IFTA-2 is a conserved cilia protein involved in pathways regulating longevity and dauer formation in Caenorhabditis elegans

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Accepted 25 July 2006

Journal of Cell Science 119, 4088-4100 Published by The Company of Biologists 2006
doi:10.1242/jcs.03187

Summary

Defects in cilia are associated with diseases and developmental abnormalities. Proper cilia function is required for sonic hedgehog and PDGFRαx signaling in mammals and for insulin-like growth factor (IGF) signaling in Caenorhabditis elegans. However, the role of cilia in these pathways remains unknown. To begin addressing this issue, we are characterizing putative cilia proteins in C. elegans that are predicted to have regulatory rather than structural functions. In this report, we characterized the novel cilia protein T28F3.6 (IFTA-2, intraflagellar transport associated protein 2), which is homologous to the mammalian Rab-like 5 protein. We found that, unlike the intraflagellar transport (IFT) genes, disruption of ifta-2 does not result in overt cilia assembly abnormalities, nor did it cause chemotaxis or osmotic avoidance defects typical of cilia mutants. Rather, ifta-2 null mutants have an extended lifespan phenotype and are defective in dauer formation. Our analysis indicates that these phenotypes result from defects in the DAF-2 (insulin–IGF-1-like) receptor signaling pathway in ciliated sensory neurons. We conclude that IFTA-2 is not a ciliogenic protein but rather is a regulator of specific cilia signaling activities. Interestingly, a mammalian IFTA-2 homolog is also found in cilia, raising the possibility that its function has been conserved during evolution.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/119/19/4088/DC1

Key words: C. elegans, Cilia, IFT, Rab-like, Insulin-IGF signaling

Introduction

Cilia are microtubule-based organelles found on most cells in the mammalian body and on many eukaryotic organisms. They are assembled from the basal body through a highly conserved process called intraflagellar transport (IFT). IFT was first described in flagella of Chlamydomonas as a bidirectional transport system that directs movement of large protein complexes called IFT particles between the base and tip of the flagella (Kozminski et al., 1993; Scholey, 2003). Anterograde and retrograde movement of the particle are mediated through the activity of kinesins and a cytosolic dynein motor protein, respectively. IFT is required for delivery of proteins necessary for cilia assembly and maintenance, and for cilia function and signaling activity.

In mammals, cilia can be motile or immotile (primary cilia), and they have a wide range of functions (Davenport and Yoder, 2005). Motile cilia found on regions such as the lung epithelium or ependymal cells in the brain are essential for fluid movement. The primary cilium, by contrast, has sensory functions and has recently been implicated as a mechanosensor on epithelia that line the tubules and ducts of the kidney, pancreas and liver (Prætorius and Spring, 2001). In addition, primary cilia are needed for normal cellular responses to ligands such as platelet-derived growth factor A (PDGF-A) and sonic hedgehog (Shh), and their dysfunction has recently been implicated in human diseases, including polycystic kidney disease (PKD), obesity and diabetes (Haycraft et al., 2005; Huangfu et al., 2003; Schneider et al., 2005; Beales, 2005; Eley et al., 2005; Saunier et al., 2005).

In contrast to the ubiquitous nature of cilia in mammals, C. elegans cilia are present only on neurons. Of the 302 neurons in the adult hermaphrodite, 60 are ciliated and many extend off dendrites into the environment through small pores in the cuticle where they sense environmental conditions (Ward et al., 1975; Ware et al., 1975; White et al., 1986). Mutations in genes required for cilia assembly in C. elegans lead to a variety of phenotypes including defects in chemotaxis and osmotic avoidance and to an increased lifespan (Apfeld and Kenyon, 1999; Culotti and Russell, 1978; Dusenbery et al., 1975; Starich et al., 1995). In several cases, cilia-mediated signaling pathways are conserved between C. elegans and mammals. This is evidenced by the localization of polycystin-1, polycystin-2, and the nephropathosis proteins nephrocystin-1 and nephrocystin-4 to cilia or the base of cilia in both organisms (Barr et al., 2001; Winkelbauer et al., 2005; Yoder et al., 2002; Mollet et al., 2002; Mollet et al., 2005; Otto et al., 2002; Otto et al., 2000; Otto et al., 2003). Although there are no overt cilia morphology abnormalities associated with mutations in these genes, defects in cilia signaling activity result in cystic
pathologies in the mammalian kidney and abnormal mating behavior, chemotaxis defects and extended lifespan in *C. elegans* (Barr et al., 2001; Winkelbauer et al., 2005; Yoder et al., 2002).

Longevity regulation in *C. elegans* requires normal cilia formation and function and nearly all of the characterized *C. elegans* cilia mutants have an extended lifespan phenotype. The connection between cilia dysfunction and increased longevity is the requirement for cilia in the DAF-2 (insulin–IGF-1-like) receptor signaling pathway (Apfeld and Kenyon, 1999). The molecular regulation of insulin–IGF-1-like receptor signaling is highly conserved across species including *Drosophila*, *C. elegans* and mammals, and in all of these organisms, insulin–IGF-1-like receptor signaling influences lifespan, growth, metabolism and reproduction (Clancy et al., 2001; Holzenberger et al., 2003; Tatar et al., 2001). Furthermore, the regulation of lifespan in *C. elegans* requires sensory neurons and these same neurons are involved in reception of environmental signals inducing formation of the dauer, an alternative phase of the life cycle that *C. elegans* enter when unfavorable conditions are encountered (Riddle and Albert, 1997). This dauer form allows for long-term survival and resistance to harsh environmental conditions. Ciliated sensory neurons are required for proper dauer formation and, accordingly, several cilia and IFT mutants are either unable to form dauers or form dauers constitutively under normal conditions (Ailion and Thomas, 2000; Apfeld and Kenyon, 1999).

