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BIOLOGY OF MULTICILIATED CELLS

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Abstract

Multiciliated cells (MCCs) are specialized in fluid propulsion through directional beating of myriads of superficial motile cilia, which rest on modified centrioles named basal bodies. MCCs are found throughout metazoans, and serve functions as diverse as feeding and locomotion in marine organisms, as well as mucus clearance, cerebrospinal fluid circulation, and egg transportation in mammals. Impaired MCC differentiation or activity causes diseases characterized by severe chronic airway infections and reduced fertility. Through studies in *Xenopus* and mouse mainly, MCC biology has made significant progress on several fronts in recent years. The gene regulatory network that controls MCC specification and differentiation has been deciphered to a large extent. The enigmatic deuterosomes, which serve as centriole amplification platforms in vertebrate MCCs, have started to be studied at the molecular level. Principles of ciliary beating coordination within and between MCCs have been identified.

Introduction

Multiciliated cells are defined by the presence at their surface of few dozens to hundreds of cilia, which beat coordinately to generate robust polarized hydrodynamic forces (Fig. 1). Each cilium is templated by a basal body (BB), a modified centriole, which harbors two asymmetric appendages, a rootlet that plunges into the cytoplasm, and a basal foot that points in the beating direction (Fig. 1A). Through their interaction with cytoskeletal elements, these appendages link BBs together, thus coordinating cilia orientation [1]. MCCs can be found as individual cells (e.g. cycad plant male gametes or *Paramecium*) or organized into specialized epithelia in metazoans [2]. In small-sized aquatic invertebrate organisms, MCCs are mainly involved in locomotion, whereas in vertebrates, MCCs help propel biological fluids at the surface of specialized tissues. Three main models have helped advancing our knowledge of

fundamental principles of MCC biology, the ciliated epidermis of the *Xenopus* embryo (Fig. 1B-E), the airway mucociliary and the brain ependymal (Fig. 1 B'-E') epithelia of the mouse. Production of efficient directional fluid flows depends on several parameters integrated in time and space during development, as well as during regeneration. First, a correct number of MCCs must be produced, second a correct number of functional cilia must be assembled in each MCC, third cilia must coordinate their beating orientation within individual and between neighboring MCCs to produce robust and regular strokes. In humans, disruption of cilia production or beating in MCCs leads to hydrocephalus, respiratory and fertility pathologies [1]. In this review, we shall focus on recent findings regarding the transcriptional control of MCC differentiation, the mechanisms of centriole synthesis, and the multiscale organization of polarized ciliary beating.

Establishment and maintenance of MCC identity

In all models, MCC fate adoption appears to be triggered by Notch pathway inhibition, which launches a specific gene regulatory network (GRN), whose key nodes are named *Gemc1* (*Gmnc*), *Mcidas* (*Multicilin*), *Myb*, *FoxJ1* and *Rfx2/3* [1] (Fig. 2). BMP pathway inhibition can also turn on the MCC GRN in *Xenopus* and in cultures of human airway epithelium [3]. *Gemc1* and *Mcidas* belong to the Geminin family of nuclear factors, and together with E2F family transcription factors are necessary and sufficient to trigger multiciliogenesis [1,4]. Both *Gemc1* and *Mcidas* can activate *Myb*, which promotes centriole multiplication, as well as *FoxJ1* and *Rfx2/3*, which coordinately control motile ciliogenesis [1,5]. A recent study revealed that in mouse, MCC fate is controlled by *Gemc1*, while *Mcidas* acts downstream to initiate centriole amplification, providing a rationale for the sequential involvement of two related genes that until now appeared to share the same activity [6]. The core MCC GRN has recently been

