The conserved centrosomal protein FOR20 is required for assembly of the transition zone and basal body docking at the cell surface

Anne Aubusson-Fleury1,2,*, Michel Lemullois1,2, Nicole Garreau de Loubresse1,2, Chloé Laligné1,2, Jean Cohen1,2, Olivier Rosnet3, Maria Jerka-Dziadosz4, Janine Beisson1,2 and France Koll1,2

1CNRS, Centre de Génétique Moléculaire, UPR3404, 91198 Gif-sur-Yvette, France
2Université Paris-Sud, 91405 Orsay, France
3Centre de Recherche en Cancérologie de Marseille, INSERM UMR1068, CNRS UMR7258, Institut Paoli-Calmettes, 13273 Marseille Cedex 09, France
4Polish Academy of Sciences, M. Nencki Institute of Experimental Biology, Department of Cell Biology, 3 Pasteur Street, 02-093 Warsaw, Poland

*Author for correspondence (anne.aubusson@cgm.cnrs-gif.fr)

Accepted 14 May 2012
Journal of Cell Science 125, 4395–4404
© 2012, Published by The Company of Biologists Ltd
doi: 10.1242/jcs.108639

Summary
Within the FOP family of centrosomal proteins, the conserved FOR20 protein has been implicated in the control of primary cilium assembly in human cells. To ascertain its role in ciliogenesis, we have investigated the function of its ortholog, PtFOR20p, in the multiciliated unicellular organism Paramecium. Using combined functional and cytological analyses, we found that PtFOR20p specifically localises at basal bodies and is required to build the transition zone, a prerequisite to their maturation and docking at the cell surface and hence to ciliogenesis. We also found that PtCen2p (one of the two basal body specific centrins, an ortholog of HsCen2) is required to recruit PtFOR20p at the developing basal body and to control its length. By contrast, the other basal-body-specific centrin PtCen3p is not needed for assembly of the transition zone, but is required downstream, for basal body docking. Comparison of the structural defects induced by depletion of PtFOR20p, PtCen2p or PtCen3p, respectively, illustrates the dual role of the transition zone in the biogenesis of the basal body and in cilium assembly. The multiple potential roles of the transition zone during basal body biogenesis and the evolutionary conserved function of the FOP proteins in microtubule membrane interactions are discussed.

Key words: FOR20, FOP, Centrin, Cilium, Paramecium, Ciliogenesis

Introduction
Cilia are highly conserved eukaryotic cell appendages present as either motile or non motile organelles (Carvalho-Santos et al., 2011; Bornens, 2012). They constitute cell compartments involved in reception and signal transduction (Bloedgood, 2010). Any defect in their functions leads to dramatic consequences at the level of the organism, as illustrated by developmental disorders and/or ciliopathies in human (Hildebrandt et al., 2011). Cilia are generally assembled from a nine fold-symmetrical microtubular template, the basal body which is docked at the cell surface, either transiently as in most metazoan tissues where the older centriole docks at the cell surface to generate a primary cilium as the cell enters the G0 phase (Sorokin, 1968; Kobayashi and Dynlacht, 2011), or permanently. In this second case, basal bodies may be assembled de novo, during epithelium differentiation (Sorokin, 1968; Lemullois et al., 1988; Laoukili et al., 2000), in flagellates assembled de novo, during epithelium differentiation (Sorokin, 1968; Kobayashi and Dynlacht, 2011), or permanently. In this second case, basal bodies may be assembled de novo, during epithelium differentiation (Sorokin, 1968; Lemullois et al., 1988; Laoukili et al., 2000), in flagellates like Naegleria (Fulton and Dingle, 1971), in ciliate cystic cycle (Grimes, 1973), in male gametes as in Marsilea vestita (Hart and Wolniak, 1998), or be assembled close to parental ones, as during the division in ciliates (Dippell, 1968; Allen, 1969). In all cases, ciliogenesis is a multi-step process: basal body assembly involves nucleation, elongation, maturation of the microtubular scaffold, docking at the cell surface and growth of a cilium.

