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Abbreviations used: BB, basal body; CC, connecting cilium; FP: flagellar pocket; IFT, intra flagellar transport; PC: primary cilium.

Abstract:
The primary cilium (PC) is present on most cell types in both developing and adult tissues in vertebrates. Despite multiple reports in the sixties, the PC was almost forgotten for decades by most of the cell biology community, mainly because its function appeared enigmatic. This situation changed ten years ago with the key discovery that this fascinating structure is the missing link between complex genetic diseases and key signalling pathways during development and tissue homeostasis. A similar misfortune might have happened to an original membrane domain found at the base of PC in most cell types and recently termed the “ciliary pocket”. A morphologically-related structure has also been described at the connecting cilium of photoreceptors and at the flagellum in spermatids. Its organization is also reminiscent of the flagellar pocket, a plasma membrane invagination specialized in uptake and secretion encountered in Kinetoplastid protozoa. The exact function of the ciliary pocket remains to be established but the recent observation of endocytic activity coupled to the fact that vesicular trafficking plays important roles during ciliogenesis brought excitement in the ciliary community. Here, we have tried to decipher what this highly conserved membrane domain could tell us about the function and/or biogenesis of the associated cilium.

Introduction:

Cilia and flagella (Box 1) are highly conserved structures among eukaryotes where they are involved in essential functions including cell locomotion, fluid movements and extracellular environment sensing. They are assembled from the centrosome which associates with the plasma membrane through distal appendages of the mother centriole which then forms the basal body (BB, Fig.1). Elongation of the microtubule doublets forming the "9+0" or "9+2" axonemes and selective import of ciliary proteins in the ciliary membrane are mediated by a highly conserved process called intraflagellar transport or IFT (Kozminski et al., 1993). IFT particles are protein complexes that are transported along the axoneme from the BB region to the tip by kinesin anterograde motor(s) and recycled to the BB by dynein retrograde motor(s) (review in (Satir and Christensen, 2007; Pedersen and Rosenbaum, 2008)) The IFT machinery is thought to select cargoes in the cytoplasm and then to access the cilium/flagellum after having crossed a nuclear pore-like region formed by the transition fibres and the transition zone (Box 2), by a mechanism that has been unveiled recently (Dishinger et al., 2010).

The "9+0" primary cilium (PC) is conserved among vertebrates and found in most quiescent or differentiated cells. It is usually described as a non-motile cellular antenna at the cell surface that controls key signalling pathways through sensing mechanical stresses or molecules present within the extra-cellular milieu. Its crucial role has been highlighted by the identification of the genes mutated in genetic disorders such as polycystic kidney diseases, nephronophtisis, and Meckel-Gruber, Joubert or Bardet-Biedl syndromes. The mutated genes all encode proteins that are localized to PC or its BB. The complexity and diversity of the observed phenotypes in patients suffering from Bardet-Biedl syndrome, including cystic kidney, progressive blindness and obesity, illustrates the diversity of processes controlled by PC. The signalling functions of PC are mediated by several receptors or transmembrane proteins specifically targeted to cilia, including G protein coupled receptors involved in light or odour sensing, the molecular machinery associated to the Sonic hedgehog (Shh) pathway, and polycystins involved in flux sensing (review in (Berbari et al., 2009; Lancaster and Gleeson, 2009)).

This general scheme of PC function emerged from initial studies carried out in the green algae *Chlamydomonas* that identified *IFT88,* the first gene encoding an IFT protein (Pazour et al., 2000). It turned out to be the gene mutated in a mouse model for polycystic disease (Tg737 mice) and led to the elucidation of the role of the PC in kidney epithelial cells (review in (Pazour and Rosenbaum, 2002)) where it controls tubules formation (planar polarity) through sensing of the urine flux (review in (Fischer and Pontoglio, 2009)). In these cells, the whole length of the PC is present within the lumen of tubules ((Latta et al., 1961); Fig.1).

However, since the sixties, multiple transmission electron microscopy (TEM) studies showed that the positioning of PC and the membrane organization at their base are very different in most of the examined cell types. In fact, PCs were rather described as being partially intracellular, found within an invagination of the plasma membrane surrounding the proximal part of the cilium (Munger, 1958; Barnes, 1961; Sorokin, 1962; Sorokin, 1968). The invaginated plasma membrane was called the ciliary sheath and the ciliary membrane was defined as the ciliary shaft (Barnes, 1961; Sorokin, 1962). These initial observations made in chick, mouse and rat developing tissues were extended to most examined cell types (see reference therein and Fig.S1). This difference in the membrane organization at the base of the PC could correspond to distinct functional properties or could be the result of different ciliogenesis pathways among cell types.

Here, we are focussing on the description of this intriguing membrane domain and discussing its possible functions in relation with the PC. The pocket adopts distinct configurations according to the type of cilia and shows fascinating structural and functional relationship with the flagellar pocket, a similar membrane domain found at the base of the flagellum of Trypanosomatids.

The ciliary pocket: a morphological definition

Trypanosomatids, e.g *Trypanosoma* or *Leishmania,* are protozoan parasites mostly known as responsible for several tropical diseases such as sleeping sickness, Chagas disease or kala-azar. Moreover, they have become a popular model to study ciliogenesis and flagellum motility, especially to identify and to investigate genes associated to primary ciliary dyskinesia (Baron et al., 2007; Duquesnoy et al., 2009). The flagellar pocket (FP) is an invagination of the plasma membrane in which the flagellum is deeply rooted ((Langreth and Balber, 1975); Fig.2)). The FP is a very important structure for Trypanosomatids since it is the unique site of endocytosis and exocytosis (secretion and recycling), as vesicles cannot fuse to or bud from the rest of the plasma membrane due to the presence of a dense sub-pellicular microtubule corset (Sherwin and Gull, 1989). The top of the pocket is marked by the presence of the flagellar pocket collar, a highly differentiated cytoskeleton and membrane-associated structure that forms a diffusion barrier (review in (Landfear and Ignatushchenko, 2001; Field and Carrington, 2009).

Several groups recently characterized a similar membrane domain at the base of PC and called it the “ciliary pocket” (Molla-Herman et al., 2010; Rohatgi and Snell, 2010) or “cilium pit” (Rattner et al., 2010). The term “ciliary pocket” was of course chosen because of the morphological similarity with the FP (compare Figs. 1 and 2). Indeed, as explained above, the PC of many different cell types were found partially “intracellular” with the BB docked at the cytoplasmic end of an invagination of the plasma membrane ((Barnes, 1961; Sorokin, 1962); Fig.S1; Fig. 1, C and D). The proximal region of the cilium is therefore surrounded by the membrane of the pocket that adopts a bubbling shape and which, in most cases, tends to rapidly narrow closer to the ciliary membrane (Fig. 1D, blue). The length of the pocket varies between different cell types ((Barnes, 1961; Sorokin, 1962) and our unpublished observations) but the distal part of the cilium usually emerges in the extracellular milieu (Fig.1D). In most cases, the overall structure is symmetric relative to the axis defined by the BB and the axoneme (also see below).

