Crystal structure of the intraflagellar transport complex 25/27

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The cilium is an important organelle that is found on many eukaryotic cells, where it serves essential functions in motility, sensory reception and signalling. Intraflagellar transport (IFT) is a vital process for the formation and maintenance of cilia. We have determined the crystal structure of *Chlamydomonas reinhardtii* IFT25/27, an IFT sub-complex, at 2.6 Å resolution. IFT25 and IFT27 interact via a conserved interface that we verify biochemically using structure-guided mutagenesis. IFT27 displays the fold of Rab-like small guanosine triphosphate hydrolases (GTPases), binds GTP and GDP with micromolar affinity and has very low intrinsic GTPase activity, suggesting that it likely requires a GTPase-activating protein (GAP) for robust GTP turnover. A patch of conserved surface residues contributed by both IFT25 and IFT27 is found adjacent to the GTP-binding site and could mediate the binding to other IFT proteins as well as to a potential GAP. These results provide the first step towards a high-resolution structural understanding of the IFT complex.

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Introduction

Cilia or flagella (interchangeable terms) are membrane-surrounded microtubule-based structures that protrude from a wide range of eukaryotic cells and serve a number of important functions including cellular motility, sensory reception and signalling (Michaud and Yoder, 2006). The motile cilium enables unicellular organisms such as *Chlamydomonas reinhardtii* (Cr) to move in response to light or other environmental cues (Witman, 2009). In mammals, the motile cilium is found on sperm cells and on cells that line the fallopian tubes and the trachea of the lungs (Satir and Christensen, 2008). As the cilium protrudes from the cell

surface, it is in a perfect position to convey signals between the cell and the environment. Immotile sensory cilia are found on photoreceptor cells in the eye and on olfactory neurons and are thus sensory organelles of smell and sight (Perkins *et al*, 1986; Snell *et al*, 2004). Furthermore, cilia are important to a number of signal transduction pathways such as platelet-derived growth factor receptor α , sonic hedgehog, epidermal growth factor and 5HT₆ serotonin signalling (Brailov *et al*, 2000; Huangfu *et al*, 2003; Haycraft *et al*, 2005; Ma *et al*, 2005; Schneider *et al*, 2005). Both sensory reception and signalling in the cilium are believed to be a result of increased clustering of receptor molecules in the ciliary membrane. Due to the versatility of cilium function, a large number of genetic diseases (collectively known as ciliopathies) and developmental abnormalities are the result of non-functional cilia. In human, these diseases range from blindness, male sterility, ectopic pregnancy and polycystic kidney disease to cognitive impairment and various limb deformities (Snell *et al*, 2004; Tobin and Beales, 2007).

The axoneme, which forms the structural framework of the cilium, is built up of nine doublets of microtubules that grow from a centriole-derived basal body anchored in the cell body at the base of the cilium. The axoneme is surrounded by the ciliary membrane that is contiguous with the plasma membrane, but has a unique composition of lipids and proteins (Emmer *et al*, 2010). Transition-zone fibres are proteinaceous structures that span the distance between the plasma membrane and the microtubules of the basal body (Gibbons and Grimstone, 1960; Craige *et al*, 2010). This prevents random diffusion of macromolecules between the cilium and the cell body and effectively creates a ciliary pore that brings about the necessity of active transport in order to correctly target proteins to the cilium. This transport process was first discovered by Joel Rosenbaum and co-workers and is termed intraflagellar transport (IFT) (Kozminski *et al*, 1993). IFT not only mediates the kinesin-2-dependent targeting of structural proteins and signalling receptors to the cilium (anterograde transport) but is also responsible for the removal of ciliary turnover products in a dynein-1b/2-dependent manner (retrograde transport) (Qin *et al*, 2004). These transport processes rely on a large protein complex, the IFT complex, that is believed to mediate the contacts between ciliary cargo proteins and the motor proteins that facilitate transport (Piperno and Mead, 1997; Cole *et al*, 1998; Piperno *et al*, 1998). The complete IFT complex contains at least 20 different proteins and has been shown to dissociate into two different sub-complexes, namely IFT-A and IFT-B, that are involved in retrograde and anterograde transport, respectively (Piperno and Mead, 1997; Cole *et al*, 1998). To date, six protein subunits of the IFT-A (IFT43, 121, 122, 139, 140 and 144) and 14 of the IFT-B (IFT20, 22, 25, 27, 46, 52, 54, 57, 70, 72/74, 80, 81, 88 and 172) complexes have been identified (Cole *et al*, 1998; Piperno *et al*, 1998; Luckner *et al*, 2005; Folliot *et al*, 2009; Lehtreck *et al*, 2009; Wang *et al*, 2009; Fan *et al*, 2010). In addition to the IFT complex, another large macro-

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molecular complex, the BBSome, may participate in IFT (Nachury *et al*, 2007; Lechtreck *et al*, 2009). The BBSome appears to physically bridge the IFT-A and -B complexes in *Caenorhabditis elegans* (Ou *et al*, 2005) and has been suggested to function as a coat for the sorting of membrane proteins to cilia (Jin *et al*, 2010). IFT appears to be a universal process as genes encoding IFT subunits are conserved in almost all ciliated eukaryotic organisms ranging from single-celled algae to humans (Jekely and Arendt, 2006). The importance of the IFT complex is illustrated by the fact that mutations in IFT genes lead to perturbation of cilium formation in organisms as diverse as green alga, nematodes, fruitfly, zebrafish, mouse and human (Cole *et al*, 1998; Murcia *et al*, 2000; Pazour *et al*, 2000; Han *et al*, 2003; Sun *et al*, 2004; Tsujikawa and Malicki, 2004; Pedersen *et al*, 2005; Beales *et al*, 2007). Furthermore, knockouts of the IFT core proteins IFT88 and IFT172 in mice are embryonically lethal, which demonstrates that the IFT process is essential for development (Murcia *et al*, 2000; Huangfu *et al*, 2003).