Like in the mammalian insulin signaling pathway, insulin–IGF-1-like signaling in *C. elegans* through DAF-2 activates AGE-1 [the *C. elegans* ortholog of the phosphoinositide 3-kinase (PI 3-K) p110 catalytic subunit] (Morris et al., 1996). This leads to phosphorylation of the serine/threonine kinases AKT-1 and AKT-2, whose activity regulates the phosphorylation of DAF-16, a transcription factor of the HNF-3/forkhead family, homologous to human forkhead box O1A (FOXO1A) (Ogg et al., 1997; Paradis et al., 1999; Paradis and Ruvkun, 1998). In the phosphorylated state, DAF-16 is excluded from the nucleus, leading to wild-type expression levels of DAF-16 target genes. In the absence of an activated *daf-2* signaling pathway or in *C. elegans* strains with mutations in *daf-2*, *age-1* or *akt*, DAF-16 remains in an unphosphorylated state and accumulates in the nucleus, resulting in an increased lifespan (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). The longevity and dauer phenotypes in the *daf-2* or *age-1* mutants can be suppressed by mutations in *daf-16* (Kenyon et al., 1993; Albert et al., 1981; Dorman et al., 1995; Lin et al., 1997; Vowels and Thomas, 1992). In addition, mutations that disrupt IFT increase lifespan similar to that seen in *daf-2* mutants, and cause constitutive dauer phenotypes at 27°C (Apfeld and Kenyon, 1999; Ailion and Thomas, 2000). The analysis of *daf-2*IFT double mutants indicates that the lifespan is not extended further than either mutant alone, suggesting that IFT and DAF-2 act in the same pathway. Furthermore, the increased lifespan in IFT mutants is associated with nuclear localization of DAF-16 and, as seen in the *daf-2* mutants, the longevity can be suppressed by loss of *daf-16* (Lin et al., 2001).

The current theory holds that cilia located on sensory neurons detect environmental conditions, such as food availability or stress, and elicit a secondary signal controlling dauer formation, metabolism, stress resistance and lifespan by modulating DAF-16 localization and activity. To further understand the mechanism by which cilia-generated signals may influence lifespan, it is important that we identify and characterize novel factors that function in the cillum but do not directly influence cilia assembly or morphology. Here, we characterize a novel cilia-localized protein (T28F3.6) that was identified through genomic sequence and microarray-based searches for genes regulated by the ciliogenic transcription factor DAF-19 (Blacque et al., 2005; Colosimo et al., 2004; Efimenko et al., 2005). Many of the DAF-19 target genes characterized to date have been found to encode proteins involved in IFT. T28F3.6 encodes a Rab-like protein named IFTA-2 (IFT-associated protein 2). Our characterization of *C. elegans* with mutations in *ifta-2* demonstrates that IFTA-2 is not required for formation of most – if not all – cilia or for localization or movement of IFT proteins in the cilia. In addition, *C. elegans* lacking IFTA-2 have normal chemotaxis and osmotic avoidance, in contrast to the abnormal chemotaxis and osmotic avoidance phenotypes typically seen in *C. elegans* with defects in cilia morphology. Rather, *ifta-2* mutants display abnormalities in dauer formation and exhibit an extended lifespan that is not further extended in *ifta-2;daf-2* mutants. Both of these phenotypes are dependent on *daf-16*. Together, these data indicate that IFTA-2 is a novel component of the *daf-2* pathway that functions in cilia of sensory neurons to regulate a pathway involved in longevity and dauer formation, possibly in response to environmental cues detected by the cillum.

**Results**

**IFTA-2 encodes a putative Rab-like protein**

The *C. elegans* protein T28F3.6 (IFTA-2, intraflagellar transport associated protein 2) was identified as part of a search for genes coordinately regulated by the ciliogenic transcription factor DAF-19. IFTA-2 has putative homologs in several organisms including human (GenBank accession number AAH38668), mouse (GenBank accession number NP 808349), and *C. briggsae* (GenBank accession number CAE57646). The highest sequence similarity in vertebrates is with the Rab-like 5 (RabL5) protein. IFTA-2 shares 31% identity and 50% similarity with human RabL5, 24% identity and 44% similarity with mouse RabL5, and 59% identity and 70% similarity with the *C. briggsae* homolog. The predicted protein product of *C. elegans* IFTA-2 is 252 amino acids long. It has an estimated molecular mass of 28.8 kDa and an isoelectric point of 4.4.

