The intraflagellar transport component IFT88/polaris is a centrosomal protein regulating G1-S transition in non-ciliated cells

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[1]Institut Cochin, Département Génétique et Développement, [2]INSERM, U567, [3]CNRS, UMR8104 and [4]Université Paris 5, Faculté de médecine R. Descartes, UM 3, Paris, F-75014 France
[5]INSERM U785. Université Paris XI, CHB Paul Brousse, Villejuif Cedex, F-94804 France
[6]Laboratoire de Biologie du Cycle Cellulaire et de la Motilité, UMR144 Institut Curie/CNRS, 12 rue Lhomond, Paris cedex 05, F-75248 France
*Author for correspondence (e-mail: desdouets@cochin.inserm.fr)

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[6]Laboratoire de Biologie du Cycle Cellulaire et de la Motilité, UMR144 Institut Curie/CNRS, 12 rue Lhomond, Paris cedex 05, F-75248 France
*Author for correspondence (e-mail: desdouets@cochin.inserm.fr)

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Summary

Loss of normal primary cilia function in mammals is linked to proliferative diseases, such as polycystic kidney disease, suggesting a regulatory relationship between cilia and cell cycle. The primary cilium expressed by most mammalian cells is nucleated from the elder centriole of the centrosome. The relationship between centrosome and cilia suggests that these structures share functions and components. We now show that IFT88/polaris, a component of the intraflagellar transport, remains associated to the centrosome in a proliferative state. IFT88/polaris is tightly associated with the centrosome throughout the cell cycle in a microtubule- and dynein-independent manner. IFT88/polaris tetratricopeptide repeat motifs are essential for this localization. Overexpression of IFT88/polaris prevents G1-S transition and induces apoptotic cell death. By contrast, IFT88/polaris depletion induced by RNA interference promotes cell-cycle progression to S, G2, and M phases. Finally, we demonstrate that IFT88/polaris interacts with Che-1, an Rb-binding protein that inhibits the Rb growth suppressing function. We propose that IFT88/polaris, a protein essential for ciliogenesis, is also crucial for G1-S transition in non-ciliated cells.

Key words: Polaris, Centrosome, Cilium, Intraflagellar transport, G1-S transition

Introduction

The centrosome functions as an organizing center for cytoskeletal components, especially microtubules (Doxsey, 2001; Nigg, 2002; Salisbury et al., 1999). A vertebrate centrosome comprises two barrel-shaped centrioles which are embedded within a protein-dense matrix known as the pericentriolar matrix (PCM). By nucleating and anchoring microtubules, centrosome influences most microtubule-dependent processes, including cell shape, polarity, adhesion and motility, as well as intracellular transport and positioning of organelles (Bornens, 2002; Fukasawa, 2002; Rieder et al., 2001). The centrosome also has a crucial role in cell division, mediating assembly and organization of the mitotic spindle that is required for correct chromosome segregation, and the positioning of the cleavage plane during cytokinesis (Doxsey, 2001; Piel et al., 2001). Consistent with this role, centriole duplication is coordinated with the cell cycle (Hinchcliffe and Sluder, 2001). Centrosomes are also the precursors of primary cilia, nonmotile sensory organelles found on most vertebrate cells (Badano et al., 2005; Quarmby and Parker, 2005). Typically, a single cilium projects from each cell, emerging from the elder centriole of the centrosome that has become a basal body (Sorokin, 1962). The presence of a cilium is associated with the establishment of polarity and differentiation of the cell (Wheatley et al., 1996). Thus, cilia are most abundant on non-proliferating G0 cells and entry into the cell cycle is preceded by ciliary resorption, whereas exit from mitosis is accompanied by ciliary assembly, a relationship that may reflect the use of the basal bodies and/or centrioles as mitotic spindle poles (Rieder et al., 1979).

Primary cilia assembly occurs by a process called intraflagellar transport (IFT) that was first described in *Chlamydomonas* (Kozminski et al., 1993; Rosenbaum and Witman, 2002). This process has recently become the focus of intense research interest owing to its association with human disease and developmental abnormalities, including polycystic kidney disease (PKD), hepatic and pancreatic defects, blindness and obesity, as well as skeletal patterning abnormalities and randomization of the left-right body axis (Hildebrandt and Otto, 2005). The first evidence that defective cilia are the proximal cause for PKD originated with the discovery that the gene mutated in the Oak Ridge polycystic kidney (*Tg737*^{orpk}) mouse (Moyer et al., 1994) is homologous to a gene essential for ciliary assembly in *Chlamydomonas*, *ift88* (Pazour et al., 2000) and in *C. elegans, osm-5* (Haycraft et al., 2001; Qin et al., 2001). Whereas *Tg737*^{orpk} mutants are viable, complete knockout of the mouse *Tg737* gene (*Tg737*$^{\Delta 2\text{-}3\beta\text{-}gal}$) is embryonic lethal (Murcia et al., 2000). Homozygous *Tg737*$^{\Delta 2\text{-}3\beta\text{-}gal}$ mutants die in early to mid-gestation and exhibit neural tube defects, random left-right

body axis specification and a pronounced expansion of the anterior-posterior (AP) limb bud axis. In *Tg737*^{orpk} and *Tg737*$^{\Delta 2\text{-}3\beta\text{-}gal}$ mutant mice, cilia are severely malformed or absent (Murcia et al., 2000; Pazour et al., 2000; Yoder et al., 2002a). In fact, in mammals, IFT88/polaris, the protein encoded by *Tg737*, is a core component of the intraflagellar transport machinery and is required for the formation of all cilia (Murcia et al., 2000; Pazour et al., 2000).

Growing evidence suggest a link between cilia, centrosome and proliferating disorders. Here, we show that the IFT protein IFT88/polaris, essential for cilia function, is also crucial for cell cycle progression. We demonstrated by immunofluorescence and biochemical analysis in proliferating cells, either ciliated or not, that IFT88/polaris is tightly associated with the centrosome throughout the cell cycle. Expression of a series of deletion constructs revealed the presence of two independent centrosome-targeting domains. Moreover, we tested whether IFT88/polaris participates in the regulation of cell cycle progression. Our results show that altered levels of IFT88/polaris concentration influence the G1-S cell cycle progression.

Results

Subcellular localization of IFT88/polaris in proliferating ciliated or non-ciliated cells

IFT88/polaris is known as an IFT protein localized to basal body and axoneme of cilia from *Chlamydomonas* to mammals (Cole et al., 1998; Pazour et al., 2000; Qin et al., 2001). To provide insight into its cellular distribution in proliferating ciliated cells (resorpted cilia) or in non ciliated cells, we generated polyclonal antiserum against the C-terminal region of the protein. As expected, in quiescent ciliated cells IFT88/polaris was localized to the basal body and along the axoneme (Fig. 1A). We investigated IFT88/polaris localization in proliferating cells either ciliated or not (ciliated cells, RPE-1 and CHO; non-ciliated cells, HeLa, 2A1.6) (shown for RPE-1 cells, Fig. 1B). Endogenous IFT88/polaris was found at one or two perinuclear focal points that colocalized with γ-tubulin in cells in G1 or G2 phase (Fig. 1B). This localization was confirmed biochemically (Fig. 1C). Low-speed Triton-X-100-soluble and -insoluble fractions of unsynchronized 2A1.6 cell lysates were prepared as described previously (Moudjou and Bornens, 1994) and submitted to western blot analysis together with centrosome sucrose-gradient preparations. IFT88/polaris was detected both in the Triton-insoluble fraction and, to a lesser extent, in the Triton-soluble fraction (Fig. 1C, left panel). Clearly, IFT88/polaris was as enriched in the centrosomal fraction as γ-tubulin (Fig. 1C, left panel). Exposure of the centrosomal fraction to various solubilizing treatments, including detergents and chaotropic agents, was done to assess the strength of this association. We found that IFT88/polaris, as well as γ-tubulin, can partly be solubilized in 2D buffer and about half of the protein can be solubilized in 8M urea (Fig. 1C, right panel). These results demonstrated a tight association