Based on primary sequence information, most subunits of the IFT complex appear to contain protein–protein interaction domains such as tetratricopeptide repeats, β -propeller or coiled-coil domains that likely mediate contacts within the IFT complex as well as contacts to cargo and motor proteins (Jekely and Arendt, 2006). However, two of the IFT-B core subunits (IFT22 and IFT27) show significant sequence identity to members of the small guanosine triphosphate hydrolyase (GTPase) superfamily and may well serve regulatory roles in IFT. IFT27 was predicted to be an Rab-like GTPase and shown to bind GTP in a previous study (Qin *et al*, 2007). A complete knockdown of CrIFT27 with siRNA is lethal, whereas a partial depletion of IFT27 leads to shorter flagella (Qin *et al*, 2007). Recently, IFT25 was identified as a component of the IFT-B complex in *Chlamydomonas* (Lechtreck *et al*, 2009; Wang *et al*, 2009) as well as in mouse (Follit *et al*, 2009) and has been demonstrated to interact with IFT27 in *Chlamydomonas* (Wang *et al*, 2009), mouse (Follit *et al*, 2009) and human (Rual *et al*, 2005). IFT25 and IFT27 are conserved in many ciliated organisms with the notable exception of *C. elegans* and *Drosophila melanogaster* (Lechtreck *et al*, 2009). Another interesting exception is *Tetrahymena thermophila* that appears to have lost IFT25, but retained IFT27 (Shida *et al*, 2010). Although IFT-B complexes are known to pre-assemble in the cell body before entering the cilium, sedimentation experiments have shown that IFT-B from the cell body contains sub-stoichiometric amounts of IFT25/27 (Wang *et al*, 2009). A significant portion of IFT25 and IFT27 is pre-assembled in an IFT25/27 sub-complex that appears to associate with the rest of the IFT-B complex only upon entrance into the flagellum. These observations have led to the suggestion that IFT25/27 could be involved in the regulation of IFT initiation at the base of the cilium (Wang *et al*, 2009).

Electron tomographic reconstructions of the *Chlamydomonas* flagellum represent the most detailed structural study of IFT complexes to date (Pigino *et al*, 2009). Although these reconstructions provide a first view of how IFT complexes assemble into larger particles (so-called IFT-trains), the resolution is not sufficiently high to model individual IFT proteins. There is thus a paucity of knowledge about the molecular basis for IFT in ciliary assembly and function. To this end, we have determined the crystal

structure of the IFT25/27 sub-complex. The structure reveals a conserved interaction interface between the two components as well as a calcium-binding site in IFT25 and a GTP-binding site in IFT27. We show that IFT27 has very low intrinsic GTPase activity and displays micromolar affinity for GDP and GTP. Additionally, by mapping the degree of surface conservation onto the complex structure, we identify a surface patch that likely mediates the interaction to a putative GTPase-activating protein (GAP) and/or to other proteins of the IFT complex.

Results

IFT25/27 structure characterization

Initial recombinant expression and purifications of full-length CrIFT25 and CrIFT27 from *Escherichia coli* showed that CrIFT25 can be purified as a soluble protein, but CrIFT27 has a tendency to aggregate and is lost during the purification. However, CrIFT27 is significantly stabilized upon co-expression with CrIFT25. We thus co-expressed and purified the full-length CrIFT25/27 complex (Supplementary Figure S1A), but did not obtain any crystals. CrIFT25 contains an approximately 55 residue long C-terminal glycine-rich tail that is predicted to be disordered (DRIPPRED server, <http://www.sbc.su.se/~maccallr/disorder/>). This C-terminal extension of IFT25 appears to be unique to the *Chlamydomonas* protein and is not conserved in other IFT25 orthologues (Supplementary Figure S2). Consequently, a truncated version of CrIFT25 (IFT25 Δ C, residues 1–135) was co-expressed with CrIFT27 and the CrIFT25 Δ C/27 complex was purified and crystallized (Supplementary Figure S1B). The structure of the complex was determined from two different crystal forms at resolutions of 2.6 and 2.8 Å, respectively. Both crystal forms show a heterodimer of the CrIFT25 Δ C/27 complex (Figure 1), which is in accordance with the molecular weight of the complex in solution, estimated as 37 kDa using static light scattering. The structures from the two different crystal forms are very similar as they superpose with a root mean square deviation (r.m.s.d.) of 0.6 Å over all C α atoms. As typically seen in small GTPases crystallized in the absence of nucleotide, the switch I region (residues 39–55) and part of the G4 region (residues 138–140) of IFT27 did not have any interpretable electron density and were not included in the model. Further data collection and refinement statistics are given in Table I.

IFT25 is a calcium-binding protein with a jelly-roll fold

IFT25 displays a jelly-roll fold consisting of nine β -strands that are organized into two anti-parallel sheets stacked on top of each other (Figure 1). The CrIFT25 structure is similar to a previously determined structure of human IFT25 (Ramelot *et al*, 2009) as they superpose with an r.m.s.d. of 0.7 Å over 95% of the C α atoms (Supplementary Figure S3A). When compared with all structures in the protein data bank (pdb) using the DALI server (Holm and Sander, 1993), the IFT25 structures are most similar to the galactose-binding domain of bacterial sialidases, superposing with an r.m.s.d. of 1.6 Å over >90% of the C α atoms (Gaskell *et al*, 1995; Boraston *et al*, 2007). Although IFT25 shares a conserved calcium-binding site with sialidases, the putative galactose-binding site is poorly conserved in IFT25. Indeed, the IFT25/27 complex structure is incompatible with galactose binding as

Figure 1 Structure of the IFT25ΔC/27 complex. Cartoon representation of the overall structure of IFT25ΔC/27 with IFT27 and IFT25 shown in green and slate, respectively. The calcium ion bound to IFT25 is shown as an orange ball. The two views of the complex are related by a 180-degree rotation around the horizontal axis. The switch I and G4 regions of IFT27 are disordered in the structure and represented with dotted lines in the figure. The N- and C-termini of both proteins are indicated.