Rab proteins are small GTPases that belong to a subclass of the Ras superfamily (Wennerberg et al., 2005). Classification of IFTA-2 as a putative Rab protein is based on sequence alignment of G domains, which are found in most Rab proteins and are involved in GTP/GDP binding (Fig. 1). The conserved G1 domain (GxxxxGKT/S) is found at amino acids 35-42, the G2 domain (xTx) at amino acids 80-82, and the G3 domain (DxxG) at amino acids 119-122. In contrast to G1-3, the G4 domain (N/TKxD) is not as highly conserved in IFTA-2 as the G2 domain (xTx) at amino acids 80-82, and the G3 domain (DxxG) at amino acids 119-122. Although this is not common, there are examples of Rab proteins that differ from the canonical sequence. For example, Rab15 lacks the G4 domain and Rab39 lacks the N/T in the consensus N/TKxD G4 domain (Wennerberg et al., 2005). Finally, IFTA-2 does not contain a
prenylation domain (CCxx, xCxC), usually located at the C-terminus of many Rab proteins. Based on these characteristics found in most Rab proteins, IFTA-2 is likely to be a member of the Rab-like subclass and is most similar to human and mouse RabL5.

IFTA-2 localizes to cilia and is transported along the axoneme

To analyse the expression pattern of *ifta-2*, we created transgenic *C. elegans* strains with the putative *ifta-2* promoter (343 nucleotides prior to translational start site) and the sequence encoding the first 49 amino acids of IFTA-2 fused to GFP. As shown previously for several other DAF-19 target genes, *ifta-2* was expressed exclusively in ciliated sensory neurons in both the head and the tail of the adult hermaphrodite (Fig. 2A). We saw no expression outside of these neurons.

To evaluate where IFTA-2 localizes in ciliated sensory neurons, we generated a construct consisting of the *ifta-2* genomic region, including 400 nucleotides of the promoter, fused in-frame with GFP (IFTA-2::GFP). Transgenic *C. elegans* strains were generated and localization of the IFTA-2::GFP was analysed by fluorescence microscopy in anesthetized worms. The data show that IFTA-2::GFP is present at the base of cilia and within the cilia axoneme of the ciliated amphid and phasmid sensory neurons in the adult hermaphrodite (Fig. 2B). This localization is reminiscent of previously characterized IFT proteins in *C. elegans* including OSM-5, CHE-13 and XBX-1 (Haycraft et al., 2003; Haycraft et al., 2001; Schafer et al., 2003). IFTA-2::GFP can also be detected moving along the cilium axoneme (supplementary material Movie 1). Furthermore, our analysis of the RabL5 homolog in mouse renal epithelium indicates that it also localizes to the primary cilium raising the possibility of conserved function (Fig. 2E).

A missense mutation in the G1 domain alters localization of IFTA-2

Rab proteins act as molecular switches that cycle between GDP- and GTP-bound states that control their cellular localization and activity (Stenmark and Olkkonen, 2001). Thus, mutations that result in preferential binding of GTP or GDP affect the function and localization of the Rab protein. We used this property to further investigate the function of IFTA-2 as a cilia-localized Rab-like protein. We engineered two individual missense mutations in the putative GTP binding domains of IFTA-2 (Fig. 1). These domains were identified based on homology with previously characterized motifs in Rab1 and Rab5 and should alter the GDP/GTP
binding preferences. The T42N missense mutation generated in the G1 domain is predicted to preferentially bind GDP over GTP and thus remain in the inactive state. By contrast, the D123L mutation in G3 is predicted to disrupt GTP hydrolysis and thus should remain in the active state. Both of these mutations was found to differentially affect the localization of IFTA-2::GFP in the sensory neurons. The IFTA-2(T42N) mutant protein was delocalized throughout ciliated sensory neurons such that the protein appears diffuse throughout the cell bodies and dendrites, but was restricted from the cilia (Fig. 2C). The T42N mutation does not appear to directly affect a cilia-targeting domain because expression of a GFP fusion consisting of the N-terminal 49 amino acids including the entire G1 domain did not localize to the cilia (Fig. 2A). In contrast to the T42N mutation, localization of the IFTA-2(D123L) mutant protein was indistinguishable from the wild-type protein in that it also was found in cilia and could be seen migrating along the axoneme (Fig. 2D and data not shown). Although it is possible that D123L represents a dominant-negative version or a protein with enhanced activity, we did not detect a difference in its cilia localization or IFT movement compared with wild type or any overt phenotypes associated with this line. Furthermore, we attempted to detect GTP/GDP binding of the wild-type and mutant forms of the proteins but were unsuccessful using in vitro assays (data not shown). Although we have not been able to confirm that IFTA-2 binds GTP, the effect of these mutations on protein localization patterns are similar to that seen in other Rab family members (Alvarez et al., 2003; Wennenberg et al., 2005).

Disruption of ifta-2 does not alter cilia morphology or IFT
The discovery of a novel Rab-like protein in the cilia that migrates with the IFT particle raised the possibility that IFTA-2 has a role in cilia formation. To test this possibility, we obtained two independent deletion mutants in ifta-2 from the National BioResource Project in Japan. By sequence analysis of the genomic region isolated from these mutants, we found that the first strain, FX1724 ifta-2(tm1724), contains a deletion spanning nucleotides 9-902 (cosmid nucleotide numbers 21763-20868) resulting in a shift in the reading frame. As such, it is predicted to result in a protein containing only the first two amino acids of IFTA-2 and probably represents a null mutation (data not shown). The second strain, FX1725 ifta-2(tm1725), contains a deletion that begins at nucleotide 447 (cosmid nucleotide number 20956) and ends after the translational stop resulting in a truncation of the IFTA-2 protein at amino acid 148, shortly after the G3 domain (data not shown). For subsequent phenotype analyses, we used the ifta-2(tm1724) allele as it is predicted to result in a loss of all IFTA-2 protein.