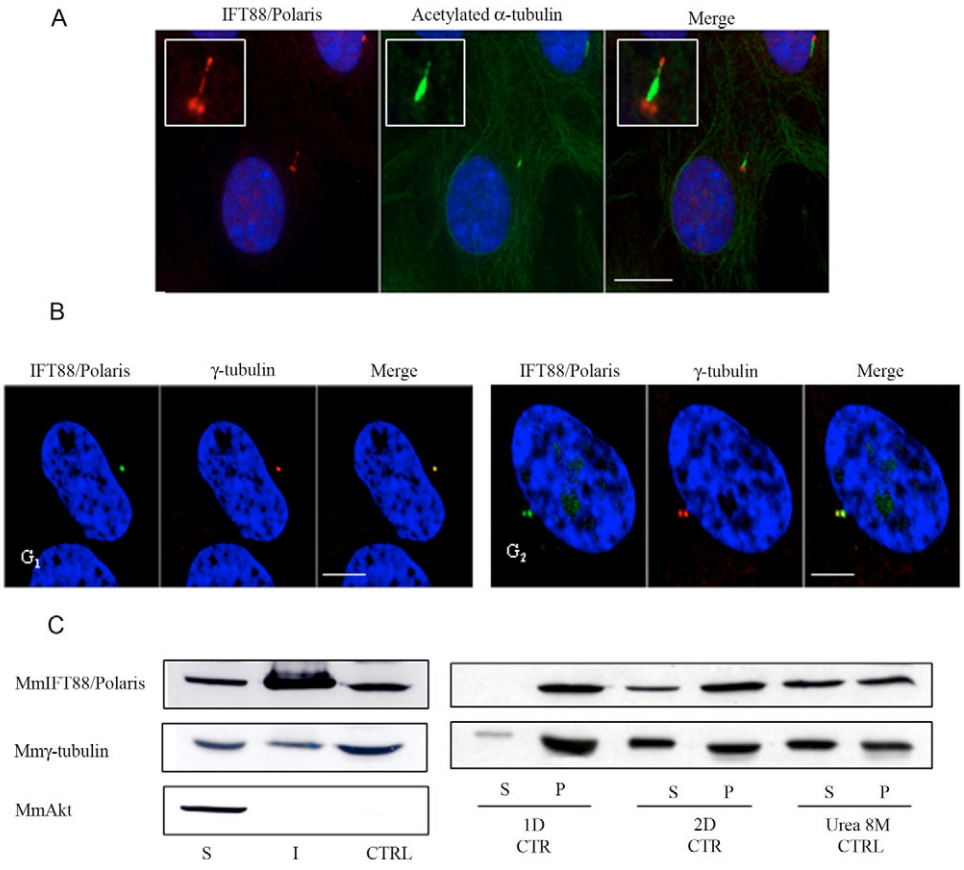

Fig. 1. IFT88/polaris localizes to basal bodies and axonemes in quiescent ciliated cells and to centrosomes in proliferating cells. (A) RPE-1 cell immunostained with an antibody against acetylated-tubulin (6-11B-1, green) to label the axoneme of the cilium, and with anti-IFT88/polaris (740, red) to stain IFT88/polaris which was localized to the axoneme and the basal body. Insets, higher magnifications of cilium. Ciliary assembly was induced by culturing RPE-1 cells in medium supplemented with 0.25% serum for 48 hours. Bar, 10 μm. (B) Confocal immunofluorescence microscopy analysis, using anti-IFT88/polaris 740 (green) and anti-γ-tubulin (GTU88, red), were performed on exponentially growing RPE-1 cells. Bars, 5 μm. Nuclei in A and B are stained with Hoechst 33342 dye. (C) Left panel, western blot analysis of Triton-X-100-soluble (S) and -insoluble (I) protein fractions from 2A1.6 cells, and of a highly enriched centrosomal fraction (CTR). Right panel, biochemical extraction of centrosome-associated IFT88/polaris. Supernatant (S) and pellet (P) of centrosomal fractions obtained under different extraction conditions (see Materials and Methods) were immunoblotted with anti-IFT88/polaris (02078), anti-γ-tubulin (GTU88) and anti-Akt antibodies. Detection of Akt, a well-characterized cytoplasmic protein, was used as a negative control to verify the purity of the centrosomal fraction.

of IFT88/polaris with the centrosome, which can only be disrupted in denaturing conditions.

IFT88/polaris localizes to the centrosome throughout the cell cycle, independently of the microtubules network and the dynein motor

We next examined whether IFT88/polaris has a cell-cycle-dependent behaviour. For that, we used HeLa HC1 cells that constitutively expressed the centriole protein centrin1 fused to GFP (Piel et al., 2000). During G1, IFT88/polaris was present at the proximal ends of maternal centrioles (Fig. 2). In early S phase, centrioles begin to duplicate, and by G2/M, duplication is usually completed. At G2-M transition process, IFT88/polaris was still localized at the proximal ends of the mother and the daughter centrioles (Fig. 2; G2, prophase cells). By metaphase, when centrosomes become mature, IFT88/polaris staining was brighter at centrosomes than at any other cell cycle stage; by telophase IFT88/polaris staining reached its lowest levels (Fig. 2). We next asked how IFT88/polaris assembles at centrosomes, which serve as nucleation and organizing sites for cytoplasmic microtubules. In nocodazole-treated cells, IFT88/polaris remained centrosomal, as did the core centrosomal protein γ-tubulin (Fig. 3). We also investigated whether centrosomal assembly of IFT88/polaris depends on dynein-dynactin molecular motor by overexpressing a mutant of $p150^{glued}$ that antagonizes the function of the motor in vivo. We confirmed that overexpression of Dsred-$p150^{217\text{-}548}$ in HeLa cells causes PCM1 mislocalization (Fig. 3). By contrast, as found for γ-tubulin, the centrosomal localization of IFT88/polaris was not affected in cells overexpressing Dsred-$p150^{217\text{-}548}$ (Fig. 3). These data demonstrate that IFT88/polaris is assembled at the centrosome throughout the cell cycle in a microtubule- and dynein-independent manner.