enriched with additional regulators that branch at distinct points (Fig. 2). Among those, the chromatin factor Trrap appears to function downstream of Notch and upstream of Mcidas in human airway MCCs [7]. Trrap was identified through a pool-based shRNA screen, demonstrating the value of unbiased phenotypic searches to identify additional MCC regulators. The p53 family member, p73, was shown to regulate about 100 ciliary genes, including FoxJ1 and Rfx2/3, and p73 mutant mouse display typical features linked to MCC dysgenesis such as hydrocephalus, sterility and chronic airway inflammation/infection [8,9]. Interestingly, it was suggested that p73 marks some basal cells in airway epithelium for MCC differentiation, lending to it a role as a pioneer or competence factor [8]. Further supporting the importance of p73, it was also reported that it regulates ependymal MCC polarity through maintenance of actin and microtubule cytoskeletal networks [10]. It is important to mention that the MCC identity is inherently labile, as its maintenance requires constant FoxJ1 transcriptional activity, at least in murine ependymal MCCs [11].

Centriole synthesis in MCCs

A key step of MCC differentiation is the large-scale production of centrioles that will subsequently be converted into BBs to template motile cilia. In vertebrate MCCs, it is achieved via two pathways: the parental centriole-dependent pathway, which is analogous to the duplication pathway active during cell cycle, but can produce 10-20 centrioles in MCCs; the deuterosome-dependent pathway, which produces 80-90% of total MCC centrioles [1,12] (Fig. 3). Deuterosomes comprise non-centriolar electron-dense structures that can support the growth of single or multiple procentrioles according to their size, which may increase over differentiation time [13-15]. Despite its initial description in the late 70's, the deuterosome remained virtually unexplored for over 40 years. Recent molecular studies started to unveil

the composition of *Xenopus* and mouse deuterosomes [13-16]. These studies have shown that the architecture of this organelle is based on a set of scaffolding proteins including Deup1 at its core, Pericentrin, γ -tubulin and Cep152 at its periphery. The three latter proteins are well-known components of the pericentriolar material (PCM) [17], which underlines an unexpected link between deuterosome and PCM. Refined mapping of Deup1, Pericentrin and γ -tubulin revealed the existence of nested subdomains in deuterosomes that require further investigation [14]. For instance, it will be important to understand how these domains are assembled and evolve over deuterosome lifetime. In addition, the functional relevance of such organization for deuterosome function remains to be addressed.

Deciphering the sub-cellular origin of the deuterosome may provide interesting cues on how it is built. Questioning the long-held belief that centriole synthesis by deuterosomes occurs *de novo* (i.e. independently from centrosomal centrioles), live imaging of newly synthesized centrioles suggested that in cultured mouse ependymal cells, deuterosomes are seeded by the daughter centrosomal centriole [15]. However, using both chemical and genetic approaches, three independent studies now suggest that formation of deuterosomes and production of multiple centrioles can occur in absence of parental centrioles (Zhao et al., bioRxiv doi.org/10.1101/373662; Nanjundappa et al., bioRxiv doi.org/10.1101/478297; Mercey et al., bioRxiv doi.org/10.1101/503730). Thus, it is unclear whether deuterosomes are seeded by or transit through parental centrioles, which can only be resolved by live imaging of deuterosomes and centrioles in parallel. Seeking for the mechanisms of deuterosome construction, it is interesting to remember that this organelle is the second entity observed during mass centriole production, the first one being fibrous granules (FGs). Apart from early EM studies that described them as electron dense granules that condensate to form deuterosomes, FGs remain marginally characterized [12]. A notable exception is the

identification of PCM1, a specific marker labelling FGs [18], but for which no role in multiciliogenesis could be detected [19]. However, a recent study showed that: i. Deup1 and PCM1 proteins co-localize during the initial steps of MCC differentiation; ii. after MCC transcriptional program is launched, E2F4, a co-activator of Multicilin, shuttles from the nucleus to the cytoplasm where it localizes in the PCM1 positive cloud of material and participates to deuterosome formation [20]. Therefore, further (re-)evaluating the role of FGs during MCC differentiation is likely to provide information about the construction and/or function of the deuterosome.