The organization of the pocket was further characterized in primary cells or in cultured model cell lines including hTERT-RPE1 cells, one of the most common cellular models for ciliogenesis (Molla-Herman et al., 2010; Rohatgi and Snell, 2010; Rattner et al., 2010). We more precisely defined the ciliary pocket as the membrane domain going from the docked BB to the region where the axoneme emerges in the extracellular milieu ((Molla-Herman et al., 2010); Fig.1, blue). The ciliary pocket (Fig.1D, blue) is therefore a sub-domain of the plasma membrane that surrounds the proximal part of the cilium. It is continuous with, but functionally distinct, from the ciliary membrane (Fig.1D, red) thanks to a diffusion barrier that limits membrane and protein exchanges and likely corresponds to the ciliary necklace (Fig.1D, green; Box 2).

This definition differs a little bit from the one of the FP which is limited to the “bubbling” part of the membrane proximal to the BB. Indeed, there is no evidence for the presence of a structure similar to the flagellar pocket collar in mammalian cells (Molla-Herman et al., 2010), and BILBO-1, the protein that is required for the formation of this structure, is specific to Trypanosomatids (Bonhivers et al., 2008). In addition, in mammalian cells, endocytic structures were identified all along the ciliary pocket (Molla-Herman et al., 2010; Rattner et al., 2010), indicating that the ciliary pocket does form a homogenous functional domain from the BB to the cilium exit site.

A possibly related membrane domain has been reported at the connecting cilium (CC) of photoreceptor cells. At the apical face of the inner segment, the CC corresponds to the proximal part of a modified PC (De Robertis and Lasansky, 1958) which adopts all the characteristics of an elongated ciliary necklace/transition zone ((Rohlich, 1975); Box 2). The distal part of the modified PC is organized as membrane stacks enriched in rhodopsin and is involved in light sensing (review in (Insinna and Besharse, 2008)). In contrast with the pocket found at PC, this microdomain looks asymmetric, since the membrane of the inner segment preferentially interacts with one “side” of the CC (De Robertis and Lasansky, 1958; Tokuyasu and Yamada, 1959; Watanabe et al., 1999). Moreover, a ciliary pocket is also present at the base of motile cilia of ependymal cells (Brightman and Palay, 1963; Molla-Herman et al.,

2010) and at the base of the flagellum in elongating spermatids (Fawcett and Ito, 1958; Molla-Herman et al., 2010), highlighting its presence at the base of all main cilia subtypes.

Biogenesis of the ciliary pocket:
In the case of the FP of Trypanosomatids, the new flagellum is assembled from the pro-basal body that matures upon cell cycle progression and directly docks onto the pre-existing pocket. The new flagellum (asterisks on the corresponding BB) elongates into this pocket (Fig.2, D1-4) but curiously it is found in an anterior position relative to the old one and then needs to rotate before it can be associated to the new FP (Lacomble et al., 2010). In the absence of a fully elongated new flagellum resulting from the inhibition of IFT, a smaller FP can be formed. However, it displays dramatically reduced endocytic activity (Absalon et al., 2008). This indicates a close dialogue between flagellum elongation and FP formation.

Even if growth of PC axoneme also likely controls elongation of the ciliary pocket (see below), the situation is different for mammalian cilia that are assembled *de novo* from the centrosome and in the absence of a pre-existing pocket. Despite a similar morphology, the pockets present at the base of many PC, of the CC or of the flagellum of elongating spermatids could be generated by different mechanisms.

The ciliary pocket as a consequence of the intracellular ciliogenesis pathway:
In his key analysis of "primary ciliogenesis", Sorokin proposed two distinct pathways depending on the cell type (Sorokin, 1962; Sorokin, 1968). First, in epithelial cells in the developing lung, the centrosome would migrate toward the apex of the cell and dock to the cytoplasmic leaflet of the apical plasma membrane. The cilium grows then from this docked BB directly in the extracellular milieu without forming or associating to a ciliary pocket (Fig.1A). In non-epithelial cells (fibroblasts, smooth muscle cells), the centrosome first docks onto a cytoplasmic vesicle (the primary ciliary vesicle) of uncharacterized origin and the cilium grows from the docked BB inside the vesicle which then elongates with the help of incoming vesicles (secondary ciliary vesicles). The distal part of the cilium finally emerges in the extracellular milieu after docking and fusion of the elongated vesicle with the plasma membrane (Fig.1B).

Even if the models proposed by Sorokin 40 years ago remain to be definitively proved by complementary approaches, they are still widely accepted and are used as a framework for further studies (review in (Barr, 2008)). The absence of a ciliary pocket at the base of PC in kidney tubules epithelial cells (Latta et al., 1961) is in agreement with a direct docking of the BB to the apical plasma membrane during ciliogenesis in this cell type. However, the fact that some ciliary pocket-associated PC could be found in kidney cells *in vitro* (Molla-Herman et al., 2010) suggested that ciliogenesis can also follow the intracellular pathway in these cells. The absence of a pocket in most individual cells could be the consequence of short-lived intracellular stages followed by complete emergence of the cilium at the cell surface resulting from the full release of the cilium from its pocket (Fig.1B'). Such a possibility remains open since, to our knowledge, detailed TEM analysis of ciliogenesis in kidney tubules epithelial cells is still lacking. In addition, even if this was not indicated in his model, Sorokin did mention in the text that he observed docking of vesicles to the BB and small pocket-like structures at the base of forming PC in lung epithelial cells (Sorokin, 1968). These observations are in agreement with the existence of a unique ciliogenesis pathway that would always start by the "intracellular" mode followed by different kinetics in the complete or partial emergence of the cilium at the cell surface.

The assembly of motile cilia in multi-ciliated cells follows a more complicated process as basal bodies are first amplified in the cytoplasm of differentiating cells through the so called "acentriolar" pathway. Then the dozens of neo-formed basal bodies dock to cytoplasmic vesicles before their fusion to the apical plasma membrane (Sorokin, 1968). Data obtained recently in ciliated cells of the frog epidermis support Sorokin's model of a vesicular intermediate during the ciliogenesis of motile cilia (Park et al., 2008). The presence of ciliary pockets at the base of motile cilia of ependymal cells (Brightman and Palay, 1963; Molla-Herman et al., 2010) is also in agreement with vesicular intermediates during

ciliogenesis in this specific cell type. The fact that pockets were not found at the base of other cells presenting motile cilia is again likely linked to differences in kinetics and final complete release of cilia from their pocket, similarly as for PC (see above). The identification of markers for the vesicles that dock to the basal bodies such as the exocyst component sec8 (Park et al., 2008) should help to characterize such short lived stages of ciliogenesis.