Recently it was found that FOR20, a protein conserved in most ciliated organisms, is involved in the assembly of the primary cilium (Sedjâï et al., 2010). FOR20 belongs to the family of FOP-related proteins (Azimzadeh et al., 2008), which is defined by the presence of two N-terminal domains, the LisH domain, necessary for the dimerisation yielding the functional form of the protein, and the TOF domain, required in addition to LisH for centrosomal localisation, and a Pro-Leu-Leu (PLL) domain of unknown function (Sedjâï et al., 2010). In this family, four subgroups can be recognised: (1) the FOP proteins, identified as oncogene partners associated to the centrosome (Andersen et al., 2003; Delaval et al., 2005; Popovici et al., 1999), required for microtubule anchoring (Yan et al., 2006) and cell cycle progression (Acquaviva et al., 2009); (2) the centrosomal OFD1 proteins, whose mutations cause oral facial digital diseases in humans (Ferrante et al., 2001); (3) the land plant TONNEAU 1 protein, required for the normal cortical cytoskeleton of microtubules and pre-prophase band formation at the division plane (Azimzadeh et al., 2008); (4) a subgroup of shorter proteins corresponding to FOR20. Proteins of this subfamily are the most evolutionarily conserved. In RPE1 cell lines, HsFOR20p localises at the centrioles and the pericentriolar satellites, close to the centrosome and its depletion has been shown to affect ciliogenesis. However, the primary effect of HsFOR20p deficiency remains
unclear. In order to obtain information about the function of the HsFOR20p, we investigated the role of its ortholog, PtFOR20p, in Paramecium, a multi-ciliated unicellular organism that offers the possibility to follow the lineage of each basal body over cell generations (Iftode et al., 1989), hence to detect and analyse any defects in the mechanisms of basal body assembly and ciliogenesis. We found that, in Paramecium, GFP-PtFOR20 localises at the transition zone of all basal bodies, whether ciliated or not, and that its depletion induces defects in basal body maturation and docking. Although the protein localised at the distal part of docked basal bodies, as previously noted in Tetrahymena (Kilburn et al., 2007), its presence could be detected very early during basal body assembly. Furthermore, PtCen2p, the ortholog of the human centriolar centrin 2 (Ruiz et al., 2005), is required for the recruitment and/or stabilisation of PtFOR20p at the basal body and both proteins are essential for building the transition zone. In contrast, PtCen3p, the ortholog of the mammalian centrin 3, is only required for docking the basal body at the cell membrane. Our findings shed new light on the assembly of the transition zone as well as on the docking of basal bodies in Paramecium and explain the ciliogenesis defects observed in HsFOR20p depleted human cells. Finally, we observed that PtFOR20p still marks the site of basal body attachment at sites of programmed disassembly-reassembly, and thus might provide a cortical pattern landmark.

Results

Two PtFOR20p-encoding genes are present in Paramecium

First described as small FOP-like proteins (Azimzadeh et al., 2008), FOR20 (FOPNL) was subsequently identified as a centrosomal protein involved in primary cilium assembly (Sedjaï et al., 2010). This protein, which is detected only in ciliated organisms, has similarities to the N-terminal part of the FOP proteins and presents the three specific sequence features, the TOF and LisH domains and the PLL motif. Using Blast homology search, two FOR20 orthologs, derived from the last whole genome duplication (Aury et al., 2006) were identified in the Paramecium genome. Multiple alignment of the two PtFOR20 proteins, which differ by a single amino acid, with numerous other FOR20 proteins show that this protein is strongly conserved through evolution (supplementary material Fig. S1). The analysis of transcriptome microarray data which are conserved through evolution (supplementary material Fig. S1).

Both GFP-PtFOR20 and GFP-HsFOR20 localise at basal bodies in Paramecium

In order to localise PtFOR20p in living cells, we examined transformants expressing the GFP-tagged protein. We also examined the localisation of the human protein. In both PtFOR20p and HsFOR20p expressing cells, which exhibited a slightly reduced growth rate (2–3 instead of 3–4 divisions per day at 22°C) but no abnormal morphology, discrete spots of fluorescence were aligned at the cell surface, following a pattern similar to that of the basal bodies (Fig. 1A). Double-labelling with the ID5 antibody, which decorates the basal bodies (Ruiz et al., 1999), showed that this surface pattern of the GFP signal precisely overlapped that of the basal bodies (Fig. 1B). When probed with the monoclonal antibody 17E2 directed against HsFOR20p (Sedjaï et al., 2010), only the HsFOR20p expressing cells reacted, yielding the same dotted cortical pattern as revealed by the ID5 labelling (not shown). In contrast, control cells were negative, indicating that the endogenous Paramecium protein did not cross-react with the antibody.

The GFP-PtFOR20 pattern does not correlate with ciliation

Because FOR20 had been shown to control ciliation in human RPE1 cell (Sedjaï et al., 2010), we examined the spatial correlation between the presence of PtFOR20p and cilia. In their vegetative stage, the cells are densely ciliated but not all basal bodies bear a cilia (Fig. 1C). Double labelling with two antibodies specific for basal bodies and cilia respectively is necessary to ascertain the ciliated or non-ciliated status of basal bodies. On the ventral surface, single and paired basal bodies alternate along the antero-posterior rows. A careful examination

![Image](http://paramecium.cgm.cnrs-gif.fr/cgi/browse/microarray)
shows that, although all the basal bodies are labelled by GFP-PtFOR20, the anterior basal bodies of some pairs are not ciliated (Fig. 1C): PtFOR20p is thus associated with both ciliated and non-ciliated basal bodies. This observation means that, if FOR20 is necessary for cilium assembly, as shown for RPE1 cells, its presence at the tip of a basal body is not sufficient, at least for motile cilia in *Paramecium*.

**GFP-PtFOR20 localises to the transition zone**

To ascertain the precise localisation of PtFOR20p in the basal bodies, we compared two sets of cells: (1) control cells double-labelled with ID5 and an antibody directed against the epiplam, a sub-membrane cytoskeletal layer segmented into units in the centre of which the basal bodies are inserted (Nahon et al., 1993), and (2) GFP-PtFOR20 expressing cells double-labelled with an anti-GFP and the anti-epiplam antibody (Fig. 2A,B). Transverse optical sections show that the ID5 and anti-GFP labelling do not colocalise: while the ID5 labelling extends beneath the epiplam (Fig. 2A), the GFP labelling is confined at the level of the epiplam (Fig. 2B), indicating that PtFOR20p localises at the distal part of the basal bodies.

