RFX3 governs growth and beating efficiency of motile cilia in mouse and controls the expression of genes involved in human ciliopathies

Loubna El Zein1, Aouafet Ait-Lounis2, Laurette Morlé1, Joëlle Thomas1, Brigitte Chhin1, Nathalie Spassky3, Walter Reith2 and Bénédicte Durand1,*

1Université de Lyon, Lyon, F-69003, Université Lyon 1, CNRS, UMR5534, CGMC, Centre de Génétique Moléculaire et Cellulaire, Villeurbanne, F-69622, France
2Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, CMU, 1 rue Michel-Servet, CH-1211 Geneva, Switzerland
3INSERM U711, Hôpital Salpêtrière, 47 Boulevard de l'hôpital, 75013 Paris, France

*Author for correspondence (durand-b@univ-lyon1.fr)

Accepted 21 June 2009
Journal of Cell Science 122, 3180-3189 Published by The Company of Biologists 2009
doi:10.1242/jcs.048348

Summary
Cilia are cellular organelles that play essential physiological and developmental functions in various organisms. They can be classified into two categories, primary cilia and motile cilia, on the basis of their axonemal architecture. Regulatory factor X (RFX) transcription factors have been shown to be involved in the assembly of primary cilia in Caenorhabditis elegans, Drosophila and mice. Here, we have taken advantage of a novel primary-cell culture system derived from mouse brain to show that RFX3 is also necessary for biogenesis of motile cilia. We found that the growth and beating efficiencies of motile cilia are impaired in multiciliated Rfx3−/− cells. RFX3 was required for optimal expression of the FOXJ1 transcription factor, a key player in the differentiation program of motile cilia. Furthermore, we demonstrate for the first time that RFX3 regulates the expression of axonemal dyneins involved in ciliary motility by binding directly to the promoters of their genes. In conclusion, RFX proteins not only regulate genes involved in ciliary assembly, but also genes that are involved in ciliary motility and that are associated with ciliopathies such as primary ciliary dyskinesia in humans.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/122/17/3180/DC1

Key words: Axonemal dyneins, Cilia, Mouse primary cell cultures, Primary ciliary dyskinesia, RFX proteins

Introduction
Cilia play key roles in various organisms ranging from cell or fluid motility to cellular responses to environmental cues (for reviews, see Eggenschwiler and Anderson, 2007; Singla and Reiter, 2006). In the last decade, numerous studies have highlighted the importance of cilia in human health and the consequences of ciliary dysfunction in several human diseases (for reviews, see Bisgrove and Yost, 2006; Fliegauf et al., 2007; Marshall, 2008). Tremendous effort has also been devoted to the identification of proteins involved in cilia assembly (Avidor-Reiss et al., 2004; Broadhead et al., 2006; Keller et al., 2005; Li et al., 2004; Ostrowski et al., 2002; Pazour et al., 2005; Stolc et al., 2005), leading to the establishment of gene lists compiled in freely available databases [cilia proteome or ciliome data bases (Gherman et al., 2006; Inglis et al., 2006)]. Recent work has added probable new candidates to these primary lists (Hayes et al., 2007; Lonergan et al., 2006; McClintock et al., 2008; Ross et al., 2007; Stubbs et al., 2008; Yu et al., 2008). These studies used complementary approaches based on proteomic and transcriptomic methods as well as comparative genomic strategies. Several studies took advantage of the specificity of the regulatory factor X (RFX) family of transcription factors for a well-defined DNA motif: the X-box (Emery et al., 1996b). This approach was pioneered by studies in Caenorhabditis elegans and was subsequently extended to other comparative studies and models.

RFX transcription factors have been shown to govern ciliogenesis in C. elegans and Drosophila, and this property has been instrumental in both organisms for identifying novel genes involved in ciliogenesis (Avidor-Reiss et al., 2004; Blacque et al., 2005; Chen et al., 2006; Dubruille et al., 2002; Efimenko et al., 2006; Efimenko et al., 2005; Haycraft et al., 2003; Haycraft et al., 2001; Li et al., 2004; Schafer et al., 2003; Swoboda et al., 2000). In mice, RFX3 has been shown to regulate primary ciliary growth in the embryonic node and in the pancreas (Ait-Lounis et al., 2007; Bonnafe et al., 2004). In both systems, RFX3 was found to regulate at least one gene coding for a molecular motor component of the intraflagellar transport (IFT) apparatus: the dynein light chain gene Dync2li1. In addition, RFX3 deficiency leads to hydrocephalus that is associated with defects in the differentiation of the subcommissural organ and choroid plexuses (Baas et al., 2006).