Table 1 Data collection and refinement statistics

<i>C. reinhardtii</i> IFT25ΔC-IFT27	Native 1	Native 2
Data collection		
Wavelength (Å)	0.973	0.973
Space group	P2 ₁	P2 ₁
Cell dimensions		
a, b, c (Å)	44.1, 96.2, 66.9	50.0, 68.3, 93.8
α, β, γ (deg)	90.0	92.4
Resolution (Å)	50–2.59 (2.59–2.74)	50–2.8 (3.00–2.80)
R _{sym}	0.090 (0.56)	0.099 (0.65)
I/σI	12.6 (2.2)	14.3 (2.5)
Completeness (%)	0.973 (0.848)	0.993 (0.920)
Refinement		
Resolution (Å)	50–2.59	50–2.80
No. reflections	16 992	15 717
R _{work} /R _{free}	0.212/0.269	0.236/0.317
No. atoms		
Protein	4310	4689
Ligand/ion	2 Ca ²⁺	1 Cl [−] and 2 Ca ²⁺
Water	32	27
B-factors		
Protein	35	47
Water	36	40
r.m.s. deviations		
Bond lengths (Å)	0.011	0.007
Bond angles (deg)	1.5	1.2

Highest resolution shell is shown in parenthesis.

the C-terminal helix of IFT27 occupies the putative galactose-binding site (Supplementary Figure S3B). It thus appears that, although IFT25 and sialidases are clearly evolutionarily related, they have diverged in function. The calcium ion bound to CrIFT25 is coordinated by the side chains of amino acids D30, T35 as well as by several main-chain carbonyls (Figure 2A). These calcium-binding residues are completely conserved in IFT25 orthologues and it is thus very likely that IFT25 proteins from other species also bind calcium (Supplementary Figure S2).

Prior to its characterization as an IFT complex protein, IFT25 was classified as a member of the family of small heat shock proteins (sHSPs) of the α-crystallin fold and named Hsp16.2 (Bellyei *et al*, 2007) or HSPB11 (Kampinga *et al*, 2009). IFT25 could thus have a chaperone function in preventing ciliary proteins or turnover products from aggregating.

Indeed, we observe that CrIFT25 increases the solubility of IFT27 when co-expressed in *E. coli*. However, when the IFT25 structure is compared with all structures in the pdb, no significant similarity with structures of the sHSPs family is found. Furthermore, the α-crystallin fold, which is a defining feature of sHSPs (Kappe *et al*, 2010), and the jelly-roll fold of IFT25 have only a superficial resemblance and do not share a common topology. This is also reflected by the low sequence identity of 6–15% between IFT25 and sHSPs. sHSPs are known to assemble into large oligomers required for their chaperone activity (Bagneris *et al*, 2009). In agreement with Ramelot *et al* (2009), we find that full-length CrIFT25 (Supplementary Figure S1A), truncated CrIFT25ΔC (Supplementary Figure S1B) as well as full-length human IFT25 (data not shown) all behave as monomers in solution during size exclusion chromatography (SEC) experiments. Thus, as previously pointed out by de Jong and co-workers, IFT25 should not be classified as a member of the family of sHSPs containing the α-crystallin fold (Kappe *et al*, 2010).

IFT27 is structurally similar to Rab8 and Rab11 but has no prenylation site

IFT27 adopts the classical fold of an Ras-like small GTPase that catalyses the hydrolysis of GTP to GDP and inorganic phosphate. GTPases are switch molecules that exist in an active GTP-bound form or an inactive GDP-bound form. The active form can bind downstream effectors, whereas the inactive form cannot. The regulation of the two forms is achieved by specific GAPs and guanine-nucleotide exchange factors (GEFs) (Vetter and Wittinghofer, 2001). In this way, GTPases can regulate a number of important cellular processes including cell-cycle control, transport processes and sensory reception. The overall structure of IFT27, when compared with all the structures in the pdb, most closely resembles structures of the Rab family of small GTPases. Rab proteins constitute the largest sub-family within the super-family of small GTPases with >60 members in human and have important roles in membrane trafficking (Stenmark, 2009). It is noteworthy that the five sequence signatures of Rab proteins are relatively well conserved in IFT27 proteins (denoted RabF1–F5 in Figure 3). Of all the Rab structures determined to date, the CrIFT27 structure is most similar to that of Rab8 (r.m.s.d. of 2.6 Å for 84% of the Cα atoms) and Rab11 (r.m.s.d. of 2.4 Å for 71% of the Cα atoms). Interestingly, both Rab8 and Rab11 are known to be involved in ciliogenesis (Nachury *et al*, 2007; Knodler *et al*, 2010). This suggests that ciliary Rabs could have arisen by gene

Figure 2 Structural characterization of the IFT25ΔC/27 interface. (A) Close-up view of the protein-protein interface of the IFT25/27 complex. IFT27 secondary structure elements that do not engage in interactions with IFT25 but hinder the view of the interface have been removed for better visualization. Residues involved in calcium coordination (D30 and T35) or forming the interaction interface between the two proteins are shown in a stick representation and labelled. The salt bridge between R22_{IFT25} and E191_{IFT27} and the hydrogen bond between E122_{IFT25} and Y95_{IFT27} are indicated with dashed lines. (B) Pull-down experiments with interface mutants. GST-tagged wild-type IFT27 (GST-27^{WT}) or mutant IFT27 (GST-27^{mut}, V194R) was co-expressed with C-terminally truncated but otherwise wild-type IFT25 (25ΔC^{WT}) or mutant IFT25 (25ΔC^{mut}, V38R, T40R and T125E triple mutant) and pulled down using GSH beads. Whereas both GST-27^{WT} and GST-27^{mut} effectively pull down 25ΔC^{WT}, they do not pull down 25ΔC^{mut}, demonstrating that the interface between the two proteins has been disrupted. To ensure that 25ΔC^{mut} is expressed and soluble, the protein was pulled down separately using the His-affinity tag on IFT25 (right panel).

