads to the expansion of the Crb complex and loss of polarity, which can be rescued by the concomitant loss of the Crb complex (Bilder et al., 2003; Tanentzapf & Tepass, 2003). The discrete membrane domains are interdependent not only during epithelial establishment but also during its maintenance. For example loss of adherens junctions proteins E-Cad and Arm leads to loss of Crb and apical polarity via the disruption of the lateral spectrin and actin cytoskeleton (Tanentzapf et al., 2000), therefore resulting in a complete loss of polarity.

5.4 Regulation of the basolateral Lgl complex

Scrib stabilizes the AJ complex in the junctional domain, but Lgl localization is accurately restricted to the basolateral domain by aPKC. Lgl, aPKC and Par-6 can interact, leading to the phosphorylation of Lgl by aPKC at three conserved serine residues in mammalian cells and *Drosophila* (Betschinger et al., 2003). Phosphorylation inactivates Lgl on the apical side and inhibits its binding to the plasma membrane in both mammalian and *Drosophila* epithelial cells by changing the conformation of the protein (Betschinger et al., 2005; Plant et al., 2003). Lgl and aPKC seem to mutually regulate each other, since loss of Lgl (leading to an overproliferation phenotype) can be suppressed by concomitant loss of aPKC in *Drosophila* (Rolls et al., 2003) (Fig. 7). In mammalian cells, Lgl phosphorylation by aPKC is furthermore facilitated by the concomitant interaction of Lgl with P32 (Bialucha et al., 2007). Thus, the interplay between junctional and basolateral proteins maintains distinct membrane domains in polarized epithelial cells.
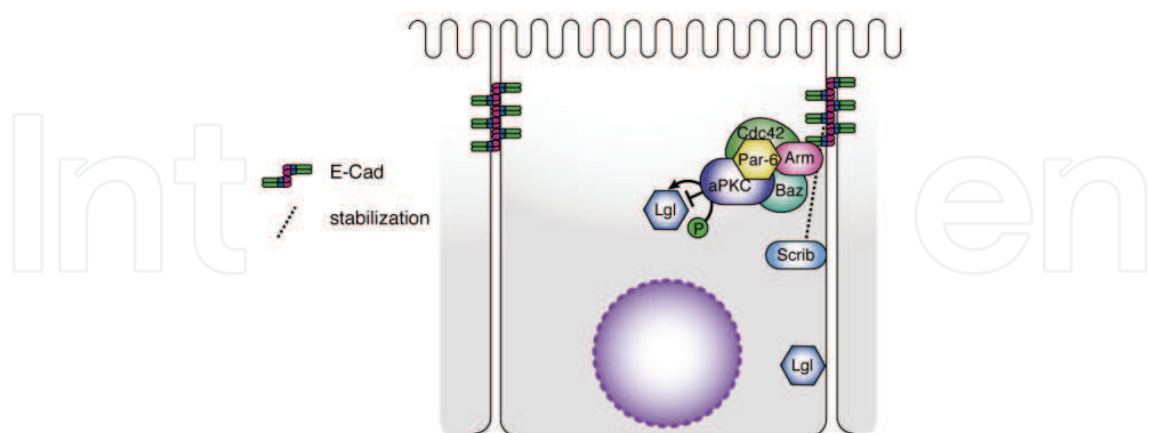

Fig. 7. Regulation of basolateral Lgl and stabilization of E-Cad by Scrib in *Drosophila* epithelial cells.

aPKC phosphorylates Lgl, which impedes the binding of Lgl to the cortex in the junctional and apical domain. Scrib leads to the stabilization of E-Cad. Arm: Armadillo; Baz: Bazooka; Lgl: Lethal giant larvae; Scrib: Scribble.

5.5 Regulation of epithelial polarity via TGF-β and EGF signaling

TGF-β and EGF signaling pathways are two out of several important signaling pathways needed for the correct development of an organism. They have an influence on epithelial polarity and can lead for example to epithelial to mesenchymal transition (EMT). Therefore, the inhibition of both pathways is important for epithelial polarity maintenance.

Transforming growth factor beta (TGF-β; Decapentaplegic, Dpp in *Drosophila*) controls proliferation and cellular differentiation in most cells and TGF-β signaling can lead to loss of cell polarity: in mammalian cells, the TGF-β downstream effector Rho controls EMT by changing the actin cytoskeleton (Ozdamar et al., 2005), whereas the downstream transcription factors of the Snail family concomitantly lead to down-regulation of claudins, occludin, E-Cad and Crb (Xu et al., 2009). Therefore, to maintain epithelial polarity, Par-6 interacts with the TGF-β receptor, which phosphorylates Par-6 so that it can recruit the E3 ubiquitin ligase Smurf1. Smurf1 leads to degradation of Rho and thus to a block of EMT. Snail protein activation is blocked by Smad6 and Smad7, thereby preventing down-regulation of TJ and apical proteins.