Since IFTA-2 localizes to cilia and moves along the cilia axoneme similar to other IFT proteins, we first evaluated whether there was a defect in cilia assembly in these mutants using the dye-fill assay. In C. elegans cilia that extend through pores in the cuticle into the environment are able to uptake fluorescent dye, which can be visualized in the dendrites of the ciliated sensory neurons (Perkins et al., 1986). Most IFT mutants, such as osm-5, che-13 and xbx-1 are unable to take up dye because of the malformed or absent cilia (Haycraft et al., 2003; Haycraft et al., 2001; Schafer et al., 2003). In contrast to these IFT mutants, both ifta-2 mutant strains absorbed fluorescent dye as well as wild-type worms, indicating that there were no gross structural abnormalities in the cilia of the amphid or phasmid neurons (data not shown).

Next we wanted to assess whether mutations in ifta-2 had
any effect on IFT proteins in the cilia similar to that recently demonstrated for bbs-7 and bbs-8, two genes involved in coordination of the IFT particle (Blacque et al., 2004). For this analysis, we crossed the ifta-2(tm1724) strain with several transgenic lines that express different subunits of the IFT particle tagged with a fluorescent protein. We then analysed cilia morphology and looked for effects on localization and movement of the IFT particle in the cilia axoneme. This analysis included the complex B IFT protein OSM-5::GFP, which is involved in anterograde movement, and the complex A IFT protein CHE-11::GFP and a dynein light intermediate chain protein XBX-1::YFP, both of which are involved in retrograde movement. Although we have not evaluated all the sensory cilia, our analysis revealed that cilia morphology on the amphid and phasmid neurons was not affected by loss of IFTA-2 and that all three proteins localized properly to the cilia and underwent IFT along the axoneme of the ifta-2(tm1724) mutants as seen in the wild-type controls (Fig. 3A and data not shown).

In reciprocal crosses, we also evaluated whether IFT was required for localization of IFTA-2 in cilia and for its movement along the axoneme. For this analysis IFTA-2::GFP was crossed into C. elegans with mutations in osm-5(m184), che-3(e1124), and che-11(e1810). We found that although these mutants have truncated or nearly absent cilia, IFTA-2::GFP still localized to the base of and within the cilia axoneme (Fig. 3B). Together, these reciprocal crosses indicate that IFTA-2 is not essential for cilia formation or maintenance.

Analysis of cilia-related phenotypes in ifta-2 mutants

Although IFTA-2 is not involved in ciliogenesis, the localization and movement of IFTA-2 in the cilia raised the possibility that it has a role in cilia-mediated signaling. Cilia function is required in C. elegans for normal responses to chemoattractants and repellents and is important for regulating entry into the dauer phase of the life cycle and controlling lifespan (Apfeld and Kenyon, 1999; Vowels and Thomas, 1992; Dusenberg et al., 1975; Starich et al., 1995). We assessed whether the ifta-2 mutants have defects in sensory cilia functions that are also seen in C. elegans mutants with cilia abnormalities. We did not detect differences between wild-type and ifta-2(tm1724) mutants in assays that measure osmotic avoidance or chemoattractant response to benzaldehyde (data not shown). However, we did detect a significant increase in lifespan of ifta-2(tm1724) mutants compared with controls (Fig. 4A, Table 1). To confirm that the longevity was caused by disruption of ifta-2, we constructed transgenic lines expressing the wild-type IFTA-2::GFP in the ifta-2(tm1724) mutant background. Our analysis of these lines indicated that there was a significant rescue of the lifespan phenotype. Rescue was observed in three of four IFTA-2::GFP strains analysed (Fig. 4B, Table 1, and data not shown). The failure to rescue the phenotype in the fourth line may be associated with mosaicism because this line did not transmit the transgene to its progeny as efficiently as the other lines. However, all four lines show IFTA-2::GFP expression similar to wild type (supplementary material Fig. S1). This correction of the lifespan phenotype indicates that mutation in ifta-2 is responsible for lifespan extension and, therefore, ifta-2 is required for wild-type longevity.

IFTA-2 functions as part of the daf-2 pathway and depends on daf-16

Extensive work in C. elegans has uncovered a conserved

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![Fig. 3.](Fig. 3. (A) OSM-5::GFP, CHE-11::GFP, and XBX-1::YFP localize to cilia in both wild type and ifta-2(tm1724) mutants. (B) IFTA-2::GFP localizes properly to cilia in wild-type, osm-5(m184), che-11(e1810), and che-3(e1124) worms. In A and B, upward arrowhead indicates base of cilia and arrow indicates cilia axonemes. In B, downward arrowheads indicate malformed cilia.)
pathway involved in lifespan regulation that involves signaling through the insulin–IGF-1-like receptor (DAF-2), the PI 3-K AGE-1, several serine/threonine kinases (including AKT-1, AKT-2 and PDK-1) and the HNF-3/forkhead transcription factor DAF-16 (Kenyon, 2005). Genetic analysis has shown that many of the long-lived mutants, such as the IFT cilia mutants, function as part of the DAF-2 pathway. However, the connection between cilia and DAF-2 has not been fully elucidated.