We show here that RFX3 is a key player in the formation of motile multicia in a primary-cell culture system derived from mouse brain. RFX3 deficiency affects both ciliary growth and ciliary beat frequency (CBF) in this culture system. In addition to the previously known Dync2li1, RFX3 was found to regulate the orthologs of genes involved in primary ciliary dyskinesia (PCD) in humans. We show that RFX3 binds to the promoters of the genes encoding two axonemal dyneins involved in ciliary motility. We also demonstrate that RFX3 regulates Foxj1 expression by binding to its promoter. Our results thus show that mammalian RFX proteins regulate genes involved in ciliary assembly per se but also control genes involved in ciliary motility. In addition, our work validates a novel cell culture system for functional studies on genes implicated in ciliogenesis in mice.
Results
In vitro differentiation of ciliated ependymal cells derived from E18.5 mouse embryos
During mouse embryogenesis, Rfx3 is strongly expressed in a subset of cerebral ventricular cells (Baas et al., 2006). After birth, the expression of Rfx3 is maintained in multiciliated ependymal cells lining the cerebral ventricles (Fig. 1A). This timing and pattern of expression is in agreement with the fact that Rfx3 is expressed in the multiciliated ependymal cell lineage from progenitors to the fully mature stage (Spassky et al., 2005). When backcrossed onto a pure C57BL/6 genetic background, no Rfx3−/− pups survived more than a few days after birth. It was hence very difficult to assess ciliary motility or growth in vivo in these mice, because ciliogenesis of ependymal cells is only completed after birth in mice (Spassky et al., 2005). To evaluate the function of Rfx3 in ependymal ciliogenesis, we took advantage of a neural-stem-cell culture system established from mouse embryos (Fig. 1B). In this system, embryonic day (E)18.5 embryonic mouse brains are dissected, the lateral ventricular zones are dissociated and cells are plated on a laminin substrate. Upon confluence, neural stem cells are separated from neurons or oligodendrocytes on the basis of cell adhesive properties by vigorous overnight shaking of the cultures and are then replated at a defined density (see Materials and Methods). The selected cells express the neuronal-stem-cell marker nestin (supplementary material Fig. S1A) and the astrocyte cell marker GFAP (supplementary material Fig. S1B). A single primary cilium is observed on the majority of cells (70%) at confluence (Fig. 1C, arrows). The cell cultures are highly homogenous after the selection procedure. Only low residual numbers of oligodendrocytes or neuronal cells remain after this selection procedure (generally less than 1%, but always less than 5%). Serum starvation induces the stem cells to differentiate into a monolayer of ciliated cells as demonstrated by staining for the tight-junction marker ZO1 and the cilia marker anti-glu-α-tubulin (supplementary material Fig. S1D).

These cells express CD24, a cell surface marker of ependymocytes (Calàora et al., 1996) (supplementary material Fig. S1E). Cells progressively develop motile cilia. After 20 days of serum starvation, numerous motile cilia are visible on the majority of cells (Fig. 1C; supplementary material Fig. S1C and Movie 1). No changes are observed in cell number during differentiation, as estimated by counting nuclei before and after differentiation. The only cells that die during the first 4 days of serum deprivation are the few (<1%) contaminating neuronal or oligodendritic cells that are easily distinguished and not included in the cell count at day 0.

Rfx3 is expressed in ependymal cell culture
RFX3 expression was visualized by immunostaining of the cell cultures. Nuclear RFX3 was evident at day 0 before serum starvation in 95-99% of the cells (Fig. 1C; Fig. 2A, around 1% of the cells never express RFX3). We observed a reproducible increase in RFX3 expression during serum starvation. Because anti-RFX3 antibodies do not allow the detection of RFX3 on western blots, RFX3 protein was quantified by confocal microscopy. A significant increase in RFX3 immunoreactivity was observed after 20 days of serum deprivation (Fig. 2B). To determine whether the in-vitro-induced ciliogenesis is accompanied by an increase in cilia-specific gene expression, we performed a kinetic analysis of the expression of a series of representative ciliary genes (Fig. 2C). Statistical significance of the variations at each stage relative to day 0 was evaluated by two-tailed Student’s t-test. We observed a small (2.5-fold) but significant increase in Rfx3 messenger levels (P<0.05 for day 8 to 20). We also observed that the expression of mRNA for genes known to be necessary for cilia assembly, such as Dynein1 or Bbs4, were increased at least 1.5-fold. Known motility genes such as Dnahc5, Dnahc9 and Dnahc11 were induced more than threefold after serum starvation. Expression of the transcription-factor-encoding Foxj1 gene was strongly increased during the first days of serum starvation but diminished after 8 days of differentiation. Maximal induction for all

Fig. 1. RFX3 is expressed in ciliated ependymal cells in vivo and during ependymal ciliogenesis in vitro. (A) RFX3 is expressed in ciliated ependymal cells lining the ventricles in postnatal mouse brains. The image shows the lateral ventricle of an adult mouse brain stained for RFX3 (red) and βIV tubulin (green). Scale bar: 50 μm. (B) Schematic representation of the cell culture protocol. E18.5 embryonic brains were dissected, the cortical lateral hemispheres (light grey) were dissociated and cells were cultured as indicated. (C) Representative images of ependymal cell cultures before (C1, day 0) and after (C2-C6: days 4, 8, 12, 16 or 20, respectively) serum deprivation. RFX3 (red), cilia (green) and nuclei (blue) were visualized by immunostaining. Note that cells first harbor a primary cilium (C1, arrows) and progressively develop multiple cilia (C2-C6, arrowheads). At 20 days after serum deprivation, most cells carry a tuft of cilia (C6). Approximately 1% of the cells never express RFX3. Scale bars: 20 μm.
of the other analyzed ciliogenic genes was observed after 8 days of serum starvation. This timing precedes the maximum density of cilia and ciliated cells in the culture (Fig. 1C; supplementary material Fig. S1C). Kif3a gene expression was induced weakly, whereas expression of the control cell-proliferation marker gene Mki67 was reduced during serum starvation (not shown).

Rfx3-deficient ependymal cells show a strong reduction in ciliary growth
To assess the function of Rfx3 in ciliogenesis, we compared parallel cell cultures obtained from wild-type and Rfx3–/– littermates. We determined by immunolabeling that no nuclear RFX3 protein was detected in Rfx3–/–-derived cells (Fig. 3A). We did not observe any
difference in expression of the precursor-specific marker GFAP or nestin before differentiation (supplementary material Fig. S1A,B), suggesting that RFX3 has no major effects on the overall cell-selection procedure. We observed a small difference in the number of primary cilia before differentiation in Rfx3−/− samples compared with wild type (Fig. 3B). This is in agreement with our previously published observation that RFX3 controls primary-ciliary growth, even though the effect is less pronounced in this cell culture system compared with the previously described in vivo situations (Ait-Louis et al., 2007; Bonnafé et al., 2004).