Imaging centriole and deuterosome markers in real-time and on fixed samples revealed three successive phases: templating of procentrioles, growth of procentrioles and disengagement of mature centrioles from deuterosomes and parental centrioles [13-15,21] (Fig. 3). Molecular regulators and effectors operating in those phases have been identified. A set of major cell cycle regulators including CDK2, CDK1, PLK1 and APC/C control transitions between the different phases [21,22]. Highlighting more detailed mechanistic aspects of the disengagement phase, a recent study reported the existence of a proteolytic cascade involving the Separase protease and CDC20B to release centrioles from deuterosomes [14]. These complementary findings opened large avenues to understand deuterosome-mediated centriole production. For instance, the implication of cell cycle kinases calls for the identification of their targets in MCCs. Furthermore, it would be interesting to determine whether – similar to what happens during the cell cycle [23] – those kinases are counteracted by specific phosphatase activities.

MCC polarized beating, a multi-step and multiscale problem

Multiciliated epithelia are specialized to ensure the vectorial transport of particles or cells along their surface. Their effective function relies on the coordination of ciliary beating within individual cells and at the tissue level. This is achieved through a multistep process involving events occurring both at the sub-micrometer scale and at the scale of the entire organ or organism.

Centrioles newly released in the cytoplasm are transported towards the apical surface. Meanwhile, they are converted into BBs by the addition of rootlet and basal foot appendages [1] (Fig. 3). The precise mechanisms of BB maturation and transport remain poorly understood but seem to implicate acto-myosin and the formation of vesicles at the distal appendages of BBs [24,25].

Once they arrive to their destination, BBs organize at the apical surface. Their distribution varies between different types of MCCs. In *Xenopus* epidermis, BBs are evenly dispersed at the surface of the MCC [26] (Fig. 1E). In contrast, BBs are organized in rows covering the entire apical surface in mouse tracheal MCCs [27]. In mouse ependymal MCCs, BBs are clustered in an off-centered patch, through a process called translational polarity (Fig. 1D'). Within this patch, BBs are organized in rows [28,29] (Fig. 1E'). Whether such variations in BB organization underlie functional differences remains to be determined. In all types of MCCs, BBs of a given cell are oriented in the same direction. This peculiar organization, referred to as rotational polarity, is essential to ensure the coordinated beating of cilia within individual MCCs.

Both distribution and orientation of BBs relies on their tight interaction with apical cytoskeletal elements that differentially contribute to the geometry of the array. Cortical actin in MCCs is organized in two structurally distinct apical and sub-apical networks [26,27,30,31]. The apical pool is necessary for docking of BBs to the cell membrane [24,26]. The sub-apical network connects BBs and is necessary for their correct spacing [26,32]. Microtubules

connecting BBs play an instrumental role in their alignment and orientation [26,27]. Finally, a network of intermediate filaments is also observed unsheathing BBs, although its precise functional contribution remains to be determined [27,31].

Interplay between those cytoskeletal elements and their interacting proteins results in the BB organization described above [33-37]. Among those, some unexpected proteins were identified. For instance, FAK, Paxillin and Vinculin - well-known focal adhesion proteins - associate with BBs and their striated rootlet to form ciliary adhesion complexes that connect BBs to the actin cytoskeleton [34,35]. Likewise, the well-characterized chromatin modifier WDR5 acts as a scaffolding protein binding to BBs and modulating apical actin in MCCs [37]. Beyond its role in BB organization, the apical cytoskeleton of MCCs is thought to be important for propagation of the metachronal wave of ciliary beating [26]. It also confers a mechanical resistance to BBs to sustain the shear stress imposed by ciliary beating in mature MCCs [30,38]. Ciliary activity itself provides an important feedback on BB organization. Although initially imperfectly coordinated, cilia beating instructs weak directional flow that improves coordination of BB polarity, which in turn reinforces the flow [39,40]. However, the precise range of action of such hydrodynamic feedback remains to be evaluated. In addition, cilia motility is necessary for apical actin meshwork assembly, thereby reinforcing BB anchoring at the apical surface [30].