Epidermal growth factor (EGF) signaling also plays an important role in the regulation of cell growth, proliferation and differentiation and it can modulate mammalian TJ formation, which allows for concerted dissociation and re-establishment of cell-cell adhesion essential for morphogenesis. Two different pathways achieve this modulation. EGF signaling activates Src (sarcoma) family kinases that phosphorylate TJ proteins, including ZO-1, ZO-2, occludin, E-Cad and Par-3, leading to positive and negative regulation of TJ formation in mammalian cells (Chen et al., 2002; Shen et al., 2008). The down-regulation of E-Cad by Src promotes EMT because it alters E-Cad trafficking by redirecting E-Cad from a recycling pathway to a lysosomal pathway (Shen et al., 2008). In both mammalian and *Drosophila* cells, EGF signaling also induces the MAPK (mitogen-activated protein kinase) pathway which leads to E-Cad and claudin expression and subsequent translocation of junctional proteins from the cytoplasm to cell-cell contacts (O'Keefe et al., 2007; Wang et al., 2006). E-Cad and EGF receptor interact at cell-cell contacts to negatively regulate the MAPK pathway in mammalian cells, suggesting a general negative feedback loop to regulate adhesion and junctional integrity (Qian et al., 2004). These results suggest that EGF signaling needs to be tightly controlled to promote either EMT or the stabilization of junctions and they emphasize the need of a strict regulation of signaling pathways in the cell in order to maintain epithelial integrity.

5.6 Regulation of epithelial polarity via phosphatidylinositol signaling

Recent studies integrate phosphatidylinositol-phosphate signaling to polarization in both mammalian and *Drosophila* epithelial cells. Phosphatidylinositol-phosphate signaling was mainly known to regulate cell size by interacting with the insulin receptor, but Martin-Belmonte, von Stein and colleagues propose that it also enhances polarity establishment.

PDZ domains can bind to phosphatidylinositol lipid membranes and *Drosophila* Baz binds to both, phosphatidylinositol lipid membranes and to the phosphatase PTEN (phosphatase and tensin homolog), thereby possibly recruiting this protein to the apical domain (von Stein et al., 2005). PTEN converts PIP3 (Phosphatidylinositol-(3,4,5)-triphosphate) to PIP2 (Phosphatidylinositol-(4,5)-bisphosphate) and this leads to Cdc42 recruitment via Annexin-2 and subsequently to aPKC recruitment to the apical domain in mammalian cells (Martin-Belmonte et al., 2007). PIP3 is produced by activation of PI3K (phosphatidylinositol-3

kinase) at the adherens junctions, as shown in *Drosophila* epithelial cells, which locally activates Cdc42 and recruits more E-Cad to the junctions (von Stein et al., 2005). This suggests a general model where the Par complex recruits PTEN by binding to phosphatidylinositol lipid membranes, and where PTEN converts PI3K-produced PIP3 to PIP2. This might mediate the establishment of epithelial polarity by the recruitment of Cdc42, aPKC and E-Cad (Fig. 8).

PIP2 and PIP3 regulate furthermore the actin cytoskeleton: PIP2 binds to actin-associated proteins that link the actin cytoskeleton to the plasma membrane or to proteins that are involved in the initiation of *de novo* actin polymerization, and PIP3 activates WASp family proteins (like WASp, WAVE and WASH proteins) and the Arp2/3 complex via interaction with Rho GTPases (Fig. 8). These results point toward a spatio-temporal fine-regulation of Cdc42 recruitment during polarization and a coeval regulation of cell polarity establishment and cytoskeleton controlled by the balance between PIP2 and PIP3, leading to an enhanced effect for both Cdc42-mediated pathways.
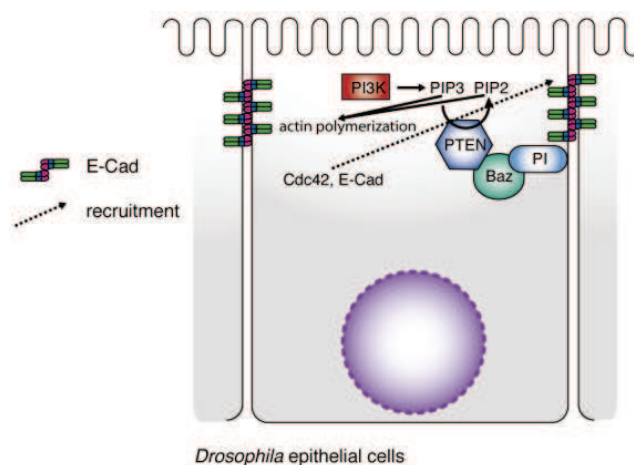

Fig. 8. Regulation of epithelial polarity via phosphatidylinositol signaling.

