Hypoxia Regulates Assembly of Cilia in Suppressors of *Tetrahymena* Lacking an Intraflagellar Transport Subunit Gene

Jason M. Brown,^{*†} Noah A. Fine,[‡] Gautham Pandiyan,^{*} Rupal Thazhath,^{*} and Jacek Gaertig^{*§}

^{*}Department of Cellular Biology, University of Georgia, Athens, Georgia 30602; and [‡]Department of Biology, York University, Toronto, Ontario, Canada M3J 1P3

Submitted March 21, 2003; Accepted April 2, 2003
Monitoring Editor: Paul Matsudaira

We cloned a *Tetrahymena thermophila* gene, *IFT52*, encoding a homolog of the *Chlamydomonas* intraflagellar transport protein, IFT52. Disruption of *IFT52* led to loss of cilia and incomplete cytokinesis, a phenotype indistinguishable from that of mutants lacking kinesin-II, a known ciliary assembly transporter. The cytokinesis failures seem to result from lack of cell movement rather than from direct involvement of ciliary assembly pathway components in cytokinesis. Spontaneous partial suppressors of the *IFT52* null mutants occurred, which assembled cilia at high cell density and resorbed cilia at low cell density. The stimulating effect of high cell density on cilia formation is based on the creation of pericellular hypoxia. Thus, at least under certain conditions, ciliary assembly is affected by an extracellular signal and the Ift52p function may be integrated into signaling pathways that regulate ciliogenesis.

INTRODUCTION

Intraflagellar transport (IFT) is a bidirectional motility that occurs within flagella as well as both motile and nonmotile cilia (Rosenbaum *et al.*, 1999; Sloboda, 2002). During IFT, protein complexes are transported between the cell body and the tips of axonemes (Kozminski *et al.*, 1993). Anterograde IFT is believed to be driven by kinesin-II motors moving along outer doublet microtubules (Kozminski *et al.*, 1995; Signor *et al.*, 1999). In *Chlamydomonas*, *Tetrahymena*, and mouse, loss-of-function mutations showed a requirement for kinesin-II in assembly of cilia and flagella (Walther *et al.*, 1994; Nonaka *et al.*, 1998; Brown *et al.*, 1999b). Kinesin-II mutations also affected some nonmotile cilia, including chemosensory neurons in *Caenorhabditis elegans* and connecting cilia in mouse retinal rod cells (Shakir *et al.*, 1993; Marszalek *et al.*, 2000). Cytoplasmic type dyneins are thought to be the motors that return IFT complexes, kinesin-II, and perhaps other ciliary components to the cell body. In *Chlamydomonas*, the dynein light chain mutant, *fla14*, and the dynein heavy

chain mutant, *dhc1b*, both have extremely shortened flagella with tips filled with IFT materials, supporting the role of dynein in retrograde IFT (Pazour *et al.*, 1998; Pazour *et al.*, 1999). In the *C. elegans che3* dynein mutant, IFT is specifically blocked in the ciliary portion of chemosensory neurons (Signor *et al.*, 1999).

In *Chlamydomonas* the IFT particles are composed of complex A, containing four proteins, and complex B, containing 11 proteins (Piperno and Mead, 1997; Cole *et al.*, 1998). Many of the identified IFT polypeptides revealed similarity with potential counterparts in other organisms. The complex B subunits p52, p88, and p172 of *Chlamydomonas* are homologous to the *C. elegans* proteins OSM-6, OSM-5, and OSM-1, respectively, all of which are implicated in the function of the nematode chemosensory cilia (Cole *et al.*, 1998). A mutation in the mouse homolog of *IFT88*, *Tg737*, caused murine polycystic kidney disease associated with shortening of cilia in the kidney cells (Pazour *et al.*, 2000), as well as affected the formation of mouse photoreceptor outer segments (Pazour *et al.*, 2002).

Despite considerable progress in the identification of the molecular components of IFT and growing evidence of their essential contribution to the assembly of cilia and flagella, the specific biochemical function of IFT is unclear. Currently, the best supported hypothesis is that IFT particles serve as platforms that carry axonemal components from the cell body to their sites of incorporation at the tips of the growing axoneme (Rosenbaum *et al.*, 1999; Marszalek and Goldstein, 2000; Sloboda, 2002). However, so far it has not been possi-

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-03-0166. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-03-0166.

[†] Present address: Department of Biology, Young Harris College, Young Harris, GA 30582.

[§] Corresponding author. E-mail address: jgaertig@cb.uga.edu.

Abbreviations used: IFT, intraflagellar transport; MAC, macro-nucleus; MIC, micronucleus; WT, wild-type.

ble to establish a direct molecular connection between the IFT particles, motors, and axonemal structural subunits. Thus, the molecular function of IFT beyond movement of IFT particles remains undocumented.

We showed that knocking out two partly redundant *Tetrahymena* kinesin-II genes, *KIN1* and *KIN2*, led to loss of existing cilia and inability to assemble new cilia. Unexpectedly, kinesin-II null cells also underwent frequent arrests in cytokinesis (Brown *et al.*, 1999b). Localization studies and observations of living cells led us to propose that kinesin-II is not directly involved in cleavage furrow constriction (Brown *et al.*, 1999b). Instead, we suggested that loss of motility in kinesin-II null mutants blocks the final separation of daughter cells, which involves a series of rotational movements facilitating cell fission (rotokinesis) (Brown *et al.*, 1999a).

Herein, we report cloning of a *Tetrahymena* homolog of IFT52. The IFT52 component of complex B is required for assembly of flagella in *Chlamydomonas* (Brazelton *et al.*, 2001), and its homolog OSM-6 is involved in assembly of sensory cilia in *C. elegans* (Collet *et al.*, 1998). Elimination of IFT52 from *Tetrahymena* cells led to a phenotype identical to the kinesin-II null phenotype, including lack of cilia and inability to complete cytokinesis. Unexpectedly, we isolated spontaneous IFT52 null suppressors that have short cilia at low cell densities and grow longer cilia at higher cell densities. We show that this effect of high cell density can be mimicked by creating hypoxic conditions. Thus, suppressor cells at high cell density influence each other mainly by creating pericellular hypoxia, which in turn stimulates assembly of cilia. To our knowledge, this is the first report on isolation of suppressors of a mutation in the IFT gene and on implicating extracellular signaling in ciliary assembly in a way that involves a known IFT component.

MATERIALS AND METHODS

Culture Growth and Conjugation

Cells were grown in either SPP (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% EDTA-ferric sodium salt) or MEPP (2% proteose peptone, 2 mM sodium citrate, 1 mM ferric chloride, 12.5 μ M cupric sulfate, 1.7 μ M folic acid) (Orias and Rasmussen, 1976) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B. Culture growth and conjugation were done as described previously (Brown *et al.*, 1999b).

IFT52 Cloning and Sequence Analysis

Polymerase chain reaction (PCR) was used to amplify a homologue of IFT52 from total *Tetrahymena* DNA. Degenerate primers were designed from published peptide sequence alignments (Cole *et al.*, 1998). These primers amplified a 750-base pair fragment with an open reading frame homologous to IFT52. A 3.4-kb *Hind*III fragment was subsequently cloned that encompasses ~1.2 kb of coding region along with two introns and the 3'-noncoding region. A cDNA (#213) was located in the *Tetrahymena* expressed sequence tag database (Fillingham *et al.*, 2002; <http://www.cbr.nrc.ca/reith/tetra/tetra.html>) and was found to be identical to the corresponding genomic sequence. Multiple sequence alignments were prepared using the PILEUP program in the University of Wisconsin (Madison, WI) GCG software (UWGCG). Shading was created with Boxshade software (http://www.ch.embnet.org/software/BOX_form.html). Sequence similarities were calculated using BESTFIT (UWGCG).