To generate a directional fluid flow and sustain the correct function of the organ, ciliary beating must be coordinated at the tissue-level. This is achieved through a planar cell polarity driven multistep process [29,41-44]. The initial cue that establishes organ-level polarity remains unknown in mouse ciliated epithelia. In contrast, it was shown that the mechanical strain transmitted from the mesoderm to the ectoderm during *Xenopus* gastrulation caused alignment of microtubule arrays along the rostro-caudal axis, which secondarily led to PCP

protein asymmetric distribution along the same axis. Through this global polarization of the epithelium, individual MCCs acquire polarized membrane identity. This generates a compass in each MCC that is used to orient the BB array, thereby allowing cilia beating direction to align with global tissue polarity [29,43,45]. Unexpectedly, it was reported that maintenance of tissue-level polarity in airways requires the presence of a sufficient number of MCCs for cell-cell transmission of PCP polarization [46]. Consequently, diseases that cause loss of MCCs, such as cystic fibrosis, are characterized by poor tissue polarity, which may worsen mucociliary clearance [46].

While tissue- and cell-level scales of organization start to be well described [41], sub-micrometer scale events remains to be understood. For instance, structural and molecular chirality of BBs need to be resolved at very high resolution to understand their function (N'Guyen et al., [biorXIV doi.org/10.1101/487330](https://doi.org/10.1101/487330)). Since BB structural organization varies between different types of MCCs, such studies will also help to understand functional differences between MCCs. At the opposite end of the spectrum, MCC activity needs to be integrated at the millimeter-scale to generate effective flows. Here, mechanical interactions between cilia and the fluid they propel become highly significant [47]. To fully understand ciliated epithelium biology, future studies should aim at bridging sub-micrometer to millimeter scales, and at integrating molecular, structural and mechanical dimensions.

Conclusions

The field of MCC biology has advanced at fast pace in recent years. However, beyond the findings described above and the questions they open, many issues remain to be addressed.

A first problem regards the degree of universality of the principles identified so far. For instance, it is unclear whether MCCs in invertebrates use deuterosome-like structures to amplify their centrioles. In opposition to this idea, *Mcidas* and *Deup1* genes appear to be absent in invertebrate genomes. This issue may receive light from planarians, which lack centrosomes, but make full-fledged epidermal MCCs required for locomotion [48](Thi-Kim et al., bioRxiv doi.org/10.1101/324822). On a different note, the various steps driving centriole biogenesis were reported to be highly synchronous in individual cultured mouse ependymal MCCs [15,21]. It is unclear whether such synchrony applies to all types of MCCs, or reflects the peculiar organization of ependymal cilia into tufts of relatively constant size.

In future, it will be important to address the architecture and the biophysical nature of the deuterosome. Compartmentalization is used by cells to concentrate biochemical reactions. In most organelles, biochemical reactions are constrained by a membrane. Alternatively, organelles that lack delimiting membrane such as the PCM are formed through complex phase-separation mechanisms to create small volumes for specific biochemical reactions in the cytoplasm [49]. Such liquid-like organelles have recently been reported in MCCs to serve as platforms of assembly of axonemal dynein arms to ensure ciliary beating [50]. It is tempting to speculate that based on its shared features with the PCM, the deuterosome may also comprise a phase-separated organelle, which would concentrate elementary parts and enzymes to build up multiple centrioles.

An overarching question in the field concerns the control of the final number of centrioles present in individual MCCs. Although this number can be highly variable in *Xenopus* epidermal MCCs or mouse tracheal MCCs, and less so in mouse ependymal MCCs, it always scales to the apical area of the cell (unpublished results; Nanjundappa et al., bioRxiv doi.org/10.1101/478297). Understanding such scaling represents a challenging but fascinating

question, with fundamental as well as biomedical implications, for instance to better fight cancer cells, which are often characterized by excess number of centrioles.