Baz can bind to PI and to PTEN. PTEN mediates PIP3 to PIP2 conversion, which leads to the recruitment of Cdc42 and E-Cad. PIP3, which is generated by PI3K activity in the apical domain, and PIP2 lead to actin polymerization at the junctions. PI: phosphatidylinositol; PIP3: Phosphatidylinositol-(3,4,5)-triphosphate; PIP2: Phosphatidylinositol-(4,5)-bisphosphate; PI3K: phosphatidylinositol-3 kinase; Baz: Bazooka.

5.7 Regulation of epithelial polarity via the acto-myosin cytoskeleton

Cell polarity also requires fine regulation of the cytoskeleton. Actin is needed for the furrow-canal formation during cellularization in *Drosophila* and AJ proteins are linked to the actin cytoskeleton. Furthermore, spectrin has a crucial role in anchoring Crb at the apical domain (Tanentzapf et al., 2000). In general, the regulation of the actin cytoskeleton is dependent on the activity of Rac and Cdc42, which both lead to actin nucleation upon their activation. Downstream of Rac and Cdc42 are WASp family proteins, which are activated through binding to the Cdc42 and Rac-binding domain (CRIB). WASp protein activity leads to actin nucleation by activation of the Arp2/3 complex. In mammalian cells, the binding of Cdc42-GTP and PIP2 to WASp synergistically enhances the activation of the protein (Parsons et al., 2005) and WASp upregulates the GEF activity of the Cdc42-

specific GEF intersectin (Malacombe et al., 2006), suggesting a positive feedback loop between Cdc42 and WASp activity. Cdc42 is also directly linked to epithelial polarity via the junctional Par complex (see 5.2). Downstream of Cdc42 is furthermore PAK1, which promotes microtubule formation, thus localizing actin nucleation and microtubule formation to the same confined space (Parsons et al., 2005), possibly needed for correct plasma membrane identity in polarized cells. Cell polarity is furthermore maintained by the AMP-activated protein kinase (AMPK), which alters the acto-myosin cytoskeleton in response to energetic stress situations. High AMP levels lead to a conformational change of the protein and Par-4 phosphorylates AMPK to activate it as indicated by biochemical data (Hawley et al., 2003). In *Drosophila*, Dystroglycan, which is localized at the basal domain, where it interacts with the extra-cellular matrix protein Perlecan, transduces a signal from the cellular energy sensor AMPK to myosin II, thus activating myosin II and regulating AB polarity (Mirouse et al., 2009). To conclude, regulation of both the actin cytoskeleton and myosin II by GTPases and polarity proteins seems to be indispensable for maintaining AB polarity.

6. *In vivo* studies on epithelial cells using *Drosophila*

As depicted above, most of our hitherto knowledge of epithelial polarity stems from genetic and biochemical studies performed in cell culture or on model systems. Though extensive, these two approaches cannot reveal all mechanisms underlying the process of polarization. Cell polarity is not only needed for the establishment and maintenance of the single cell, but also for the correct development of the whole multicellular organism. Some events, like spatio-temporal protein or organelle localization can only be captured when analyzing a whole, developing, epithelium. Whole tissue analysis can be easily performed using *Drosophila*: epithelia can be imaged *in vivo* by confocal microscopy (see Fig. 1) while the animal develops. This imaging techniques and the vast genetic tools available (e.g. gene knock-down or over-expression, expression of fluorescent tagged proteins in the living fly) have allowed for the dissection of new mechanisms regulating epithelial polarity.

6.1 Junction-cytoskeleton interaction dissected *in vivo*

The interaction of α-Catenin with E-Cad and the cortical actin control both stability and remodeling of adhesion. How this occurs is, as mentioned in section 2.4., not elucidated. Live imaging of *Drosophila* embryos expressing fluorescent E-Cad and actin (F-actin) revealed that E-Cad is not evenly distributed around the adhesion belt in epithelial cells as previously expected. Stable microdomains intersperse with mobile domains. Laser nano-ablation of actin and FRAP (Fluorescence Recovery After Photobleach) and photo-conversion experiments for E-Cad show that the stability and mobility of these microdomains depend on two actin populations, a stable network and one that rapidly turns over (Cavey et al., 2008).