After serum starvation, we observed drastic differences in the number of multiciliated cells between wild-type and Rfx3−/− samples (Fig. 3A,C,D). We quantified the total number of ciliated cells harboring either one cilium or multiple cilia in each sample after 2 weeks of serum starvation (Fig. 3C). To verify that there was no bias resulting from cell-density variations in the cultures due to heterogeneous seeding, we plotted the results as a function of the cell density for each photographed field and the linear regression was calculated. This cell density can vary considerably between fields within one cell culture, even though seeding density was controlled for each experiment. As observed on Fig. 3C, cell density impacted equally on the number of monociliated after serum deprivation in Rfx3−/− samples (Fig. 3D). By contrast, we observed that cell density had a strong impact on the growth of multiple cilia in Rfx3+/+ cells, whereas multiple ciliary growth was not dependant on cell density in Rfx3−/− samples (Fig. 3C, P=3.03×10−10). In addition, the total number of multiciliated cells in Rfx3−/− samples was reduced compared with wild type, independently of cell density (Fig. 3D). Thus, Rfx3 is necessary for ciliogenesis in ependymal cells and controls both the overall frequency of ciliated cells and the number of cilia per cell. After differentiation, we did not observe any difference between the two genotypes in the expression of the ependymal-specific marker CD24 or the cell-junction marker ZO1 (supplementary material Fig. S1D,E), suggesting that defective ciliogenesis does not result from impaired cell-cell contact formation or cell differentiation.

We noticed that cilia were markedly shorter in Rfx3−/− cell cultures. We therefore estimated the mean ciliary length of motile cilia on multiciliated cells using confocal microscopy. Three-dimensional (3D) reconstructions were used to visualize the cilia and verify the accuracy of the length measurements. As shown in Fig. 4, ciliary length was significantly reduced in Rfx3−/− cells as compared with wild type. The mean ciliary length for Rfx3−/− cells is 5.5±1.9 μm, which was 57% shorter than for Rfx3+/+ samples (12.9±1.8 μm). Statistical significance was evaluated using a two-sample Student’s t-test. The difference in ciliary length was statistically significant (P<2.2×10−16). Thus, Rfx3 is required for the growth of motile cilia in ependymal cells.

**Rfx3-deficient ependymal cells show a reduction of ciliary motility**

By videomicroscopy recordings using a high-speed camera, we assessed ciliary motility in cell cultures derived from wild-type and Rfx3−/− embryos. Striking differences were observed in slow-speed videos of the cells (see representative examples in supplementary material Movies 2 and 3). We precisely quantified the mean CBF for each cell. Cells were selected randomly in the cultures and the measurement was performed for six to nine fields for each culture (for each field the measurement was performed for one to three cells). To check that the calculated CBF differences were not affected by environmental factors, we measured CBF on samples issued from two different litters (n=seven wild-type and n=four Rfx3−/− samples) on three different days, at either room temperature or 37°C, and in a random sample order for each day and condition (Fig. 5A). The CBF differences between wild-type and Rfx3−/− samples were evident in all of the different situations, even though the absolute CBF value varied between conditions (Fig. 5A). We next repeated the measurements for cultures derived from four additional sets of litters (Fig. 5B). For each litter, the CBF was significantly reduced in Rfx3−/− cells (total number of samples: 17 wild type and 11 Rfx3−/−). The overall mean CBF for Rfx3−/− cilia was 13.3 Hz, and the overall mean CBF for Rfx3+/+ (wild-type) cilia was 20.14 Hz for all samples. The CBF difference between Rfx3−/− and Rfx3+/+ was statistically significant for all the samples (P<0.05). Thus, Rfx3 deficiency alters the motility of cilia in on-in-vitro-differentiated
primary ependymal cells. We also observed that cilia beating was more frequently asynchronous in Rfx3–/– cells compared with wild-type cells (compare supplementary material Movies 2 and 3), although a few cells showing asynchronous beating were generally observed even in wild-type samples (data not shown).

Finally, we compared by transmission electron microscopy the ultrastructure of cilia between Rfx3–/– and wild-type cultures. We did not observe defects in the basal-body ultrastructure in Rfx3–/– samples (supplementary material Fig. S2). Whereas numerous cilia were visualized in transverse sections in wild-type samples, only a few transverse sections of cilia could be observed in the mutant samples. However, it was difficult to visualize stereotyped ultrastructural defects in the mutant cilia and no clear mechanistic conclusions could be drawn from this ultrastructural analysis. Cilia with normal architecture were visualized in the mutant samples but cilia with perturbed microtubule arrangements were frequently observed. These defects seemed to affect mainly the distal region of the cilia (supplementary material Fig. S2), suggesting improper axonemal elongation in the mutant cilia. It should be noted that aberrant cilia were also occasionally observed in wild-type samples.

Ciliary genes are downregulated in Rfx3-deficient ependymal cells

RFX proteins regulate a similar set of genes involved in ciliogenesis in C. elegans and Drosophila. Several RFX target genes identified in these two organisms are involved in IFT (Avidor-Reiss et al., 2004; Blacque et al., 2005; Chen et al., 2006; Efimenko et al., 2006; Efimenko et al., 2005; Haycraft et al., 2003; Haycraft et al., 2001; Laurencon et al., 2007; Li et al., 2004; Schafer et al., 2003; Swoboda et al., 2000). In addition, the expression of Drosophila and nematode orthologs of genes involved in the Bardet-Biedl syndrome (BBS) is also regulated by RFX proteins (Ansley et al., 2003; Blacque et al., 2005; Chen et al., 2006; Efimenko et al., 2005; Laurencon et al., 2007). We therefore investigated whether RFX3 also regulates the expression of these conserved ciliary genes in mice. We analyzed a selection of IFT and BBS genes (Fig. 6). The expression of Dyn2li1 was significantly downregulated in Rfx3–/– samples. Bbs4 expression was slightly reduced in the mutant samples compared with wild type in each litter, but this difference was not statistically significant (P=0.125) when all wild-type and mutant samples were compared together. By contrast, Bbs2 expression was not affected in Rfx3–/– samples. The control Rps9 gene encoding ribosomal protein 9 showed no significant difference in expression between wild-type and Rfx3–/– samples.