duplication from an ancestral ciliary Rab-like GTPase. However, despite the large degree of overall structural similarity between IFT27, Rab8 and Rab11, the C-termini of the proteins are highly divergent. A typical hallmark of Rab proteins is the presence of a C-terminal prenylation motif consisting of one or, typically, more cysteines that upon modification allows for membrane association. Such a prenylation site can be found in both Rab8 and Rab11, but as previously noted (Qin *et al*, 2007) is not present in IFT27 (Figure 3). Interestingly, the other identified Rab-like GTPase of the IFT complex, IFT22 (also known as RABL5 or IFTA-2), is also missing a prenylation motif (Figure 3) (Adhiambo *et al*, 2009).

Structural organization of the IFT25/27 complex

The IFT25ΔC/27 structure displays an elongated shape with dimensions of 80 Å × 40 Å × 25 Å. The complex has a mixed hydrophobic/hydrophilic interface with a buried surface area of 1070 Å². Nine residues from IFT25, contributed from two loop regions (residues 36–42 and 117–125; Figure 2A), mediate binding to IFT27 (Figure 2A). These two loops are very well conserved among IFT25 orthologues and are thus likely to mediate IFT27 interactions in other species. The calcium-binding loop of IFT25 (residues 30–35) precedes the first of the two IFT27-interacting loops and is in close proximity to the interaction interface (Figure 2A). Calcium signalling is known to occur in the cilium and recent findings show that the levels of calcium influence anterograde IFT (Besschetnova *et al*, 2010). One possibility is that calcium directly regulates IFT via the calcium-binding site of IFT25. To assess the effect of calcium in complex stability, IFT25/27 was treated with a large excess of the calcium chelator EGTA and subjected to SEC. The results show that calcium binding does not have any apparent effect on the stability of the IFT25/27 complex (Supplementary Figure S4B). Consistently, neither the D30A nor the T35A single point mutations affect IFT25/27 complex formation in pull-down experiments (data not shown). However, it cannot be ruled out that calcium levels have an effect on the IFT25/27 complex *in vivo*.

IFT27 contributes residues from several different regions to the IFT25-binding surface. The residues involved in complex formation are in general better conserved among IFT25 orthologues than IFT27 orthologues. More specifically, CrIFT27 residues from β-strand β3 (F72), the C-terminal α-helix α5 (F186, E191, V194, F197 and C201) as well as the loop connecting α2 and β4 (Y89 and Y95) contribute to the protein complex interface (Figures 2 and 3). A substantial part of the interaction is thus mediated by the C-terminal α-helical extension of IFT27 that is not typically found in the GTPase core domain. Interestingly, this C-terminal α-helical extension is conserved in IFT27 orthologues, where IFT25 is conserved but not in *T. thermophila*, where IFT25 is lost, indicating that α5 of IFT27 could be specific for the interaction with IFT25 (Figure 3). Notably, of the eight CrIFT27 residues that interact with IFT25, five are aromatic and engage in hydrophobic as well as stacking interactions with residues from IFT25. The two tyrosines Y89 and Y95 are located towards the end of the switch II region (Figure 3), indicating that IFT25/27 complex formation could depend on the nucleotide state of IFT27. To test this possibility, we carried out pull-down experiments with different nucleotide states of IFT27 (Supplementary Figure S5A). The conclusion

Figure 3 Conserved sequence motifs and IFT25-interacting residues of IFT27. Multiple sequence alignment of IFT27 proteins from differences species with CrIFT22 and the GTPases Rab11 and Rab8. The secondary structure derived from the IFT27 structure presented here is indicated above the sequences. Important sequence motifs of small GTPases such as switch regions, G-protein-specific sequences (G1-5) as well as Rab-specific residues (RabF1-5) are marked below the sequence. IFT27 residues that interact directly with residues from IFT25 are coloured green. The catalytic glutamine conserved in most GTPases, but not in IFT27 or IFT22, is coloured in blue and the prenylation sites at the C-termini of Rab8 and Rab11 are shown in pink. Hs: *Homo sapiens*, Mm: *Mus musculus*, Xl: *Xenopus laevis*, Dr: *Danio rerio*, Cr: *Chlamydomonas reinhardtii*, Tt: *Tetrahymena thermophila*.

from this experiment is that IFT27 can interact with IFT25 irrespectively of nucleotide state. This is consistent with the fact that Y89 and Y95 are not well conserved among IFT27 orthologues, indicating that they are not crucial for IFT25 interaction. The three phenylalanine residues of CrIFT27 (F72, F186 and F197) that are engaged in IFT25 binding are well conserved among IFT27 proteins from different species but not conserved in Rab8 or Rab11 sequences (Figure 3). This suggests that these residues are specific for the IFT25/27 interaction. In summary, the conservation of interface residues across species makes it highly probable that the structure of the CrIFT25 Δ C/27 complex reported here can serve as a model for IFT25/27 complexes from other species.