IFT88/polaris TPR domains are essential for centrosome targeting

Polaris is known to have an evolutionary conserved role in ciliogenesis (Han et al., 2003; Haycraft et al., 2001; Murcia et al., 2000; Pazour et al., 2000; Qin et al., 2001; Yoder et al., 2002b). To circumvent any phenotypes due to defects of cilia, all the following experiments have been performed in non-ciliated cells. To define sub-regions of IFT88/polaris involved in centrosomal targeting, we generated deletion mutant constructs fused to GFP either at the N-terminus or the C-terminus (mutants Δ1 to Δ7, see Materials and Methods for details) and expressed these truncated polypeptides into transiently transfected HeLa cells. As shown in Fig. 4A, the full-length sequence of IFT88/polaris contains ten copies of a tetratricopeptide repeat (TPR), with three copies located towards the N-terminus and seven repeats located closer to the C-terminus. Sequence analysis indicated also the presence of two putative coiled-coil domains. At low expression levels, exogenous full-length IFT88/polaris localized almost exclusively to the centrosome (Fig. 4B, left panel). At higher expression levels, multiple dots of various sizes were detected in cells (Fig. 4B, right panel) as previously described for the overexpression of other centrosomal proteins (Kim et al., 2004; Mayor et al., 2002); nevertheless, of several dots present, only one stained positive for γ-tubulin (Fig. 4B, right panel, Arrow). By analyzing the localization of truncated mutants, we demonstrated that mutants Δ2, Δ3, Δ6 and Δ7 were successfully recruited to the centrosome (Fig. 4C, example for Δ6). Like the full-length protein, these fusion proteins were

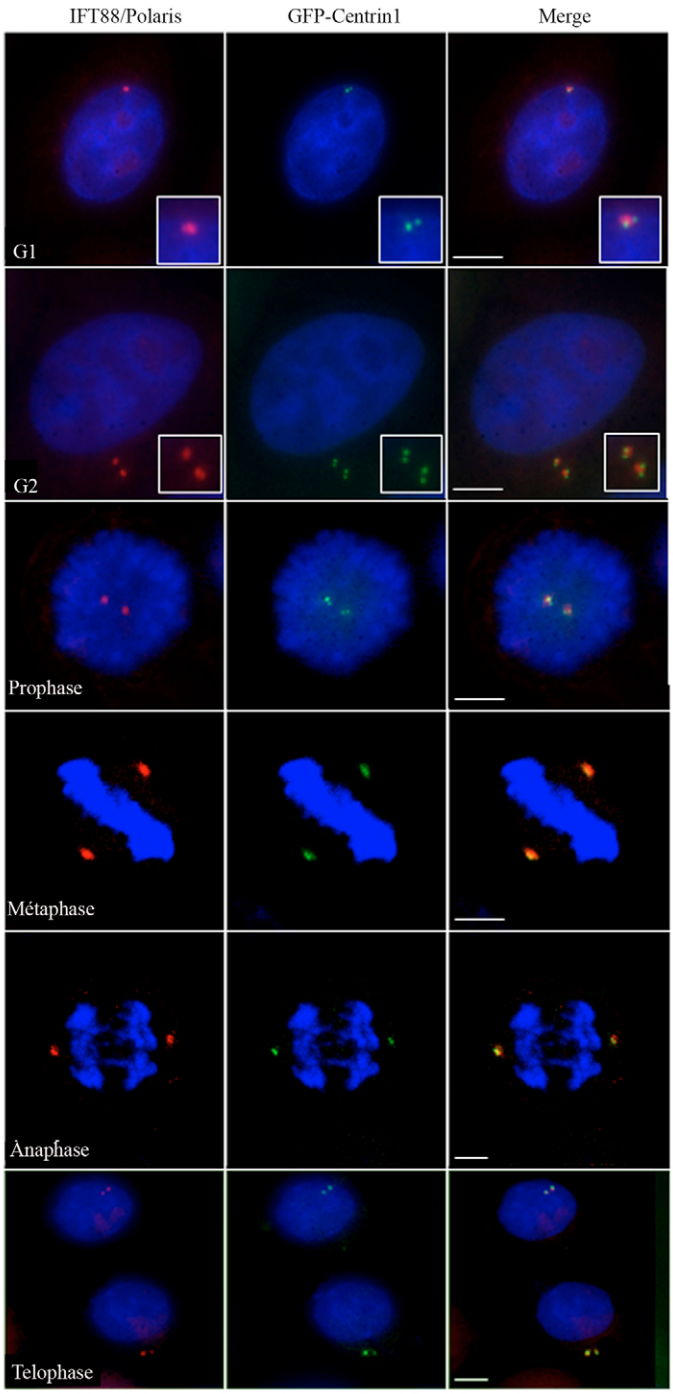

Fig. 2. IFT88/polaris is localized to the centrosome throughout the cell cycle. Immunofluorescence images of endogenous IFT88/polaris in HeLa cells at different stages of cell cycle that stably express GFP-centrin1. Right panel, merged images of IFT88/polaris (red), centrin1 (green), and nuclei (blue) from cells in G1, G2, prophase, metaphase, anaphase and telophase. Insets, higher magnifications of centrioles stained for GFP-centrin1 (left) and IFT88/polaris (anti-IFT88/polaris 740, middle) in cells at G1 and G2. Bars, 5 μm.