The localisation of PtFOR20p was then investigated at the ultrastructural level by immunolabelling of GFP-PtFOR20 expressing cells. The ultrastructure of the transition zone, located between the basal body and the 9+2 region of the cilium of *Paramecium* has been well described (Dute and Kung, 1978). This transition zone comprises three plates: a proximal thick terminal plate, a thin intermediate plate and a distal axosomal plate on which lies the axosome. The structure of the distal part of anchored but non-ciliated basal bodies is less well described. Fig. 3A shows that it displays almost the same morphology as ciliated ones, but that the region between the intermediate plate and the axosomal plate is thinner suggesting that ciliogenesis involves a reorganisation of this region. In GFP-PtFOR20 expressing cells, we did not detect any defect in the morphology of either ciliated or non-ciliated basal bodies, except for a slight increase of the terminal plate thickness (Fig. 3B) indicating that the expression of the GFP-tagged protein has little or no effect on basal body ultrastructure. By immunolabelling of whole permeabilised cells (pre-embedding) and of sections (post-embedding), we detected the GFP-labelling at the level of the transition zone, close to the microtubular scaffold (Fig. 3C,D). In post-embedding processing, immunogold particles were detected on about 24% of basal bodies, in most cases just above the terminal plate (Fig. 3C, supplementary material Table S1). After pre-embedding processing, 38% of basal bodies were decorated, with a labelling restricted to the external and apical part of the terminal plate (Fig. 3D, supplementary material Table S1). On transverse sections, in both cases, the gold particles were detected at the level of microtubule doublets, close to fibres linking these doublets to the membrane (Fig. 3C,D). The presence of this membrane in the pre-embedding protocol (Fig. 3D), which includes a treatment with Triton X100, shows that it is detergent resistant at that level. This particularity has already been observed in *Chlamydomonas* where this membrane portion endowed with specific biochemical properties has been called ‘transition membrane’ (Kamiya and Witman, 1984). Altogether, these results show that PtFOR20p is located at the level of the transition zone. A similar localisation at the tip of the basal bodies was described both in *Tetrahymena* by electron microscopy for the homologous protein called Bbc20 (Kilburn et al., 2007) and in mouse trachea epithelia by immunofluorescence (Sedjai et al., 2010). In *Giardia*, a protein homologous to Bbc20/PtFOR20 is detected along the intracytoplasmic part of axonemes, suggesting an interaction with microtubule doublets (Lauwaet et al., 2011). The fact that, at the ultrastructural level, the labelling was observed only on a fraction of basal bodies by pre-embedding labelling on whole permeabilised cells as well as by post-embedding labelling on sections of non permeabilised cells, suggests that the protein is not abundant and/or located on discrete structures, thus not found on all longitudinal sections of basal bodies. A similar discrete labelling has previously been reported in *Chlamydomonas* for Cep290, another protein of the transition zone (Craigie et al., 2010). This situation might explain why no labelling was detected at the base of the primary cilium in RPE1 cells (Sedjai et al., 2010).

**PtFOR20p is detected early in basal body assembly**

During cell division in *Paramecium*, basal bodies duplicate according to a precise spatio-temporal pattern (Iftode et al., 1989), allowing unambiguous identification of pre-existing and newly assembled basal bodies. A new basal body assembles next to and anterior to its mother, and elongates as it tilts up to dock at the cell surface (Dippell, 1968). The time of appearance of PtFOR20p with respect to these steps was followed in cells expressing GFP-PtFOR20 by double labelling with ID5 and an anti-GFP antibody. In dividing cells, spots of GFP-PtFOR20 were detected close to the proximal part of parental basal bodies, at the site of new basal body assembly, before the developing basal bodies could be detected by ID5 (Fig. 4A). As ID5 is specific for polyglutamylated tubulin, it can be concluded that PtFOR20p is associated with the nascent basal bodies before their polyglutamylation.

**PtFOR20p stably associates with the cell surface**

During division, the duplication of basal bodies and epiplam units starts at the cell equator and spreads as a wave towards the two cell poles (Iftode et al., 1989). However, in the ‘invariant anterior field’ (Fig. 1C, supplementary material Fig. S2), where...
all epiplastic units harbour two basal bodies, these units do not duplicate but, as the wave of basal body proliferation reaches them, the anterior basal body of each pair disappears and a new one is reassembled and anchored at the same place (Romero and Torres, 1993) (supplementary material Fig. S2). Surprisingly, when the anterior basal body of each pair disappears, the GFP-PtFOR20 labelling still marks its site (Fig. 4B). This observation suggests that, once PtFOR20p is anchored at the membrane after basal body docking, it may become dynamically independent of the microtubule shaft and may guide the anchoring of a newly assembled basal body.

Depletion of PtFOR20p prevents basal body docking at the cell surface

In order to analyse its role in the process of basal body and cilium assembly, PtFOR20p was depleted by RNAi in wild-type and in GFP-PtFOR20-expressing cells (see Materials and Methods). In both types of cells, a strong effect was observed within two divisions, yielding poorly ciliated, abnormal small cells with non-functional oral groove and an altered basal body pattern. In addition, many basal bodies were detected inside the cells (Fig. 5A).

