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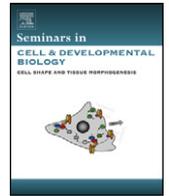
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Review

Effectors of tridimensional cell morphogenesis and their evolution

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ABSTRACT

One of the most challenging problems in biology resides in unraveling the molecular mechanisms, hard-wired in the genome, that define and regulate the multiscale tridimensional organization of organs, tissues and individual cells. While works in cultured cells have revealed the importance of cytoskeletal networks for cell architecture, *in vivo* models are now required to explore how such a variety in cell shape is produced during development, in interaction with neighboring cells and tissues. The genetic analysis of epidermis development in *Drosophila* has provided an unbiased way to identify mechanisms remodeling the shape of epidermal cells, to form apical trichomes during terminal differentiation. Since hearing in vertebrates relies on apical cell extensions in sensory cells of the cochlea, called stereocilia, the mapping of human genes causing hereditary deafness has independently identified several factors required for this peculiar tridimensional organization. In this review, we summarized recent results obtained toward the identification of genes involved in these localized changes in cell shape and discuss their evolution throughout developmental processes and species.

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1. Introduction

Every animal cell is characterized by a specific tridimensional organization, directly linked to its function within the whole organism. Indeed there is a huge diversity in the size and shape of individual cells, and the ways they establish physical contacts between each other, well illustrated when comparing meter long neurons, discoid small erythrocytes or densely packed epithelial cells. Various human pathologies are linked to failures in a proper

control of cell shape, including hereditary sensory disorders or blood diseases, and defects in cell shape and adhesion impinge on many cancers. The general question of morphogenesis can be formulated as which are the mechanisms that define a subpopulation of cells to achieve a specific behavior [1]. While it is well established that the morphological differentiation of our cells relies on differential genome expression, little remains known on which factors directly remodel the cell shape and the way their action is finely coordinated.

Genetic analyses in *Drosophila* have provided a rich source of information about the molecular players involved in tissue morphogenesis. Early developmental processes depend on collective cell reorganization. For instance, studies on mesoderm invagination, germ band extension and dorsal closure in *Drosophila* have shown the importance of apical constriction, asymmetric cortical

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tension and lamellipodia/filopodia formation, relying on reorganization of the actin cytoskeleton [2–5] and junction complexes [6].

Another important but yet poorly understood aspect of morphogenesis consists in the localized tridimensional shaping of the apical side of epithelial cells that occurs at later developmental stages [7,8]. This leads to the production of polarized cell morphologies, characterized by a variety of cell extensions. This review focuses on terminal differentiation of the *Drosophila* epidermis and of the sensory part of the inner ear in vertebrates, two cell types that produce highly differentiated apical extensions required for their respective functions.

2. Morphological differentiation of *Drosophila* epidermal cells

The *Drosophila* embryonic epidermis is composed of a layer of post mitotic cells that become patterned by cascades of transcription factors and signaling pathways, ultimately determining their morphological fate. The tissue comprises cells with a smooth or “naked” surface and cells producing apical extensions involved in larval locomotion, called ventral denticles and dorsal hairs, collectively referred to as trichomes [7]. Epidermal cells that will form trichomes modify their apical region, from square-like to a rectangular shape extending along the dorso-ventral axis (Fig. 1A and B), and align into parallel columns [9,10]. This is achieved through junctional conversion, relying on actomyosin contractility and remodeling of polarity complexes, including Disc large [10], Lethal giant larvae and Disheveled [11].

Trichome cells then accumulate F-actin at the posterior margin of each cell where cytoplasmic protrusions begin to elongate [9,12] (Fig. 1B). In the mean time, epidermal cells start secreting cuticle, a complex exoskeleton composed of chitin, various proteins and lipids (reviewed in [13]). At the end of embryogenesis, cuticle layers become thicker and harder and microfilaments supporting extensions eventually disassemble. Fully differentiated trichomes present a specific size, shape and orientation depending on their position along the body (Fig. 1C and D).

3. Genetic determinants of epidermal cell morphogenesis

The stereotyped arrangement of naked *versus* trichome cells has been widely used as developmental readout in genetic screenings, allowing the discovery of numerous genes establishing the trichome pattern.

Signaling pathways including Wnt, EFG-r, Hedgehog and Notch control epidermal cell fate [14], at least in part through regulating the expression of a common gene, *shavenbaby* (*svb*) [7,15] (Fig. 2A). *Svb* encodes a transcription factor specifically expressed in trichome cells and required for their morphological differentiation. The ectopic expression of *svb* is sufficient to promote trichome formation when artificially expressed in smooth cells [15,16], showing that *Svb* governs the transcriptional program of trichome morphogenesis.