The ciliary pocket as post-ciliogenesis events:

The ciliary pocket could alternatively be formed as a consequence of inward movement of the BB toward the nucleus (Sotelo and Trujillo-Cenoz, 1958). This hypothesis cannot be formally ruled out in the case of PC and could account for the formation of ciliary pockets at the base of the flagellum of elongating spermatids. In addition to this BB retraction model, the formation of the CC pocket of photoreceptor cells likely occurs as a consequence of complex membrane remodelling of the apical surface during differentiation of both inner and outer segments.

The sperm flagellum

The initial assembly steps of the sperm tail (flagellum) take place in round spermatids but are not well described in vertebrates and especially in mammals, where studies mainly focused on the formation of the acrosome or on later steps of tail development. Depending on the authors and the cell types investigated, the axoneme is thought to either grow from the BB in the cytoplasm or directly from the BB docked to the plasma membrane. In addition, even in the case of cytoplasmic intermediates, it is not clear if the flagellum grows directly in the cytoplasm or within a vesicular compartment. Interestingly, data obtained in the water mold *Allomyces arbusculus* (Renaud and Swift, 1964) as well as in insects (Phillips, 1970) provide strong evidences indicating that the flagellum is assembled within a vesicular structure, following a pathway very similar to the intracellular pathway described above (discussed in (Renaud and Swift, 1964)).

In currently accepted schemes of spermiogenesis in vertebrates (Fawcett and Bedford, 1979), the flagellum first fully emerges at the surface of round spermatids (Fig.3, A1). At later stages, in elongating spermatids (Fig.3, A2-4), the proximal part of the flagellum is found within a micro-environment that has been described as an invagination of the plasma membrane and called the “flagellar canal” (Fawcett et al., 1971). The pocket-like domain becomes longer during maturation of spermatids (Fig.3, A3-4) but is finally resorbed (Fig.3, A5-6). The base of the pocket on which the future annulus is docked, detaches from the distal centriole (Fig.3, A5) and migrates toward the distal end of the flagellum (Fig.3, A6) by poorly characterized mechanisms. This region will form the annulus, a septin-based diffusion barrier that separates the mid piece, where mitochondria surround the axoneme, from the distal end of the flagellum (review in (Caudron and Barral, 2009)). The pocket at the base of the flagellum is therefore a transient structure during spermiogenesis. Its generation is not linked to the ciliogenesis pathway but likely results from two concomitant events (Discussed in (Burgos and Fawcett, 1956; Fawcett et al., 1971)), including migration of the BB toward the nucleus (Fig.3, A2; green arrow) and caudal extension of the cell body during elongation of spermatids (Fig.3, A2-3; gray arrows).

The connecting cilium

Ciliogenesis in photoreceptor cells remain poorly characterized but evidences indicate that the CC is assembled in the cytoplasm similarly as PC in fibroblasts with images indicating the presence of a pocket at the base of the CC once it has reached the apical membrane that will become the inner segment (Greiner et al., 1981). Thereafter, the CC emerges at the apical surface in a configuration very similar to the one found for PC in kidney cells (Fig.3, B1), with the exception of its distal region which enlarges and differentiates to form the outer segment (De Robertis and Lasansky, 1958; Galbavy and Olson, 1979). At later stages, when the outer segment is mature (Fig.3, B4), a ciliary pocket-like domain is present at the base of the CC (De Robertis and Lasansky, 1958; Tokuyasu and Yamada, 1959; Watanabe et al., 1999). This domain was first call the periciliary ridge complex in the frog (Peters et al., 1983)

and, due to structural differences (Watanabe et al., 1999), the periciliary complex (Maerker et al., 2008) or periciliary membrane complex (Yang et al., 2010) in mammals. The precise mechanisms by which this pocket-like structure is formed are not established but it is usually assumed that the extension of the inner segment apical membrane toward the developing outer segment may favour its contact with the CC and further organizes this domain (Fig.3, B1-4). In addition, the fact that the CC is positioned laterally at one “edge” of the inner segment might explain the fact that this pocket is asymmetric, interacting preferentially with one side of the CC contacting the inner segment membrane (Fig.3, B4).

Interestingly, proteins implicated in the Usher syndrome, a deafness and blindness disorder, were recently involved in the biogenesis of the CC-associated pocket. Usher proteins and their partners form a protein network including both transmembrane and cytoplasmic proteins, which was first characterized for its role in hair cells, in the formation of filamentous structures which links the kinocilium, another modified cilium, to the neighbouring stereocilia (review in (Saihan et al., 2009)). In photoreceptor cells, proteins of this network are localized either in the CC (myosin VIIa and RPGR) or at the region of the apical inner segment membrane that faces the CC (VLGR1, Usherin, whirlin and SANS) (Liu et al., 2007; Maerker et al., 2008; Yang et al., 2010) where the transmembrane proteins VLGR1 and Usherin likely form the meshwork of filaments described in the lumen of this partially open pocket (Watanabe et al., 1999; Maerker et al., 2008) (Fig.3, B4). The functional loss of these proteins results in the disorganization of the periciliary region and outer segment degeneration (Liu et al., 2007; Maerker et al., 2008; Yang et al., 2010), showing that they are implicated in the formation and/or stabilization of this post-ciliogenesis-formed pocket.

Interestingly, the organization of the periciliary membrane region resembled that of trypanosome flagellum which asymmetrically interacts with the cell surface through a specialized structure found at the cell body side and called the flagellum attachment zone (Kohl and Gull, 1998). This structure is specific of Trypanosomatids and is crucial for the global organization of the organism, including morphogenesis and cytokinesis (Kohl et al., 2003). The Usher protein network might play a similar function in cell organisation relative to the cilium (compare Figs. 2A and 3B). Finally, whether Usher proteins are implicated in the organization/stabilization of other cilia-associated pockets is an open question. Indeed, physical interactions between the pocket membrane and that of the cilium might impede complete release of cilia in the extracellular milieu and therefore stabilise the pocket. Therefore, differential tissue expression patterns or subcellular localizations of these proteins can explain the presence or absence, or differential aspect of cilia-associated pockets.

Function(s) for the ciliary pocket?

Despite its presence at PC in most cell types, the possible functions of the ciliary pocket remained to be clarified. Several data obtained in trypanosomes, cultured cell lines and photoreceptor cells suggest that the pocket may act as a cilium-associated platform for both in and out vesicular trafficking and for interactions with the actin-based cytoskeleton.