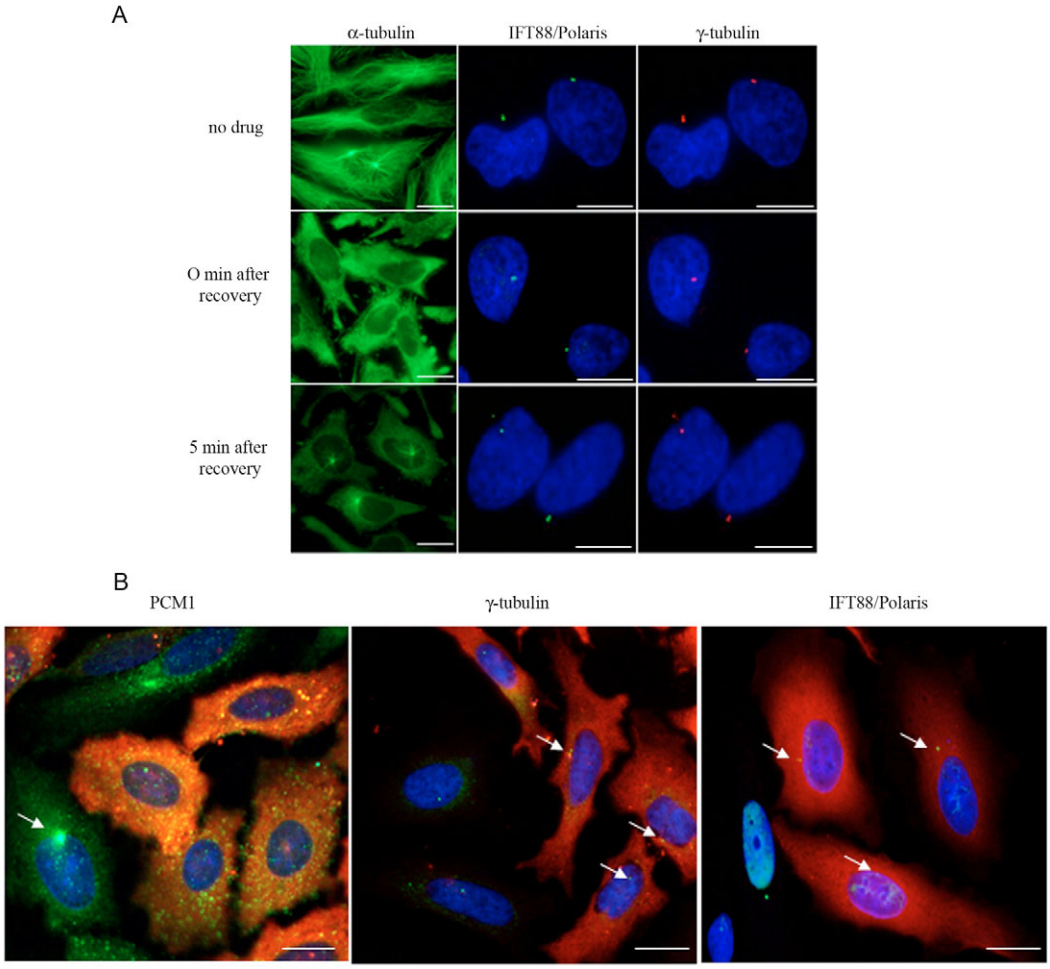

Fig. 3. Centrosomal assembly of IFT88/polaris is not dependent on polymerized microtubules or the dynein-dynactin molecular motor. (A) Hela cells were either mock-treated (no drug) or treated with nocodazole for 1 hour and allowed to recover for 0 or 5 minutes. Cells were stained with α-tubulin antibody (methanol fixation, Tub2.1, green) to follow depolymerization (0 minutes) or polymerization (5 minutes after recovery) of microtubules, or co-stained with anti-IFT88/polaris 740 (PFA 4% fixation, green) and anti-γ-tubulin (PFA 4% fixation, red) antibodies. IFT88/polaris and γ-tubulin localizations were analyzed on cells treated or not with the nocodazole (n=200). Bars, 10 μm. (B) HeLa cells were transiently transfected with DsRed-p150$^{217\text{-}548}$, which inhibits dynein-dynactin motor function, and co-stained with antibodies against PCM1, γ–tubulin or IFT88/polaris (green). The left panel shows untransfected cells with expected PCM1 localisation (greens staining, arrow) and transfected DsRed-p150$^{217\text{-}548}$ cells with PCM1 mislocalization (scattered staining). Middle and right panels show that, after expression of plasmid DsRedp150$^{217\text{-}548}$, centrosomal localization in transfected cells is unchanged for γ-tubulin and IFT88/polaris (arrows). Bars, 10 μm.

identified as multiple dots with one that colocalized with γ-tubulin (Fig. 4C, example for Δ6) when expressed at high levels. By contrast, mutants Δ1 and Δ5 were distributed diffusely in the cytoplasm, like the GFP control protein, and colocalization with γ-tubulin was not observed (Fig. 4C, example for Δ5). Finally, distribution of mutant Δ4 was visible in the cytoplasm as multiple dots, but more irregular in shape and did not colocalize with γ-tubulin (Fig. 4C). From these experiments, we concluded that the two TPR domains were independently essential to target IFT88/polaris to the centrosome.

Overexpression of IFT88/polaris prevents entry into S phase and induces apoptotic cell death

Since no late stages of mitosis were observed in transfected cells overexpressing IFT88/polaris, we sought to determine whether its ectopic expression caused changes in cell cycle progression. C-Nap1, a protein involved in centrosome cohesion, was used as control. Expression of GFP–C-Nap1 resulted in the formation of multiple dots without altering mitosis progression (Mayor et al., 2002). Nocodazole treated cells expressing only GFP or GFP–C-Nap1, showed a prominent accumulation of phases G2 and M (without treatment 16% of cells, with nocodazole 40%) (Fig. 5A). By contrast, the proportion of cells at G2/M was not significantly changed in cells expressing GFP-IFT88/polaris treated or not with nocodazole (19% or 21%, respectively, Fig. 5A). Thereafter, we determined whether its ectopic expression changed the progression into S phase. IFT88/polaris overexpression dramatically decreased the fraction of BrdU-positive cells at 36 hours post transfection (Fig. 5B, 4% compare with 25% and 32% for GFP and GFP–C-Nap1, respectively). The impairment of G1-S transition was confirmed by thymidine block experiments (Fig. 5C). In a final

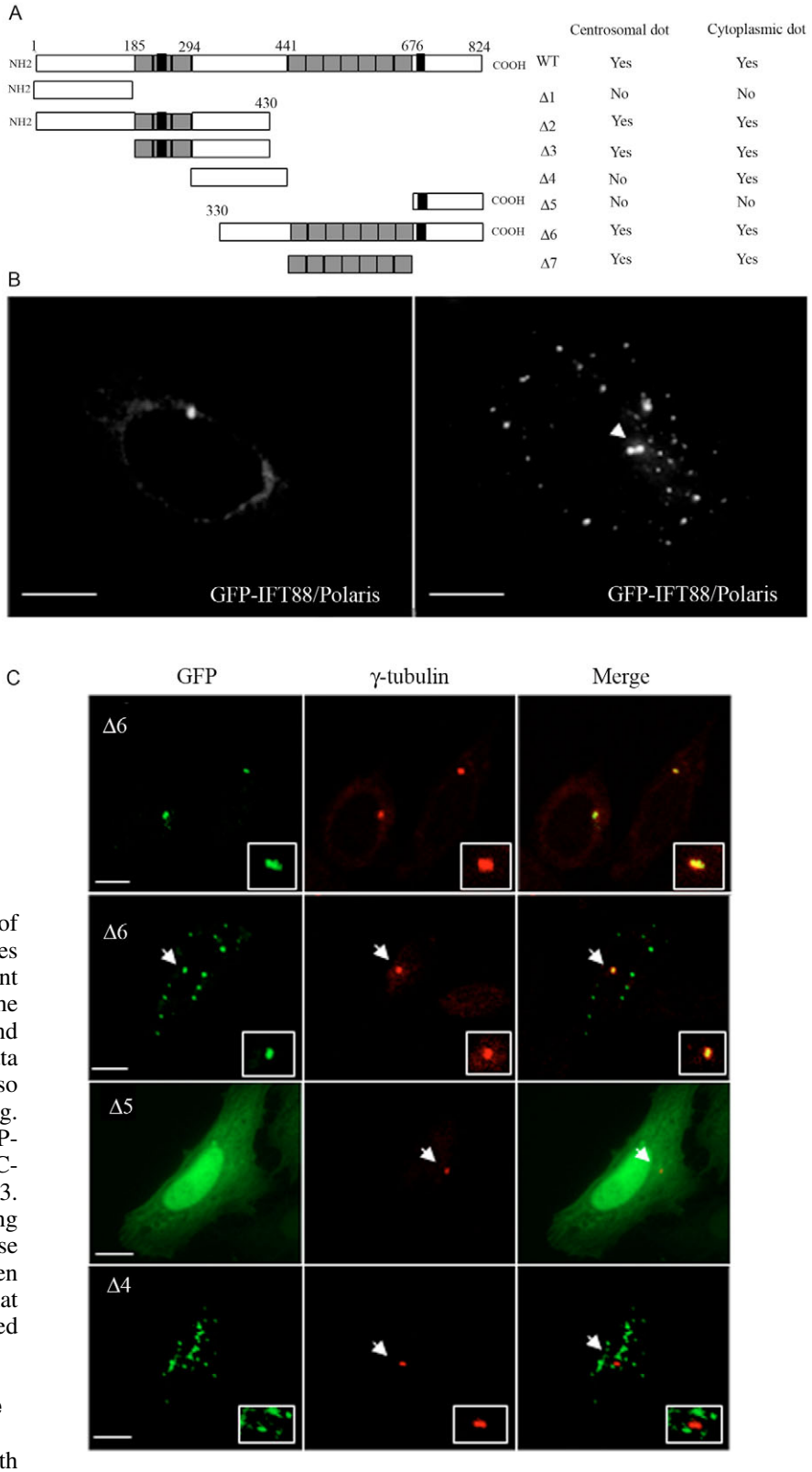

Fig. 4. IFT88/polaris is targeted to the centrosome by its TRP domains. (A) Deletion constructs. Numbers indicate the positions of amino acids, black boxes designate the predicted coiled-coil domain of IFT88/polaris, grey boxes designate the tetratricopeptide repeat domains (TPR). Results of the immunolocalization of GFP-tagged polypeptides are summarized on the right. (B) HeLa cells were transiently transfected with GFP-IFT88/polaris full-length and, 24 hours later, processed for immunofluorescence microscopy (GFP staining). Depending on the expression level, ectopic IFT88/polaris expression resulted in the formation of a single dot (left panel) or multiple dots (right panel). The arrowhead points to the dot containing the centrosome (γ-tubulin labeling, data not shown). Bars, 10 μm. (C) Phenotypes observed after ectopic expression of GFP-tagged truncated IFT88/polaris polypeptides (green). Transfected cells were stained with γ-tubulin (red) 24 hours post transfection. Although polypeptides Δ1, Δ4 and Δ5 produced proteins that were not found at the centrosome (see Δ4 and Δ5), other polypeptides containing TRP domains (Δ2, Δ3, Δ6 and Δ7) produced proteins that were recruited to the centrosome (see Δ6). Depending on expression levels, ectopic expression of mutants Δ2, Δ3, Δ6 and Δ7 resulted in the formation of a single dot (Δ6 first panel) or of multiple dots (Δ6, second panel). Bars, 10 μm.