Supporting this conclusion, modifications of *svb* expression have led to the diversification of trichome patterns in various clades of insects [17–20]. For example, the restriction of *svb* expression in dorsal cells has evolved the trichome pattern in *Drosophila sechellia*, in which several rows of dorsal hairs are replaced by naked cuticle [18,19]. Transcription of *svb* in the embryonic epidermis is directed by seven cis-regulatory modules (CRM), driving overlapping expression in subsets of cells [21,22]. These apparently redundant CRMs actually ensure a robust *svb* expression in varying environmental or developmental conditions [21]. Consistently, the modification of several independent CRMs has been required to evolve *svb* expression and thus the trichome pattern across species

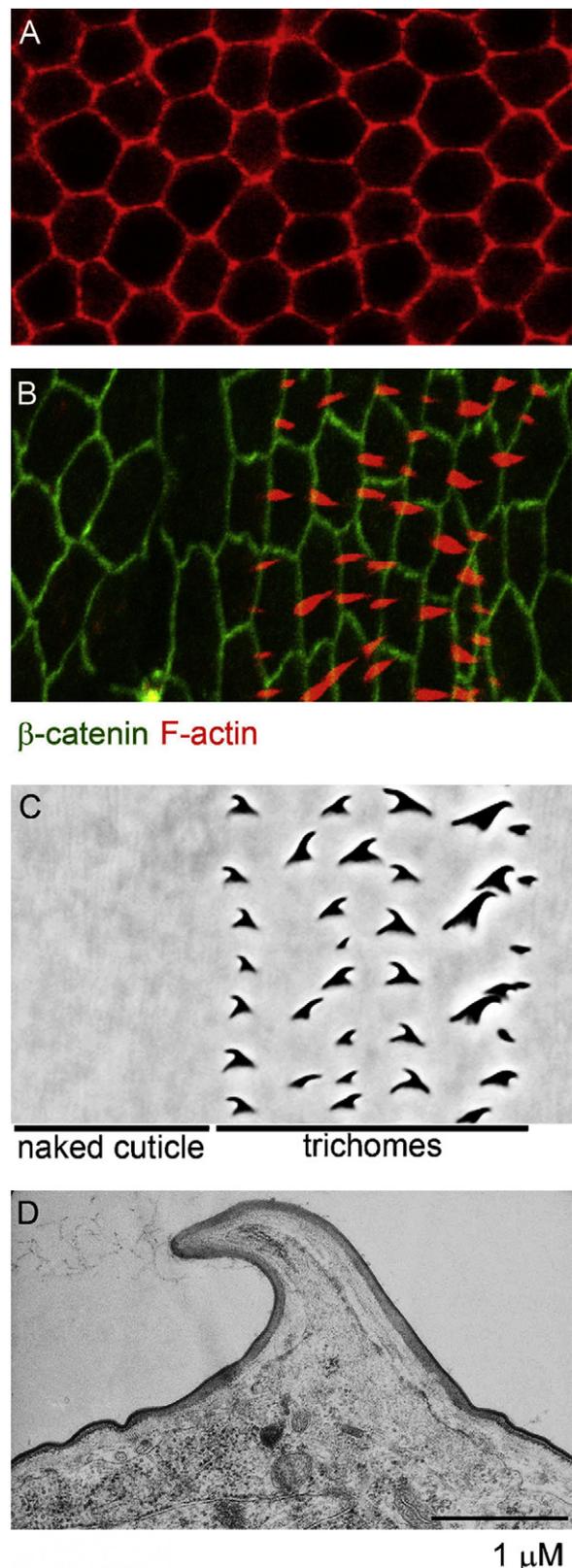


Fig. 1. Morphology of epidermal cells in the *Drosophila* embryo. Confocal images of presumptive epidermal cells (7 h after egg laying – AEL, F-actin is in red) (A) and differentiating epidermal cells (14 h AEL) stained for F-actin (red) and α -catenin (green) (B). (C) Cuticle of an abdominal segment showing the stereotyped pattern of naked cuticle and trichomes in the ventral region of a first instar larva (24 h AEL). (D) Electron micrograph showing the apical organization of a ventral trichome cell; fourth row of an abdominal segment (20 h AEL).