An endocytic membrane domain?

The presence of clathrin-coated pits is one of the most striking features of the ciliary pocket (Fonte et al., 1971; Poole et al., 1985; Haycraft et al., 2005; Molla-Herman et al., 2010; Rattner et al., 2010), whose functionality has been demonstrated recently (Molla-Herman et al., 2010), indicating that the ciliary pocket is a specialized endocytic membrane domain (Fig.1, C-E). The link between cilia and endocytosis appears to be a conserved feature among many ciliated organisms. For example, the FP of Trypanosomatids is even the unique site for endocytosis and exocytosis activity, presumably because the presence of a dense sub-pellicular network of microtubules underlying the rest of the plasma membrane prevents vesicle fusion or budding (review in (Landfear and Ignatushchenko, 2001; Field and Carrington, 2009)). In *Paramecium* and *Tetrahymena*, pinocytosis is also mediated by clathrin-coated pits that correspond to the parasomal sacs described close to the base of cilia (Allen, 1969). These structures do correspond to coated pits that are not randomly distributed at the cell surface, with a unique pit localized very close to each BB (Nilsson and

van Deurs, 1983; Allen et al., 1992; Wiejak et al., 2004; Elde et al., 2005). Finally, even if the endocytic pathway of *Chlamydomonas* has not been characterized, clathrin localizes at the base of flagella where internalization occurs (J.Z. Rappoport, personal communication).

Therefore, clathrin-mediated endocytosis is restricted to the plasma membrane region close to cilia and flagella in numerous protists. However, the situation is different in many cell types of metazoan species: even if coated pits are easily detected at the ciliary pocket and appear to be enriched compared to the rest of the plasma membrane, the total endocytic capacity of this micro-domain remains minor relative to the rest of the cell surface (Molla-Herman et al., 2010). Hence, the ciliary pocket is certainly not the preferential site for global endocytosis. We propose that the pocket-associated endocytic activity could be specifically involved in the internalization of ciliary components during either recycling of membrane proteins and lipids or removal of excess incoming material (see below). Such a pathway could also be used to clear the excess of ciliary membrane proteins that were not properly targeted to cilia or to restrict the access of specific plasma membrane proteins to the pocket. While pocket-associated coated pits are able to internalize transferrin (Molla-Herman et al., 2010), a widely used marker of clathrin-dependent internalization, it still remains to be determined whether they are involved in the internalization of specific cargoes. Recent evidences indicate that ciliary proteins undergo very slow exchanges, if any, with the rest of the cell (Hu et al., 2010), what might explain the difficulty to observe internalization events of cilia-associated membrane proteins (Smoothened or somatostatin type 3 receptor, our unpublished data). The identification of the cargoes internalized at the ciliary pocket would definitively contribute to a better understanding of its endocytic function.

Endocytic profiles were detected at the ciliary pocket of motile cilia of ependymal cells and at the flagellum of mice spermatids (Molla-Herman et al., 2010), also suggesting internalization events. In the case of spermatids, it has been shown that vesicles containing an internalized marker from the surface (cationic ferritin) surround the proximal part of the flagellum, suggesting that this region is an active site of endocytosis and/or that it receives internalized material through polarized recycling (Segretain and Roussel, 1988; Segretain, 1989). Finally, in the case of photoreceptor cells, evidence for active endocytosis at the periciliary membrane is still lacking.

An exocytic membrane domain?

In trypanosomes, the FP is the unique site of secretion and recycling from endosomes (Field and Carrington, 2009). In cells bearing a PC, the BB also exerts the classical functions of the centrosome. The pocket is at a unique position since it is very close to the anchoring site of microtubules (Fig.1F), ie subdistal appendages of the BB, what could allow easy and specific targeting of vesicles that are transported by the cytoplasmic dynein complex. The Golgi apparatus and the endocytic recycling compartment are positioned around the BB (Fig. 1G and see for example: (Barnes, 1961; Poole et al., 1997; Follit et al., 2006; Yoshimura et al., 2007) and other populations of endosomes as well as lysosomes have been localized close to the ciliary pocket (Segretain, 1989; Rattner et al., 2010).

Recent reviews focused on the comparison between the PC environment and the immune synapse, a specialized structure formed in T lymphocytes upon interaction with antigen bearing target cells and involved in signalling functions as well as polarized secretion of cytolytic granules (Baldari and Rosenbaum, 2010; Griffiths et al., 2010). This comparison was initially raised by the fact that the IFT machinery is implicated in polarized recycling of the T cell antigen receptor to the immune synapse (Finetti et al., 2009). It was also sustained by the observation that during immune synapse formation, the centrosome migrates to a position just underneath the synapse (Stinchcombe et al., 2006), resembling a frustrated ciliogenesis event, a fascinating observation when one keeps in mind that T lymphocytes were thought to be one of the rare cell types unable to build a PC. These data support the proposal that the PC could be involved in polarized secretion events. This hypothesis was mainly suggested from studies in connective tissues. In both chondrocytes and tenocytes (cells found within tendons), PC are aligned along the axis of the matrix they are in (Poole et al., 1985; Poole et al., 1997; de Andrea et al., 2010; Donnelly et al., 2010), suggesting that

they would be implicated in the polarized secretion of extracellular matrix components. In this context, the ciliary pocket could act as a docking platform for secretory vesicles whose contents could be released in the pocket lumen before reaching the extracellular milieu, acting then like a reservoir (Poole et al., 1985). The latter proposal could explain the presence of ciliary pockets of variable diameter depending on the cell types (Poole et al., 1985; Molla-Herman et al., 2010).

In addition to secretion events, the ciliary pocket may act as a preferential site for docking and fusion of vesicles transporting ciliary membrane proteins. While this hypothesis remains to be tested for PC, evidences obtained in photoreceptor cells clearly indicate that the periciliary region acts as a preferential site for docking of Golgi-derived vesicles (Tai et al., 1999) associated to IFT proteins such as IFT20 and IFT52 (Sedmak and Wolfrum, 2010) and likely involved in the transport of cargoes en route for the outer segment. Even if there is strong evidences for a specific role of the ciliary pocket in the docking and fusion of vesicles containing cargoes for cilia, it has to be stressed that such events could occur at cilia that do not present a pocket, in a specific region of the plasma membrane found at the base of PC (review in (Nachury et al., 2010)).

Possible interactions with the actin-based cytoskeleton

Both actin and microtubules have been implicated in specific and distinct functions at the FP of trypanosome. Disruption of actin leads to an enlargement of the FP (Garcia-Salcedo et al., 2004), a phenotype similar to clathrin knock-down (Allen et al., 2003) and in agreement with a conserved role for actin polymerisation in endocytosis (review in (Lanzetti, 2007)). However, visualization of a clear actin network has so far not been possible. Tomography studies revealed the presence of an array of 4 microtubules at the inner face of the FP, that appears to define a channel in which ligands en route from the cell surface could be visualized (Gadelha et al., 2009). Whilst a functional validation of the role of these four microtubules is yet to be provided, microtubules appear to be tightly associated to the cytoplasmic surface of the FP (Lacomble et al., 2010).