From the structure of the IFT25/27 complex presented here, it should be possible to introduce structure-based mutations of interface residues to break up the complex and verify the interaction biochemically. To this end, we introduced several point mutations of interface residues in both IFT25 and IFT27 and tested for complex formation in pull-down experiments with co-expressed proteins (Figure 2B). The pull-down experiments were performed using either GST-tagged IFT27 (Figure 2B) or CBP-tagged IFT25 (Supplementary Figure S5B) and carried out using co-expressed rather than purified proteins because of the low solubility of IFT27 when expressed in the absence of IFT25. Several single point mutations were introduced in both IFT27 (V194R, GST-27^{mut} in Figure 2B) and IFT25 Δ C (V38R or T40R, data not shown) but did not prevent complex formation in the pull-down experiment. Consequently, an IFT25 Δ C mutant protein was tested where three small non-charged residues were mutated to large charged residues (IFT25 Δ C^{mut} in Figure 2B, V38R, T40R and T125E triple point mutant). IFT25 Δ C^{mut} is well expressed and soluble (see Ni pull down in Figure 2B) but does not form a complex with IFT27 demonstrating that the interface has been effectively disrupted. This is also the case in the reciprocal pull down using CBP-tagged IFT25 to precipitate IFT27 (Supplementary Figure S5B). These data provide a biochemical verification of the structural organization of the IFT25/27 complex.

IFT27 binds GTP/GDP with micromolar affinity and has very low intrinsic GTPase activity

The crystal structure of the IFT25 Δ C/27 complex presented here allows us to analyse and compare the active site configuration of IFT27 to that of well-studied GTPases such as Ras (Figure 4A). As the IFT25 Δ C/27 crystals represent the nucleotide-free state of IFT27, the switch I region is disordered and not modelled. However, the positions of the phosphate-binding P-loop as well as the α -helix (α 1) preceding switch I are well conserved in the structure (Figure 4A). Additionally, the mapping of sequence conservation onto the surface of the IFT25 Δ C/IFT27 structure reveals a conserved surface area at the GTP-binding pocket (Figure 5). Important nucleotide-binding motifs such as N/TKXD (G4), DXXG (G3) and GXXXXGKS/T of the P-loop are completely conserved in IFT27 (Figure 3). Specificity for guanine versus adenine is provided by the NXXD motif of G4 (Espinosa *et al*, 2009), which is conserved in IFT27 orthologues (see Figure 3), indicating that IFT27 binds GTP but not ATP (confirmed experimentally; Figure 4C; Supplementary Figure S6). The sequence and structure of IFT27 are thus compatible with

a competent GTP-binding site. Interestingly, the other Rab-like protein of the IFT complex B (IFT22/RabL5) does not have G4 conserved (as previously noted by Adhiambo *et al* (2009)) and may not be specific for GTP.

GTP hydrolysis by GTPases relies on the coordination of a water molecule for the in-line attack on the γ -phosphate (Pai *et al*, 1990). Different families of GTPases utilize different catalytic residues to achieve GTP hydrolysis. In case of the Ras, Rho and Ran GTPases, a conserved glutamine in the switch II region (Q61 in human Ras; Figure 4A) was found to coordinate the nucleophilic water molecule (Pai *et al*, 1990; Rittinger *et al*, 1997; Seewald *et al*, 2002). Mutation of Q61 is oncogenic in Ras and lowers the intrinsic GTPase activity by 8–10-fold (Der *et al*, 1986). Other GTPases such as Sar1 (Bi *et al*, 2002) and elongation factors Tu (Voorhees *et al*, 2010) utilize a catalytic histidine for the purpose of orienting a nucleophilic water molecule. The catalytic glutamine of Ras is conserved in most Rab proteins but appears to be involved in GAP binding rather than catalysis as demonstrated for Rab33, where the catalytic glutamine is instead provided *in trans* by the Rab-GAP (Pan *et al*, 2006). In Rap proteins, the Q61 residue is replaced by a threonine that is involved in Rap-GAP binding and not in catalysis (Chakrabarti *et al*, 2007). For Rap proteins, the water molecule needed for hydrolysis is instead coordinated by an Asn-thumb coming from the Rap-GAP (Daumke *et al*, 2004; Scrima *et al*, 2008). Despite a conserved fold and GTP-binding motifs, GTPases thus have different ways of achieving GTP hydrolysis. The catalytic glutamine of Ras is poorly conserved in IFT27 orthologues and is found to be a serine in the case of CrIFT27 (S79; Figures 3 and 4A). The other GTPase of the IFT complex B, IFT22, also has a serine (S67; Figure 3) at the position of the catalytic Q61 of Ras. Since the switch regions of IFT27 are likely to undergo significant conformational changes upon nucleotide binding, it is currently not clear if S79 could participate in the positioning of a nucleophilic water molecule. It is noteworthy that the IFT25 protein is located >10 Å from the nucleotide-binding pocket of IFT27 and is thus unlikely to contribute any nucleotide binding or catalytic residues.

To test if IFT27 can bind and hydrolyse GTP, we carried out GTP binding and hydrolysis assays using purified CrIFT25 Δ C/27. To measure the intrinsic GTP hydrolysis activity of IFT27, the release of inorganic phosphate upon conversion of GTP to GDP was followed (Figure 4B). This experiment shows that IFT25 Δ C/27 has very low but measurable intrinsic GTPase activity. The reaction rate under the conditions of the assay is $2.1 \times 10^{-3} \text{ min}^{-1} \pm 2.9 \times 10^{-5} \text{ min}^{-1}$, which is very low but comparable with the intrinsic rate of other small GTPases (Simon *et al*, 1996; Scheffzek and Ahmadian, 2005). As a positive control, the interferon-induced large GTPase IIGP1 with a reaction rate of 2 min^{-1} was used in the GTPase assay (Uthaiiah *et al*, 2003; Ghosh *et al*, 2004). As for most large GTPases, IIGP1 has robust GTPase activity without the assistance of a GAP (Ghosh *et al*, 2004). The GTP hydrolysis can be attributed to the GTPase site of IFT27 as mutations known to affect GTP binding or catalysis significantly change the reaction rate of the IFT25 Δ C/27 complex. The CrIFT25 Δ C/27(S30N) mutant, where GTP binding is impaired, displays a significantly lower reaction rate ($1.5 \times 10^{-3} \text{ min}^{-1} \pm 1.5 \times 10^{-4} \text{ min}^{-1}$) than wild-type (WT) complex (Figure 4B). CrIFT27 has a