It has become clear that centriole synthesis during the cell cycle and in MCCs share many molecular and regulatory principles. This leads to the idea that centriole duplication on the one hand and centriole mass production on the other hand are indeed extreme cases of one and the same biological process: centriole biogenesis. The question then becomes: what variations shape those pathways such that only one or hundreds of centrioles are produced, and can such variations account for centriole over-duplication in cancer ?

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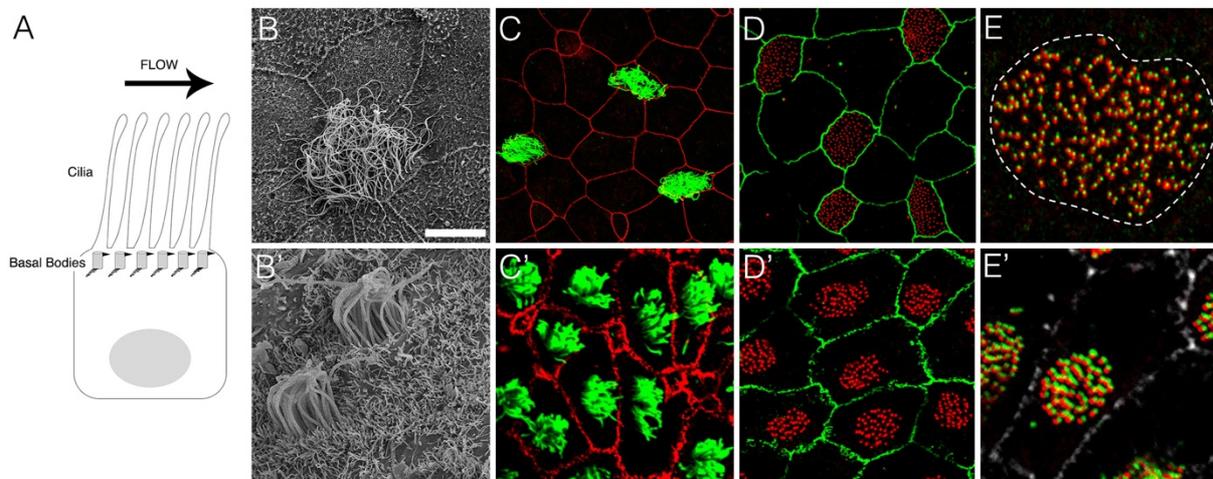


Figure 1: Multiciliated cells in *Xenopus* epidermis and mouse ependyma

(A) Scheme depicting MCC organization. (B, B') Scanning Electron Microscope micrographs of MCCs from *Xenopus* epidermis (B) and mouse ependyma (B'). (C-E') Confocal pictures of fluorescent staining on *Xenopus* epidermis (C-E) and mouse ependyma (C'-E'). (C, C') Staining for the junction marker ZO1 (red) and the cilia marker acetylated- α -tubulin (green). (D, D') Staining for the junction marker ZO1 (green) and BB markers centrin (D) and FOP (D') (red). (E, E') Zoom on individual MCCs stained with centriole marker centrin (E) or FOP (E') and basal foot marker γ -tubulin (red). Scale bar: 10 μ m (B), 25 μ m (C, D), 5 μ m (E, F, I), 2,5 μ m (G, H).