The presence of microtubules along the cytoplasmic side of the ciliary pocket has been reported in some (Rattner et al., 2010) but not all cell types ((Molla-Herman et al., 2010); Fig.1F). Therefore, the functional relationship between the ciliary pocket and the microtubule-based cytoskeleton remains to be better characterized. In contrast, data obtained in different cell types showed that the actin-based cytoskeleton does establish specific contacts with the cilium via the ciliary pocket (Molla-Herman et al., 2010; Rattner et al., 2010)).

Actin cables formed by arrays of actin filaments were found docked at the inner face of the pocket in various cell types, including those with motile cilia (Molla-Herman et al., 2010; Rattner et al., 2010). Such a specific actin organization could fulfil at least three functions. First, it could act as a structural element involved in forming and maintaining the local environment of the ciliary pocket. Disruption of actin filaments in cultured cells results in modification of the pocket's shape that becomes wider, with loss of the tight contacts between the ciliary and the pocket membranes ((Rattner et al., 2010), our unpublished data). Second, an actin network could participate to vesicle trafficking to and from the pocket, in association with the endocytic and exocytic activities rehearsed above. In the case of spermatids, local actin organization has not been reported but the base of the pocket serves as a docking site for the future annulus that contains septins (Ihara et al., 2005; Kissel et al., 2005), proteins that also interact with actin (review in (Caudron and Barral, 2009)). Third, local actin dynamics could be associated to new sensory function. Live cell analysis revealed two modes of actin dynamics at the ciliary pocket: docking of static actin cables, mostly at the proximal region, and dynamic sites of transient polymerisation at the distal part. Intriguingly, actin polymerization events corresponded to deformation of the distal region of the cilium, without apparent movement of the BB area (Molla-Herman et al., 2010). Such events could be sensed by PC as mechanical stress and could lead to signal transduction, a function suggested for PC in connective tissues (Poole et al., 1985; Poole et al., 1997).

In multi-ciliated cells, the actin-based cytoskeleton has been implicated in the migration of BBs to the apical membrane and in cilia positioning at the apical surface. The mechanisms by which actin controls BBs migration to the apical membrane is not clearly understood but it might be linked to the dishevelled and planar polarity pathway (review in (Vladar and Axelrod, 2008)). Another possibility is that the actin based-cytoskeleton controls migration of the vesicles on which BBs are docked, before their fusion with the apical membrane, in a process similar to "classical" secretion events (review in (Lanzetti, 2007)). Disruption of actin filaments during ciliogenesis leads to the presence of assembled cilia within large cytoplasmic vesicles (Sandoz et al., 1988), as if all the vesicles had fused together but not with the apical membrane.

Until very recently, there was little evidence for a direct role of actin or of the actin-based cytoskeleton in cilia function or biogenesis. Myosin VIIa, which is responsible for Usher syndrome type 1b, has been localized to the CC and implicated in cargo transport to the outer segment (see above). Actin itself has been localized to the proximal region of motile cilia of oviduct cells (Sandoz et al., 1982), in the flagellum of spermatozoon (Behnke et al., 1971) and in the CC of photoreceptor cells (Chaitin et al., 1984) but its function in cilia was not further investigated.

Recent evidences from a siRNA screen in mammalian cells revealed that ciliogenesis is tightly controlled by proteins regulating actin polymerization. To summarize these unexpected results, increasing actin polymerization state inhibits ciliogenesis whereas relaxing the actin cytoskeleton using low doses of actin depolymerizing drugs increases cilium length and could rescue ciliogenesis defects in different cellular models (Kim et al., 2010). Similar observations were obtained in two different studies, the first aiming to elucidate the function of the actin regulatory protein MIM in the sonic-hedgehog pathway (Bershteyn et al., 2010), and the second, studying relationship between cell shape/contractility and ciliogenesis (Pitaval et al., 2010). These data show for the first time that actin dynamics somehow regulates ciliogenesis and/or cilium length. This might be linked to the role of actin assembly state in controlling membrane tension. Finally, even if actin filaments are absent from cilia, one cannot exclude direct functions of monomeric actin or short actin filaments at cilia. The interaction of actin filaments with the ciliary pocket membrane might provide other clues to better understand the exact role of actin at cilia.

Conclusion/perspective:

The data summarized in this review stem from studies carried out many years ago by morphologists who were discovering the fascinating world of the inside of cells, revealing the diversity of cellular organelles. They stressed the striking resemblance of the local environment found at the base of cilia and flagella between vertebrate cells and unicellular models, underlining the great interest to study various organisms (Review in (Vincensini et al., in press)). Ciliary or flagellar pockets are found at many different types of cilia and whatever the pathway responsible for their formation, they appear involved in similar processes of vesicular trafficking (endocytosis and/or exocytosis), whose cargoes identification will help to define the trafficking function. Pockets also interact with the actin-based cytoskeleton, in relationship with the key role of actin assembly in ciliogenesis.

In addition to these shared and evident properties, the fact that some cilia do not present a pocket raises the question of which specific advantages are provided by such a structure? Much can be learnt from comparative studies between situations where the cilium is rooted in a ciliary pocket or directly docked to the plasma membrane. In the case of *C. elegans*, even if the base of sensory cilia is an active site of endocytosis (O. Blacque, personal communication), there is no evidence for the presence of a pocket-like structure (wormimage.com, (Perkins et al., 1986)). However, sensory cilia are often found within a specific environment formed by surrounding cells and that could be assimilated to a pocket-like structure (review in (Inglis et al., 2007)). The presence of cilia within a depression might then be linked to their sensory functions, for example in response to gradient of diffusing molecules. Finally, another hypothesis is a possible role of the ciliary pocket in cilia positioning. In polarized cells, cilia are present at the apical membrane and PC are not

randomly positioned even in non-polarized cells where they are parallel to the adherent surface (Albrecht-Buehler, 1977; Kaplan et al., 2010; Molla-Herman et al., 2010). The presence of the proximal part of the cilium within a deep invagination of the plasma membrane that establishes contacts with the actin cytoskeleton could also be linked to the close position of the BB and the nucleus ((Sorokin, 1968), our unpublished results).

We realize that this review probably provides more questions than answers, but we do hope that this will help to design new types of experiments to better understand the signification of the ciliary pocket.