Figure 4 IFT27 is lacking a catalytic glutamine residue. **(A)** Comparison of the GTPase sites of GDP-AlF₃-bound Ras (pdb code 1WQ1) and nucleotide-free IFT27. IFT27 is coloured green, Ras light brown and the bound nucleotide is shown as black sticks. The magnesium ion and the catalytic water are shown as a purple and red ball, respectively. The catalytic Q61 of Ras and equivalent residue of IFT27 (S79) are shown as sticks and labelled. Switch regions and the P-loop are also labelled. **(B)** GTPase assay. The release of inorganic phosphate upon GTP hydrolysis is followed using the EnzCheck Phosphate kit (Invitrogen) by absorbance measurements at 360 nm. Uncatalysed GTP hydrolysis in buffer as well as catalysed reactions using 500 μM of WT or mutant IFT25ΔC/27 complex over a time course of 90 min are shown. The concentration of GTP is in all cases 500 μM. As a positive control, 25 μM of the large GTPase IIGP1 is seen to hydrolyse all of the GTP in approximately 10 min. Whereas the GTP-binding mutant S30N is seen to reduce the GTPase activity, introduction of a glutamine at the position of S79 of IFT27 increases the activity significantly. Each of the curves representing WT or mutant IFT25ΔC/27 catalysed GTP turnover is the average of two independent experiments. **(C)** ITC measurements. ITC was used to measure the affinity of IFT25ΔC/27 for GDP and GTP nucleotides. WT IFT25ΔC/27 is seen to bind both GTP and GDP with micromolar affinities. The occupancies are close to one, demonstrating that only one nucleotide-binding site is present in the complex. The nucleotide binding can be attributed to the GTPase site of IFT27 since a point mutation (S30N) in the P-loop of IFT27 abolishes binding to GTP (right panel).

serine (S79) at the position of the catalytic glutamine (Q61) that in the Ras protein coordinates a water molecule for the nucleophilic attack on the γ -phosphate of GTP. To explore if the residue at this position is also important for the catalytic activity of IFT27 we introduced an S79Q mutation and tested the CrIFT25ΔC/27(S79Q) mutant complex in GTPases assays. As seen from Figure 4B, the S79Q mutant has significantly higher activity (reaction rate of $5.4 \times 10^{-3} \text{ min}^{-1} \pm 5.4 \times 10^{-4} \text{ min}^{-1}$) than WT protein, indicating that glutamine at position 79 in CrIFT27 is better suited to coordinate the

catalytic water than serine is. To summarize, CrIFT25ΔC/27 has very low but measurable intrinsic GTPase activity suggesting that if the cellular function of IFT27 requires GTP hydrolysis, a yet unidentified GAP is likely to be involved.

To measure the affinities of CrIFT25ΔC/27 for GDP and GTP nucleotides, isothermal titration calorimetry (ITC) was carried out. The results of these experiments show that the complex binds GTP with a K_d of 19 μM and GDP with a K_d of 37 μM (Figure 4C). The observed nucleotide binding is specific for the GTPase site of IFT27 as the S30N mutant

Figure 5 Surface conservation of the IFT25/27 structure. (Top) Cartoon representation of the IFT25 Δ C/27 complex in a similar orientation as for Figure 1. Residues 24–38 of IFT27 that partly block the view of the GTP-binding pocket have been omitted from the figure for better visualization. (Bottom) Surface representation of the IFT25 Δ C/27 complex in a similar orientation as for Figure 1. The sequence conservation between different species of IFT27 and IFT25 (same species as for the alignment in Figure 3; Supplementary Figure S2) are mapped onto the surface of the IFT25/27 heterodimeric structure. Variable residues are coloured white, whereas conserved residues are shown in orange according to the colour bar. In addition to a highly conserved GTP-binding pocket of IFT27, a neighbouring surface patch, extending across the IFT25/27 dimer, is highly conserved and likely to mediate protein–protein interactions.

does not display any GTP binding in our ITC experiment (Figure 4C). This micromolar affinity for GDP/GTP is much weaker than what is observed for other small GTPases of the Ras superfamily that typically display picomolar to nanomolar affinities for nucleotides (Simon *et al*, 1996; Esters *et al*, 2001). Interestingly, large GTPases that also have K_d 's for GDP in the μ M range typically do not have an identified GEF and may well function without exchange factors (Uthaiiah *et al*, 2003). Given the low affinity of IFT27 for GDP, it is plausible that IFT27 could fulfil its biological role without a cellular GEF. The conclusions from the biochemical analyses are that IFT27 binds GTP/GDP with micromolar affinity and has very low intrinsic GTPase activity.

Discussion

The IFT25/27 structure displays a conserved surface patch that could mediate protein–protein interactions

IFT25/27 is a sub-complex of the larger 14-subunit IFT-B complex and was found to associate with a core of IFT-B proteins after washing away salt labile subunits with 300 mM NaCl (Luckner *et al*, 2005). In addition to IFT27, this IFT-B core was originally shown to contain IFT subunits IFT46, 52, 72/74, 81 and 88 (Luckner *et al*, 2005). IFT25 was not identified as an IFT subunit in the Luckner *et al* (2005) study, but was recently shown to belong to the IFT-B and not the IFT-A complex in co-immunoprecipitation and co-sedimentation experiments, consistent with its direct interaction with IFT27 (Follit *et al*, 2009; Lechtreck *et al*, 2009; Wang *et al*,

2009). As we find that IFT25 Δ C/27 forms a stable complex in the presence of 300 mM NaCl (Supplementary Figure S4C), IFT25 should also be counted as part of this IFT-B core complex. It follows that the IFT25/27 complex must present a surface interface mediating the association with other IFT-B core subunits. Although there is limited data available on how IFT25/27 associates with the rest of the IFT-B core, chemical cross-linking and MALDI-TOF analysis have indicated a possible interaction between IFT27 and IFT81 (Luckner *et al*, 2010).