Drosophila genes involved in ciliary motility have been found to share an X-box in two Drosophila species (Laurencon et al., 2007). Among these motility genes, two axonemal dynein genes, CG9492 and CG3723, are under Rfx control in Drosophila (Laurencon et al., 2007) (our unpublished results). Their orthologues in mammals are Dnah5 and Dnah9, respectively. We analyzed the expression of these genes in our cell culture system, together with Dnahc11, a paralog of Dnahc9 that is also homologous to CG3723. The expression of Dnahc5, Dnahc11 and Dnahc9 was strongly downregulated in Rfx3–/– cell cultures, and these genes are thus under Rfx control in mice (Fig. 6). Because Dnahc11 and Dnahc9 are under the control of Foxj1 (Brody et al., 2000; Chen et al., 1998; Stubbs et al., 2008; Yu et al., 2008), we investigated whether Rfx3 might also regulate the Foxj1 gene. We observed a modest but significant reduction in Foxj1 expression (P=0.0428) in Rfx3–/– samples (Fig. 6). We can therefore not exclude that downregulation of Dnahc11 and Dnahc9 expression in Rfx3–/– samples could be an indirect consequence of the minor reduction in Foxj1 expression.

Ciliary genes are direct targets of RFX3 in ciliated ependymal cells

To determine whether RFX3 directly regulates ciliary gene expression, we performed chromatin immunoprecipitation (ChIP) experiments. Crosslinked chromatin from large-scale cultures of ependymal cells from wild-type newborn pups was immunoprecipitated with anti-RFX3 antibodies. Immunoprecipitates were then amplified by quantitative PCR using primers situated upstream of the expected transcription start sites of six of the candidate RFX3 target genes identified by our expression analysis (see above). Primers situated at randomly selected downstream positions within the genes were used as negative controls. Our results demonstrated that the promoters of Bhs4, Dyn2li1, Dnahc11 and Dnahc9 are selectively enriched in the RFX3 ChIP samples and are thus bound by RFX3 in the cultured ependymal cells (Fig. 7A). Analysis of the promoter sequences of these genes in several mammalian genomes revealed the presence of highly conserved X-boxes situated immediately upstream of the
transcription start sites of the Dnahc9, Dyn2li1 and Bbs4 genes (Fig. 7B). No conserved X-box was evident in the promoter proximal region of the Dnahc11 gene. However, a strongly conserved X-box was observed immediately downstream (~80 nucleotides) of the transcription start site in several mammalian genomes. Enrichment of the Dnahc11 promoter in our ChIP experiments could be the consequence of RFX3 binding to this downstream site. No binding of RFX3 to the Dnahc5 promoter was detected despite the fact that it contains an X-box-like sequence situated upstream of the transcription start site. Alignment of the X-box motifs found in the promoters analyzed in this study reveals that the Dnahc5 X-box contains changes at highly conserved nucleotides of the X-box consensus site, suggesting that these residues are crucial for the binding of RFX3 (Fig. 7C). Dnahc5 is thus either regulated indirectly by RFX3 or controlled by an X-box motif that remains to be localized but would have to be situated outside of the 2-kb upstream region that was analyzed by ChIP. Foxj1 harbors a strongly conserved X-box in its proximal promoter and the Foxj1 promoter is strongly enriched in the RFX3 ChIP. Together with the small but significant reduction in Foxj1 expression observed in Rfx3−/− cells, these results suggest that binding of RFX3 is required for optimal Foxj1 expression.

Discussion

The results presented here demonstrate that RFX3 regulates the number, growth and motility of cilia on cultured mouse ependymal cells. We identified genes encoding three axonemal dyneins as novel RFX3-regulated genes. RFX3 binds to the promoters of two of these axonemal dynein genes in vivo. RFX3 therefore regulates two types of genes implicated in cilia biology: genes involved in ciliary assembly (Dyn2li1, Foxj1 and Bbs4) and genes involved in ciliary motility (Dnahc11, Dnahc9 and Dnahc5). This work sets the stage for the identification of target genes of ciliogenic transcription factors in multiciliated mammalian cells.

Transcriptional control of ciliogenesis

A large set of RFX-regulated genes have been defined in Drosophila and C. elegans. Many of these genes are involved in IFT (Avidor-Reiss et al., 2004; Blacque et al., 2005; Chen et al., 2006; Efimenko et al., 2005; Haycraft et al., 2003; Schafer et al., 2006; Efimenko et al., 2005; Haycraft et al., 2003; Haycraft et al., 2001; Laurencon et al., 2007; Li et al., 2004; Reiss et al., 2004; Blacque et al., 2005; Chen et al., 2006; Efimenko et al., 2005; Haycraft et al., 2003; Haycraft et al., 2001; Laurencon et al., 2007; Li et al., 2004; Schafer et al., 2003; Sloboda et al., 2000). These studies have shown that all known...
orthologs of Bbs genes are tightly regulated by RFX factors in Drosophila and C. elegans. We show here that expression of the Bbs4 gene is not strongly dependent on RFX3 in cultured epidermal cells, despite the fact that RFX3 does bind to the Bbs4 promoter in vivo. One possible explanation is that other RFX factors might compensate for the absence of RFX3. Although there is only one RFX transcription factor in C. elegans, and only two in Drosophila, there are seven RFX transcription factors in mammals (Aftab et al., 2008; Emery et al., 1996a). Rfx2 has been shown to regulate ciliogenesis in the zebrafish (Liu et al., 2007). A role of Rfx1 and Rfx4-Rfx7 in ciliogenesis has so far not been demonstrated. It is possible that functional redundancy between RFX factors is responsible for the mitigated role of RFX3 in Bbs4 expression. The absence of an effect on Bbs2 expression in Rfx3−/− cells could also reflect a redundancy between RFX factors. Alternatively, it is possible that the requirement for specific RFX factors varies according to the target gene. In this respect, it is not known whether RFX proteins can distinguish between different sets of target genes. RFX1, RFX2 and RFX3 bind to DNA as homo- or heterodimers, and all three bind to the same X-box motifs (Reith et al., 1994). There is currently no experimental evidence suggesting that there are differences in binding affinity or specificity between different RFX dimers.