To understand how intracytoplasmic basal bodies and altered basal body pattern were generated, the steps leading to this phenotype were further analysed by double-labelling of wild-type cells with antibodies specific for basal bodies and for the epiplastic. The first phenotypic effects were detected at the first division under inactivation conditions. The new basal bodies were slightly mis-positioned in the epiplastic, more anterior than normal and occasionally between two units (Fig. 6B). These observations demonstrate that: (1) PtFOR20p depletion does not prevent the assembly of new basal bodies and (2) the GFP-tagged protein is stably incorporated into the basal bodies, since it remains associated with parental basal bodies over at least two cell generations.

After two divisions, in both wild-type and GFP-PtFOR20-expressing cells, the same phenotype was observed: many basal bodies, all devoid of GFP were present within the cytoplasm (not shown). The only basal bodies retaining the GFP signal, presumably the parental ones, were anchored at the surface (Fig. 5B). These observations demonstrate that: (1) PtFOR20p depletion does not prevent the assembly of new basal bodies and (2) the GFP-tagged protein is stably incorporated into the basal bodies, since it remains associated with parental basal bodies over at least two cell generations.

At the ultra-structural level, many basal bodies, whether undocked or partially docked at the membrane, were found next to the parental ciliated basal bodies (Fig. 7A). Particularly significant are the upper images of Fig. 7A which account for the slight mis-positioning observed at the photonic microscope level (Fig. 6B). Free basal bodies were also present deep in the cytoplasm. These internal basal bodies all exhibit a distal end

![Fig. 3. PtFOR20p localises to the transition zone.](image)
closed with structures resembling an incomplete transition zone. In some internal basal bodies, microtubules extending beyond the distal end could also be observed. As intracytoplasmic basal bodies are never found in control cells, these observations demonstrate that PtFOR20p is essential for basal body docking at the cell surface and/or for completion of its maturation before docking. Such observations indicate that, as already described by Dippell (Dippell, 1968), some structures of the transition zone are assembled before basal body anchoring to the cell surface. In addition, in such undocked basal bodies, depletion of PtFOR20p allows microtubules to extend beyond the incomplete transition zone.

PtFOR20p and PtCen2p, but not PtCen3p, are necessary to build the transition zone

Undocked intracytoplasmic basal bodies have previously been observed under depletion of PtCen2p and Ptcen3p (Ruiz et al., 2005), the two Paramecium basal body specific centrin isoforms, orthologs to the mammalian centriolar centrins HsCen2 and HsCen3 (Middendorp et al., 1997; Bornens and Azimzadeh, 2007). In order to compare the roles of these two proteins with that of PtFOR20p, we re-investigated their localisation and the effects of their depletion in Paramecium. GFP-PtCen2p which localises within the basal body cylinder (Ruiz et al., 2005) was shown here to be more abundant at its distal part, at the level of the terminal plate (supplementary material Table S1). The same localisation has also been reported in Tetrahymena (Stemm-Wolf et al., 2005). A new observation was also that, under depletion of PtCen2p, many basal bodies not properly docked or undocked were longer than normal (Fig. 7B).

While the majority of GFP-PtCen3 is localised outside the centriolar cylinder, anteriorly and apposed to its proximal end (Ruiz et al. 2005), a faint labelling is also detected at the terminal plate (supplementary material Table S1). A novel interesting observation is that, under PtCen3p depletion, all internal basal bodies develop a distal structure similar to the transition zone of non-ciliated anchored basal bodies, with terminal, intermediate and axosomal plates (Fig. 7C).

These observations, taken together with our results on PtFOR20p localisation and depletion presented above, allow us to propose the following sequence in basal body biogenesis: PtCen2p acts first in basal body elongation and is essential for the initiation of the transition zone assembly (Fig. 7B, supplementary material Table S2). This step is upstream of the action of PtFOR20p, which is involved in the completion of a structural cap evoking intermediate or axosomal plates (Fig. 7A, supplementary material Table S2). Last in the sequence is Ptcen3p, whose major role is not in the building of the transition zone, but in the docking of the mature basal body to the cortex.

In order to investigate more precisely the functional interactions between PtFOR20p and the centrins during basal body assembly, GFP-PtFOR20p expressing cells were depleted for either Ptcen2p or Ptcen3p. In both cases, the GFP-PtFOR20p signal remained associated with the parental basal bodies over generations. But while depletion of Ptcen2p induced a loss of GFP-PtFOR20p in all new basal bodies, the GFP fluorescence was
retained on all basal bodies under depletion of PtCen3p (Fig. 8). These results show that PtCen2p, but not PtCen3p, is necessary for the recruitment and/or stabilisation of PtFOR20p at the basal body. Accordingly, in GFP-PtCen2-expressing cells, the GFP signal associated with the basal bodies did not disappear after PtFOR20 depletion (data not shown).

**Discussion**

Ciliogenesis requires the assembly and maturation of the basal body, its docking at the cell surface and the growth of the ciliary axoneme. In this paper, we demonstrate that, in the multiciliated model *Paramecium*, the PtFOR20 protein is recruited very early in the course of basal body biogenesis, cooperates with PtCen2p to build the transition zone, and is required, in conjunction with PtCen3p for basal body docking at the cell surface. These results identify successive steps of basal body biogenesis, as well as multiple roles of the transition zone.