set of experiments, we studied the outcome of these cells. Morphological evaluation of cultures using Hoechst 33342 staining and fluorescent microscopy revealed a marked increase in the number of cells with nuclear condensation and fragmentation 48 hours after transfection (data not shown). Caspase 3 activity was also examined on transfected cells. As shown on Fig. 5D, only 4% of cells transfected with the GFP-control vector and 6% transfected with GFP–C-Nap1, expressed the activated form of caspase 3. By contrast, 40% of cells overexpressing IFT88/polaris were positive for cleaved caspase 3 at 48 hours post transfection (Fig. 5D). Taken together, the above results demonstrated that cells overexpressing IFT88/polaris arrested before S phase and then died by apoptosis.

IFT88/polaris silencing promotes cell cycle progression to S, G2 and M phases

These results prompted us to analyze the growth characteristics of cells depleted from IFT88/polaris. Two non-overlapping IFT88/polaris small interference RNAs (siRNAs) (siPol1 and siPol2) were tested and produced similar results. Cells transfected with siPol1or siPol2 showed a specific reduction of about 60% of IFT88/polaris expression compared with control (scramble) transfected cells (Fig. 6A, left panel). We also confirmed by immunofluorescence staining that IFT88/polaris was greatly reduced at the centrosome in most cells (Fig. 6A, right panel). In contrast to cells overexpressing IFT88/polaris, reduction of the endogenous protein activated entry into S phase as

demonstrated by BrdU labeling (from 21.1%±0.4 in control cells to 45.6%±2.3 in siPol1 cells at 48 hours, Fig. 6B). In fact, cells expressing reduced levels of IFT88/polaris protein exhibited an increased rate of proliferation (Fig. 6C). Furthermore, flow cytometric analysis for DNA content showed that inhibition of IFT88/polaris resulted in a significant reduction in cells in G1 (70.8%±2 with scramble siRNA, 50.3%±2.7 with siPol1) and a significant increase in cells in S phase (18.1%±3.6 with scramble siRNA, 31.1%±3.4 with siPol1) and in G2 and M phases (11.1%±1.9 with scramble siRNA, 18.6%±1 with siPol1) (Fig. 6D). In conclusion, IFT88/polaris silencing promoted cell-cycle progression to S and G2/M phases.

Identification of IFT88/polaris downstream signaling partners involved in cell cycle regulation

To identify IFT88/polaris targets in cell-cycle regulation, we performed a yeast two-hybrid screening. The mouse full-length IFT88/polaris protein was used to screen a human fetal liver cDNA library. From the 3×10^6 yeast transformants obtained, we selected six clones that fulfilled our selection criteria. One of these clones contained the cDNA encoding amino acids 392 to 558 of Che-1 protein. Che-1 is known to interact with Rb protein and this binding affects the growth-suppressing function of Rb by relieving its inhibition of E2F1 transcriptional activity essential for G1-S transition (Bruno et al., 2002; Fanciulli et al., 2000). To test the specificity of this interaction in mammalian cells, we immunoprecipitated extracts from HEK293T cells that had been transfected with Myc-tagged full-length IFT88/polaris plasmid, with anti-Myc or anti-Che-1 antibodies. We demonstrated that Myc-tagged full-length IFT88/polaris could be coimmunoprecipitated by endogenous Che-1 (Fig. 7A, upper panel) and conversely, endogenous Che-1 could be coimmunoprecipitated with full-length IFT88/polaris (Fig. 7A, lower panel). More importantly, we observed interaction between endogenous IFT88/polaris and Che-1 (Fig. 7B). In order to understand the mechanism of action of IFT88/polaris at the G1-S transition, we analyzed whether the interaction between Che-1 and Rb is affected by IFT88/polaris overexpression. As shown in Fig. 7C, a reduced amount of Rb was immunoprecipitated by Che-1 in the cells overexpressing IFT88/polaris. Taken together, these results demonstrated that IFT88/polaris interacted with Che-1 and, thus, modulated its binding to Rb protein.