To evaluate whether IFTA-2 functions as part of the insulin–IGF-1-like lifespan-regulation pathway, we performed genetic epistasis analysis between the \textit{ifta-2} (tm1724) mutant and other mutants known to function in the DAF-2 lifespan-regulation pathway. We first tested whether IFTA-2 functions with DAF-2 or in a parallel pathway to regulate longevity. Similar studies were conducted with \textit{eat-2}, which functions in parallel to \textit{daf-2} because \textit{eat-2;daf-2} double mutants display significantly longer lifespan than either mutant alone (Lakowski and Hekimi, 1998). This is in contrast to the results obtained for \textit{IFT;daf-2} (e.g. \textit{osm-5;daf-2}) double mutants, which do not display additive effects (Apfeld and Kenyon, 1999). Thus, we compared the lifespan of \textit{daf-2(e1370)} mutants, \textit{ifta-2(tm1724)} mutants and \textit{daf-2(e1370);ifta-2(tm1724)} double mutants, and found that lifespans of all three are not statistically different. These data support the hypothesis that \textit{ifta-2} and \textit{daf-2} act in a common pathway (Fig. 4C, Table 1). One caveat of the interpretation of these data is that the \textit{ifta-2} lines used have not been outcrossed.

Data from Apfeld and Kenyon have also shown that the increased lifespan phenotype in IFT mutants, such as \textit{osm-5} and \textit{che-13}, is dependent on the function of \textit{daf-16} (Apfeld and Kenyon, 1999). Thus, to further demonstrate that \textit{ifta-2} is part of the DAF-2–DAF-16 pathway, we analysed the lifespan of \textit{ifta-2(tm1724);daf-16(mu86)} double mutants. As seen with the mutants disrupting IFT, the \textit{ifta-2(tm1724);daf-16(mu86)} double mutants have a significantly reduced lifespan compared with the \textit{ifta-2(tm1724)} strain and were similar to the \textit{daf-16(mu86)} mutants alone (Fig. 4D, Table 1). Thus, the \textit{ifta-2(tm1724)} longevity phenotype is dependent on \textit{daf-16} function. This finding is identical to that reported for the IFT, \textit{daf-2} and \textit{age-1} mutants, demonstrating a role for IFTA-2 in this pathway and that it probably involves functions in the cillum.
To further evaluate the ifta-2(tm1724) phenotype and the connection with cilia, we analyzed whether the longevity observed in the IFT mutants, which are known to disrupt the daf-2 signaling pathway, were further affected by loss of ifta-2. Our analysis indicates that the lifespan of osm-5(m184);ifta-2(tm1724) double mutants was not significantly different than the original ifta-2(tm1724) single mutants (Fig. 4E, Table 1). However, lifespan in the double mutant is longer than in the single mutant osm-5(m184) alone, suggesting that ifta-2 is epistatic to osm-5. This result might be expected if a low level of DAF-2 signaling is remaining in the IFT mutants were abrogated by loss of ifta-2 function. Alternatively, cilia might process signaling activity that both negatively and positively influences lifespan.

In light of the role of cilia in the DAF-2–DAF-16 lifespan pathway, we wanted to evaluate whether any components of the DAF-2 pathway were found in the cilia axoneme and whether ifta-2 had a role in their subcellular localization. We performed immunofluorescence analysis of fixed worms using antibodies against DAF-2 and AGE-1 to determine the subcellular distribution of these proteins in wild type and ifta-2 mutants. Interestingly, our data indicate that both DAF-2 and AGE-1 are concentrated in the cilia in a similar pattern to IFTA-2 (Fig. 5). DAF-2 and AGE-1 were also present diffusely in other regions of the sensory neurons that do not overlap with IFTA-2 as well as in non-ciliated cells that do not express IFTA-2. We also determined that disruption of ifta-2 does not alter DAF-2 or AGE-1 localization in the cell (Fig. 5). Thus, these data indicate that IFTA-2 is not required for the ciliary targeting of these proteins and that the longevity in the ifta-2(tm1724) mutants is not caused by mislocalization of the DAF-2 receptor. In addition, because DAF-2, AGE-1 and IFTA-2 colocalize in cilia, these data further support the idea that they function in a common lifespan pathway that is initiated in the cilium.

Finally, previous work has shown that the transcription factor DAF-16 is localized primarily to the cytoplasm and to a lesser extent the nucleus of wild-type worms. Under normal circumstances, activation of the DAF-2 pathway leads to phosphorylation of DAF-16 and its exclusion from the nucleus (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). However, in many of the long-lived mutants, such as daf-2(e1370) and the cilia mutants daf-10 and osm-3, the population of worms showing DAF-16 enrichment in the nucleus is dramatically increased due to inhibition or loss of the daf-2 signaling activity (Henderson and Johnson, 2001; Lin et al., 2001). Thus, we evaluated whether there was an increase in the population of ifta-2(tm1724) worms that exhibited DAF-16 nuclear accumulation. Using a DAF-16::GFP transgenic line, we compared DAF-16::GFP in age-matched wild-type and the cilia mutants daf-2 and daf-16. Our data show that in the ifta-2(tm1724) mutant worms there is an increase in the population of C. elegans with strong nuclear localization (Fig. 6). We did not see this marked increase in nuclear localization in wild-type controls. These data indicate that loss of ifta-2 alters DAF-16 localization similar to loss of daf-2 or IFT function and further support the genetic epistatic analysis data described above.