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Definitions:

Box1:
In humans, cilia and flagella can be differentiated by the structure of their axoneme. They are all made of 9 peripheral doublets of microtubules corresponding to the elongation of two of the microtubule triplets of the basal body. Most motile cilia and flagella possess a central pair of microtubules, a structure defined as the "9+2" axoneme. In contrast, primary cilia do not present this central pair of microtubules and therefore are called "9+0". They lack dynein arms and do not appear to possess intrinsic motility properties. A striking exception to this rule is the nodal cilium that displays a 9+0 configuration but possesses outer dynein arm and is capable of rotational motility.

Box2:
The ciliary necklace (Gilula and Satir, 1972) is a highly organized domain of the ciliary membrane which lies above the attachment site of the transition fibers, i.e. the distal appendages of the basal body/mother centriole, that attach to the cell membrane just as it becomes the ciliary membrane. It is comprised of the cup-shaped structures linking the microtubules doublets of the very distal region of the basal body to the ciliary membrane and their intramembrane components that appear as intramembraneous particles (IMPs) in freeze-fracture. This region containing the Y links (the two dimensional appearance of the cups) is part of the more complex transition zone in 9+2 axonemes which also corresponds to the very proximal part of the axoneme between the end of the basal body and the appearence of the two central microtubules. The ciliary necklace is also present in 9+0 primary cilia and it likely contains the recently identified septin-based diffusion barrier. Altogether, these structures form a nuclear pore-like region through which all the exchanges between the cilium and the rest of the cell takes place.

Figure legends

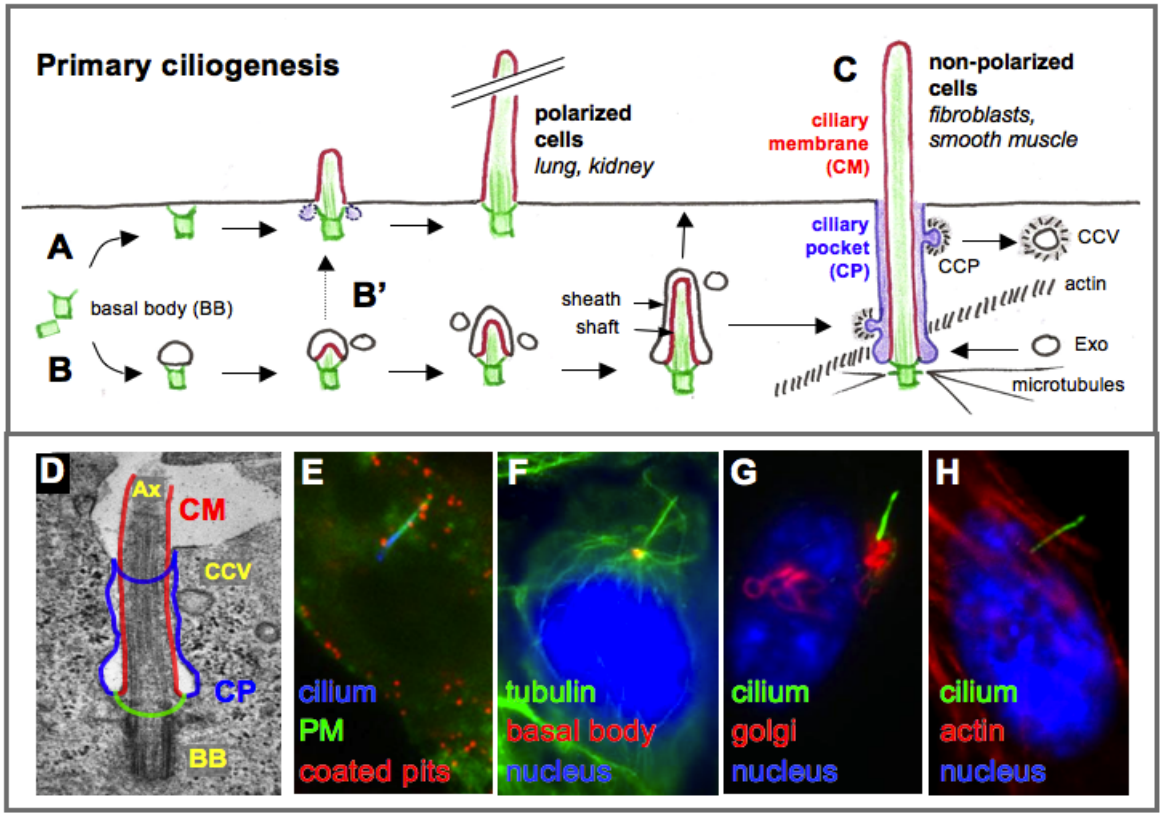

Fig. 1: Cell-type dependent ciliogenesis pathways.
Sorokin (1968) defined two pathways to generate a primary cilium depending on the cell types, the "extracellular" pathway in epithelial cells (**A**) and the "intracellular" pathway in fibroblasts (**B**). In the extracellular pathway (**A**), the mother centriole of the centrosome (green) directly docks to the plasma membrane and the cilium (membrane in red) grows directly in the extracellular milieu. In the intracellular pathway (**B**), a primary ciliary vesicle interacts with the distal appendages of the mother centriole, the axoneme grows then within this vesicle which elongates thanks to the fusion with incoming secondary vesicles. The elongated vesicle then forms the ciliary sheath surrounding the ciliary shaft, the latter corresponding to the ciliary membrane (red). The sheath docks and fuses with the plasma membrane, allowing the distal part of the cilium to reach the extracellular milieu. The ciliary pocket (blue) acts as a specific endocytic membrane domain from where clathrin-coated pits and vesicles form (CCP, CCV) and also involved in the interaction with the actin-based cytoskeleton. It could also serve as a platform for the docking of vesicles coming from the secretory pathway (Exo) or from endosomes (**C**). Ciliogenesis might also follow the intracellular pathway in polarized cells but with shorter intracellular growth stages, a faster fusion event with the plasma membrane and/or complete extrusion of the cilium (B') resulting in cilia without or with a transient ciliary pocket. **D.** TEM picture of the primary cilium in RPE1 cells showing the ciliary pocket highlighted in blue (CP), the ciliary membrane in red (CM) and the diffusion barrier region in green. **E-H**. Immunofluorescence images of RPE1 cells showing respectively clathrin-coated pits, microtubules, the Golgi apparatus and actin filaments distribution relative to the primary cilium. In E, PM is for the plasma membrane stained with GFP marker.