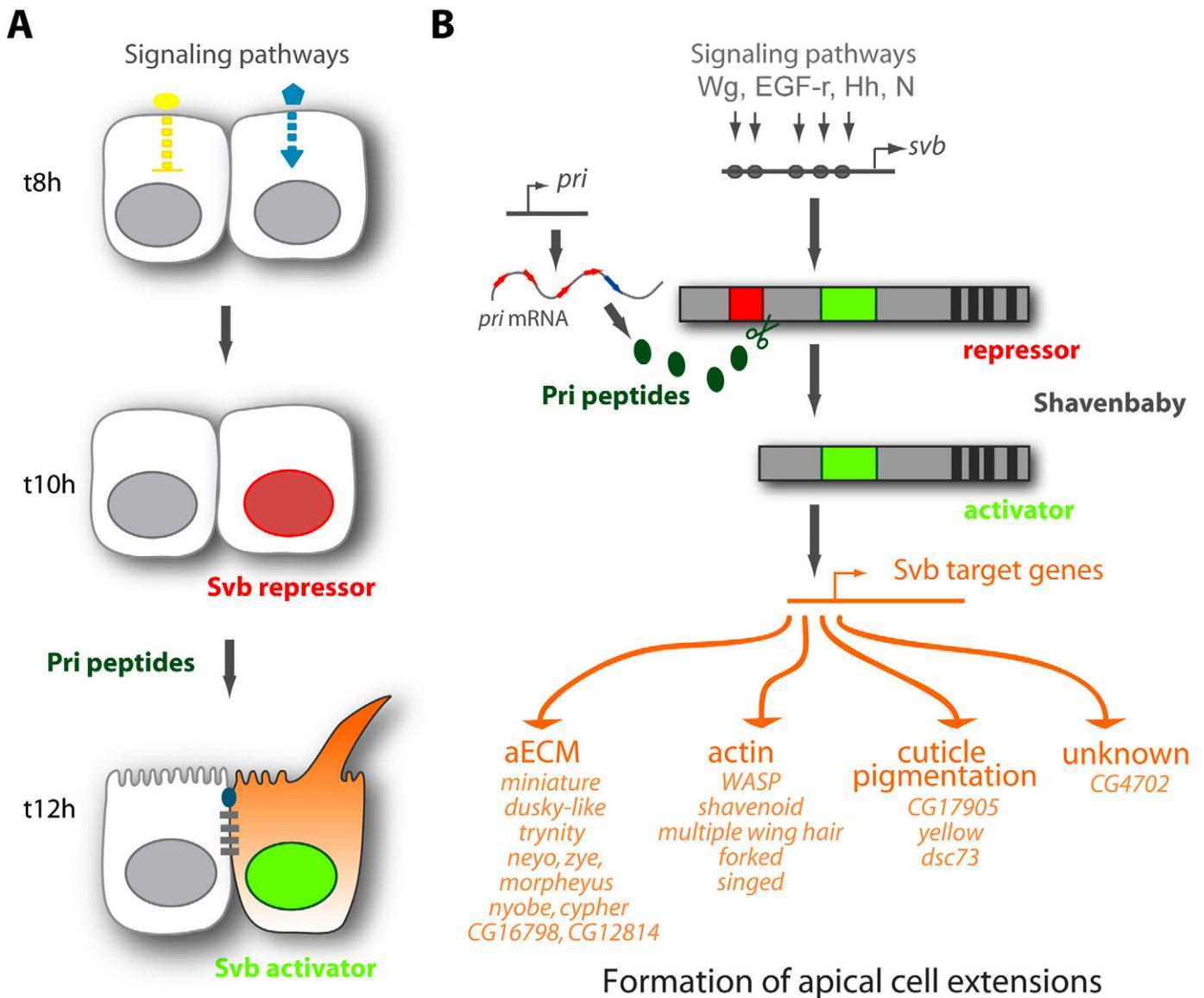


Fig. 2. Genetic networks regulating trichome differentiation. (A) In response to signaling pathways, a subset of epidermal cells turn on expression of *shavenbaby*, which initially produces a transcriptional repressor (red). *Pri* expression provides a temporal control, converting *Svb* into an activator (green). This triggers the transcription of *Svb* downstream targets (orange), encoding cell effectors of trichome formation. (B) Spatial cues are integrated by the cis-regulatory modules directing *svb* transcription in the proper pattern of trichome cells. *pri* mRNA is a polycistronic transcript encoding 4 peptides of 11–32 aa in length. These peptides induce a post-translational cleavage of the *Svb* transcription factor, releasing the N-terminal region that contains a repressor domain. Once activated, *Svb* directly triggers the transcription of a battery of target genes, remodeling different cellular compartments to achieve localized changes in the shape of epidermal cells.

[22]. Thus, both developmental and evolutionary data show that *svb* plays a pivotal role in determining which epidermal cells form trichomes.