Table 1. Statistics of the experiments described in Fig. 4

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<th>Median (days)</th>
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<th>Mean life span ± s.e.m. (in days)</th>
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<td>16.6±0.65</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td></td>
<td>(ifta-2(tm1724);</td>
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</tr>
<tr>
<td></td>
<td>osm-5(m184))</td>
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<tr>
<td></td>
<td></td>
<td>65</td>
<td>22</td>
<td>(20,22)</td>
<td>23.2±0.92</td>
<td>&lt;0.0001*</td>
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<tr>
<td></td>
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<td>73</td>
<td>22</td>
<td>(20,24)</td>
<td>24.0±0.77</td>
<td>&lt;0.0001*</td>
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<tr>
<td>4D</td>
<td>Wild type</td>
<td>52</td>
<td>19</td>
<td>(19,19)</td>
<td>19.9±0.30</td>
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<tr>
<td></td>
<td>(ifta-2(tm1724);</td>
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<td></td>
<td></td>
<td></td>
<td>0.0568</td>
</tr>
<tr>
<td></td>
<td>daf-16(mu86)</td>
<td></td>
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<td></td>
<td>55</td>
<td>20</td>
<td>(19,20)</td>
<td>20.1±0.29</td>
<td>&lt;0.0001*</td>
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<tr>
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<td>55</td>
<td>20</td>
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<td>19.9±0.30</td>
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<tr>
<td>4E</td>
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<td>(21,23)</td>
<td>22.3±0.64</td>
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<td></td>
<td>osm-5(m184)</td>
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<td>20.0±0.48</td>
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<tr>
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<td></td>
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<td>23</td>
<td>(23,24)</td>
<td>24.1±0.60</td>
<td>&lt;0.0001*</td>
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P values <0.05 were deemed statistically significant (indicated by asterisks); CI confidence interval.
ifta-2 mutants display defects in dauer formation

In addition to lifespan, the DAF-2–DAF-16 pathway also regulates dauer formation. We next evaluated whether ifta-2(tm1724) mutants exhibit a dauer phenotype. Previous work has shown that daf-2(e1370) mutants exhibit a constitutive dauer phenotype (Daf-c) at 25°C. IFT mutants such as osm-5(m184) and daf-10(e1387) display a defective dauer phenotype (Daf-d) at 20°C and 25°C but a constitutive dauer phenotype at 27°C (Ailion and Thomas, 2000; Apfeld and Kenyon, 1999). To further assess the connection between ifta-2, IFT and the daf-2 pathway, we evaluated whether ifta-2(tm1724) showed dauer formation at 20°C, 25°C and 27°C. As seen for IFT mutants, ifta-2(tm1724) hermaphrodites displayed a Daf-d phenotype at 20°C and 25°C but a Daf-c phenotype at 27°C (Table 2). Although these mutants were not all Daf-c at 27°C, we found that 25-30% of ifta-2(tm1724) were constitutive dauers at approximately 42-46 hours posthatching. This was similar to positive controls osm-5(m184) and daf-10(e1370), which exhibited 15-20% and 5-10% dauers, respectively. Less than 1% of the worms formed dauers at 27°C in wild-type and daf-16(mu86) strains. Furthermore, the Daf-c phenotype at 27°C was suppressed in ifta-2(tm1724);daf-16(mu86) double mutants. These data mimic the rescue of ifta-2(tm1724) lifespan by daf-16(mu86). Both lifespan and dauer formation are regulated by the DAF-2–DAF-16 pathway, so these data further confirm that ifta-2 functions through this pathway.

Discussion

Cilia have recently become the center of intense research efforts in mammalian systems because of their association with numerous diseases, including polycystic kidney disease, and obesity, and their involvement in important signaling pathways, such as the Shh and PDGFβ pathways (Haycraft et al., 2005; Huangfu et al., 2003; Schneider et al., 2005). In C. elegans, cilia also have crucial functions, and their loss on sensory neurons results in altered metabolism and growth, defects in chemosensation, abnormal male mating responses, defects in dauer formation and lifespan extension (Apfeld and Kenyon, 1999; Barr et al., 2001; Dusenbery et al., 1975; Starich et al., 1995; Vowels and Thomas, 1992). Importantly, genes involved in those pathways that regulate many of these effects are highly conserved in mammals, suggesting that the connection between cilia and disease can be further assessed using C. elegans as a convenient model.

In this regard, we became interested in a cilia protein T28F3.6 (now called IFTA-2) in C. elegans that shares strong homology with human and mouse Rab-like proteins of unknown function. We reasoned that this Rab-like protein could play a regulatory role in cilia and, thus, characterizing

Table 2. Dauer assays

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Summary of dauer phenotypes</th>
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<tr>
<td></td>
<td>20°C</td>
</tr>
<tr>
<td>N2</td>
<td>+</td>
</tr>
<tr>
<td>ifta-2(tm1724)</td>
<td>Daf-d</td>
</tr>
<tr>
<td>osm-5(m184)</td>
<td>Daf-d</td>
</tr>
<tr>
<td>daf-10(e1387)</td>
<td>Daf-d</td>
</tr>
<tr>
<td>daf-2(e1370)</td>
<td></td>
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<tr>
<td>daf-16(mu86)</td>
<td>Daf-d</td>
</tr>
<tr>
<td>ifta-2(tm1724);daf-16(mu86)</td>
<td>Daf-d</td>
</tr>
</tbody>
</table>

Dauer assays were performed at three temperatures at least three independent times with approximately 50-100 animals per experiment. N2, daf-2(e1370), daf-10(e1387) and daf-16(mu86) served as controls. daf-c, dauer constitutive; daf-d, dauer defective; +, wild type for dauer formation.
in other cilia proteomes including a comparative genomics search and mass spectrometry analyses of the lung cilia in mammals and the *Chlamydomonas* flagella (Li et al., 2004; Ostrowski et al., 2002; Pazour et al., 2005). In agreement, our analysis of IFTA-2 indicates that it localizes to cilia and moves along the cilium axoneme similar to previously characterized IFT proteins, such as CHE-13 and OSM-5, as well as the IFT-associated proteins BBS-7, BBS-8 and XBX-1 (Blacque et al., 2004; Haycraft et al., 2003; Haycraft et al., 2001; Schafer et al., 2003).