**PtFOR20p is an essential constituent of the transition zone required for basal body anchoring to the membrane**

Immunofluorescence and immunogold labelling on GFP-PtFOR20 expressing cell lines show that PtFOR20p is stably incorporated at the transition zone of basal bodies. By definition, this region is localised between the basal body and the cilium, its distal and proximal ends being clearly defined only in motile cilia by the transition between triplets and doublets of microtubules and the beginning of the two central axonemal microtubules, respectively. In contrast, in non-motile cilia with the 9+0 architecture, this area remains weakly delimited and recent observations suggest that the whole cilium could correspond to a transition zone (Gluenz et al., 2010). In motile cilia, the transition zone displays a variable architecture depending upon the species; nevertheless some ultrastructural elements conserved during evolution can be recognised, in particular the transition fibres and the Y links which connect the microtubule doublets to the membrane at the level of the ciliary necklace (reviewed by Fisch and Dupuis-Williams, 2011). In *Paramecium* cells expressing GFP-PtFOR20, the immunogold labelling is found on microtubule/membrane links, at the level of microtubule doublets, that is on the transitional fibres and/or the Y links described by Dute and Kung (Dute and Kung, 1978). This observation fits well the proposed microtubule-associated functions of the LisH domain (Ames and Ponting, 2001).

The localisation of PtFOR20p at the transition zone of assembled cilia is in agreement with the observed impairment of basal body docking at the cell surface after PtFOR20p depletion and can account for the phenotype observed after HsFOR20p depletion in RPE1 cells (Sedjaï et al., 2010). The biogenesis of the primary cilium is a two step process, leading first to the assembly of a short axoneme as the mother centriole binds the ciliary vesicle, and then to axoneme elongation after docking of the centriole at the cell surface (Sorokin, 1962; Rohatgi and Snell, 2010). HsFOR20p depletion could thus interfere with primary cilium assembly by preventing the second

---

**Fig. 5. Depletion of PtFOR20p prevents basal body docking at the cell surface.** (A) Confocal section at the intracytoplasmic level of a control cell (left) and a cell after two divisions under PtFOR20p depletion (right), double labelled with ID5 (red) and anti-epiplasm antibody (grey). Many internal basal bodies are detected after depletion, whereas none are detected in the control cell. Arrow indicates oral apparatus. (B) The surface of a GFP-PtFOR20-expressing cell after three divisions under PtFOR20p depletion, labelled with ID5 (red) and the anti-GFP antibody (green). The basal body pattern is completely disorganised. Arrow indicates abnormal oral apparatus. The GFP signal is detected on very few basal bodies (right inserts), which correspond to the labelled basal bodies of the parental cell, assembled before depletion. Scale bar: 20 μm (inserts ×2).

**Fig. 6. Depletion of PtFOR20p alters basal body positioning at the cortex.** Projections of optical sections acquired at the level of the dorsal side of the cells double labelled with ID5 (green) and the anti-epiplasm antibody (red). (A) Control dividing cell. (B) Cell in the first division during depletion. No abnormality is detected in the global morphology of the depleted cell (B) compared with the control (A). High magnification of the future fission line shows that the new basal bodies, which appear in the centre of the unit, close to parental basal bodies in the control cell (insert A), are less precisely localised within the epiplastic units and often far from parental basal bodies after PtFOR20p depletion (arrows). Scale bar: 20 μm (inserts: ×6).
step of the process. In addition, the reduction of ciliary length observed in ciliated cells could also be explained by a defect in the assembly of the microtubule/membrane links, known to control the intraflagellar transport (Craige et al., 2010; Williams et al., 2011).

Beyond the nine triplet nucleation by the ancestral centriole/basal body set of core proteins (Carvalho-Santos et al., 2011; Cottee et al., 2011), the above functional and cytological dissection circumscribes four steps in basal body biogenesis: elongation of the triplet microtubules, capping as an initiation of transition zone assembly, maturation of the transition zone, then basal body docking. Each of these steps can be identified by the sequential requirement of PtCen2p, PtFOR20p and PtCen3p.

PtCen2p is required to initiate the transition zone assembly and is involved in the control of basal body elongation. Long basal bodies were often observed after PtCen2p depletion, showing that PtCen2p is required for control of basal body length. In addition, after PtCen2p depletion, the cap which closes the distal part of the basal body is missing. This strongly suggests that PtCen2p is necessary to recruit cap proteins, which in turn would be required to control pro-basal body elongation and assembly of the transition zone. A similar role of this centrin in transition zone assembly has already been noted in Chlamydomonas in the case of the centrin mutant vfl2 (Jarvik and Suhan, 1991). However, a role of this protein in the control of basal body elongation has not yet been documented. In this context, it will be interesting to test the interaction of PtCen2p with other proteins shown to control centriole length such as POC1, POC5 or OFD1 (Keller et al., 2009; Azimzadeh et al., 2009; Singla et al., 2010).

The respective roles of PtFOR20p, PtCen2p and PtCen3p identify four steps in basal body assembly

Fig. 7. Differential ultrastructural effects of PtFOR20p, PtCen2p and PtCen3p depletion on basal body maturation and docking. (A) Upon PtFOR20p depletion, undocked or partially docked basal bodies are detected close to the cell surface (top panel) or deep in the cytoplasm (bottom panel). A partial terminal plate can be detected on partially docked basal bodies on the side where they attach to the cell surface, and on intracytoplasmic basal bodies (arrows); in the latter case, microtubule extensions are often detected on the free, uncapped side of the basal body. Vestigial structures resembling intermediate and axosomal plates are detected on all basal bodies (arrowheads). (B) Upon PtCen2p depletion, basal bodies longer than normal were often observed, as in the top panel where the new basal bodies (arrows) are longer than their parents (to the right). In addition, their transition zones are abnormal (arrows). Many intracytoplasmic long basal bodies without or with an incomplete (arrow) transition zone are also detected (lower panel). (C) Upon PtCen3p depletion, the partially undocked and the intracytoplasmic basal bodies have an associated transition zone in which the three plates (terminal, intermediate and axosomal) can be identified (arrows). Scale bars: 200 nm.