Two groups have identified a novel player required for trichome formation, called *tarsal-less* or *polished-rice* (*pri*) [23,24]. Like in the absence of *svb*, trichomes are replaced by naked cuticle in *pri* mutants [24]. *pri* expresses a 1.5 kb long polyadenylated RNA, which comprises only five small ORFs (smORFs). These smORFs have been conserved throughout considerable evolutionary distances, e.g. in silk moth, beetles and even crustaceans [25]. The first four *pri* smORFs encode 11–32 aa peptides, which share a LDPTGQ/TY motif. Compelling evidence support that the activity of *pri* is mediated by these short peptides, which are able to diffuse over several cell diameters within epithelial tissues [23,24].

Although *pri* and *svb* are both required for trichome formation, their expression is mutually independent [23,24]. Indeed, *Pri* peptides switch the transcriptional activity of the *Svb* protein,

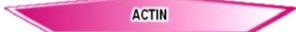
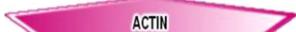
from a repressor to an activator. This is achieved through a post-translational cleavage releasing the N-terminal region of *Svb*, which comprises a repressor domain [26] (Fig. 2B). In the absence of *pri*, *Svb* thus persists as a transcriptional repressor, preventing the expression of the whole set of its target genes (see below). It has been proposed that *Pri* provides a temporal control of epidermal differentiation, determining when the *Svb* transcription factor turns into an activator, and thus triggers the program of trichome production [26].

4. Cellular effectors involved in epidermal cell shape changes

Consistently with the actin reorganization that occurs in trichome cells, several related factors have been reported to accumulate in growing extensions (Table 1). This includes the two main actin nucleator complexes Arp2/3 and the formin Diaphanous, ENA, APC [9,16] or nonmuscle Myosin II Heavy Chain. The transcription

Table 1

Cellular factors involved in epidermal morphogenesis. Schematic representations are taken from Prosite or SMART, numbers refer to references cited. Mwh, Multiple wing hair; Dia, Diaphanous.

		Schematic representation	In vitro assays	Drosophila			Vertebrate cochlea
				Trichome	Wing hair	Bristle	
Actin nucleation	Arp2		[41]	[41]			
	Arp3		[41]				
	Wasp		[41]	[26]	[41]	[40]	
	Toca1		[41]		[42]		
	Dia		[41]	[71]		[49]	
	Mwh			[11]	[27,28]		
Microfilament organization	Fascin		[41]	[11]		[38]	
	Espin			[11]		[39]	
	Whirlin					[66,75]	
Motors	Myosin VI					[67]	
	Myosin XV					[65,70]	
Membrane interaction	Ezrin, Radixin, Moesin					[67]	
PCP	Frizzled			[41]	[67]	[41]	
	Dachsous			[41]	[41]		
Apical ECM	Miniature			[26]	[43]		
	Trynity			[32]			
	Tectorin					[57,76]	