IFT52 Knockout

The plasmid pIFT52-1, carrying the 3.4-kb genomic *Hind*III fragment of IFT52, was digested at the unique *Bsu*361 site of the coding region. Blunt ends were created, and the *neo2* cassette (Gaertig *et al.*, 1994) was inserted creating pIFT52-1bsuneo2. For germline disruption of IFT52, 15 μ g of pIFT52-1bsuneo2 digested with *Hind*III to release the insert was used to coat 6 mg of 1- μ m gold particles (Bio-Rad, Hercules, CA). CU428.1 and B2086.1 cells conjugating for 3 to 4.5 h were bombarded every 30 min with coated particles by using the biolistic gun (Cassidy-Hanley *et al.*, 1997). Bombarded cells were incubated in SPP medium at 30°C and selected with 120 μ g/ml paromomycin 5.5 h after the last shot. A single transformant heterozygous in the germline for IFT52::*neo2* was brought to homozygosity and knockout heterokaryons were constructed (Cassidy-Hanley *et al.*, 1997). To bring the phenotype to expression, two knockout heterokaryon strains (UG7G5 and UG7G6) were induced to conjugate and individual mating pairs were isolated into MEPP.

Rescue Transformation with Green Fluorescent Protein (GFP)-tagged IFT52

In an attempt to construct a cadmium-inducible system for expression of IFT52, the IFT52 cDNA was amplified with addition of *Hind*III and *Bam*HI sites to the 5' and 3' ends of the coding region, respectively. The resulting fragment was used to replace the coding region of pMTTG1 (Shang *et al.*, 2002), creating pMTTIFT52B2. In pMTTIFT52B2, the cadmium-inducible *MTT1* promoter (Shang *et al.*, 2002) controls the IFT52 coding region and both are flanked by the 5' and 3'-untranslated regions of the *Tetrahymena* *BTU1* gene, allowing insertion into the *BTU1* locus as described previously (Gaertig *et al.*, 1999). To create a GFP-tagged IFT52, PCR was used to add a *Hind*III site to the 5' end and *Mlu*I, *Nco*I, and *Bam*HI sites to the 3' end of the IFT52 cDNA coding region. The resulting fragment was digested with *Hind*III and *Bam*HI and inserted into pMTTG1 to create pMTTIFT52-3enz. A *Mlu*I-*Nco*I-flanked GFP fragment (Haddad and Turkewitz, 1997) was inserted into digested pMTTIFT52-3enz to create pMTTIFT52GFP, which encodes an IFT52 protein with a C-terminal GFP tag. IFT52 Δ cells grown in MEPP were biolistically bombarded with the 3.4-kb genomic IFT52 fragment released from pIFT52-1, the 5'BTU1-IFT52coding-3'BTU1 (pMTTIFT52B2), or the 5'BTU1-5'MTT1-IFT52coding-GFP-3'BTU1 fragment released from pMTTIFT52GFP. No drug selection was required because only transformants recovered motility and normal cytokinesis and therefore could grow on the SPP medium.

Immunocytochemistry and Electron Microscopy

Cells were prepared for confocal analysis as described previously (Brown *et al.*, 1999b) with some modifications. Briefly, cells were isolated into drops of 10 mM Tris pH 7.5 on poly-L-lysine-coated coverslips. For staining with SG serum against total *Tetrahymena* tubulin (Guttman and Gorovsky, 1979), cells were simultaneously fixed and permeabilized and were then air dried at 30°C. For staining with anti-GFP rabbit polyclonal antibodies (BD Biosciences Clontech, Palo Alto, CA), cells were permeabilized for a few seconds in 0.5% Triton X-100 in the PHEM buffer with protease inhibitors (0.5 μ g/ml leupeptin, 10 μ g/ml E-64, 10 μ g/ml chymostatin, 12.5 μ g/ml antipain) and 1 μ M paclitaxel, followed by fixation with an equal volume of 2% paraformaldehyde in PHEM and air drying at 30°C. Nuclei were stained with either propidium iodide or 4,6-diamidino-2-phenylindole (DAPI). An MRC 600 (Bio-Rad) confocal microscope was used for imaging. Ciliary lengths were measured on individual confocal sections using Scion Image (Scion, Frederick, MD). For consistency, cilia were measured using the widest section through the nucleus for a given Z-series. Scion Image was also used to measure the total length of the cell periphery on the section from which ciliary lengths were measured by using the freehand tool to trace the cell periphery and the Measure Accumulated Perimeter