To explore which part of the IFT25/27 complex could potentially be responsible for IFT-B core binding, we mapped the degree of sequence conservation of IFT25 and IFT27 orthologues onto the surface of the IFT25 Δ C/27 crystal structure (Figure 5) (Landau *et al*, 2005). This method assumes that surface residues engaged in protein–protein interactions will show a higher degree of conservation than residues that are not. Two surface areas of the IFT25 Δ C/27 structure, located on the same side of the complex, show a high degree of conservation (encircled in Figure 5). One of these areas maps solely to IFT27 and overlaps with the GTP-binding site. Adjacent to this, a second, somewhat larger patch is found to display a surface of highly conserved residues. This surface area has residues contributed by both IFT25 and IFT27 and is a good candidate for a protein–protein interaction surface. Part of this conserved surface overlaps with the Rab-GAP-binding site as seen in an Rab33-Rab-GAP crystal structure and could thus be conserved for the purpose of Rab-GAP binding (see Figure 6 and the following section).

Figure 6 Model of putative Rab-GAP binding to IFT25/27. Surface representation of the IFT25/27 heterodimer with the sequence conservation mapped onto the surface as for Figure 5. The model for the putative binding of Rab-GAP to the IFT25/27 complex is shown in blue as ribbon. The model has been derived by superimposing a transition-state structure of Rab33 (the Rab33-GDP-AlF₃-Rab-GAP complex) (pdb code 2G77) onto IFT27 of the IFT25/27 complex. GDP-AlF₃ from the Rab33 structure is displayed as sticks. Both the GDP-AlF₃ and the Rab-GAP binding to IFT25/27 represent models and are not experimentally determined structures.

However, a significant portion of the surface area is not predicted to be involved in Rab-GAP binding and could thus mediate interactions between IFT25/27 and the IFT-B core complex, although additional biochemical and structural data are required to verify this prediction.

Is IFT27 GTP hydrolysis activated by a Tre-2, Bub2 and Cdc16 domain containing GAP?

Small Ras-like GTPases are known to function as switch molecules cycling between an active GTP-bound form and an inactive GDP-bound form. Small GTPases in general have low intrinsic GTP hydrolytic activity and thus usually require the activation by a GAP for robust GTP turnover. Once GTP is hydrolysed, typical affinities for GDP in the picomolar to nanomolar range mean that the GDP-bound form of the GTPase requires the assistance of a GEF to exchange the GDP for GTP (Simon *et al*, 1996; Esters *et al*, 2001). IFT27 appears to be an unusual small GTPase in the sense that it exhibits micromolar affinity rather than sub-nanomolar affinity for GDP (Figure 4C). In this respect, IFT27 more closely resembles large GTPases like IIGP1 that also exhibit micromolar affinities for GDP/GTP (Uthaiah *et al*, 2003; Ghosh *et al*, 2004). Since many large GTPases, such as IIGP1, appear to function without a GEF, it is possible that the low affinity of IFT27 for GDP allows this small GTPase to efficiently exchange GDP for GTP without the assistance of a GEF.

Although IFT27 may function without a GEF, its very low intrinsic GTPase activity indicates that a GAP is needed for robust GTP turnover. Different families of GAPs serve as activators for the different families of GTPases (Scheffzek and Ahmadian, 2005). Although the details of GAP-mediated activation appear to differ between different GAPs, the insertion of one or more residues into the GTPase active site seems to be a common feature to facilitate hydrolysis (Scheffzek and Ahmadian, 2005). In the case of Ras, Ras-GAP contributes

a so-called arginine finger to the GTPase site, thereby increasing the rate of hydrolysis by several orders of magnitude (Scheffzek *et al*, 1997). Of the different Ras-like GTPase families, the sequence and structure of IFT27 most closely resembles that of Rabs. Rab-like GTPases appear to rely on GAPs that possess a Tre-2, Bub2 and Cdc16 (TBC) domain for efficient GTP hydrolysis (Pan *et al*, 2006). The mechanism of activation by TBC domain containing Rab-GAPs was elucidated by the crystal structure of a trapped GDP-AlF₃ transition-state intermediate of Rab33 in complex with the Rab-GAP Gyp1p (Pan *et al*, 2006). This study demonstrates that Gyp1p functions via a dual-finger mechanism where, in addition to the classical arginine finger, a glutamine is inserted *in trans* into the active site of Rab33 (Pan *et al*, 2006). Since IFT27 does not have the catalytic glutamine conserved, it is thus conceivable that it is also activated by a TBC domain containing GAP that provides the missing catalytic glutamine *in trans*. To test if the IFT25ΔC/27 structure is compatible with Rab-GAP binding, the Rab33-Gyp1p structure was superimposed on the IFT25ΔC/27 structure (Figure 6). This superposition shows that the TBC domain containing GAP complements the surface of the IFT25ΔC/27 structure without any major clashes. It is also noteworthy that the predicted GAP-binding surface partly overlaps with the highly conserved surface patch and covers both IFT27 and IFT25. BLAST searches with Gyp1p against the *Chlamydomonas* genome reveal the presence of seven TBC domain containing GAPs (*E*-value < 10⁻³) that could be possible candidates for an IFT27 GAP (Supplementary Table S1). Of the seven candidates, one candidate (gene accession code XP_001699577.1) was shown to localize to the cilium (Pazour *et al*, 2005) but, curiously, does not contain the usual Rab-GAP catalytic motifs (see Supplementary Table S1). Further research is thus required to identify the potential GAP specific for IFT27. The cellular localization of such a GAP will likely provide important insights into the function of IFT25/27 in IFT.