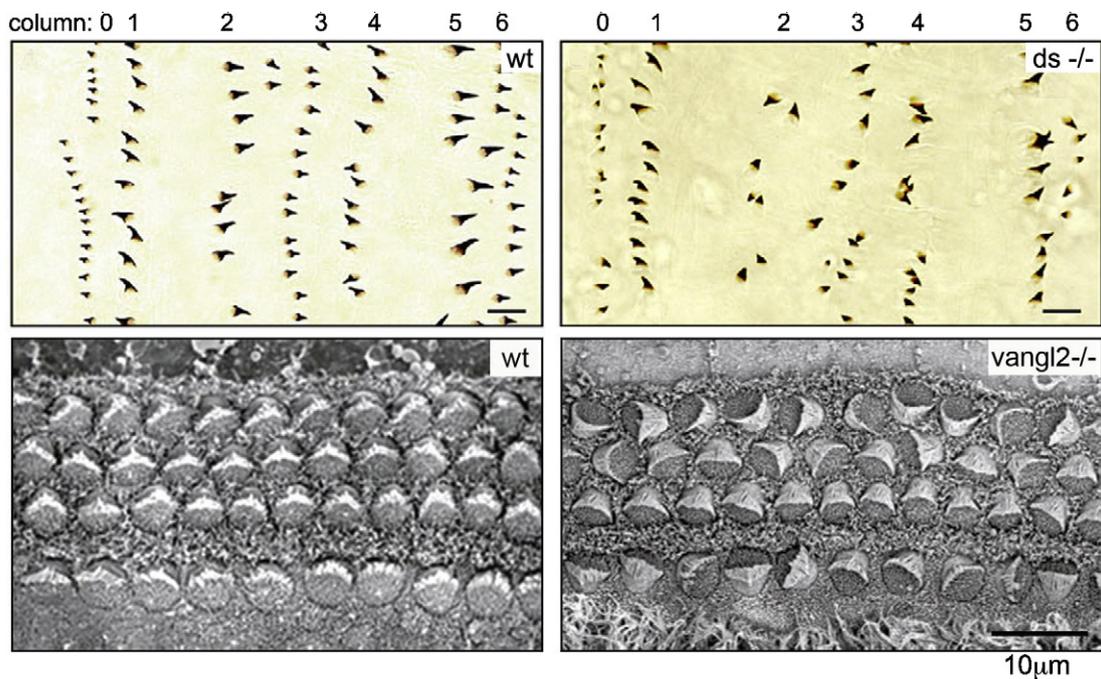


Fig. 3. Planar cell polarity and organization of apical cell extensions. (A) Cuticle of wild type (left) and *ds* mutant (right) larvae. Pictures are from [37]. (B) Scanning electron micrographs of cochlea in wild type and *vangl2* mutant mice [67]. Scale bars: 10 μm .

of these genes is mostly ubiquitous throughout embryonic tissues, as expected for components of basic actin machineries.

The identification of *Svb* target genes in the embryonic epidermis has provided, however, an unbiased means to identify additional factors controlling cell shape remodeling. *Svb* drives the transcription, specifically in trichome cells, of various cytoskeletal regulators directly involved in trichome formation [27]. *WASP* is an activator of the Arp2/3 complex that promotes formation/elongation of actin filaments. *Multiple wing hairs* encode a formin-related protein that locally inhibits ectopic hair initiation [28,29]. *Shavenoid* encodes a fast evolving protein, putatively involved in remodeling the actin cytoskeleton and playing a critical role in trichome morphogenesis [27,30]. *Singed* and *Forked* are actin bundling proteins that localize in growing trichomes where they play non-redundant roles in organizing microfilaments into parallel arrays [12,27].

Beside actin regulators, *Svb* also triggers the expression of membrane-anchored extracellular proteins, including eight proteins comprising a zona pellucida domain (ZPD) [27,31]. The ZPD is a polymerization module promoting assembly of ZP proteins into extracellular fibers (reviewed in [32,33]). ZP proteins are expressed in various epithelia and organize specialized ECMs involved in cell shape and function [34]. In *Drosophila* trichome cells, they display differential distribution along the extension, revealing an unexpected sub-compartmentalization of the apical region [31]. Each ZP protein links the plasma membrane to cuticle layers in a restricted region of the apical domain and mutants for ZP genes display distinct abnormalities in trichome shape [27,31]. These data therefore show that the apical matrix constitutes an extracellular scaffold required for shaping the trichomes.

In the course of epidermal differentiation, cuticle becomes locally modified to regulate the exoskeleton mechanical properties. For example, the catecholamine pathway produces quinone species, mediating cuticle hardening (sclerotization) and tanning (melanization). Several enzymes of the pathway are expressed in trichome cells, and at least one gene, *yellow*, is regulated by *Svb* [27]. Several studies have uncovered additional genes activated by

Svb in trichome cells, including cuticle components [35], enzymes and membrane proteins [27,31].

The individual inactivation of a given *svb* target gene does not prevent trichome formation. Instead, it leads to specific defects in trichome shape. The simultaneous inactivation of several target genes led to cumulative defects in shape, ultimately leading to a reduction in trichome number [27]. This suggests that trichome formation relies on the collective action of various classes of effectors, acting together as a developmental module that reorganizes apical cell shape. Supporting this conclusion, expression of the set of trichome-specific effectors is lost in epidermal cells that have evolved a naked morphology in *D. sechellia* [27].

The planar cell polarity (PCP) pathway is required for positioning and orientation of embryonic trichomes (Figs. 1B and 3). PCP relies on two systems, *Frizzled* (*Fz*) and *Dachsous* (*Ds*) that interact differently between tissues, such as the adult wing, eye or abdomen [36]. In the embryo, trichome polarity is mainly controlled by the *Ds* pathway, whilst the *Fz* system may play a subtler role [9,37,38]. Although *multiple wing hairs* and *shavenoid* interact with PCP components in adult tissues [29,30,39], it is not yet clear whether *Svb* controls the expression of *bona fide* PCP genes in the embryonic epidermis. However, supernumerary trichomes formed upon ectopic expression of *svb* are not properly implanted within the apical surface [40], suggesting that at least some PCP cues are independent of *svb*.