Rfx genes are expressed differentially in mammalian tissues. For example, Rfx3 is expressed more strongly in the brain whereas Rfx2 is expressed strongly in the kidney (Reith et al., 1994). Rfx4 also exhibits a specific expression pattern (Blackshear et al., 2003; Zhang et al., 2007). The differential expression patterns of RFX transcription factors might help to fine tune ciliogenesis in different cell types. We have shown here that RFX3 is required for the generation of motile cilia in epidermal cells. We have not been able to establish whether RFX3 is also involved in the growth and motility of cilia in the upper airways of the mouse, because Rfx3-deficient mice die at birth and ciliogenesis is completed only postnatally in mice (Toskala et al., 2005). However, RFX3 is expressed strongly in ciliated cells of the upper airways towards the end of embryogenesis and throughout postnatal life (our unpublished observations), suggesting that RFX3 could also play a role in ciliogenesis in airways in vivo. We cannot, however, exclude that other RFX transcription factors are also implicated in ciliogenesis of motile cilia in the upper airways.

In addition to genes required for ciliary assembly, we show here that RFX3 regulates several genes encoding axonemal dyneins. A search for X-box motifs led to the identification of several axonemal dynein genes having conserved X-boxes in two Drosophila species (Laurencon et al., 2007). We show here that the expression of the Dnahc9, Dnahc11 and Dnahc5 genes is downregulated in Rfx3-deficient epidermal cells. Dnahc11 and Dnahc5 are involved in cilia motility in mice and humans (Bartoloni et al., 2002; Ibanez-Tallon et al., 2002; Olbrich et al., 2002; Schwabe et al., 2008; Supp et al., 1997). Dnahc9 encodes a dynein that is associated with human motile respiratory cilia and exhibits a perturbed distribution in certain patients with PCD (Carson et al., 2002; Fliegauf et al., 2005). FOXJ1 has been shown to be implicated in ciliogenesis of motile cilia in vertebrates. Foxj1 expression increases dramatically during in vitro ciliogenesis in multiciliated cells (Ross et al., 2007; You et al., 2004), and Foxj1-deficient mice are characterized by the absence of motile cilia (Blatt et al., 1999; Brody et al., 2000; Chen et al., 1998; Tichelaar et al., 1999; Whitsett and Tichelaar, 1999). Foxj1 has been shown to be sufficient for driving motile cilia assembly in Xenopus and zebrafish (Stubbs et al., 2008; Yu et al., 2008). Although Foxj1 is not sufficient to drive ectopic ciliary growth in mouse epithelial cells (You et al., 2004) and only a few mouse target genes of this transcription factor have been reported (Brody et al., 2000; Chen et al., 1998; Gomperts et al., 2004; Huang et al., 2003), more than 100 target genes have been identified in Xenopus (Stubbs et al., 2008; Yu et al., 2008). Dnahc11 expression depends on Foxj1 in mice (Brody et al., 2000; Chen et al., 1998) and Dnahc9 expression depends on Foxj1 in both Xenopus and zebrafish. Interestingly, Dnahc9 was confirmed to be a direct Foxj1 target in these animals, and we have shown that Dnahc9 and Dnahc11 are direct targets of RFX3 in mice. These observations suggest that RFX and FOXJ1 proteins regulate a common set of ciliary motility genes. RFX3 and FOXJ1 might cooperate to regulate Dnahc9 and Dnahc11 expression. In addition, we observed a small but significant reduction in Foxj1 expression (P=0.0428) in Rfx3-deficient cultures, which could amplify the consequences of the loss of Rfx3 function on Dnahc9 and Dnahc11 expression in our cell-surface system.

The HNF1β transcription factor has been shown to be important for ciliogenesis in mouse kidneys (Gresh et al., 2004). ChIP experiments performed with whole kidney tissue led to the identification of HNF1β-binding sites in several ciliogenic genes (Gresh et al., 2004) involved in ciliary assembly or function. However, there is no evidence for a function of HNF1β in multiciliated cells. Recently, the Noto transcription factor has been shown to regulate ciliogenesis in mice, and seems to function upstream of both Rfx3 and Foxj1 in the embryonic node (Beckers et al., 2007). It would be of particular interest to determine whether Noto is involved in the development of multicilia and identify its direct target genes.

We have shown here that our ependymal cell-surface system is suitable for large-scale ChIP experiments, permitting the identification of direct target genes of ciliogenic transcription factors. Multicilia only start to differentiate in the mouse brain and lung epithelia as of E18.5 (Spasovska et al., 2005; Toskala et al., 2005). Our cell-surface system allows the isolation of large numbers of ciliated cells from E18.5 embryos and thus permits the analysis of the function of late-embryonic-lethal genes in multiciliated-cell differentiation.