Fig. 8. Recruitment of PtFOR20p requires PtCen2p. After PtCen2p depletion, some newly assembled basal bodies (arrows) lack the associated GFP-PtFOR20 signal, whereas after PtCen3p depletion, all basal bodies (in red) have their associated GFP-PtFOR20 cap (green). Scale bar: 2 μm.

FOR20 in basal body docking
PtFOR20p is required for transition zone maturation
PtCen2p depletion prevents the appearance of PtFOR20p in basal bodies, indicating that the former protein is required for the recruitment/stabilisation of the latter. Accordingly, under PtFOR20p depletion, basal body biogenesis is stopped at a later step, when basal bodies exhibit a normal length and are closed with a structure resembling an incomplete transition zone. Ultrastructural data suggest that it is the terminal plate which is the most affected, but molecular data for the incomplete transition zone would be necessary to confirm this hypothesis. The fact that upon PtFOR20p depletion microtubules of intracytoplasmic basal bodies are able to grow beyond the cap suggests that recruitment of PtFOR20p is necessary to control microtubule elongation. The presence of the LisH domain suggests that, as proposed for OFD1 (Singla et al., 2010), PtFOR20p could regulate the dynamics of basal body microtubules.

PtCen3p is required for basal body docking of fully mature basal bodies
Undocked basal bodies resulting from PtCen3p depletion have a normal length and a fully assembled transition zone, at least according to ultrastructural criteria. These results show that the transition zone can be assembled independently of basal body docking at the cell surface. These undocked basal bodies result from a defect in the tilting up movement of the new basal body toward its docking site at the cell surface, in agreement with the fact that PtCen3p has been mainly localised outside the basal body at its proximal part (Rui et al., 2005) (M.J.-D. et al., unpublished results). The same localisation has been observed in another ciliate, *Paraurostyla* (Lemullois et al., 2004). We show here that, in addition to this proximal localisation, some PtCen3p is also detected distally, within the basal body at the transition zone. In ciliated episthelia, HsCen3p has been exclusively detected at the distal part of basal bodies (Loukili et al., 2000). This distal localisation suggests that this protein could also be required for basal body docking at the cell surface. PtCen3p could thus be involved in different functions in association with diverse centrin-binding proteins, such as SFI1 (Kilmartin, 2003) or gPOC5 (Azimzadeh et al., 2009) whose homologs exist in *Paramecium* (Gogendeau et al., 2007).

The basal body as a cell compartment
It is striking that proteins localised at the transition zone of the basal body are recruited very early during its biogenesis, as demonstrated here for PtFOR20p, and in other studies for VIF1 in *Chlamydomonas* (Silflow et al., 2001), and a 210kD protein in *Spermatoopsis similis* (Lechtreck et al., 1999). This observation is paralleled by ultrastructural data showing that nascent basal bodies display a distal cap at the very beginning of their assembly (Dippell, 1968; Allen, 1969). These molecular and ultrastructural observations strongly suggest that the distal cap corresponds to the first step of transition zone assembly. The nascent basal body would thus be equipped with a nascent transition zone.

A growing set of data shows that the mature transition zone acts as a barrier or a filter for the cilium compartment (Craige et al., 2010; Williams et al., 2011; Garcia-Gonzalo et al., 2011) (for a review, see Ishikawa and Marshall, 2011). Moreover, it maintains basal body integrity by preventing entry of ciliary specific components: in the centrin vfl2 mutant of *Chlamydomonas* which has a defective transition region, the central pair of the cilium runs into the lumen of the basal body (Jarvik and Suhan, 1991). In the same way, as soon as assembled, the nascent transition zone could act as a diffusion barrier to create a nascent basal body compartment, thus maintaining its molecular individuality until its docking at the surface. The CP110 cap complex assembled at the distal part of the elongating cilium in mammalian cells (Kleylein-Sohn et al., 2007) might fulfil a similar function. Accordingly, the internal texture of centrioles and basal bodies differs from that of the surrounding cytoplasm (for a review, see Azimzadeh and Marshall, 2010) suggesting that these two cellular spaces, inside and outside the centriole/basal body compartment, would not have the same composition.

FOP-like proteins as components of an ancestral module involved in microtubule membrane interaction
Our results show that, in *Paramecium*, once assembled, the FOR20 protein remains at the cell surface after basal body disappearance, and thus could act as a structural landmark for subsequent morphogenetic events as for example during reciliation after injury of epithelia (Carson et al., 1981). Such a structural landmark has already been observed for FASS, a centrosomal protein conserved in land plants and involved in the assembly of the pre-prophase band (Wright et al., 2009), a transient cortical microtubular array required for determination of the subsequent cell division plane in plants.