The general view emerging from these studies is that trichome differentiation requires the transcriptional regulation of an unexpectedly large number of cell effectors, which switch or finely modify the activity of basic cellular machineries. This occurs in a pre-differentiated tissue, which has already established a polarized organization (apicobasal and planar) and mature cell junctions, both being a prerequisite for trichome formation. Therefore, the control of cell shape involves generic factors (such as Arp2/3), tissue specific polarity determinants and junction complexes, and a surprising diversity of effectors that are specifically expressed in a restricted array of cells. In addition to the cytoskeleton, these results highlight the overlooked importance of remodeling

different cell compartments including membrane domains and the extra-cellular matrix for the fine sculpting of cell extensions.

5. A conserved effector module for epithelial cell extensions?

Cell effectors of embryonic trichomes are also expressed in other *Drosophila* epithelial cells that produce apical extensions, such as adult wing hairs or sensory bristles (Table 1). For example, WASP, Forked and Singed are involved in bristle development [41–43]. The large size of bristles (400 μm) facilitates their analyses, showing regionalized activity of these proteins for the formation of large bundles of actin filaments. Each wing cell forms a unique hair, with a stereotyped polarity at the distal vertex of the apical region. Genetic analyses of wing hair orientation have led to the discovery of the PCP pathway and of many of its components. *Shavenoid* is also expressed in wing cells, where it is required for the formation of apical hairs [30]. Singed accumulates in the growing extensions supporting wing hairs and its absence alters their proper shaping. *Multiple wing hairs* are renowned for its function during wing hair morphogenesis [28,29] where it determines the position of cell extension within the apical region and regulates its elongation [44].

Lee et al. have developed an elegant approach to study *in vitro* the formation of cell extensions, using frog egg extracts and lipid bilayer [45]. They show that the initiation of filopodia-like extensions is primarily determined by the local accumulation of Toca1 and N-WASP, together with the Arp2/3 complex and actin.

Interestingly, Toca1 and WASP were recently shown to be involved in wing hair morphogenesis [46]. As observed *in vivo*, elongation of filopodia-like *in vitro* then requires Diaphanous and Fascin [45]. Therefore the core of proteins identified *in vivo* for their role in epithelial cell remodeling appears sufficient to promote membrane protrusions in a reconstituted assay.

Furthermore, several ZPD proteins have a documented role in wing morphogenesis. Miniature was initially identified as a mutation leading to small wing size, a phenotype resulting from defects in apical cell flattening and actin organization [47]. Additional ZPD proteins, Dusky [47], Papillote, Piopio and Dumpy [48] are required for wing morphogenesis, the three latter impairing microtubule organization when missing in wing cells. Interfering with Dusky and Dusky-like activity also impairs bristle formation [49,50].

Despite the different morphologies observed between *Drosophila* tissues, formation of apical cell extensions thus requires a similar set of cell effectors, ranging from factors promoting assembly of actin filaments and regulating their organization, to proteins mediating specific interaction between architectural elements and the membrane compartment(s).

6. Evolutionary aspects

The cochlea is a region of vertebrate inner ears specialized in hearing. Sensory part of the cochlea is composed of supporting cells interspersed with two types of sensory hair cells forming apical extensions, the stereocilia (Fig. 4), supported by actin bundles [51].