A

Hs CGI 53	1	~ ~ ~ ~ ~ N E K E L R S T I L F N A Y K K E I F T T N G Y K S M Q K K L R S N W K I Q S L . K D E I T S E K L
MmNGD5	1	~ ~ ~ ~ ~ N E K E L R S T I L F N A Y K K E V F T T N T G Y K S L Q K R L R S N W K I Q S L . K D E I T S E K L
Cr I FT52	1	~ ~ ~ ~ ~ M E E P G A E E V R . . I L F S T A K G E S F H K A G F N Q L F R R L R S T Y R P D K V D K D D F I L D T L
Tt RFT1	1	~ ~ ~ ~ ~ N S G E Y . S I I V F N A S K K E A G N P S T N I K I I K Y K E T Y Q C G R N I E D . F I Q D R I
Ce OSM6	1	~ ~ ~ ~ ~ M P P F S D E K M T N R S I G R K V L I D Q S L Q Q Q I S L I S G F R G V A R H L K S V L T V E . I N T E P I N L N G I
DmCG9 59 5	1	~ ~ ~ ~ ~ N M H R K L K V N V N V E Q N D A E . L R M E R I
Hs CGI 53	51	N G V K L V I T A G P R E K F T A A E F E I L K K Y L D . T G G D V L V M L G E G G E S R F D T N I N F L L E Y G I M
MmNGD5	51	I G V K L V I T A G P R E K F T A A E F E V L K K Y L D . S G G D I L V M L G E G G E S R F D T N I N F L L E Y G I M
Cr I FT52	54	R S A H I L V L G G P K E K F T A P E V D M L K F V K . N G G S I L I I N S E G G E K A G I N I N Y F L E C G M S
Tt RFT1	50	K N A S I V I F F G P K E N S I R E E F D A L K O Y L E . S G G R V L V S S E G G H K N R I N I N F F L E C Y G I S
Ce OSM6	60	E D V R M I I P Q P K T S G T G E I E A I V K F V E . F G G S L M I I S G E G G E R Q . . S I N E M I A K Y G I T
DmCG9 59 5	25	A R V E I F V I A G F Q D R F I E D F D M L K H Y V E V C G G S L V I L L G E G G E P E F N I N I N V F F L E C Y G I Y
Hs CGI 53	110	V N N D A V V R N V Y L K Y F P K E A L V S G V L N R E I S R A A G S A V L A I D E E
MmNGD5	110	V N N D A V V R N V Y Y K Y F P K E A L V S D G V L N R E I S R A A G S A V P G V I D E E
Cr I FT52	113	V N N D A V V R T T H Y K Y L P K E V L I S D G I L N R A V I T C A G S L N S N D D I F R V
Tt RFT1	109	I N N D G V V R T A H Y K Y F P K E T Y V H S G I L N E B V T R V A N G L P K E T K R P Q N T F L Q N V I G K D D E
Ce OSM6	116	V N K D S V I R T V E L K Y F P K E A L V A N G V I N R A I A V A A K K N V H L L K L D I E V Y Y D F S D E K Y K .
DmCG9 59 5	85	I N G I T V V R P F Y Y K N F P K E C I V G G V V C E S M V R H L L K L D I E V Y Y D F S D E K Y K .
Hs CGI 53	156	S S G N N A . . . Q A L T F V Y P F G A T L S V . M K P A V A V L S T G S V C F P I N R P I L A E Y H S K N C G
MmNGD5	156	N S G N N A . . . Q A L T F V Y P F G A T L S V . M K P A V A V L S T G S V C F P I N R P I L A E Y H S K N C G F
Cr I FT52	162	S R G P Q A F D G T G L E Y V E P F G A T L S V . Q K P A V P V L S S C K I A Y P N R P V G A V V . . A C P G Y
Tt RFT1	169	D E Y Q K E Q S R V G I D F V A E G A T I V . Q Q P A F A I L G S C P L S Y P S N R P S A I V Q T K N
Ce OSM6	155	. S T E Q K H N S Q A L S E I Y P Y G G T I D V N N R V S N V L S S G S T S F P T S R P V A A H E T I S L N E M K K K
DmCG9 59 5	138	 I H E Q Y P Y G A T I N S . E P A N V I L I T G P V Y P F N R P L V G Y F T N G K G
Hs CGI 53	208	G K L A V L G S C H M F S T Q Y L D K E E N S K I M D V V F Q W L T . G D I H L N C I D A D P E I S D Y V M L P Y
MmNGD5	209	G K L A V L G S C H M F S D Q Y L D K E E N S K I M D . V V F Q W L T . G D I H L N C I D A D P E I S D Y T M V P D
Cr I FT52	216	G R I A V L G S C A M F D D K Y L D K E E N S K I M D F . F E K F I K P H S K I Q L N D I D A E P D V S D L K L P D
Tt RFT1	223	G R L A V I G S F E M F T D E V F D N E D N S K I F D F I K Y L L N E C E F . . . E F S P K E P D V . E Y F K V P D
Ce OSM6	214	G R V Q V G S V S M F H D T M I D K E E N G K I F D T F V . E F I V N G . . L E L N T I D A A E P E I N D Y T N I P D
DmCG9 59 5	181	G K I L A V G S G V I V H D K Y L Q D K T D A M F E Y L I K . . I L G G D E I T Y S H L D F N D V E L S I N K H F T D
Hs CGI 53	267	T A T I S K R N R E C L O E S T . . . E I P R D F T I L F E L S I E Q L D T T S F H S V I E A H E Q L N V K H E P L Q L
MmNGD5	267	T A T I S E Q I R V C L O E G L . . . E N P R D F T I L F E L S I Y Q L D T T C L F K V I K A H E Q L N V K H E P L Q L
Cr I FT52	275	T A S I A D K L N C L O E I D . . . D V P R D W T S L F E D S L E K F D T G I I P E A V S L Y E K I G V K K G Q L N I
Tt RFT1	279	I A E I A D N L K S C L O E S T . . . P L F P S K O L E N T D L E K Y D V D I V P E A V K L Y E T I G V R E P I A L
Ce OSM6	271	H I H N S Q Q I K Y G M Y E G E L D Q A I S S D F M K I M T S I H S F N L K H W M T I R L Y E A L N L S P P I T L
DmCG9 59 5	239	I G F I A D M P K A C T I D S . I G T D M T D E K Q M E M R I C R I S N R I L K D V M D T Y E Q I F V K Y E P I K I
Hs CGI 53	324	I Q P Q F E T P L F T L C . . . P A V F P P S E R E L P P P P L E L F D L D E T E S S E K A R L A G I T
MmNGD5	324	V Q P Q F E M P L T A L Q L R S S L R V S G S C R P L P W S C I T ~ ~ ~ ~ ~
Cr I FT52	332	I P P S F E T P L P L C . . . P A V F P T I R E P P P A L E L F D L D E S F A S E T N R L A S I T
Tt RFT1	336	I V P Q F E T P L L G L . . . V S A V F P P I L K E L A P S L E L F D L D D E F A S E K V R L A Q L I
Ce OSM6	331	V E P Q F E L P M P P F C . . . P A V F P P T R Q E L P P P L E L F D L D E Q F S S P E I Q L S Q L A
DmCG9 59 5	298	I N P Q F E I P L E N V Q L . . . A T F P P T R S E P S A P P L E L Y D L D E T E S G A R S Q L A H M I G Q V I Q A L Q
Hs CGI 53	373	 N K C . T E E D L E F Y V R K C G D I L G V T S K L P K D Q Q D A K H I T E H V F F Q V V E F K K . . . L N
MmNGD5	357	 N K C H G E E D L E V Y I M E A C H I L G L K L . . . Q E N A N A K H V S E V F R R I A Q Y K V G S L G L G
Cr I FT52	381	 N K C N N S E . I D V Y I K E S G D I L G V T D K V . K N K H D A K A I R Y V L E E L I N E K L N N ~ ~ ~
Tt RFT1	385	 N R . S E E D I I F F I E K A G E I T G I S A E L T R S E R T P K K I I E L A V S K L N L F K R S N M D G E
Ce OSM6	380	 N R . S E E D I I F F I E K A G E I T G I S A E L T R S E R T P K K I I E L A V S K L N L F K R S N M D G E
DmCG9 59 5	355	S K E P Q R R A L N Q R E L E N V I K E C A R I T A I V D E . . R Q D N A A R E I N I V A R Q I S Y R P Y A E D ~ ~
Hs CGI 53	423	Q E H D I D T S E T A F Q N N F ~ ~ ~ ~ ~
MmNGD5	357	~ ~ ~ ~ ~
Cr I FT52	433	Q T L D S N G Q T L P A A N Q F G D Q F E L ~ ~ ~ ~ ~
Tt RFT1	435	~ ~ ~ ~ ~
Ce OSM6	434	L E V A S A F D I G E H D A H Q S F N Q G E E M D E Q L F S D I D E F D D L
DmCG9 59 5	411	~ ~ ~ ~ ~

B

C

Figure 1.

macro. The total number of measurable cilia was divided by length of cell periphery to calculate measurable cilia/ μm .

For electron microscopy (EM), cells were washed with 10 mM Tris, pH 7.5 and fixed in 4% glutaraldehyde in 10 mM Tris buffer at 4°C for 1 h, washed three times with 10 mM Tris, and postfixed in 4% osmium tetroxide for 1 h at 4°C. Cells were embedded in Epon after dehydration in graded steps from 30 to 100% ethanol. Sections were stained with uranyl acetate and lead citrate and were visualized on a 100CXII transmission electron microscope (JOEL, Tokyo, Japan).

Phenotypic Analysis

For dilution experiments, IFT52 Δ sm1 cells growing in MEPP with shaking at 160 rpm at 30°C were washed and resuspended in fresh MEPP at a concentration of $3 \times 10^5/\text{ml}$. Serial dilutions were prepared and cells were incubated in 10 ml on Petri plates (10 cm diameter) with or without gentle shaking (30 rpm) either at 30°C or at room temperature. Cells were scored on an inverted microscope using a 10 $\times$ objective (100 $\times$ total magnification) for motility and presence of the multiple subcells indicative of cytokinesis failures. Cells were scored as motile if there was clear cell body displacement. To account for growth of cells without successful cytokinesis, average number of cortical subcells per milliliter was calculated. First, average number of cells per 10 $\times$ field of view on an inverted microscope was converted to cells per milliliter by calibrating the microscopic counts using a Beckman Coulter (Fullerton, CA) model ZF cell counter. Average subcells per cell was calculated and multiplied by average cells per milliliter to obtain subcells per milliliter. Stimulation of ciliary assembly between strains was addressed by mixing IFT52 Δ sm1 cells with IFT52 Δ 10 or wild-type (OC21-12) cells in different proportions. For stimulation by mutant cells, motility was quantified as described above. For filter assays, 1.5 ml of potential stimulator cells were separated from 0.5 ml of responder cells by a 3- μm pore filter (using Transwell inserts, 12 mm diameter; Corning Costar Corp., Cambridge, MA) inserted in a 24-well tissue culture plate well. After 24-h incubation, responder cells (from the inserts) were scored for motility and subcells per chain. Statistical significance was assessed with one- and two-tailed *t* tests.