Further *in vivo* studies using FRAP and nano-ablation show that the myosin-II forces needed for actin remodeling at the junctions is not as previously assumed based on polarized activity of junctional myosin-II, but by the polarized flow of medial actomyosin pulses towards a specific junctional domain (Rauzi et al., 2010).

6.2 Planar cell polarity mechanisms dissected *in vivo*

The morphogenesis and function of an epithelial tissue relies on the precise arrangement of its constituent cells. Tissue patterning and organization during development depends on the establishment of concentration gradients of signaling molecules along the tissue that furthermore can lead to the formation of polarized structures in the plane of the epithelium (like hairs). This type of polarization of a field of cells is referred to as planar cell polarity (PCP), where the spatial information that organizes planar polarity is transmitted locally from one cell to the next (for review see Seifert & Mlodzik, 2007). Thus, epithelial tissues not only show an apico-basal polarity, but also a positional oriented appearance in the plane in order to generate polarized structures and to orient themselves in a directed fashion. An evolutionary conserved set of genes control establishment of planar polarity in flies and vertebrates, the core Frizzled/PCP (Fz/PCP) factors Flamingo (Fmi), Strabismus (Stbm), Dishevelled (Dsh), Diego (Dgo), Fat, Dachsous (Ds) and Prickle (Pk) (Jenny et al., 2005). The Fz/PCP factors polarize a field of cells along a specific axis. Local differences in Fz activity between neighboring cells provide directional information required for planar polarity, in other words a gradient of the morphogen is needed for correct PCP. As a result, wing hairs point away from the site of highest Fz activity in *Drosophila* (Adler et al., 1997).

Drosophila wing epithelial cells are irregularly arranged throughout most of development, but they become hexagonally packed shortly before hair formation. PCP proteins promote hexagonal packing in the *Drosophila* wing by polarizing membrane trafficking (Classen et al., 2005). Planar polarity arises during growth due to a cell flow that is triggered by tension arising from the wing hinge contraction. *In vivo* imaging of *Drosophila* wing epithelial cells expressing fluorescent polarity proteins Stbm and E-Cad led to these observations, that would be very difficult to dissect *in situ* or *ex vivo* (Aigouy et al., 2010).

6.3 Asymmetric cell division of epithelial cells dissected *in vivo*

Planar cell polarity proteins, amongst others, are also needed to align the mitotic spindle correctly in order to allow for oriented mitosis to occur. Live imaging of the spindle in the *Drosophila* dorsal thorax using fluorescent microtubule components have revealed the mechanism that keeps the spindle in the correct plane and correct apico-basal tilt. This positioning controls the correct asymmetric cell division needed in the mechanosensory organ precursor cell, which resides in the dorsal thorax. Mutations in either Fz/Dsh or the NuMa homolog Mud as well as mutations in Ric-8, a guanine nucleotide-exchange factor for heterotrimeric G proteins, result in the mis-orientation of the spindle during division and the subsequent mal-formation of the sensory organ (David et al., 2005, Ségalen et al., 2010). The alignment of the spindle during mitosis can only be captured using *in vivo* studies and the underlying mechanism would have stayed unknown without the use of *Drosophila* for *in vivo* imaging and the vast genetic techniques available for this model system.

7. Concluding remarks

Epithelial cell function relies in both vertebrates and invertebrates on the tight regulation of the underlying polarity protein machinery, which is highly conserved (Leibfried, 2009). This regulation has been analyzed in epithelial cells in cell culture, but an analysis in a whole living organism, integrating all cellular and environmental cues that an epithelium is exposed to, remains an interesting task, comprising the truth about epithelial function and

the regulatory networks needed for it. The use of *Drosophila* as a model system allows us to better study epithelial establishment, maintenance and plasticity in the context of a whole organism. Furthermore, green-fluorescent-protein (GFP) and its derivates give us the opportunity to analyze epithelial cell function in a spatio-temporal manner by live imaging. Thus, *Drosophila* will also in the future help to better understand epithelial establishment, maintenance and plasticity thanks to today's microscopy imaging and manipulation techniques, the extensive genetic tools available and the feasibility to study the epithelium in the context of the whole organism.

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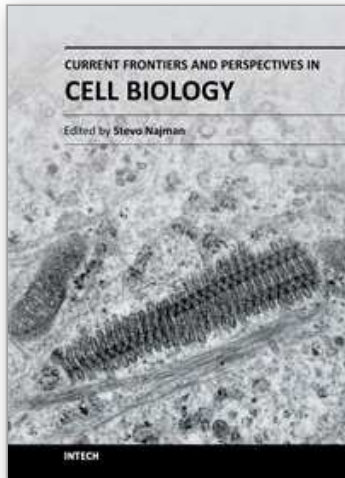
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