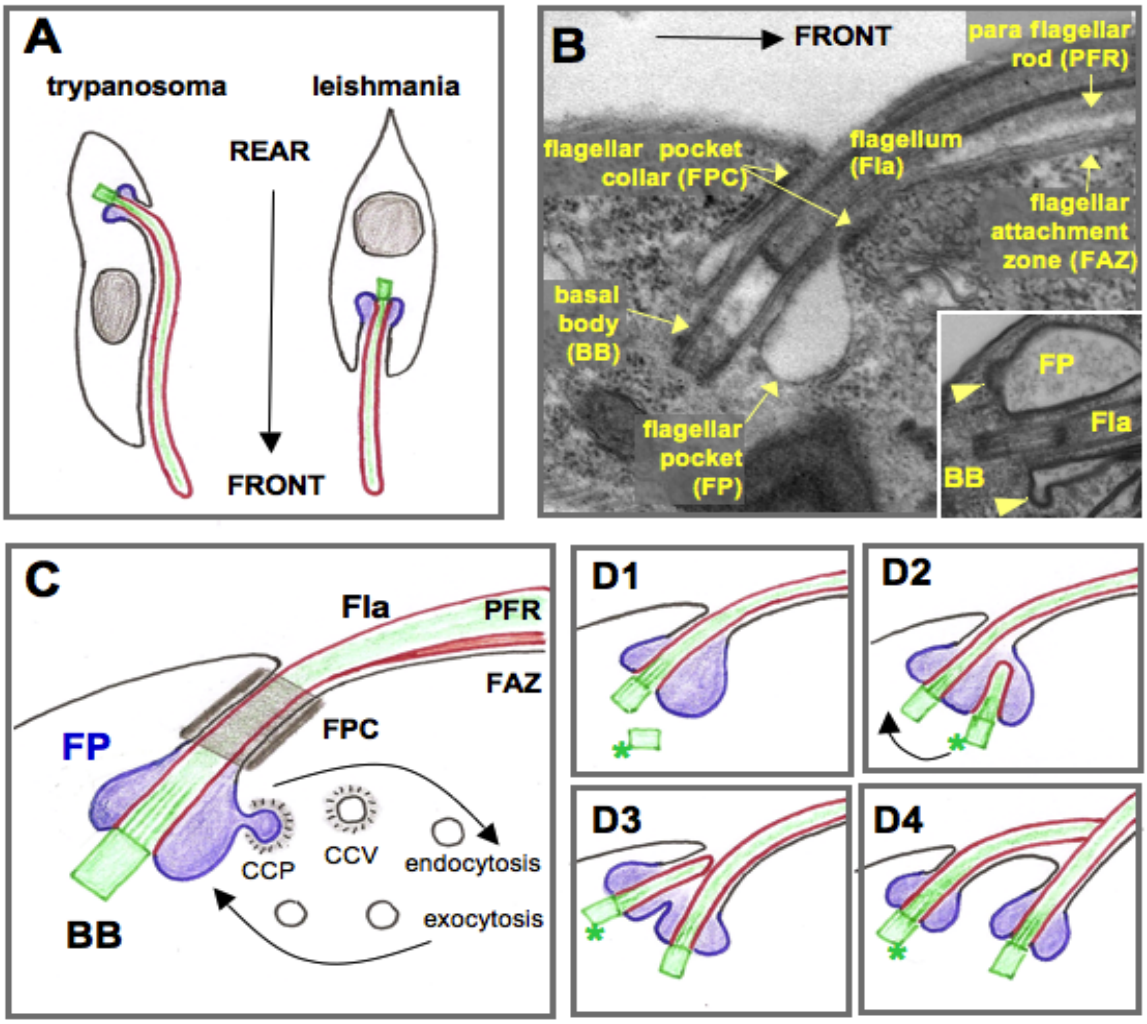

Fig. 2: The flagellar pocket of trypanosomatids
A. Trypanosomatids including *Trypanosoma* and *Leishmania* moves with their flagellum (red) in front. **B.** TEM picture of a longitudinal section through the flagellar pocket (FP) of a procyclic stage (main picture) or a bloodstream stage (inset) of *Trypanosoma brucei* showing the basal body (BB) and the flagellum (Fla), as well as the flagellar pocket collar (FPC) and the flagellar attachment zone (FAZ). **C.** The flagellar pocket (blue) is a specialized membrane domain found at the base of the flagellum (membrane in red) of Trypanosomatids which is involved in vesicular trafficking including endocytosis through the formation of clathrin coated pits and vesicles (CCP, CCV) and exocytosis. The flagellar pocket is limited at its distal end by the flagellar pocket collar (FPC), which forms a diffusion barrier between the pocket and the cell surface. Once the flagellum exits the pocket it adheres to the cell surface through the flagellar attachment zone (FAZ). **D**. During flagellum duplication, the pro-basal body (marked by a star) matures and docks to the anterior region of the pre-existing pocket, then rotates around the existing BB to reach its posterior side before it can associate to the new flagellar pocket giving rise to the new flagellum.