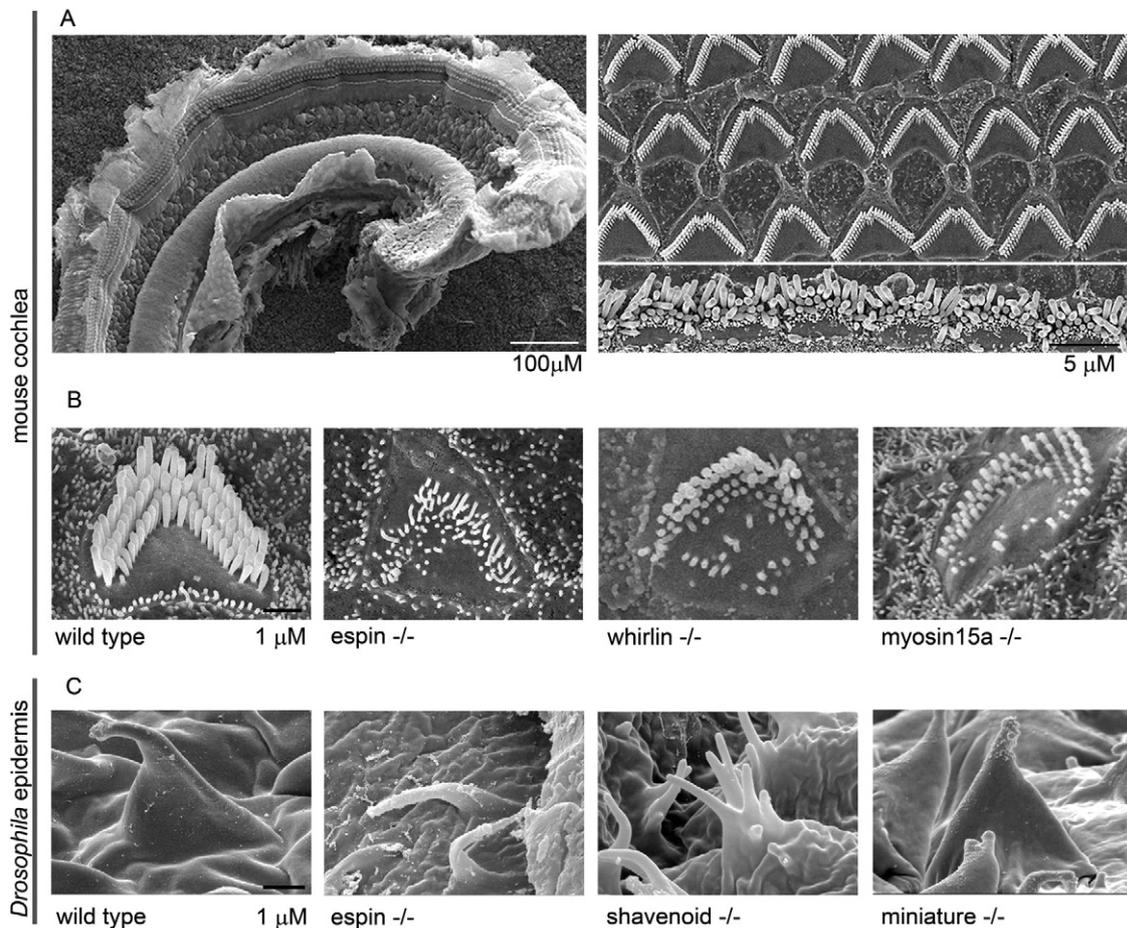


Fig. 4. Evolution of cell effectors of apical extensions. Scanning electron micrographs of sensory hair cells in the mouse cochlea (A, B) and of *Drosophila* epidermal trichomes (C). (A) Left: low magnification view of a mouse cochlea (13-month old), scale bar is 100 μm . Right: outer and inner hair cells in wild type mouse; pictures kindly provided by J. Bartles (see [55]). (B) Close ups showing cochlear outer hair cells in wild type, *espin* $-/-$ (*jerker*), *whirlin* $-/-$ (*whirler*) and *myoXVa* $-/-$ (*shaker-2*) mutant mice. Pictures were adapted from [55,78]. (C) Trichomes produced by embryonic epidermal cells in wild type, or *forked* (*espin*), *shavenoid* and *miniature* mutants.

Inner hair cells (IHC) display a single row of stereocilia and transmit electric signals to the auditory nerve. Outer hair cells (OHC) differentiate 3–4 rows of stereocilia with a staircase arrangement that amplify vibrations. Hair cells sense sound through vibrations of the tectorial membrane (TM), a specialized extracellular matrix.

Similarly to *Drosophila* trichome cells, the specification of sensory versus non-sensory hair cells involves both signaling, e.g. FGF, Hh and Notch pathways, and transcription factors such as Math1 (reviewed in [52]). However, the register of their target genes in sensory cells remains to be fully elucidated. Many factors involved in stereocilia morphogenesis have been identified from genetic analyses of human deafness and targeted gene inactivation in mice has provided animal models [8].

Pioneering studies have proposed that, besides morphological differences, the mechanisms underlying the differentiation of stereocilia, sensory bristles and more generally microvilli-like apical extensions may share common properties and players [53]. Indeed, several factors required for stereocilia belong to the core module of effectors, as defined by both genetics in flies and *in vitro* approaches (Table 1). For example, Diaphanous is linked to deafness suggesting a role in stereocilia morphogenesis [54]. The role of Espin in stereocilia formation and/or maintenance is well established and *espin* mutations cause hearing impairment in mammals [55]. Several protein isoforms are produced from the *espin* gene, displaying distinct localizations along stereocilia [56]. Interestingly, *forked*, the putative homolog of *espin* in flies, also expresses various protein isoforms [57], albeit their respective distribution within bristles or hairs is not known.