In addition to IFTA-2, other small GTP binding proteins have been identified in cilia and flagella. The first example described was the identification of four small G proteins in the green algae *Volvox carteri* and *Chlamydomonas reinhardtii* (Huber et al., 1996). In Zebrafish, a mutation in the gene scorpion results in cystic kidneys and is required for normal cilia formation (Sun et al., 2004). ARL3 is required for flagella formation in *Leishmania*, and it has been identified in *C. elegans* as a candidate cilia gene based on the presence of an X-box in its promoter (Cuvillier et al., 2000; Efimenko et al., 2005). In *Drosophila* the GTP-binding proteins ARL3 and ARL6 are expressed in ciliated sensory neurons (Avidor-Reiss et al., 2004). Likewise, in *C. elegans* ARL-6 localizes to cilia and undergoes IFT and mutations in BBS3 (the human homolog of ARL-6) are responsible for a form of Bardet-Biedl syndrome that results in cilia-dysfunction-related phenotypes, such as cystic kidneys, polydactyly and blindness (Fan et al., 2004).

![DAF-16::GFP](image)

**Fig. 6.** (A) Representative image of cytoplasmic DAF-16::GFP localization in an L1 worm. (B) Representative image of intermediate DAF-16::GFP localization in an L1 worm showing both cytoplasmic and nuclear localization. (C) Representative image of nuclear DAF-16::GFP localization in an L1 worm. (D) DAF-16::GFP worms (%) were classified as cytoplasmic, intermediate or nuclear by three researchers. Numbers are the average (to the nearest whole number) of the scores of three researchers. The proportion of nuclear, intermediate and cytoplasmic DAF-16::GFP localization for each strain was compared by the Chi2 test. *P<0.001, Chi2=9.2475.

Similarly, we found that IFTA-2 behaves like a typical Rab protein. The point mutation T42N causes delocalization of IFTA-2 throughout the ciliated sensory neurons, whereas D123L results in wild-type localization. These findings are similar for known Rab proteins including human Rab1b (Alvarez et al., 2003). The T42N mutation is particularly interesting because the homologous residue in human ARL6 was identified as a mutation in patients with Bardet-Biedl syndrome, indicating that this residue is in a functionally conserved domain thought to be crucial for GTP binding and protein activation in cilia (Fan et al., 2004).

Given the high degree of homology between IFTA-2 and the Rab family proteins, and the effects of point mutations within the nucleotide-binding domains, we believe it is likely that IFTA-2 is a GTP/GDP binding protein. However, using in vitro assays we were unable to detect GTP binding with IFTA-2 (unpublished data). This may reflect abnormal folding of the bacterially expressed protein, a requirement for an accessory factor, or the fact that very little is known about the Rab-like family of proteins and their GTP-binding properties. However, we do show that conserved point mutations in the putative GTP-binding domain alter localization of IFTA-2 as seen in other Rab proteins.

Because IFTA-2::GFP travels along the cilium axoneme similar to an IFT protein, we were surprised to find that *ifta-2* mutants behaved like the wild type for dye-filling, indicating that the cilia, at least on the amphid and phasmid neurons, are normal. This was further confirmed by analysing cilia morphology using GFP-tagged IFT proteins expressed in *ifta-2(tm1724)* mutants. This result was unexpected because most mutations in proteins associated with IFT are defective in dye-filling because of cilia abnormalities (Perkins et al., 1986; Mukhopadhyay et al., 2005; Starich et al., 1995). Although our

its function would provide new insights into the regulation of cilia-mediated signaling pathways. Here, we report that IFTA-2 functions as part of the DAF-2–DAF-16 signaling pathway regulating dauer formation and lifespan.

A recent publication has implicated *ifta-2* as encoding a candidate cilia protein based on the presence of an X-box in the promoter (Efimenko et al., 2005). The DAF-19 transcription factor recognizes the X-box and regulates expression of several *C. elegans* genes that encode cilia proteins, including those previously implicated in lifespan regulation, such as *nph-1*, *nph-4* and *tub-1* (Mukhopadhyay et al., 2005; Winkelbauer et al., 2005; Efimenko et al., 2005; Swoboda et al., 2000). Also, IFTA-2 homologs have been listed
data suggest that IFTA-2 is associated with the IFT particle, it is not required for cilia formation.

Since Rab5 is known to regulate protein trafficking, we wanted to determine whether the IFT proteins were affected by loss of ifta-2, as shown recently for several BBS proteins (Blacque et al., 2004). This was analysed using an IFT complex B protein (OSM-5::GFP), IFT complex A protein (CHE-11::GFP), and a member of the dynein complex (XBX-1::YFP). All three proteins localize normally to the cilia of ifta-2(tm1724) mutants and were transported along the axoneme like in the controls. Thus, IFTA-2 does not have a role, at least for the proteins analysed here, in regulating IFT protein entry into the cilia or loading onto the IFT particle. Similarly, we found that IFTA-2::GFP was still localized to the base of and within abnormally formed cilia in the IFT mutants osm-5(m184), che-3(e1124) and che-11(e1810), indicating they are not required for cilia localization of IFTA-2.