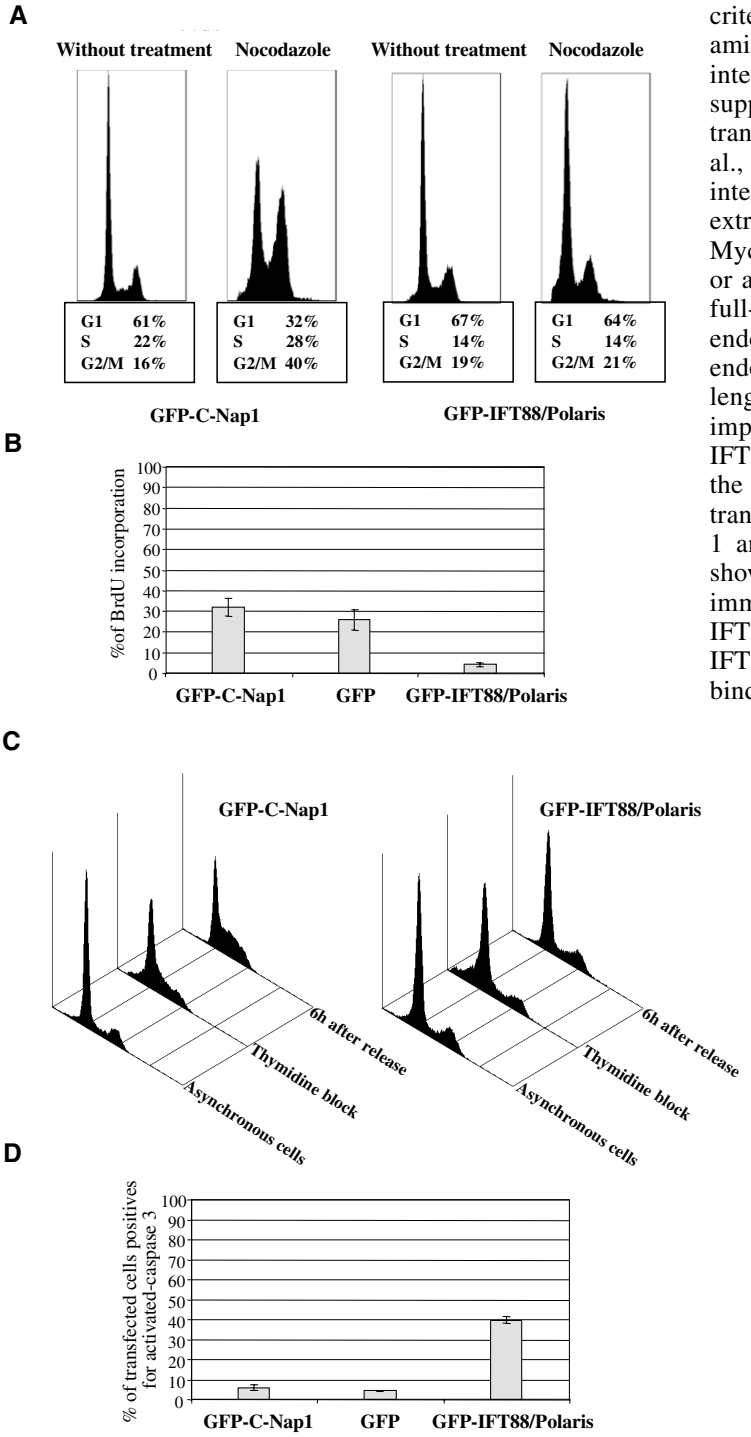

Fig. 5. Overexpression of IFT88/polaris induces an arrest in the cell cycle before S phase and an apoptotic death. (A) HeLa cells were transfected for 36 hours to express either GFP-IFT88/polaris or GFP–C-Nap1 and these cells were treated or not with nocodazole for 12 hours. Cell-cycle-phase distribution of GFP-positive cells was assessed using propidium iodide. Proportions were estimated by triplicate measurements using FACS analysis and the software Wincycle™. (B) HeLa cells were transfected for 36 hours to express GFP-IFT88/polaris, GFP–C-Nap1 or GFP. BrdU staining was analyzed on GFP-positive cells after labeling with anti-BrdU monoclonal antibody (n=150). (C) HeLa cells were transfected for 36 hours to express GFP-IFT88/polaris, GFP–C-Nap1. These cells were synchronized by thymidine block for 12 hours and released for 6 hours. Cell-cycle-phase distribution of GFP-positive cells was assessed using iodide propidium and FACS analysis. (D) Caspase-3-like activity was measured 48 hours after transfection in GFP-positive cells (n=150). Data are from three independent experiments and presented as the mean ± s.d.

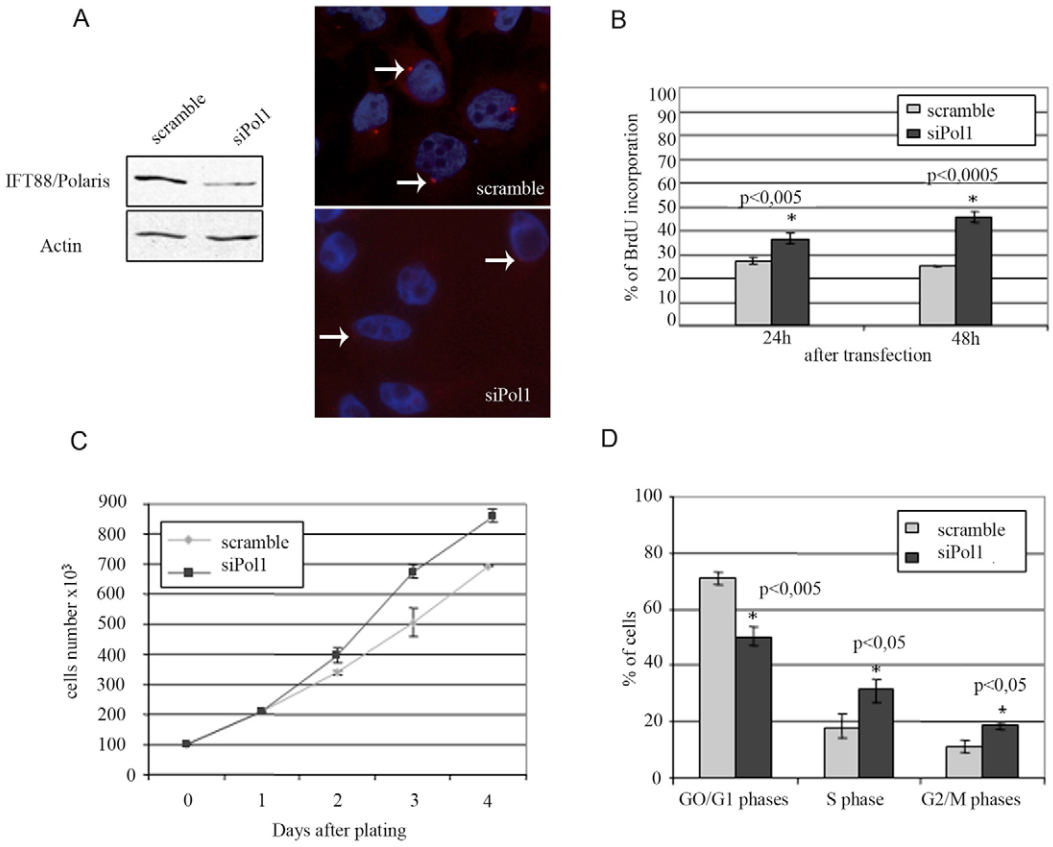

Fig. 6. IFT88/polaris downregulation causes cells proliferation. (A) Two non-overlapping IFT88/polaris siRNAs (siPol1 and siPol2) were tested and were found to produce similar results. Data obtained with siPol1 are illustrated in this figure. HeLa cells were transfected with scramble or IFT88/polaris siRNA (siPol1). Cell lysates (48 hours post transfection) were subject to western blot (left panel) using either the anti-IFT88/polaris antibody 740 (top) or an anti-actin antibody (I-19, bottom). HeLa cells transfected with scramble or IFT88/polaris siRNA (siPol1) were also immunostained (right panel) with anti-IFT88/polaris antibody 740. (B) HeLa cells were transfected with scramble or siPol1, treated with BrdU during 4 hours before the indicated time and BrdU-positive cells were analyzed (n=300). (C) Growth curves of cells transiently transfected with scramble or siPol1. (D) FACS analysis, 48 hours post tranfection. The histogram indicates the percentage of cells at a given cell cycle phase. Data are from three independent experiments and presented as the mean ± s.d.

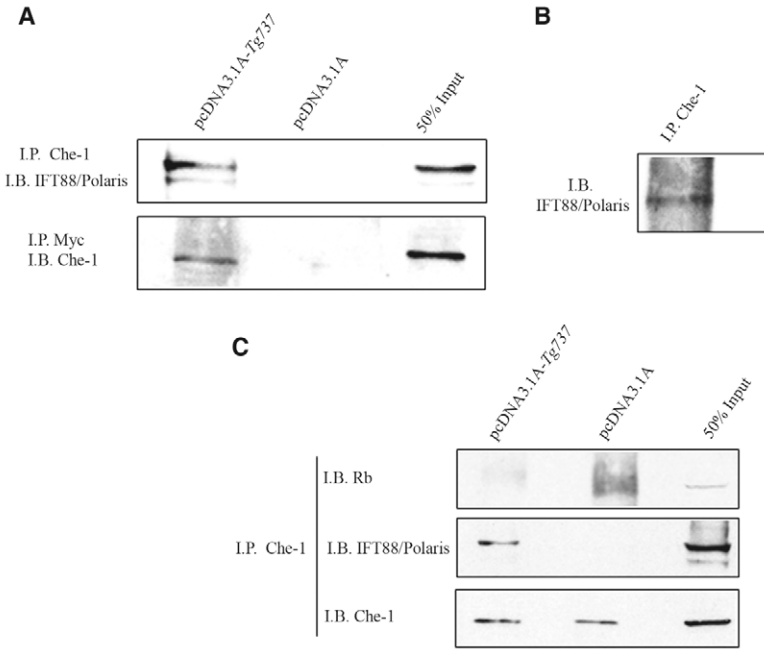

Fig. 7. IFT88/polaris interacts with Che-1 and inhibits its interaction with Rb protein. (A) HEK293T cells were transfected with control plasmid (pcDNA3.1A) or Myc-tagged full-length IFT88/polaris (pcDNA3.1A-*Tg737*). Lysates (500 μg) from transfected cells were immunoprecipitated with anti-Myc antibody or anti-Che-1 antibody and analyzed by western blot using specific anti-IFT88/polaris (740) or Che-1 antibodies. (B) Lysates (1 mg) from non transfected HEK293T cells were immunoprecipitated with anti-Che-1 antibody and analyzed by western blot using a specific anti-IFT88/polaris (740) antibody. (C) HEK293T cells were transfected with control plasmid or Myc-tagged full-length IFT88/polaris. Lysates (500 μg) from transfected cells were immunoprecipitated with anti-Che-1 antibody and analyzed by western blot using specific anti-Rb, anti-IFT88/polaris (740) or Che-1 antibodies. I.P., immunoprecipitation; I.B, immunoblot.