Several other proteins involved in centriolar and ciliary functions are conserved in acentriolar plants (Laligè et al., 2010; Hodges et al., 2011). Many of them have, or are supposed to have, a role in cytoskeletal/microtubule organisation (Gardiner and Marc, 2011; Hodges et al., 2011) suggesting that they are maintained for microtubular organisation in non-ciliated plants (Hodges et al., 2011). The member of the centrosomal FOP family conserved in land plants, TONNEAU1, is known to interact with centrin and to be required for correct assembly of the preprophase band (Azimzadeh et al., 2008). The common properties of FOR20 and TONNEAU1 suggest an ancestral evolutionarily conserved function of the FOP proteins in the control of the interactions between microtubular complexes and the cell surface.

Materials and Methods
Strains and culture conditions
Stock d4-2 of *Paramecium tetraurelia*, the wild-type reference strain, was used in RNAi experiments. The mutant nd7-1 (Skouri and Cohen, 1997) which carries a recessive monogenic mutation preventing trichocyst discharge, a dispensable function under laboratory conditions, was used for transformations. Cells were grown at 27°C in a wheat grass infusion, BHB (L’arbre de vie, L’arbre de vie, Hyperion, France) or WGP (Pines International, Lawrence, KS), bacterised with Klebsiella pneumoniae and supplemented with 0.8 μg/ml β-sitosterol according to standard procedures (Sonneborn, 1970).

Gene identification
By BLAST search, we identified two *Paramecium* genes encoding proteins homologous to FOR20, *PtFOR20a* (PTETG1700017001) and *PtFOR20b* (PTETG4900011001) (Arnaiz and Sperling, 2011). These two genes result from the last whole genome duplication which occurred in *Paramecium*, and are 92.4% identical: they code for 165 amino acid long proteins which differ by a single amino acid, at position 22.

Gene cloning
For expression of the GFP-tagged PtFOR20 protein, the entire 595 base pair long *PtFOR20a* gene was amplified from genomic DNA by PCR, using primers into which linkers containing the specific sequence of the KpmI enzyme were added. Two GGT glycine codons were also added in the sequence of the 5’ primer between the KpmI restriction sequence and the ATG sequence of the *PtFOR20*
gene. After KpnI restriction digest, the fragment was cloned into the KpnI restriction site located at the 3' end of the GFP synthetic gene that was designed by Eric Meyer and Jean Cohen (personal communication) and which had been introduced into the pPVX vector (Haynes et al., 1995), the recombinant gene being under the control of the Paramecium calmodulin regulators. After cloning, the gene was entirely sequenced to ensure that no error was introduced during the amplification. For gene silencing, only a part of the PIFOR20-b gene (from base 3 to 453) was amplified by PCR and cloned into the Ahol and HinfI site of the L4440 vector. This vector allows the synthesis of double-stranded RNA corresponding to the cloned gene from two T7 promoters. The possible off-target effect of this sequence was analysed using the RNAi off-target tool in the Paramecium genome database (http://paramecium.cgm.cnrs-gif.fr/cgi/tool/alignment/off_target.cgi) that searches the genome for stretches of 23 identical nucleotides (the size of Paramecium siRNA). The only genomic sequence matching with PIFOR20a was PIFOR20b, thus ruling out possible off-targeting effect, but indicating that both genes will be co-silenced by the use of a single RNAi vector. For Ptccn2 and PtcCon3 silencing, we used the same vectors as those described previously (Ruiz et al., 2005).

**Paramecium transformation**

nd7-1 mutant cells were transformed by micro-injection into their macronucleus of filtrated and concentrated plasmid DNA containing a mixture of the plasmids of interest (1ug/ul) isolated by SII and cut with plasmid DNA directing the expression of the nd7 wild-type gene (Skouri and Cohen, 1997). Transfectants were first screened for their ability to discharge their trichocysts and if so, further analysed. Microinjection was made under an inverted Nikon phase-contrast microscope, using a Narishige micromanipulation device and an Eppendorf air pressure microinector.

**Gene silencing**

Gene silencing was performed by the feeding method as previously described (Galvani and Sperling, 2002). Stationary wild-type cells or log-phase transformed cells were fed with the appropriate double-stranded RNA-expressing HT115 bacteria and transferred daily into fresh feeding medium as needed. Control cells were fed with bacteria transformed by the L4440 plasmid or the L4440 plasmid carrying the complete coding region of the ND7 gene.

**Fluorescence microscopy**

Immunostaining of cells was carried out as previously described (Iffide et al., 1989), except for cilia labelling, where saponin 0.5% was used instead of Triton X-100 to permeabilise the cells, with saponin 0.25% maintained in all subsequent buffers. Cells were observed under a Zeiss Axioskop 2-plus fluorescence microscope equipped with a Roper Coolsnap-CP intensifying camera with GFP filters. Images were processed with Metamorph software (Universal Imaging). Alternatively, they were observed with a Confocal Nikon eclipse TE 2000-U microscope equipped with argon and Helium-Neon lasers using the EZ-C1 3.30 software for acquisitions. Unless specified, images are projections of optical sections acquired at the level of one cell side. Projections were calculated using ImageJ (NIH) and images were treated with Photoshop CS2 (Adobe) software.