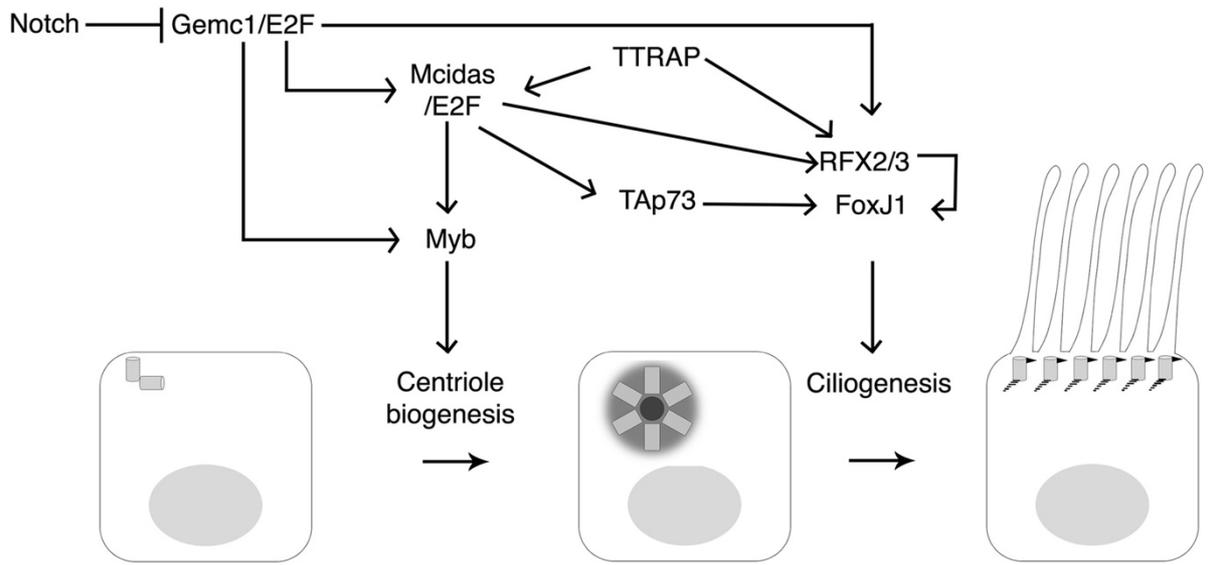


Figure 2: Gene regulatory network at play during MCC differentiation

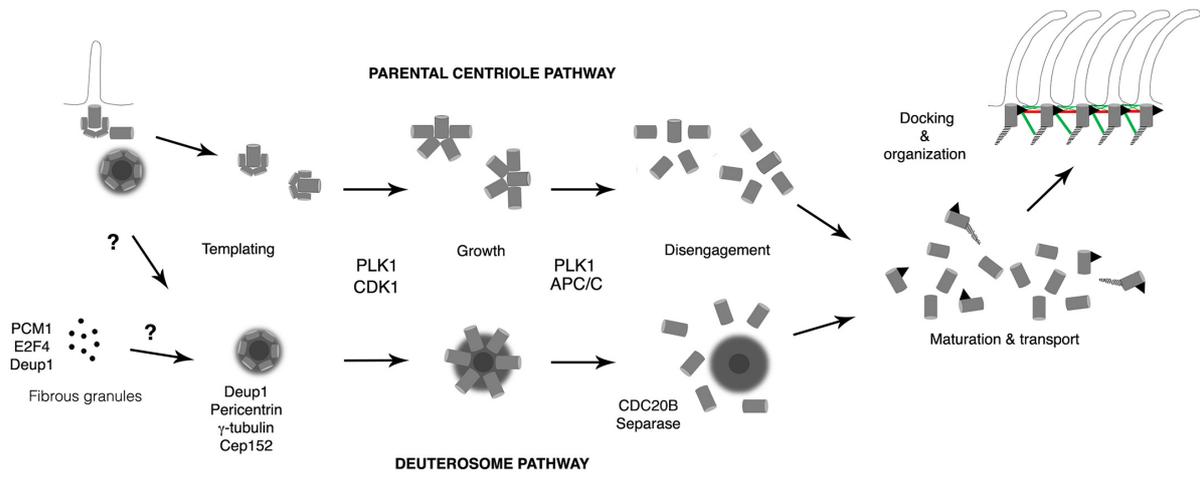


Figure 3: Centriole production and maturation in MCCs

The authors declare no conflict of interest.

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