Discussion

We have demonstrated with this study that IFT88/polaris localizes in proliferating mammalian cells at the centrosome and participates to cell cycle progression by controlling G1-S transition. In differentiated ciliated cells, IFT88/polaris is known to have an essential and evolutionarily conserved role for the formation of all cilia (Rosenbaum and Witman, 2002). Here, we show that IFT88/polaris is tightly associated at the proximal end of centrioles throughout the cell cycle. This association is dependent on IFT88/polaris TPR domains and independent of the microtubule network and the dynein-dynactin motors. IFT88/polaris centrosomal localization is conserved in non-ciliated cells, suggesting an additional role beyond the classic IFT system.

A recent convergence of data has indicated an intimate regulatory relationship between ciliogenesis and cell cycle progression. Thus, members of protein families known for their mitotic functions play roles in ciliary assembly. For instance, a distant paralog of aurora kinase is essential for flagellar disassembly in *Chlamydomonas* (Pan et al., 2004). The centrosomal protein pericentrin, essential for correct spindle organization (Zimmerman et al., 2004), forms a complex with IFT proteins and is required for assembly of primary cilia (Jurczyk et al., 2004). However, proteins that are essential for cilia function appear to be indispensable for cell cycle progression. The FA2p protein, discovered in a genetic screen for *Chlamydomonas* mutants defective in deflagellation (Finst et al., 1998), is essential for G2-M transition (Mahjoub et al., 2002). Rootletin, a major structural component of the ciliary rootlet (Yang et al., 2002), regulates centrosome cohesion and separation during the cell cycle (Bahe et al., 2005; Yang et al., 2006). Indeed, the association between cilia and proliferative diseases has already raised the idea that cystoproteins provide a functional signal relay between the cilium, the centrosome and the cell cycle. Inversin, a protein of the axoneme, basal body and centrosome, binds to the anaphase-promoting-complex protein Apc2 (Morgan et al., 2002). Moreover, the Bardet-Bield syndrome protein BBS4, a protein of the basal body and the centrosome, has been shown to target pericentriolar material 1 (PCM1) to the pericentriolar region and, therefore, to control cell division (Kim et al., 2004). Here, we show for the first time that an IFT protein controls also cell cycle progression. Recently, another IFT component, IFT20 has also been shown to localize to the centrosome; nevertheless, its knockdown has no effect on cell cycle progression (Follit et al., 2006). In our case, alteration of the levels of IFT88/polaris in non-ciliated cells induces the following cell cycle defects: (1) overexpression of IFT88/polaris interferes with the G1-S cell cycle transition leading to a higher incidence of apoptotic cell death; (2) by contrast, inhibition of IFT88/polaris expression leads to cell-cycle progression to S and G2/M phases. Previous studies report in *Tg737*^{orpk} mouse model that the pathology is associated with epithelial cell hyperproliferation (Richards et al., 1996; Zhang et al., 2005). On the basis of our findings, we propose that IFT88/polaris controls proliferation by regulating G1-S transition. Consistent with this idea, we demonstrate that IFT88/polaris interacts with Che-1, a key regulator for S phase entry. The role of Che-1 in cell proliferation is clearly linked to the growth-suppressing function of Rb (Bruno et al., 2002). Furthermore, we show that IFT88/polaris modulates Che-1 binding to Rb. Thus, we hypothesize that the overexpression of IFT88/polaris interferes with the G1-S cell cycle transition by titrating out Che-1, which is normally required at this crucial stage. It is tempting to speculate that, during cell cycle progression, IFT88/polaris, by interacting with Che-1, regulates its localization and/or its activity. It will be of interest to test this possibility and to identify other partners in order to better understand the molecular mechanisms by which IFT88/polaris controls G1-S transition.

The challenge is now to determine how dysfunction of primary cilia impacts upon processes such as cell cycle regulation. In vertebrates, primary cilia function as environmental sensors to transfer information from the extracellular space into the cell body (Davenport and Yoder, 2005). The ciliary membrane and cytoplasm are relatively isolated from cell body which offers the advantage of compartmentalization (Anderson, 1972), whereas IFT proteins allow for rapid transport of proteins between cilium and cell body (Kozminski et al., 1993). Cilia may provide information that help cells to remain in a differentiated state. Because cystoproteins change their subcellular localization according to the cell-cycle stage (Mollet et al., 2005; Morgan et al., 2002; Nurnberger et al., 2004; Sayer et al., 2006), cilia resorption may permit the redistribution of IFT proteins and/or ciliary proteins in the rest of the cell and, thus, promote cell-cycle progression. This model is fully compatible with our results on IFT88/polaris and suggests the existence of a mechanism that regulates proteins by shuttling them to the nucleus, the centrosome or to cilia.

Finally, we report a new cellular function for a component of the mammalian IFT by demonstrating that IFT88/polaris has a direct role in cell cycle progression. *C. elegans*, *Trypasoma brucei* and *Chlamydomonas* mutants for IFT88/polaris have not been shown to have defects in cell proliferation (Haycraft et al., 2001; Kohl et al., 2003; Pazour et al., 2000). This suggests that IFT88/polaris has others functions in higher eukaryotic organisms. In agreement with this idea, recent studies have demonstrated that IFT88/polaris is implicated in the regulation of the Sonic hedgehog signaling in mice (Liu et al., 2005); however, IFT mutations in *Drosophila* do not disrupt this pathway (Han et al., 2003). We can assume that polaris and, more generally, IFT proteins play roles in signaling pathways in vertebrates.

We are convinced that certain ciliary proteins are components of the cell cycle. Defining their role in this process will lead to a better understanding of the molecular mechanism involved in proliferative diseases such as PKD. Our findings may facilitate the understanding of the link between ciliary function and cellular response, and provide an entry point to define the dysfunctions associated with abnormal cilia formation.

Materials and Methods

Cell lines

HEK293T cells and HeLa cells that express or not centrin1-GFP were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, BRL) with 10% foetal bovine serum (FBS), and penicillin and streptomycin (100 international unit (IU)/ml and 100 μg/ml, respectively). Human RPE1 cells were grown in DMEM–F-12 medium supplemented as above. Mouse lymphocytes (2A1.6) were grown in RPMI medium with 10% FBS, 1% β-mercaptoethanol, 5 mM sodium pyruvate, and penicillin and streptomycin (100 IU/ml and 100 μg/ml, respectively). All the cell lines were incubated at 37°C with 5% CO_2 atmosphere.