Figure 1 (facing page). Sequence analysis and disruption of *IFT52*. GenBank accession numbers for sequence data used in comparisons are indicated in parentheses. (A) Ift52p (AY071864) is homologous to the known IFT proteins *C. elegans* OSM-6 (CAA03975) and *Chlamydomonas* IFT52 (AAL12162), as well as mouse NGD5 (AAA96241), human CGI53 (NP_057088), and *Drosophila* CG9595 (AAF52401). Black shading indicates 50% or greater identity at a given position and gray shading indicates similarity. (B and C) Southern blot analysis of germline *rft1::neo2* transformants. (B) Left, Southern blot of total genomic DNA digested with *Eco*RI and *Bsu*36I and probed with a radiolabeled 3.4-kb genomic *Hind*III fragment of *IFT52*. Right, diagram of the *IFT52* locus. Lane 1, control strain; lane 2, germline transformant. Arrow in right panel, site susceptible to *Eco*RI star activity 1.2 kb upstream of the 3' *Eco*RI site. The WT locus gives fragments of 1.3 and 4.5 kb. A knockout is expected to yield a single 7.2-kb fragment. The 4.5-kb band is present in both lanes, indicating that the germline transformant is heterozygous for *IFT52/ift52::neo2*. Fragments generated by *Eco*RI star activity are indicated with asterisks. A 5.8-kb fragment likely generated by digestion of DNA with only *Eco*RI is indicated by δ . (C) Left, Southern blot of total genomic DNA digested with *Pst*I and *Xba*I and probed with the same fragment as in B. Right, diagram of *IFT52* locus. Lanes are as in B. The WT locus gives fragments of 2.0 and 5.2 kb. A knockout is predicted to give 0.6-, 2.8-, and 5.2-kb fragments. Only the fragments larger than 0.6 kb are visible. Due to heterozygosity, the 2.0-kb fragment is present in the knockout strain. Restriction sites: E, *Eco*RI; H, *Hind*III; B, *Bsu*36I; P, *Pst*I; X, *Xba*I.

For induction of partial hypoxia, IFT52 Δ sm7 cells were grown in MEPP, washed, and suspended at 1.5×10^5 cells/ml in 3 ml of MEPP supplemented with 0.2% normocin antibiotic (InvivoGen, San Diego, CA). Cells were transferred to a 125-ml bottle with a ground glass closure covered with vacuum grease, and exposed to a stream of nitrogen gas for about a minute. As a control, another batch of cells was exposed to a stream of oxygen gas. The bottles were closed tightly and incubated at 30°C with shaking at 130 rpm.

RESULTS

Cloning a *Tetrahymena* Homolog of *IFT52*

To identify IFT52/OSM-6 homologs, genomic DNA was amplified by degenerate PCR with primers for conserved regions of the known related genes. A 750-base pair sequence with similarity to IFT52 was isolated and used to clone a 3.4-kb genomic fragment containing a partial open reading frame encoding the putative IFT52 related gene, named *IFT52*. A cDNA sequence of the *IFT52* gene was identified in the *Tetrahymena* expressed sequence tag project (Fillingham *et al.*, 2002). The *IFT52* coding region encodes a protein, Ift52p, of 434 amino acids with calculated molecular mass and pI of 49 kDa and 4.99, respectively. The predicted Ift52p protein sequence is 48% identical to IFT52 of *Chlamydomonas* and 35% identical to OSM-6 of *C. elegans* (Figure 1A).

Cells Lacking Ift52p Do Not Assemble or Maintain Cilia and Are Unable to Complete Cytokinesis

We created knockout heterokaryons (Hai and Gorovsky, 1997) with disrupted copies of *IFT52* in the transcriptionally silent micronucleus (MIC) and wild-type (WT) copies in the transcriptionally active macronucleus (MAC) (Figure 1, B and C). These heterokaryons maintain a WT phenotype during vegetative growth, because only the genes in the MAC are expressed. When heterokaryons are mated to each other, the progeny develop a new MAC from the MIC, thus expressing the *IFT52* null phenotype. Remarkably, the progeny (IFT52 Δ) lost cilia with very similar timing to the loss of cilia in previously reported kinesin-II knockouts (Brown *et al.*, 1999b). Anti-tubulin immunofluorescence revealed that most cells nearly completely lost cilia by 30 h (Figure 2). The IFT52 Δ mutants died on the standard SPP medium but could grow in MEPP, which bypasses the requirement for phagocytosis dependent on the oral cilia (Rasmussen and Orias, 1975). Many mutants consisted of multiple cortical "subcells" and sets of nuclei (Figure 2, D–F), showing that they fail to complete cytokinesis. This phenotype is essentially identical in both the temporal progression and terminal morphology to the phenotype of cells lacking kinesin-II motor subunits (Brown *et al.*, 1999b). Thus, as kinesin-II, Ift52p is required for assembly and maintenance of cilia, and both proteins likely function in the same pathway.

GFP-tagged Ift52p Localizes to Cilia

We prepared a chimeric gene encoding an Ift52p-GFP fusion. We placed the Ift52p-GFP coding region under the control of the cadmium inducible *MTT1* promoter and targeted the whole fragment into the nonessential *BTU1* locus (Shang *et al.*, 2002). Surprisingly, IFT52 Δ cells could be rescued with either the *MTT1*-IFT52 or *MTT1*-IFT52-GFP fragments without the addition of cadmium. Attempts to re-

Figure 2. Cytological analysis of IFT52Δ cells. The phenotype was induced by conjugation of knockout heterokaryon strains UG7G5 and UG7G6. Conjugation progeny were grown in MEPPA and stained with anti-tubulin antibodies (SG) and propidium iodide. Conjugation progeny were prepared for confocal microscopy 30–31 h after isolation of conjugating pairs. (A and B) Control cells before and during division, respectively. (C) IFT52Δ mutant cell nearing the end of cytokinesis. (D) Mutant cell in an early stage after the cytokinesis failure. Two MACs are evident. (E and F) Mutants after additional cytokinesis failures forming “monster” cells. Bar, 25 μ m.

move possible traces of cadmium from the medium by extensive washing and incubation in ultrapure water never resulted in a loss of motility. It is known that in the absence of cadmium, the basal level of expression controlled by the *MTT1* promoter is extremely low (Shang *et al.*, 2002). Thus, a very low level of production of Ift52p is sufficient for assembly of cilia, suggesting that either the protein is needed in small amounts or that it is very stable and can be recycled.

Staining with polyclonal anti-GFP antibodies revealed a predominant localization of Ift52p to cilia (Figure 3). In dividing cells, there was no colocalization of Ift52p-GFP

with the cleavage furrow (Figure 3, D–O). During early stages of cell division, clusters of short immature oral cilia were intensely labeled, suggesting that as for kinesin-II, IFT particle proteins preferentially accumulate within cilia in the initial phase of assembly (Figure 3, D–F).