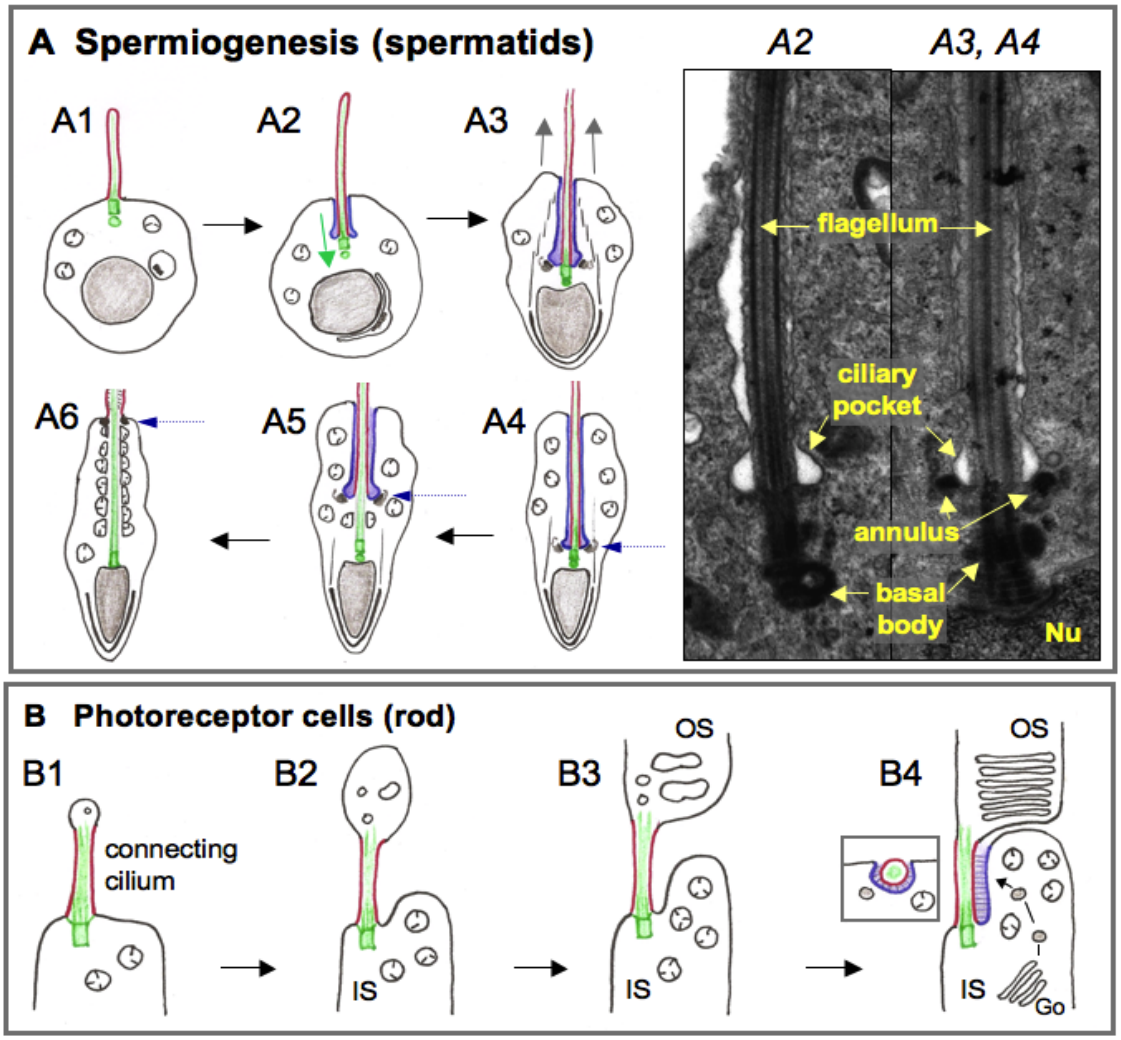

Fig. 3: Ciliary pockets of spermatids and photoreceptor cells.
A. Left, cartoon of the flagellum (membrane in red) found at the surface of round spermatids does not present a pocket. The basal body (green) migrates toward the nucleus and this inward movement likely explains the presence of a pocket (blue) at the base of the flagellum of elongating spermatids (A2). The pocket further elongates with caudal extension of the cell body, and the annulus and the chromatoid bodies dock to the base of the pocket (A3). At later stages, the base of the pocket becomes free from the basal body and is repositioned with its docked annulus toward the caudal end of the flagellum (A4-6). Mitochondria then dock to the nude "cytoplasmic" part of the axoneme to form the midpiece (A6). Right, TEM pictures obtained from sections of mice testis showing two different stages of the flagellum-associated ciliary pocket (CP). On the left, the basal body (BB) is not yet docked to the nucleus and the two centrioles are clearly visible. On the right, the BB is now close to the nucleus and the annulus/chromatoid body is docked to the base of the pocket. **B.** The connecting cilium (membrane in red) resembling a primary cilium first appears at the apical surface of photoreceptors, in a region that will become the inner segment (IS) (B1). The distal tip of the cilium enlarges to form the outer segment (OS) and the apical surface of the inner segment also grows toward the forming OS (B2-3). An asymmetric pocket (blue) is present in differentiated cells likely resulting from the interaction of the connecting cilium membrane with the adjacent apical membrane of the IS (B4). The membrane of the pocket is a docking site for IFT positive vesicles and/or containing cargos loaded vesicles coming from the Golgi.

Supplementary Figure 1:

In addition to the references cited in the text, below are listed additional examples in which the presence of a ciliary pocket like structure at the base of primary cilia has been described. This list is likely incomplete and limited to publications that we could obtain and in which we clearly found pictures confirming the presence of a pocket.

Ciliary pocket in vivo:

- **Brain:** neuroepithelial cells (Sotelo and Trujillo-Cenoz, 1958) Grueneberg ganglion neurons (mice (Brechbuhl et al., 2008)); hypohysis, (pars distalis; mice ((Barnes, 1961; Dingemans, 1969)), rabbit (Salazar, 1963)); adenohypohysis (rat (Wheatley, 1967)); hippocampus (mice (Breunig et al., 2008)); lateral ventricle B1 and E2 cells (mice (Mirzadeh et al., 2008)); choroid plexus, epithelial cells,(rat (Peters, 1979)).
- **nerves**: Schwann cells (rat (Grillo and Palay, 1963)); astrocytes (human (Sturrock, 1975)); carotid body (cat and rabbit (Biscoe and Stehbens, 1966)).
- **eye:** cornea, keratocytes (rabbit (Smith et al., 1969)); retina, pigmented epithelium (human, (Allen, 1965)); photoreceptor cells (ferret (Greiner et al., 1981)); lens fiber (mice (Sugiyama et al., 2010)).
- **endocrine tissues:** adrenal gland, chromaffin cells (mice (Coupland, 1965)); adrenal cortex (rat (Wheatley, 1967)); pancreas, beta-cells (mice (Munger, 1958)).
- **muscle:** heart, cardiomyocytes (chicks, (Rash et al., 1969)); smooth muscle cells, lung and intestine (chick and rat, (Sorokin, 1962; Sorokin, 1968)).
- **connective tissues:** chondrocytes (human, canine and equine (Cox and Peacock, 1977; Poole et al., 1985; Sotelo and Trujillo-Cenoz, 1958; Wilsman, 1978; Wilsman et al., 1980)); ligament cells (mice (Beertsen et al., 1975)); sinoviocytes (rat (Graabaek, 1984)); meniscus cells (rabbit (Hellio Le Graverand et al., 2001)); fibroblasts (rat and chick (Fonte et al., 1971; Sorokin, 1962; Sorokin, 1968), human (Schuster, 1964)).
- **reproductive organs:** oocyte follicles, granulosa and cumulus cells (guinea pig (Adams and Hertig, 1964), mice, (Molla-Herman et al., 2010)), endometrium, stromal cells (rat, (Tachi et al., 1969)).

Motile cilia and flagella:

- elongating spermatids (Molla-Herman et al., 2010)

Ciliary pocket in vitro:

- CHO cells (chines hamster ovary cells (Stubblefield and Brinkley, 1966))
- adipocytes (Marion et al., 2009)
- htert-RPE1 (retinal pigmented epithelial cells (Molla-Herman et al., 2010))
- fibroblasts (Wheatley, 1969)
- choroid plexus epithelial cells (Narita et al., 2010)
- synoviocytes (Rattner et al., 2010)
- astrocytes, (Moser et al., 2009)
- IMCD3 (inner medulla collecting duct cells (Molla-Herman et al., 2010)).
- ciliated cells of the ependyma (Molla-Herman et al., 2010)

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