RFX3-binding-site definition

The consensus RFX3-binding site was derived from experiments performed with human RFX1. The consensus RFX1-binding site was defined by in vivo site-selection experiments in yeast (Emery et al., 1996b; Gajiwala et al., 2000). On the basis of this study, X-box-motif searches in C. elegans and Drosophila, combined with functional studies, led to the identification of consensus X-box motifs in these two organisms. The function of the X-box motif was assayed directly by mutagenesis for several genes in C. elegans. In C. elegans, the consensus RFX-binding site was precisely defined with a two-nucleotide spacer between the two half sites. In this organism, X-boxes are generally situated within the first 250 nucleotides upstream of the transcription start site. Such a strict position requirement does not seem to be conserved in Drosophila (Laurencon et al., 2007) or in mammals. The potential X-box site in Dnahc11 resides downstream of the transcription start site. Large-scale ChIP experiments have suggested that transcription-factor-binding sites at this position could be underscored in the literature and might be more important than previously anticipated (Koudritsky and Domany, 2008; Tabach et al., 2007). We can, however, not exclude that the true RFX3-binding site in the Dnahc11 promoter is not the X-box motif found downstream of the transcription start site, but another highly
dissimilar RFX-binding motif. An alternative possibility is that
RFX3 is recruited by interaction with another transcription factor
that determines promoter specificity, such as FOXJ1. Combining
motif searches with large-scale ChIP data could be very informative
for defining the regulatory networks that control ciliogenesis in
multiciliated mammalian cells.

**RFX proteins and human syndromes**

Our results demonstrate that RFX transcription factors regulate two
categories of genes: those involved in ciliary assembly and in ciliary
motility. The distinction between these two categories was first
emphasized by human syndromes resulting from ciliary dysfunction,
which can also be classified into two categories corresponding to
defects in either ciliary motility or growth. Prototypical examples
are PCD and BBS, respectively. These two types of syndrome have
been considered to be clinically distinct. However, recent results
have revealed previously unnoticed overlaps between these
syndromes. For example, recent reports have highlighted the
function of BBS proteins in airway respiratory cilia (Shah et al.,
have noted a high incidence of bronchiectasis, a previously
uncharacterized manifestation of this disease (Driscoll et al., 2008).
In addition, some patients with PCD also have retnitis pigmentosa
or kidney failure (Bonneau et al., 1993; Moore et al., 2006; Osman
et al., 1991). These results suggest that genes involved in BBS might
act as modifiers of PCD. Conversely, genes known to be required
for ciliary motility could modify the severity of BBS symptoms.
In this respect, altered RFX3 function could be responsible for
variations in the severity of clinical symptoms in both types of
ciliopathy.

**Materials and Methods**

**Mouse strains**

Rfx3-deficient mice were generated as previously described (Bonnafe et al., 2004). Wild-type OF1 mouse embryos were used for kinetic studies of ciliogenesis and ChIP experiments. For studies on RFX3 function, experiments were performed with embryos derived from crosses between heterozygous Rfx3+/– adults on a C57BL/6 genetic background (Bonnafe et al., 2004). The embryonic stage was estimated on the basis of gestational time, with day 0.5 being defined as the morning when a vaginal
plug was detected. Adult mice and embryos were genotyped by PCR as described
previously (Bonnafe et al., 2004). The embryonic stage was estimated on the basis of gestational time, with day 0.5 being defined as the morning when a vaginal plug was detected. Adult mice and embryos were genotyped by PCR as described
previously (Bonnafe et al., 2004). Animal experimentation was carried out in a certified
animal-housing facility according to procedures approved by the local animal care
and experimentation authorities (Ministère Développe Recherche et Nouvelles Technologies, agreement no. 4936; Direction des Services Vétérinaires, agreement no. 69266 0602).

**Ependymal cell culture**

All reagents for cell culture were purchased from Gibco Life Technologies. Rat
deep well plates were disinfected from 18.5 days post-coitum embryos in
Hank’s medium (HBSS 1× without Ca2+ and Mg2+, 0.075% sodium bicarbonate, 0.01 M HEPES and 100 U/ml penicillin-100 µg/ml streptomycin). Cells were
trypsinized for 10 minutes at 37°C with 0.01% trypsin followed by centrifugation
for 5 minutes at 900 g. Cells were suspended mechanically in DMEM containing
100 U/ml penicillin-100 µg/ml streptomycin and 1.25 µg/ml amphotericin B, and supplemented with 10% fetal calf serum (FCS). Dissociated cells from a single brain were seeded in two laminin-coated (10 µg/ml) wells of a 24-well plate and
maintained in DMEM-10% FCS in a humidified 5% CO2 atmosphere at 37°C.
At confluence, culture trays were sealed and shaken overnight at 300 rpm (Janke and Kunkel, HS250). Adherent cells were rinsed with PBS and trypsinized. Cells were
harvested in DMEM-10% FCS and seeded on laminin-coated wells as above (two wells/single brain), or polylysine and laminin-coated coverslips (10 µg/ml each) for
immunohistochemical analysis. The minimum seeding density at this step was 5×104
cells/well in a 24-well culture tray. We used 103 seeding density/well in all the
experiments presented here. After 24 hours (for 105 cells/well seeding density) to 5
days (for lower seeding density), the medium was changed to DMEM without serum
to induce cell differentiation. For the culture of ependymal cells from OF1 mice, the
brains of ten newborn littersmates were pooled and cultured in two flasks of 25 cm2.
After shaking, the cells of each flask were distributed in 24-well tissue-culture trays or in a 100-mm diameter Petri dish coated with laminin and treated as above.