We have previously described an elaborate type of cell motility called rotokinesis, which seems to help WT cells complete cytokinesis by creating mechanical strain within the cytoplasmic bridge connecting daughter cells (Brown *et al.*, 1999a). As in kinesin-II mutants, IFT52 knockout cells are unable to undergo rotokinesis, suggesting that for both mutations the cytokinesis defect is caused by cell paralysis. To

Figure 3. Localization of Ift52p-GFP. IFT52Δ cells rescued with a BTU1-MTT-IFT52-GFP-BTU1 fragment (see MATERIALS AND METHODS) were grown in SPP medium in the absence of cadmium. Cells were fixed and stained for confocal microscopy with polyclonal anti-GFP antibodies and DAPI. Left column, anti-GFP signal; middle column, DAPI signal; right column, merged images. (A–C) Interphase cell. Ift52p-GFP is primarily in cilia. (D–F) Cell in very early stage of micronuclear division. Posterior oral apparatus has short cilia in which Ift52p-GFP is highly concentrated. (G–I) Early stage of macronuclear elongation showing a zone of intense ciliary anti-GFP labeling immediately posterior to the developing cleavage furrow. (J–O) Later stages of macronuclear division and cleavage furrow ingression showing ciliary labeling but no accumulation in the cleavage furrow or cell body. oa, oral apparatus; mac, macronucleus; mic, micronucleus; Bar, 10 μm.

further test the importance of cell motility for the completion of cytokinesis, we grew *IFT52* knockouts with and without vigorous shaking for 48 h. Cells grown without shaking were polynucleated indicating cytokinesis failures (Figure 4B). On the other hand, although cells grown with shaking did not recover motility, they had dramatically fewer cytokinesis fail-

ures (Figure 4A). Similar results were obtained for kinesin-II mutants lacking *KIN1* and *KIN2* genes (our unpublished data). These results further confirm that cleavage furrow ingression is not substantially impeded in *Tetrahymena* IFT mutants and that displacement of cells by an external mechanical force is sufficient for final separation of daughter cells.

Figure 4. IFT52 Δ cells were grown with (A) or without (B) shaking in MEPPA medium. After 48 h, cells were prepared for immunofluorescence microscopy as in Figure 2. Shown are merged grayscale images of the anti-tubulin and propidium iodide signals. Bar, 50 μ m.

Spontaneous Partial Suppressors of IFT52 Δ

The knockout strains of IFT52 used in this study were generated by mating two parental strains with isogenic MIC genomes derived by genomic exclusion (Orias and Bruns, 1976). Thus, we expected that all heterokaryon progeny cells would show the same phenotype. To our surprise, 3% ($n = 240$) of the progeny of knockout heterokaryons showed a partial suppression of the IFT52 Δ phenotype. Within the synclones (mixed progenies derived from both exconjugants of a single pair), the suppressed phenotype occurred within the first few divisions after conjugation and not all cells showed the suppressed phenotype. After subcloning, the phenotype of both suppressed and nonsuppressed cells was stable. In the suppressor clones designated as IFT52 Δ sm (semimotile), cells had short but partly functional oral cilia (unlike nonsuppressed, IFT52 Δ sm produced some food

vacuoles), and very short, scattered locomotory cilia allowing for minimal movements. The density of identifiable cilia on confocal sections of the nonsuppressed IFT52 Δ cells was only 0.07 cilia/ μ m of cell periphery compared with 0.28 cilia/ μ m in WT cells. Compared with the nonsuppressed population, in IFT52 Δ sm cells there was a dramatic increase to 0.21 cilia/ μ m. Although the measurable cilia on IFT52 Δ sm cells were slightly longer (mean length 1.2 μ m, $p = 0.000012$) than on IFT52 Δ (mean length 0.83 μ m), these cilia were still much shorter than WT (mean length 4.2 μ m) (Figure 5, A–C, and E). We did not find any suppressors among the 288 progenies of mating heterokaryons lacking kinesin-II genes (Brown *et al.*, 1999b). Thus, the observed suppression does not seem to be a general property of IFT mutants in *Tetrahymena*.

During an extended period of growth (~ 130 generations) a more complete suppression of the mutant phenotype occurred spontaneously in one of the eight IFT52 Δ sm clones (IFT52 Δ sm1) studied. We established a subclone resulting from this apparent second suppression event called IFT52 Δ mov1 (moving IFT52 Δ). These cells recovered normal cilia density (0.29/ μ m) and the measurable cilia were of intermediate length (2.9 μ m) between WT and IFT52 Δ sm1 cells ($p = 2.6 \times 10^{-20}$; Figure 5, D and E). Attempts at obtaining such clones from the remaining seven IFT52 Δ sm strains failed, despite growing some of them continuously for 20 mo.

Thin section EM revealed that most basal bodies in IFT52 Δ cells completely lacked axonemes (Figure 6A). Although some basal bodies did have associated extremely short cilia, these cilia lacked a central pair (Figure 6B). The semimotile suppressor IFT52 Δ sm1 had fewer naked basal bodies, but cilia were short with only 13% having a central pair (Figure 6C and Table 1). These short 9 + 2 cilia may be responsible for the partial recovery of motility (Figure 6, D and E, and Table 1). The further suppression in IFT52 Δ mov1 cells is correlated not only with an increase in ciliary length but also with a dramatic increase in the proportion of 9 + 2 axonemes (Figures 5D and 6F and Table 1). Thus, relatively frequent, spontaneous suppression events allow IFT52 null cells to assemble cilia without the Ift52p protein.

IFT52 Δ Suppression Occurs by a Novel Mechanism That Does Not Involve a Heritable Change in the Germline Genome

To study the genetic basis of the suppression in IFT52 Δ cells, we performed a series of crosses and analyzed segregation of suppressed and nonsuppressed phenotypes. We could not use IFT52 Δ sm clones for genetic analyses because they did not recover motility in the starvation medium and therefore would not mate. For this reason, we used the more complete suppressor, IFT52 Δ mov1 and crossed it to a WT strain. All F1 progenies analyzed had a WT phenotype. Six F1 clones after reaching maturity were crossed to each other in three combinations. A total of 408 conjugation progenies were analyzed (after exclusion of cells, which retained the parental MACs or died). Among them, 304 had WT phenotype, 103 had nonsuppressed IFT52 Δ phenotype, and 1 had the IFT52 Δ sm suppression phenotype. This result is inconsistent with the presence of a suppressor locus in the germline MIC. Rather, heterozygotes show a standard 3:1 F2

Figure 5. Cytological analysis of suppressor strains. Cells were grown in MEPP medium and prepared for confocal imaging by staining with anti-tubulin antibodies as in Figure 2. (A) Wild-type strain. (B) *IFT52Δ*10. (C) *IFT52Δsm*1. (D) *IFT52Δmov*1. (E) Quantitation of mean ciliary length ($\pm$ SE of the mean) by using the measurement macro of Scion Image software. P values above each category were generated with a paired two-tailed *t* test with the following null hypotheses: WT = *IFT52* null; *IFT52* null = *IFT52 sm*; *IFT52sm* = *IFT52 mov*. Bar, 25 μ m.

segregation ratio, and the suppression seems to occur among a small number of homozygous mutant progenies as a secondary event, possibly mediated by a genetic alteration in the newly developed somatic MAC or a nongenetic mechanism based on physiological adaptation.