Since IFTA-2 appears to play no role in cilia formation but is localized to cilia, we reasoned that it probably participates in cilia signaling. Although we found no differences between the ifta-2(tm1724) mutants and the wild type with regards to typical cilia mutant phenotypes, including osmotic avoidance and chemotaxis, the ifta-2(tm1724) mutants did exhibit a marked increase in lifespan and defects in dauer formation. C. elegans lifespan and dauer formation are influenced by several factors, including dietary restriction, signals from the germline, reactive oxygen species from mitochondrial respiration and the DAF-2 signaling pathway (Kenyon, 2005). Our analysis indicates that IFTA-2 functions as a new factor in the DAF-2 pathway. DAF-2 activity leads to phosphorylation of DAF-16 accumulation in the nucleus, and this extended lifespan of daf-2 mutants can be suppressed by mutations in daf-16. This same phenomenon exists in ifta-2 mutants because we see an increase of DAF-16 in the nucleus together with an extended lifespan.

Intriguingly, Apfeld and Kenyon showed that many cilia assembly mutants, including osm-5, che-13, che-11 and osm-1, were long-lived and disrupted the DAF-2 signaling pathway (Apfeld and Kenyon, 1999). These mutants also display Daf-d phenotype at 20°C or 25°C but a Daf-c phenotype at 27°C (Ailion and Thomas, 2000; Apfeld and Kenyon, 1999). Apfeld and Kenyon proposed that environmental cues sensed by the cilia may be involved in regulating lifespan and dauer formation. Further work showed that lifespan regulation in C. elegans involved specific gustatory and olfactory neurons (Alcedo and Kenyon, 2004). We found similar results for ifta-2 by creating double mutants with other lifespan genes. The ifta-2(tm1724) phenotype is suppressed by loss of DAF-16, whereas daf-2(e1370);ifta-2(tm1724) double mutants and osm-5(m184);ifta-2(tm1724) double mutants both displayed lifespans similar to ifta-2(tm1724) alone. Because there are no additive effects on lifespan, we propose that IFTA-2, IFT and DAF-2 act in the same pathway to regulate DAF-16 function with regard to lifespan regulation.

Previous work has shown that the transcription factor DAF-16 is present largely in the cytosol and at low levels in the nucleus in wild-type worms under normal conditions. By contrast, when any pathway leading to DAF-16 phosphorylation is perturbed (i.e. environmental stresses, loss of DAF-2 signaling or loss of cilia), DAF-16 translocates into the nucleus (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). We found that levels of DAF-16 in the nucleus were significantly elevated in ifta-2(tm1724) mutants compared with wild-type controls, further supporting an effect on the DAF-2 signaling pathway. Although this measurement was done in intestinal cells and IFTA-2 is found exclusively in ciliated sensory neurons, previous work has shown that the DAF-2 pathway has a cell non-autonomous function and probably works through an endocrine-signaling-like pathway to influence the whole organism (Apfeld and Kenyon, 1998).

Based on our studies here and additional work on lifespan regulation and dauer formation in C. elegans, we present a tentative model of IFTA-2 function in the context of the DAF-2 signaling pathway (Fig. 7). Although we believe it is unlikely, it remains a possibility that IFTA-2 functions upstream of DAF-2 in the release of DAF-2 ligands such as the insulin-related peptides. This would also result in an impaired DAF-2 signaling pathway. More likely, our data support a role for IFTA-2 in ciliated sensory neurons together with DAF-2 and AGE-1 to regulate longevity and dauer formation through DAF-16. Despite being a putative Rab-like protein, IFTA-2 does not appear to be required for transport or localization of DAF-2 or AGE-1 in the cilia. Thus, we favor the possibility

\[\text{IFTA-2 in cilia and lifespan regulation} \quad 4097\]
that IFTA-2 functions in cilia to mediate transmission of signals from DAF-2 and/or AGE-1 to AKT-1/2 that phosphorylate DAF-16 and regulate its nuclear localization. Alternatively, IFTA-2 could function in the cilia between DAF-2 and AGE-1. These possibilities will need to be further defined by conducting epistatic analyses with additional mutants in the pathway and evaluating the effect of the double mutants on dauer formation and lifespan. Examples would include using constitutively activated akt mutants that are known to rescue the dauer phenotype in age-1 mutants (Paradis and Ruvkun, 1998). This would allow us to position IFTA-2 more accurately in the pathway and these studies are currently underway.

The insulin–IGF-1-like receptor signaling pathway is highly conserved from worms to humans and disruption of this pathway in multiple organisms is known to lead to increased longevity and altered metabolism. As with other proteins in the DAF-2 pathway, IFTA-2 is conserved and our analysis indicates that its mammalian homolog (RabL5) also resides in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia. Thus, it will be interesting to assess whether RabL5 is also involved in IGF1R or insulin signaling activity in cilia.
IFTA-2 in cilia and lifespan regulation


Sun, Z., Amsterdam, A., Pazour, G. J., Cole, D. G., Miller, S. M. and Hopkins, N.