**Immunohistochemical analysis**

The anterior ependymal cells were fixed in 4% paraformaldehyde for 20 minutes at
4°C, blocked for 1 hour in 5% goat serum, 0.1% Triton X-100 in PBS at room
temperature and incubated overnight at 4°C with one of the following antibodies:
anti-RFX3 antibody (1/100) (Reith et al., 1994); anti-acetylated-α-tubulin mouse
monoclonal antibody (1/150, Sigma); anti-glutathion S-transferase mouse monoclonal antibody (clone ID5, 1/100, Synaptic Systems); mouse nestin antibody (1/400, BD Pharmingen),
rabbit anti-α-SMA antibody (1/200, Zymed Laboratories), rabbit anti-GAP antibody (1/300, DakoCytomation) and rabbit anti-Glu-tubulin (1/100, Abcys).
Immunostaining was revealed with donkey anti-rabbit biotinylated antibody (1/400, Jackson, Interchim) and Cy3-conjugated streptavidin (1/400, Interchim) or anti mouse-Alexa-Fluor-488-
conjugated antibodies (1/400, Molecular Probes). The slides were mounted in the
presence of DAPI or TOPRO3 in Vectashield (Vectors Laboratory) mounting medium.
For CD24 staining, rat anti-CD24 antibody (1/200, BD Pharmingen) was
used and no Triton X-100 was included during incubations. After incubation with
the secondary antibodies (goat biotinylated anti-rat antibody, Vector Laboratories and
Cy3-conjugated streptavidin, Interchim), cells were post-fixed in 4% paraformaldehyde for 10 minutes at room temperature and processed for other primary antibodies with 0.1% Triton X-100. Slides were visualized under an inverted Zeiss Axiovert fluorescent microscope equipped with 20× Plan-neofluor (0.5 numerical aperture) or 40× Plan-neofluor (0.75 numerical aperture) objectives. Controls in which individual primary antibodies were omitted resulted in no detectable staining. Images were acquired with a CCD camera (HQ2, Roper-Scientific) and Metaview software (MetaMorph). Image brightness and contrast were adjusted using ImageJ (NIH image) and separate panels were assembled with Photoshop 9.02 software. Capture
times and adjustments were identical for images mounted together. Quantification of immunofluorescence was performed on confocal stacks acquired with a Zeiss
LSM510 Meta confocal microscope and processed with MetaMorph software
(Molecular Devices).

**Cilia counting and density**

For quantification of cilia, different fields from wild-type or Rfx3–/– cells were photographed. Cilia were counted and classified into six categories: monocilia, 3 cilia/cell, 5-10 cilia/cell, 10-15 cilia/cell, 20-30 cilia/cell and 40 cilia/cell and above. Scatter-plot analysis was performed to report the number of monocrililated cells (first
category) or multicipililiated cells (five other categories combined) relative to the
total number of nuclei for each field for seven wild-type samples and seven Rfx3–/– samples. Correlation was calculated using a linear regression model and statistical significance of the variations between the linear regression slopes was evaluated.

**CBF measurements**

Behatiing cells were observed using an oil-immersion microscope (Leica DM-RXA) at
a magnification of 100× for room-temperature measurements and an inverted
microscope (Olympus IX-50) at a magnification of 40× for measurements in
a temperature-controlled chamber at 37°C. Beating cilia were recorded with a
digital high-speed video camera (PCO.1200hs, PCO.imaging, Germany) at a rate of 500
frames per second. The video sequences were extracted and recorded by ImageJ
followed by resclicing to visualize and measure the time period (in seconds) of
individual beating cilia. For the calculation of CBF and the statistical comparison
between Rfx3–/– and Rfx3+/– ependymal cilia, six to nine different fields were recorded
or analyzed per sample. Cilia were classified into 5 categories: 1-4 cilia/cell (n=5
samples) or 5-10 cilia/cell (n=4 samples) and analyzed in the same way on three different days (4 days between each
measurement, first measure at day 16) for all the Rfx3–/– (n=4) and Rfx3+/– (n=7)
littermates. The last measurement was performed at 37°C. A total of 407 fields were
video recorded and analyzed (one to three cells/field and one to three
cilia/cell). Statistical analysis was performed using ANOVA and a linear mixed
effect model. P-values of <0.05 were considered statistically significant. Movies from acquired
stacks were made with ImageJ software (Sorensen compression).

**Ciliary length**

Immunofluorescence images of cilia stained with detyrosinated or acetylated-α-tubulin
were acquired in 20-30 consecutive serial slices (depending on the length of cilia
using a Leica confocal microscope (TCS SP5) with a 63µ objective. The z-stack
images were flattened using Leica confocal software (LCS Lite, v2.61). Cilia length
were measured directly using a graphical pencil. 3D depictions of cilia were reconstructed using IMARIS software (Bitplane). Statistical significance of the
differences in ciliary length between wild-type and Rfx3–/– samples was evaluated
using a two sample Student’s t-test.

**Electron microscopy**

Cells were fixed in 2.0% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.35, for
45 minutes at room temperature. After extensive washing in sodium cacodylate 0.2
M, pH 7.35, cells were post-fixed in 1% Oso4 for 30 minutes, stained with uranyl acetate 1% for 30 minutes and dehydrated using a graded ethanol series. Cells
were embedded in epoxy resin. Ultrathin sections were cut with a Leica ultramicrotome.
Sectioned materials were contrasted in Leica ultrastainer in lead citrate. Sections
were observed with a Philips CM120 microscope.
Chromatin immunoprecipitation

Brains from 40 newborn pups (OF1 strain) were dissected and processed as above for cell cultures. After 12 days of serum deprivation, cells were fixed and processed for ChIP experiments as described previously (Masternak and Reith, 2002) using antibodies specific for RFX3 (Reith et al., 1994). Results were quantified by real-time PCR using the primers listed in supplementary material Table S1. PCR was performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad) and a SYBR-Green-based kit for quantitative PCR (iQ Supermix Bio-Rad). All results are presented as the mean ± s.e.m. of three independent ChIP experiments and three independent immunoprecipitations. A box motif was identified with Genepalette software (Rebeiz and Posakony, 2004) using the degenerate consensus defined for RFX proteins in mammals RYYNYYN0-3RRNRAC (Emery et al., 1996b). Each box motif was checked for sequence conservation between mammalian species using the UCSC genome browser. Only one conserved box motif was generally found in the 2-kb region upstream of the transcription start site. ChIP primers were designed at positions flanking the conserved box-sequences. For Dmha5, several pairs of primers were tested in a region covering 2-kb upstream of the transcription start site.