Maintenance of Cilia in *IFT52Δsm* Suppressor Cells Is Cell Density and Temperature Dependent

The *IFT52Δsm*1 cells could grow with shaking at 30°C to a maximal cell density of 5×10^5 cells/ml, which is consider-

ably lower than WT. In a shaken culture, the majority of cells complete cytokinesis, and cells have very limited motility, even at the maximal density. Surprisingly, we discovered that high-density cultures left unshaken gradually recovered cell motility. The extent of motility in unshaken cultures was strongly dependent on cell density (Figure 7, A–D). The absence of motility at low cell density was especially striking 9.5 h after dilution in cells diluted $20\text{--}100 \times$ ($1.5 \times 10^4\text{--}3 \times 10^3$ cells/ml). Such cultures completely lost motility by 9.5 h postdilution and remained immobile at 21.5 h (Figure 7, B

Figure 6. Electron microscopic analysis of suppressor strains. Cells prepared for EM were isolated from cultures actively growing in MEPP. (A and B) Longitudinal sections through IFT52Δ10 basal bodies. (B) A short cilium lacking a central pair is evident. (C and D) Longitudinal sections through IFT52Δsm1 basal bodies and short cilia. Some cilia in this strain have a central pair (D), whereas some do not (C). (E) Cross-sections through several IFT52Δsm1 cilia showing different degrees of completeness of central pairs. (F) Longitudinal, cross, and oblique sections through IFT52Δmov1 cilia showing that cilia are longer than in IFT52Δ10 and IFT52Δsm1 cells and most contain central pairs. Bars, A–E, 0.25 μ m; F, 1 μ m.

and C). As cell density increased in these diluted cultures over time (33.5 h), some motility returned (Figure 7D). Even after cells recovered some motility with increased cell density, dilutions that initially led to the most severe loss of motility had many more cells with evidence of cytokinesis defects (Figure 7D). The remaining seven IFT52Δsm clones also showed an identical cell density-dependent effect on motility. Cell density did not affect motility in the more advanced suppressor IFT52Δmov1 (our unpublished data).

Immunofluorescence analysis of IFT52Δsm1 cells 21.5 h after dilution revealed that the extent of motility was correlated with the density and length of cilia (Figure 8 and Table 2). Cells from a culture with an initial density of 1.5×10^5 cells/ml were covered with scattered short cilia (Figure 8A). Cells initially diluted to between 3×10^4 and 6×10^3 cells/ml maintained fewer short cilia that often seemed to be concentrated near the anterior end and oral apparatus of the

cell (Figure 8, B and C). The nearly complete absence of motility in cells that were diluted to 3×10^3 cells/ml was associated with a drastic reduction in ciliary length (Figure 8, D and E). In these cells, the density of measurable cilia was only 60% of the density in the culture that was $50\times$ more concentrated. The measurable cilia were also short, their mean length being only 48% of the mean length of cilia from cells diluted to 1.5×10^5 (Table 2).

The above-mentioned experiments were all performed at the standard temperature of 30°C. Unexpectedly, IFT52Δsm1 unshaken cells grown at room temperature (22°C) could assemble and maintain cilia and had an accompanying return to nearly normal cell motility and cytokinesis even at low initial cell density (Figure 8F and Table 2). The remaining seven IFT52Δsm clones showed the same temperature sensitivity (our unpublished data). Thus, the partial suppression of IFT52Δ phenotype is conditional and dependent on both cell density and temperature.

Finally, the effects of cell density and temperature on ciliogenesis in IFT52Δsm cells were fully reversible. Cells that initially were highly ciliated (due to growth at low temperature or high cell density without shaking) lost cilia when diluted to low density and subsequently regained motility when incubated unshaken at high cell density.

Table 1. Quantitative EM analysis of axonemal structure in null and suppressor strains

Strain	Naked basal bodies	Short axonemes lacking central pair	9 + 2 Axonemes
IFT52Δ	66	18	0
IFT52Δsm1	35	63	15
IFT52Δmov1	0	20	55

Longitudinal sections through basal bodies were scored for the presence or absence of an associated axoneme, and axonemes were scored for the presence or absence of a central pair. Numbers in table are raw numbers of basal bodies and axonemes in each category.

Imbalance between Rapid Growth Rate and Slow Ciliary Assembly Does Not Account for the Cell Density-dependent IFT52Δsm Phenotype

Increased ciliogenesis in IFT52Δsm cells at lower temperatures, and higher cell densities raised the possibility that suppressors are able to maintain longer cilia only under conditions when their growth rate is reduced. Thus, there could be an imbalance between the rate of ciliary assembly and the rate of cell growth. To address this possibility, we

Figure 7. High cell density but not slow growth rate leads to ciliation in IFT52 Δ sm1 cells. (A–D) Exponentially growing (shaken) cells ($3\text{--}5 \times 10^5/\text{ml}$) were washed and resuspended in fresh MEPPA at final dilutions of 0 $\times$ (undil), 2 $\times$, 10 $\times$, 20 $\times$, and 100 $\times$. Cells were maintained at 30°C without shaking, and live cells were scored on an inverted microscope for presence or absence of motility and for gross evidence of cytokinesis defects or normal cell shape. Shown are 3.5 h (A), 9.5 h (B), 21.5 h (C), and 33.5 h (D) after dilution. (E and F) IFT52 Δ sm1 cells were diluted to $\sim 3 \times 10^3/\text{ml}$ in modified MEPPA media containing varying concentrations of protease peptone. (E) Growth rate decreased in media containing <2% protease peptone. White bars, inoculum at $t = 0$ and dilutions at 20.5 h postdilution; black bars, dilutions at 44 h postdilution. (F) Cells from cultures in E were scored for motility and cytokinesis defects as in A–D.

diluted cells to 3×10^3 cells/ml and grew them at 30°C in several modifications of the MEPP medium containing progressively lower concentrations of protease-peptone. Although reducing the protease-peptone concentration drastically slowed the growth of IFT52 Δ sm cells (Figure 7E), it had no effect on the motility. Cells in all media showed the same dilution-dependent loss of cilia and cytokinesis defects. In fact, at later times, the cells grown at higher concentration of peptone had more motility (Figure 7F). This can be explained by the faster growth rate, which leads to higher cell density. The simplest explanation of these data is that the stimulation of ciliogenesis is directly dependent on increased cell density, suggesting that cell-cell signaling may be involved.

Ciliary Assembly in IFT52 Δ sm Can Be Stimulated by Wild-Type and Nonsuppressed IFT52 Δ Cells at High Cell Densities

To test whether cell-cell communication is involved in maintaining cilia in IFT52 Δ sm cells, we performed mixed strain experiments. When we mixed IFT52 Δ sm1 cells diluted to a concentration that inhibits ciliogenesis among the suppressor cells alone with a high concentration of WT cells, motility was clearly stimulated in the suppressor cells. To test whether the effect is mediated by ciliogenesis, we measured ciliary length on tubulin antibody-stained cells. To be sure we were measuring cilia only on mutant cells, we chose only cells that showed clear arrests in cytokinesis (Figure 9D).

Figure 8. Cytological analysis of IFT52Δsm1 dilution series and temperature shift experiment. Cells grown in MEPPA were washed in fresh media and resuspended at 3×10^5 /ml. These cells were then diluted $2\times$ (1.5×10^5 /ml) (A), $10\times$ (3×10^4 /ml) (B), $50\times$ (6×10^3 /ml) (C), and $100\times$ (3×10^3 /ml) (D and E). (F) Cells diluted $100\times$ as in D and E were grown at 22° . After 21.5 h, cells were fixed and stained as in Figure 1.

Clearly, this method prevents us from measuring cilia on cells that recover near WT assembly and motility. Even so, we found that cilia on identifiable IFT52Δsm1 cells were 39% longer when diluted and mixed with WT ($p = 8.69 \times 10^{-39}$) than when they were only diluted (Figure 9D). On the other hand, IFT52Δ (nonsuppressed) cells were unable to respond to the assembly-promoting signal generated by WT cells (Figure 9D). Similar mixed strain experiments showed that concentrated IFT52Δ cells stimulate ciliary assembly in diluted IFT52Δsm cells (our unpublished data). These results suggest that IFT52Δsm cells possess a reception ability that responds to the presence of other cells, whereas IFT52Δ cells lack such a mechanism. Thus, the suppression mechanism could involve increased sensitivity of IFT52Δsm cells to an unknown external signal.