Construction of expression vectors

Constructs encoding the different domains of IFT88/polaris were engineered by PCR using the IFT88/polaris encoding cDNA as a template (*Tg737* cDNA 5.5). PCR fragments were then subcloned into the multicloning site of eukaryotic expression vectors: (1) pEGFP-N2 (Clontech), in fusion with GFP in C-terminal, (2) pGFP-C2, in fusion with GFP in N-terminal and (3) pcDNA3.1A (Invitrogen). All constructs were sequenced to verify intact reading frames. The series of deletion constructs used in this study is listed in Fig. 4A and were cloned in pEGFP-N2 (Clontech): Δ1 (amino acids 1-185), Δ2 (amino acids 1-430), Δ3 (amino acids 185-430), Δ4 (amino acids 294-441), Δ5 (amino acids 676-824), Δ6 (amino acids 330-824), Δ7 (amino acids 441-676).

Antibodies

Two antibodies against IFT88/polaris were generated according to the protocol of Agro-Bio (La Ferté St Aubin, France). The first antibody, IFT88/polaris-02078, was generated in rabbits by using a 21-residue-long peptide (NVHLAPETDEDDLYSGFNDYN) starting at position 3 of the mouse protein. For the anti-IFT88/polaris-740, a 1050-bp *Eco*RI digestion fragment of mouse IFT88/polaris (corresponding to amino acids 474-824) was cloned into the *Eco*RI site of the glutathione-S-transferase (GST) expression vector pGEX 4T1 (Pharmacia). Large-scale protein inductions were carried out, and the GST-IFT88/polaris fusion proteins were injected into rabbits for the production of polyclonal antibody. Commercial antibodies against γ-tubulin (GTU88, Sigma), α-tubulin (B-5-1-2, Sigma), acetylated tubulin (6-11B-1, Sigma), actin (I-19, Santa Cruz), Myc (9E10, Santa Cruz), Rb (sc-50, Santa Cruz), Akt (Cell Signaling) and cleaved caspase 3 (Asp175, Cell Signaling) were also used according to the manufacturer's instructions. Secondary antibodies against rabbit and mouse IgG conjugated to Alexa Fluor-488 (Molecular Probes) or Texas Red (Jackson) were used as well.

Centrosome subfractionation

Soluble, insoluble and centrosome fractions were isolated from 2A1.6 lymphocytes as described previously (Moudjou and Bornens, 1994). Pelleted centrosomes were incubated for 1 hour at 4°C with extraction buffer (20 mM Tris-HCl pH 7.4, 2 mM EDTA) alone, with 1D buffer (extraction buffer containing 0.5% NP-40 and 0.5% deoxycholate), with 2D buffer (extraction buffer containing 0.5% NP-40, 0.5% deoxycholate and 0.1% SDS) or with 8 M urea. Proteins were then fractionated into pellet (P) and supernatant (S) by centrifugation at 10,000 ***g*** for 15 minutes. Proteins were detected by various antibodies and visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

Transfections, drug treatments, and FACS analysis

For transient transfection, HeLa cells were seeded onto glass coverslips (22×22 mm; 3×10^5 cells) and transfected with 1 μg of plasmid DNA using transfecting reagent exgen500 (Euromedex). At the indicated time point post transfection, cells were washed with PBS and fixed either with 4% paraformaldehyde (PFA) (15 minutes at 4°C) or methanol (6 minutes at –20°C). For synchronization experiments, cells were treated with either thymidine (2.5 mM, for 16 hours) or with nocodazole (165 nM, for 12 hours). For microtubule depolymerization and regrowth, HeLa cells were incubated in 25 μM nocodazole at 37°C for 1 hour. Following drug removal, cells were incubated for the indicated times to allow microtubule regrowth, and fixed and processed for immunofluorescence as described above. For FACS analysis, cells were collected by trypsinization and fixed in 70% ethanol-PBS overnight at 4°C. Cells were then incubated with RNAse (100 μg/ml) for 30 minutes at room temperature, stained with 15 μg/ml propidium iodide and analyzed on a Beckman Coulter flow cytometer using a gate on GFP-positive cells. The percentages of cells in G1 and S phases and at G2-M transition were determined using the software Wincycle™.

Immunofluorescence

Cells grown on glass coverslips were fixed either in 4% PFA for 15 minutes at 4°C and permeabilized for 2 minutes with ice cold methanol or fixed and permeabilized directly for 6 minutes with ice cold methanol. After blocking with 10% goat serum in PBS (30 minutes), cells were incubated for 1 hour with primary antibodies, washed in PBS containing 0.1% Tween 20 and incubated in secondary antibodies for 30 minutes, all at room temperature. Hoechst 33342 (0.2 μg/ml, Sigma) was included in the final wash to counterstain nuclei. Samples were mounted on slides in fluorescent mounting medium (DakoCytomation). The percentage of cells undergoing DNA synthesis was estimated by counting the number of BrdU-labeled cells using the BrdU Detection Kit II (Roche). Images were usually taken using a Nikon Statif Eclipse E600 microscope with 63× magnification, 1.4-0.7 NA PL-APO objectives, a DXM1200 cooled CCD camera (Nikon) and ACT-1 (Universal Imaging). For studies of colocalizations with centriolar markers, *z*-axis stacks were collected using a piezoelectric device mounted at the base of a 63× magnification, 1.4 NA PL-APO objective on a Leica DMRA2 microscope, a Coolsnap HQ camera controlled by Metamorph software (Universal Imaging) was used. The *z*-axis stacks were compiled as single 2D projections using ImageJ software. All images were imported into Adobe Photoshop v5.0 for contrast adjustment and figure assembly.

Knockdown of IFT88/polaris expression using siRNA

We synthesized 21-mer nucleotide siRNA duplexes (designed by oligoengine), comprised of a 19 nucleotide duplex corresponding to IFT88/polaris in HeLa cells (siPol1, position 667 from start codon: CCGAAGCACUUAACACUUA; siPol2, position 1650 from start codon: CUGAAACUUCACGCAAUCC) and dTdT overhangs at each 3′-end (Sigma-Proligo). A non-silencing siRNA-scramble was also used as control. For transfection, each duplex was mixed with oligofectamine (Invitrogen) according to the manufacturer's protocol and introduced (24 hours after plating) into cells cultured in 6-well plates. Analyses were performed 24 and 48 hours post transfection.

Yeast two hybrid screen

The entire coding sequence of mouse IFT88/polaris was subcloned into the bait plasmid pBTM116 to create pBTM116-Tg737. The *Saccharomyces cerevisiae* strain Y190, carrying *Gal4-LacZ* and *Gal4*-histidine fusion genes, was transformed with the bait construct and a human liver cDNA library (clontech) in the prey plasmid, pACT2. Transformants were first selected for histidine prototrophy and then assayed for induction of β-galactosidase activity. Plasmids from positive clones were rescued into *Escherichia coli* and re-transformed into L40 containing either pBTM116-Tg737 or pBTM116. The sequences were compared with the database using the blast program.

Immunoprecipitation

For immunoprecipitation experiments cells were lysed at 4°C for 30 minutes in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5 mM EDTA, and 0.5 mM EGTA, protease inhibitor cocktail (Roche). After high-speed centrifugation, the lysates were immunoprecipitated by standard procedures using mouse anti-Myc monoclonal antibody clone 9E10 (Santa Cruz) or as described previously with Che-1 antibody (Bruno et al., 2002).

Data analysis and statistic

All values for statistical significance represent the mean ± standard deviation (s.d.). Mean values were compared using the Student's *t*-test. All comparisons of statistical significance have *P* values of $P<0.05$.

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