Fascin is required for hearing since DBA/2J deaf mice present a Fascin-2 point mutation, in a region that directly contacts F-actin [58]. Compared to *espin* mutants in which hair defects appear during embryogenesis, stereocilia start to degenerate only after birth in DBA/2J mutants. Fascin-2 normally accumulates at the tip of tallest stereocilia [58], where it impacts on bundle length [59]. The function of vertebrate Fascin is controlled by phosphorylation of a conserved Serine that regulates its bundling properties [60], and this regulation is also occurring in flies for trichome and bristle morphogenesis [61].

In addition to collagens, α -tectorin and β -tectorin, two proteins that contain a zona pellucida domain, are necessary for TM assembly and function. Targeted inactivation of β -tectorin in mice disrupts the TM matrix and thus impairs sound transmission [62]. Mutations in α -tectorin provoke human deafness [63,64], including dominant hearing loss due to a punctual mutation (Y1870C) in the ZPD [64]. Strikingly, engineering a corresponding mutation in α -tectorin in mice [65], or even in the *Drosophila* protein Miniature [31], leads to dominant defects in hearing or epidermal morphogenesis, respectively. Structural studies establish that this mutation affects the conformation of the ZPD and impairs its homo- (and possibly hereto-) dimerization capability [66]. Therefore, despite the rapid evolutionary divergence of ZPD proteins [34], these results support that they exert their function through conserved molecular interactions.

Planar cell polarity determine stereocilia orientation through two components of the Frizzled system, Vangl2 and Scrib, homologs to *Drosophila* Vang Gogh and Scribble, respectively [67]. As observed in flies, PCP components are asymmetrically distributed at the apex of sensory hair cells, and mutant mice for PCP components display altered hair polarity [68,69].

Additional factors required for apical extensions have been uncovered in mammals. For example, Whirlin, scaffolding PDZ protein controls actin polymerization and membrane growth of stereocilia [70,71]. Several unconventional Myosins, which mediate the polarized transport of molecular machines and organelles, are required for stereocilia differentiation [70,72]. In the same vein, Ezrin, Radixin, Moesin (ERM) proteins that regulate the interaction

between actin filaments and the membrane compartment [73] accumulate in growing stereocilia [74]. ERM are highly related proteins, which are often co-expressed within a given tissue. Radixin stays expressed in sensory hair cells after birth, when Ezrin and Moesin expression progressively decreases, and consistently Radixin knocked-out mice display a late onset degeneration of stereocilia [75].

These data illustrate two important views for our understanding of morphological differentiation and its evolution in animals. First, a common theme for the formation of apical extensions is the switch of basic machineries, through the transcriptional control of a battery of various effectors and regulators, showing unexpected similarity between tissues and species. Second, the phenotypical identification of effectors may be hampered by the existence of functional redundancy between paralogs. Cytoskeletal proteins derive from a common set in primitive eukaryotes, and mammalian genomes often encode several paralogs. While diversification of protein sequences is clearly playing a role in the evolution of ZPD proteins for example [34], modifications of gene expression has also contributed to specialized morphological differentiation between tissues, as illustrated by differential expression of the three Fascin [60] and three ERM [75] genes in human.

7. Conclusion

The comparison between terminal differentiation of epithelial derivatives in flies and vertebrates identifies similar regulatory mechanisms for cell specification and common effectors of 3D morphogenesis. Although confirming the importance of cytoskeletal regulators, recent insights into the mechanisms of cell shape changes highlight the importance of different cell compartments, including membrane domains, junction complexes, extracellular matrices etc. A future challenge in understanding morphogenesis is to unravel how these different cellular elements and machineries communicate together, and with the general components of differentiated cells. Moreover these cellular processes are likely influenced by the mechanical constraints [76] exerted by the cytoskeleton, extracellular coats and other external cues [77]. In addition to top-down genetic approaches, bottom-up identification of genes regulated by transcription factors specifying a given morphological state will provide precious information. While independent identification of the same players in different tissues and species plaid in favor of the evolutionary conservation of the mechanisms of epithelial 3D remodeling, it also illustrates a puzzling paradox: how similar mechanisms can produce such diversity in the number per cell, size and shape of apical extensions. It is possible that our current view has been influenced by a will of highlighting common mechanisms. A similar trend has been noticed in the beginning of the so-called evo-devo field, initially focusing on evolutionary conserved mechanisms, but more recently engaged in tackling the question of evolutionary diversification, including that of animal forms. We believe that extensive molecular profiling of differentiated cells, in various model systems amenable to functional studies, may contribute in the next future to a better comprehension of how, besides common mechanisms, the shape of distinct apical extensions is finely tuned to fulfill their specific functions.

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