The data presented above do not distinguish between direct cell-cell contact and mediation by a diffusible factor. To distinguish between these two possibilities, we performed coculture experiments where cells potentially able to stimulate ciliary assembly were separated by a $3\text{-}\mu\text{m}$ pore filter from cells potentially able to respond to the signal. Under these conditions, direct cell contact is prevented. A

Table 2. Analysis of ciliary length and density for suppressor strain dilution series

Initial cell density (per ml)	Culture temp. ($^\circ\text{C}$)	Cilia/ μm cell periphery	Avg. length of measurable cilia $\mu\text{m} \pm \text{SEM}$ (n)	P value
1.5×10^5	30	0.25	2.9 ± 0.089 (236)	n/a
3.0×10^4	30	0.16	2.0 ± 0.112 (100)	1.0×10^{-5}
3.0×10^3	30	0.15	1.4 ± 0.051 (256)	8.5×10^{-32}
3.0×10^3	22	0.25	2.7 ± 0.076 (256)	0.018

n/a, not applicable.

Growing (shaken) IFT52Δsm cells were washed and resuspended in fresh MEPP at the indicated concentrations. After 21.5 h, cells were fixed and stained for immunofluorescence with anti-tubulin antibodies. Cilia were counted and measured on single confocal sections corresponding to the widest section through the macronucleus for a given z-series. Data are from a single representative experiment among many that were performed with very similar loss of motility in diluted cells. P values were generated using paired two-tailed *t* tests comparing means for all other dilutions to the mean for 1.5×10^5 cells/ml.

Figure 9.

stimulation of motility of diluted IFT52Δsm1 cells was observed when they were exposed to concentrated IFT52Δsm1 cells compared with incubation with diluted IFT52Δsm1 or culture medium alone. Furthermore, high concentration nonsuppressed mutants were also able to stimulate assembly on the suppressors in the filter assay (our unpublished data). Thus, the ciliogenesis promoting effect of concentrated cells in IFT52Δsm background does not require direct contact between cells.

The Ciliation-stimulating Factor Is Equivalent to Pericellular Hypoxia

We first attempted to detect a potential positive regulator of ciliogenesis in medium containing high-density cells. Unexpectedly, the spent medium from IFT52Δsm1 cells grown at high density did not stimulate assembly of cilia in IFT52Δsm1 at low cell density. Also, spent medium from WT and IFT52Δmov1 cells did not have an effect on diluted IFT52Δsm1. In contrast, we observed that any kind of spent medium decreased motility compared with fresh medium. Thus, the putative stimulatory factor could be extremely unstable. This hypothesis was reinforced by the results of shaking of suppressor cultures. We grew IFT52Δsm1 cells on a plate at low density at room temperature, either without shaking or with very gentle shaking (30 rotations/min). Slow shaking led to slower growth and less of motility (more cytokinesis defects), even in high-density cells kept at room temperature (Figure 9A). At 30°C we observed that shaken cells had fewer cytokinesis defects compared with unshaken cells. Strikingly, more motile cells were still present in the unshaken culture compared with the shaken culture (Figure 9A). This result can be explained if we assume that at 30°C, increased membrane fluidity allows even gentle shaking to help cells complete cytokinesis by bypassing rotokinesis. Thus, the results of shaking at 30°C are remarkable. Even though the culture that was shaken grew to higher cell density, these cells did not recover robust motility, as did cells grown at the same temperature without shaking. Confocal imaging showed that the decreased motility of cells in the shaking cultures was due to a dramatic

Figure 9. Effect of shaking and mixing with other strains on ciliogenesis in IFT52Δsm suppressors. (A) IFT52Δsm1 cells were diluted to 3×10^3 /ml and grown at 22°C with or without gentle shaking or were diluted to 3×10^4 /ml and grown at 30°C with or without gentle shaking. Cells were scored for motility by using a 10× objective on an inverted microscope. P values were generated for comparison of proportions using a two-proportion z-test with H_0 : shaken = nonshaken and H_a : shaken $\neq$ nonshaken ($n = 62$ for unshaken at 30 and 22°C, 50 for shaken at 30°C, and 70 for shaken at 22°C). (B) Representative IFT52Δsm1 cells 24 h after dilution to 3×10^4 /ml and growth without shaking. (C) Representative IFT52Δsm1 cells 24 h after dilution to 3×10^4 /ml and growth with gentle shaking. (D) IFT52Δsm1 or IFT52Δ10 cells were diluted to 3×10^3 /ml and incubated at 30°C without shaking and either with or without 7.5×10^4 WT cells/ml. Cells were prepared for immunofluorescence as in Figure 2. Cilia lengths ($\pm$ SE of the mean) were measured on single confocal sections ($n_{\text{cells}} = 20$ for IFT52Δsm1 and $n_{\text{cells}} = 10$ for IFT52Δ10). Numbers of individual cilia measured were 1187 for sm, 1058 for sm + WT, 126 for null, and 136 for null + WT. Indicated P values are from paired two-tailed t tests with H_0 : sm = sm + WT and H_0 : null = null + WT.

Figure 10. Hypoxia regulates assembly of cilia in IFT52Δsm suppressors. (A) IFT52Δsm7 cells were prepared at a high density of 3×10^5 cell/ml and grown without shaking in either 1- or 20-μl drops or in the 10-ml volume on a 10-cm Petri plates for 20 h and scored for motility. There is an inverse relationship between the culture volume and extent of motility. (B) IFT52Δsm7 cells were suspended at the initial density of 3×10^4 cells/ml and grown in varying volumes in the 250-ml Erlenmeyer flasks with shaking (100 rpm). The cultures were scored for the presence of motile cells after 18 h. As the culture volume increases, the fraction of motile cells also increases. (C) IFT52Δsm7 cells were prepared at the density of 1.5×10^5 cells/ml in bottles with airtight closures and subjected to a brief stream of either nitrogen or oxygen gas, or untreated (air) and left for 9.5-h incubation either shaken (130 rpm) or not at 30°C. Note high level of motility in the sample in which hypoxic conditions were created with a stream of nitrogen as well as in the normoxic sample left unshaken. Exposing cells to oxygen had an inhibitory effect on cell motility, even compared with the normoxic cells subjected to shaking. (D) Immunofluorescence image of RFTΔsm7 cells that regained motility after creation of hypoxia with a stream of nitrogen.

inability to assemble cilia (Figure 9, B and C). These data suggested that the signal acts locally and may be extremely unstable.