Online supplementary information

Supplementary material Table S1 lists the primers used for the ChIP experiments. Supplementary material Movie 1 shows OF1 cultures after 20 days of serum deprivation imaged at ten frames per second by DIC videomicroscopy and played at real speed. Supplementary material Movie 2 shows Rfx3-/- beating cilia imaged at 500 frames per second by DIC videomicroscopy and played at a tenfold reduced speed. Supplementary material Movie 3 shows Rfx3-/- beating cilia observed by DIC videomicroscopy and played at a tenfold reduced speed. Supplementary material Fig. S1 summarizes cell-culture characteristics before and after serum deprivation. Supplementary material Fig. S2 shows basal-body and cilia ultrastructure of wild-type and Rfx3-/- samples.

This work was supported by grants from the ANR Jeune-Checheur, the ANR Maladies Rare (ANR-05-MRAR-022-01) and the Région Rhône-Alpes (Programme Emergence, Programme Cible). Work in the laboratory of W.R. was supported by the Swiss National Science Foundation and the National Centre of Competence in Research NCCR-NEURO. A.A.-L. was supported by fellowships from the Association de Langue Frangaise pour l’Etude du Diabete et des Maladies Metaboliques (ALFEDIAM), the Jules Thorn Foundation and the EFSD/Lilly program. We thank Guillaume Tanniou for helpful assistance in confocal microscopy. Lastly, we thank Elisabeth Cortier, Jean-Luc Duteyrat, Annie Rivoire and Christelle Boule for excellent assistance in confocal microscopy. Lastly, we thank Elisabeth Cortier, Jean-Luc Duteyrat, Annie Rivoire and Christelle Boule for excellent assistance in confocal microscopy. Lastly, we thank Elisabeth Cortier, Jean-Luc Duteyrat, Annie Rivoire and Christelle Boule for excellent assistance in confocal microscopy. Lastly, we thank Elisabeth Cortier, Jean-Luc Duteyrat, Annie Rivoire and Christelle Boule for excellent assistance in confocal microscopy. Lastly, we thank Elisabeth Cortier, Jean-Luc Duteyrat, Annie Rivoire and Christelle Boule for excellent assistance in confocal microscopy. Lastly, we thank Elisabeth Cortier, Jean-Luc Duteyrat, Annie Rivoire and Christelle Boule for excellent assistance in confocal microscopy. Lastly, we thank Elisabeth Cortier, Jean-Luc Duteyrat, Annie Rivoire and Christelle Boule for excellent assistance in confocal microscopy. Last


Sup. Figure 1
Sup. Figure 2
<table>
<thead>
<tr>
<th>Gene (position)</th>
<th>orientation</th>
<th>sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dyn2lic (Promoter)</strong></td>
<td>forward</td>
<td>GCCGAAGGTGGAGAAGTAC</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>AGTTGGGAGGAGAATGC</td>
</tr>
<tr>
<td><strong>Dyn2lic (downstream)</strong></td>
<td>forward</td>
<td>AAC TCC GGC TAC TCT TCC</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>AGGGCTTCTGATCCCTTG</td>
</tr>
<tr>
<td><strong>Bbs4 (Promoter)</strong></td>
<td>forward</td>
<td>CTTCCACTCAACTCCCTTAG</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>ACCATCCCAAGCTCCACTTC</td>
</tr>
<tr>
<td><strong>Bbs4 (downstream)</strong></td>
<td>forward</td>
<td>CATCAGGGAGCCGTAATCAC</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>CCAGGAAACCAGGACTACAC</td>
</tr>
<tr>
<td><strong>Dnahe5 (Promoter)</strong></td>
<td>forward</td>
<td>TGGGCAGTGTGGAATGGG</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>CCATGCTGTGGGCGTTAAG</td>
</tr>
<tr>
<td><strong>Dnahe5 (downstream)</strong></td>
<td>forward</td>
<td>AAGTCCCTCAACTGCATACC</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>CCAGCTCAGCTCCATACATAC</td>
</tr>
<tr>
<td><strong>Dnahe9 (Promoter)</strong></td>
<td>forward</td>
<td>TGAGAGCTTCCACCAGTTTC</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>CTCCCTATATGGGGCACTGGAC</td>
</tr>
<tr>
<td><strong>Dnahe9 (downstream)</strong></td>
<td>forward</td>
<td>GGCTGCGGAATATCTTAGG</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>GGTTGTGAGCTTCCATGATAG</td>
</tr>
<tr>
<td><strong>Dnahe11 (Promoter)</strong></td>
<td>forward</td>
<td>CTGAATCCAGAGCCGCTAAC</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>GCAGAGACCGGGAAATCAAC</td>
</tr>
<tr>
<td><strong>Dnahe11 (downstream)</strong></td>
<td>forward</td>
<td>CGCTGCGGAATTTAAACGAGAC</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>TCTGGCAGCGATCCCTGTAG</td>
</tr>
</tbody>
</table>