**Electron microscopy**

For ultrastructural observations, the depleted and control cells were fixed in 1% (v/v) glutaraldehyde and 1% OsO4 (v/v) in 0.05 M cacodylate buffer, pH 7.4 for 30 min. After rinsing and dehydration in ethanol and propylene oxide series, they were embedded in Epon resin. For pre-embedding immunolocalisation, the immunostaining process was carried out as described for immunofluorescence using a gold-coupled instead of fluorochrome-coupled secondary antibody. Then cells were rinsed, fixed and embedded as previously described. For post-embedding immunolocalisation, cells were fixed in 3% paraformaldehyde, 0.15% glutaraldehyde, in 0.05 M cacodylate buffer, pH 7.4 at 4°C for 1 h. After washes and dehydration in ethanol, they were embedded in LR White (London Resin). Thin sections were collected on nickel grids and treated with 0.1 N HCl in 0.1 M PBS and then saturated with 3% BSA and 0.1 M glycine in PBS. Sections were incubated with anti-GFP polyclonal antibody at room temperature for 45 min. After several washes in PBS a gold-labelled anti-rabbit IgG (GAR G10, Aurion) was applied for 30 min. The grids were rinsed in PBS and distilled water, and finally stained with uranyl acetate. All ultrathin sections were contrasted with uranyl acetate and lead citrate. The sections were examined under the control of the cortex of the ciliated protozoon Tetrahymena pyriformis. J. Cell Biol. 40, 716-733. Andersen, J. S., Wilkinson, C. J., Mayor, T., Mortensen, P., Nigg, E. A. and Mann, M. (2003). Proteomic characterization of the human centrosome by protein correlation profiling. *Nature* 426, 570-574.


**Fig. S1. Multiple Alignment of the two PtFOR20p with their homologs.** The TOF and LisH-PLL motifs conserved in these FOP-like proteins are highlighted. The amino acids differing in the two *Paramecium* proteins are in red.
Fig. S2. Pattern of the basal body duplication during cell division. A, B, C: Confocal images of the μbasal bodies labelled with the ID5 antibody: cells anterior points towards the top. While globally the number of basal bodies doubles during division, duplication is not uniform and varies according to a precise regionalization, to yield the shaping of the two asymmetric halves of the mother cell into two daughter cells. Hyper duplication takes place across the division furrow; in contrast, no enlargement of the cell surface takes place in the two outlined “invariant fields”, which are passed over to the anterior and posterior division products respectively—In the anterior field (top), all basal bodies are paired (top insert A), while they are not in the posterior field (bottom insert A). In the rest of the cell, they are either paired or not without clear rule. As the cell divides, a new oral apparatus assembles close to the parental one by intense local proliferation of basal bodies. At the cell surface, proliferation of basal bodies anterior to parental ones starts at the equator and progresses as a wave towards the two poles, thus marking the future division plane (B). When the wave of duplication reaches the “invariant” anterior field, the anterior basal body of each pair disappears (top insert B). In contrast no change takes place in the posterior invariant field (bottom insert B). At a later division stage (C), when the two oral apparatuses have pulled apart and the development of the two daughter cells is in progress, paired basal bodies are again detected in the anterior field: a new basal body has been assembled anterior to the remaining parental one (top insert C), while the posterior field remained unchanged (bottom insert C). Bar: 20 μm. Inserts, ×5
Fig. S3. Decrease of the GFP signal in a GFP-PtFOR20 expressing cell during the first division after FOR20 depletion. Confocal image of the ventral side of a cell double-labelled with the anti-GFP (green) and the ID5 (red) antibodies. Bar is 20 μm. Insert: ×3. The new oral apparatus begins to assemble. The future fission line is not yet easily detected at the whole cell level, but higher magnifications show that, as depletion occurs, no GFP is associated with new basal bodies detected with ID5 assembled anterior to parental ones (arrows in the merge image).
Table S1. Percentages of gold particles observed along the basal bodies with the anti-GFP antibody labelling after pre- and post-embedding procedure on GFP-PtFOR20 expressing cells, and post-embedding on GFP-PtCen2 and GFP-PtCen3 expressing cells.

<table>
<thead>
<tr>
<th></th>
<th>PtFOR20</th>
<th></th>
<th>PtCen2</th>
<th></th>
<th>PtCen3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post-emb</td>
<td>Pre-emb</td>
<td>Post-emb</td>
<td>Post-emb</td>
<td>Post-emb</td>
<td>Post-emb</td>
</tr>
<tr>
<td>NBb</td>
<td>225</td>
<td>146</td>
<td>21</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>24%</td>
<td>38%</td>
<td>100%</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Npar</td>
<td>61</td>
<td>63</td>
<td>194</td>
<td>83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m</td>
<td>0.3</td>
<td>0.4</td>
<td>9</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TZd</td>
<td>13%</td>
<td>15%</td>
<td>0%</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TZp</td>
<td>58%</td>
<td>85</td>
<td>55%</td>
<td>20%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBd</td>
<td>21%</td>
<td>0%</td>
<td>33%</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBp</td>
<td>8%</td>
<td>0%</td>
<td>12%</td>
<td>80%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table S2. Quantification of the ultrastructural characteristics of the transition zone of intracytoplasmic basal bodies after PtCen2p, PtCen3p, and PtFOR20p depletion.

<table>
<thead>
<tr>
<th></th>
<th>Centrin 2 N=39</th>
<th>Centrin 3 N=45</th>
<th>FOR20 N=15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incomplete plates</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Less than 3 plates</td>
<td>23</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>3 plates</td>
<td>6</td>
<td>42</td>
<td>0</td>
</tr>
</tbody>
</table>