Materials and methods

Preparation and crystallization of the CrIFT25/27 complex

IFT25ΔC and IFT27 were co-expressed in the *E. coli* BL21 (DE3) Gold pLysS strain with TEV protease cleavable GST and His tags, respectively. The complex was purified by Ni²⁺ and glutathione sepharose (GSH)-affinity chromatography followed by TEV cleavage of the tags and further purification by anion exchange chromatography (MonoQ, GE healthcare). As a last step, SEC (Superdex 75, GE Healthcare) was performed in a buffer containing 10 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgSO₄, 1 mM CaCl₂ and 1 mM DTT to separate excess IFT25ΔC from the IFT25ΔC-IFT27 complex. All mutant constructs of IFT25 and IFT27 were generated using the QuickChange site-directed mutagenesis method from Stratagene and the proteins purified using the same protocol as for the WT complex. Crystals of IFT25ΔC/27 at 30 mg ml⁻¹ were obtained by sitting drop vapour diffusion at 18°C by mixing the complex with an equal volume of 20% PEG 3350 buffered with 50 mM MES pH 6.0.

Data collection and structure determination

Crystals were cryo protected using mother liquor supplemented with 35% PEG3350 before flash cooling at 100 K. Diffraction data were collected at the Swiss Light Source (SLS, Villigen, Switzerland) and processed with XDS (Kabsch, 1993). Phase information was obtained for the 2.8-Å resolution data set by molecular replacement using the coordinates of the human IFT25 structure (pdb code 1TVG) and an ensemble of four different Rab GTPase structures that were superimposed (pdb codes 2O52, 1Y2K, 2AED and 1N6H) as

search models in the program PHASER (Storoni *et al*, 2004). The model was completed by iterative cycles of model building in COOT (Emsley and Cowtan, 2004) and refinement in PHENIX (Adams *et al*, 2010). The structure from the second crystal form (2.6 Å resolution data set) was determined by molecular replacement using the model obtained from the 2.8-Å resolution data.

ITC and GTPase assay

ITC was carried out at 25°C using a VP-ITC Microcal calorimeter (Microcal, GE healthcare). Proteins and nucleotides were buffered with 10 mM Tris, 150 mM NaCl, 1 mM CaCl₂ and 5 mM MgSO₄ pH 7.5. A volume of 1.44 ml of protein was titrated with nucleotide in 45 injections of 5 µl each with 5 min intervals between injections. CrIFT25AC/27 (WT or S30N mutant) were at a concentration of 50 µM for the GTP and ATP titration and at 95 µM concentration for the GDP titration. The nucleotides were in all cases 10 × the concentration of protein in the cell. For each ITC curve, a background curve consisting of the titration of nucleotide into buffer without protein was subtracted to account for heat dilution. The ITC data were analysed using the program Origin version 7 provided by Microcal.

The GTPase activity of CrIFT25AC/27 was measured using the EnzCheck Phosphate kit (Invitrogen) at 20°C. For each assay, 500 µM GTP was added to the solutions provided in the assay kit according to the manufacturer's recommendations. The reaction was initiated by the addition of 500 µM CrIFT25AC/27 and the release of phosphate upon GTP hydrolysis monitored by following the enzymatic conversion of inorganic phosphate and 2-amino-6-mercapto-7-methylpurine riboside into ribose 1-phosphate and 2-amino-6-mercapto-7-methyl-purine by absorbance measurements at 360 nm. Absorbance measurements were taken every 6 s for a total duration of 90 min, and each curve in Figure 4B represents the average of two independent experiments. For the positive control, 25 µM of the GTPase IIGP1 was found to hydrolyse 500 µM GTP in approximately 10 min in agreement with its reported reaction rate of 2 min⁻¹ (Uthiaiah *et al*, 2003). As a negative control, the intrinsic hydrolysis of GTP in buffer (without the addition of any protein) was measured.

Pull-down experiments

His-tagged CrIFT25AC and GST-tagged CrIFT27 were co-expressed in *E. coli* BL21 (DE3) Gold pLysS cells. Cells were lysed in lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgSO₄ and 1 mM CaCl₂ and the supernatants after centrifugation were incubated with pre-blocked GSH beads to pull down GST-CrIFT27 from the lysate. Beads were washed extensively using the lysis

buffer to remove contaminants and the bound protein was eluted from the beads using lysis buffer supplemented with 30 mM glutathione. For the Ni-NTA pull downs of IFT25, lysed cells expressing the proteins were centrifuged and the supernatant incubated with Ni-NTA beads followed by extensive washing with lysis buffer before eluting with 500 mM imidazole. For the CBP pull downs shown in Supplementary Figure S5B, pre-blocked calmodulin beads were incubated with the supernatant from cells where CBP-IFT25AC and IFT27 were co-expressed. After extensive washing, proteins were eluted from the calmodulin beads using a buffer containing 20 mM EGTA. In all cases, the eluted proteins were analysed using SDS-PAGE.

Accession codes

The coordinates and structure factors have been deposited in the pdb under the accession codes 2yc2 and 2yc4.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: SB did the protein expression, purification, crystal structure determination and biochemistry under the supervision of EL. The ITC experiments shown in and Supplementary Figure S6 were done by SB under the supervision of CB. MM cloned the IFT25 and IFT27 constructs. MT assisted with purification and pull-down experiments and made Figures 4–6. SB, MT and EL wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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