If an unstable, autocrine stimulatory factor is involved, a single IFT52Δsm cell should autostimulate itself for assembly of cilia when grown in a extremely small volume. We therefore grew single isolated IFT52Δsm1 cells in 1-μl drops of medium. Unexpectedly, after 24 h (at 30°C) cells in such extremely small drops did not recover motility and invariably showed extensive cytokinesis defects ($n = 21$). Furthermore, even initially highly motile IFT52Δsm cells (grown at room temperature), lost motility within a few hours after isolation into 1-μl drops kept at 30°C. This surprising result was inconsistent with involvement of a positive, autocrine-type regulator of ciliation. Intrigued by the effect of culture volume on ciliation, we prepared IFT52Δsm1 cell suspension at high cell density and grew cells in drops of varying size. After 24 h in 1-μl drops, we observed no motility despite the fact that cells were suspended at the high cell density (3×10^5 cells/ml) (Figure 10A). However, the extent of motility increased with the increase of drop volume to 20 μl and increased further when cells were incubated in 10-ml volume on a Petri plate (Figure 10A). Thus, reducing the culture volume overrides the stimulatory effect of high cell density. A similar stimulatory effect of increased culture volume was observed when IFT52Δsm cells were grown with shaking in Erlenmeyer flasks (Figure 10B). As the volume of the culture medium increases, the surface/volume ratio decreases. This result taken together with the inhibitory effect of culture

shaking strongly suggested that either a volatile stimulatory factor is released, or a volatile inhibitory factor is acquired from the atmosphere. We therefore considered that the atmospheric oxygen was responsible for the suppression of motility in small volume and in shaken cultures, due to increased aeration. To reduce the concentration of dissolved oxygen, we briefly exposed the suspended IFT52Δsm cells to a stream of nitrogen, and incubate them in an airtight culture vessel for 9 h with shaking. Remarkably, the cell population exposed to a nitrogen stream showed a dramatic increase in cell motility, despite shaking (Figure 10C). The stimulatory effect of nitrogen stream was independent on cell density. Furthermore, the nitrogen-flashed cultures showed a reduction in the growth rate, indicating that hypoxic conditions were created (our unpublished data). The stimulating effect of hypoxia on cell motility was correlated with the increase in the length and density of cilia, as determined by immunofluorescence (Figure 10D). Importantly, the hypoxia-induced newly assembled cilia were concentrated mostly at the anterior ends of cells, the localization that also was predominant in the cells exposed to high cell density without shaking under normoxic conditions (Figure 8, A and B). These observations indicate that the effect of high cell density is equivalent to the effect of pericellular hypoxia. Thus, the inhibition of cell density-mediated suppression by shaking and small culture volume is caused by increased oxygen concentration in the medium. In accordance, cells in the culture exposed to a stream of oxygen and

grown for 9 h, showed lower motility, even compared with cells kept at normoxic conditions (Figure 10C).

DISCUSSION

We show that *Tetrahymena* has a gene, *IFT52*, that encodes a homolog of the IFT52 of *Chlamydomonas reinhardtii*, OSM-6 of *C. elegans*, and predicted proteins from *Drosophila* and mammals. Knocking out *IFT52* led to loss of cilia and defects in cytokinesis. The role of IFT52 in ciliogenesis is consistent with the involvement of OSM-6 in the assembly of chemosensory cilia in *C. elegans* (Collet *et al.*, 1998) and the requirement for IFT-52 in assembly of flagella in *Chlamydomonas* (Brazelton *et al.*, 2001). Furthermore, the entire phenotype of IFT52 null is essentially identical to the phenotype of cells lacking subunits of kinesin-II (Brown *et al.*, 1999b). These results are consistent with Ift52p being a cargo of kinesin-II. The Ift52p localizes mainly to cilia and, as in the case of kinesin-II, does not occur in the cleavage furrow (Brown *et al.*, 1999b). Furthermore, the cytokinesis defects of IFT52 and kinesin-II mutants could be overcome by vigorous shaking, indicating that the cleavage furrow progresses almost to completion so that cell fission can be accomplished by mechanical force without damaging cells. It seems that the loss of a novel type of whole cell motility, called rotokinesis, is sufficient to explain the cytokinesis defects in IFT mutants. Rotokinesis involves the persistent, unidirectional rotation of only the posterior daughter cell around an axis passing through the cytoplasmic bridge connecting the daughter cells. We have proposed that the torque generated by rotokinesis may help weaken the cytoplasmic bridge between presumptive daughter cells, thereby increasing the likelihood that cytokinesis will be successful (Brown *et al.*, 1999a). Recently, a null mutant of *FLA10*, a *Chlamydomonas* kinesin-II subunit, was found not to be affected in cell division (Matsuura *et al.*, 2002). All these data together indicate the components of IFT are not directly involved in cell division.

It was unexpected that spontaneous partial suppressor cells were identified at a relatively high frequency among progenies of the *IFT52* knockout heterokaryons. The result was surprising because both the suppressed and nonsuppressed cells were derived from crosses of two genetically identical parental strains. Given that the partial suppression phenotype occurred with such high frequency and after only a few generations, it is unlikely that it was caused by spontaneous mutations. When a new MAC is produced during conjugation, the genome undergoes extensive rearrangement, which involves deleting ~10% of MIC-specific DNA, as well as chromosome fragmentation. Importantly, these genome-processing events are sometimes nonidentical. In some cases, deletions occur at alternative sites separated by considerable distance (Howard and Blackburn, 1985; Austerberry and Yao, 1988; Chau and Orias, 1996). It has never been reported that such alternative processing could affect protein-coding genes. However, our results suggest that alternative genome processing may have contributed to the suppression in the original *IFT52Δ* strain. At the end of conjugation, each exconjugant has two MACs, which undergo genome processing events independently (Nanney and Caughey, 1953). During the first cell division after conjugation, the two MACs do not divide and are transmitted to the progeny cells, giving the so-called caryonidal lines,

which may therefore have genetically nonidentical MACs. The fact that only a subset of cells derived from the same conjugation pair of *IFT52* knockout heterokaryons showed a suppressed phenotype is consistent with a caryonidal inheritance and with the suppression mechanism originating from alternative MAC genome processing. We found that a single protein is more abundant in suppressor cilia compared with wild type (our unpublished data). Thus, alternative processing may have affected the level of expression of this or perhaps additional proteins which could compensate for loss of Ift52p.

The most surprising result was the observation that the phenotype of intermediate suppressor cells was dependent on cell density. We subsequently found that the stimulating effect of high cell density can be opposed by increasing medium aeration, either by shaking culture or by increasing the surface/volume ratio of culture medium. Furthermore ciliogenesis could be stimulated by decreasing the concentration of oxygen in the medium, even at low cell density. It seems therefore that the cell density effect is based mainly if not entirely on creation of local hypoxic conditions. The exact mode of influence of hypoxia on ciliogenesis is unknown. First, it is possible that the conformation of a molecule(s) involved in suppression is sensitive to the oxygen presence, for example due to its direct oxidation. Alternatively, the suppression could be based on induction of gene expression by hypoxia. It is now well described that hypoxic conditions lead to a response at the level of transcription of genes whose products mediate adaptation to low oxygen conditions (Semenza, 2000). Furthermore, high cell density was recently found to be responsible for induction of signaling responses typical of hypoxia in prostate cancer cells maintained under standard culture generally considered as normoxic (Sheta *et al.*, 2001). The signaling hypothesis requires an existence of sensory and signal transduction mechanisms that respond to the oxygen levels. In this regard, it is unclear whether hypoxia has any effect on the assembly of cilia in wild-type cells. We have not observed an increase in cell motility in unshaken wild-type cultures. Furthermore, the more advanced suppressor, *IFT52Δmov1*, did not show cell density effects. Thus, it seems that the suppression occurred in two stages, and only the initial stage is dependent on hypoxia. On the other side, there is evidence for existence of mechanisms that sense oxygen concentration among ciliates. When *Paramecium* cells were exposed to hypoxia, they swam along a provided temperature gradient toward lower temperature, which seems to be a survival adaptation allowing for lowering the rate of metabolism (Malvin and Wood, 1992). Thus, hypoxia can modify the parameters of ciliary beat and therefore hypoxia-responsive signaling components are likely to exist inside the cilium. It is unlikely however, that oxygen is a general regulator of ciliary assembly, because both aerobic and anaerobic species of protists can assemble the conserved 9 + 2 axoneme (van Hoek *et al.*, 2000). More likely, the suppression event might have coupled the ciliary assembly machinery to hypoxia-mediated signaling and such a direct connection may not be functioning in normal cells. Interestingly, we showed that lower temperature also leads to increased ciliation in the *IFT52Δsm* suppressors. In yeast, up-regulation of the gene encoding D9 fatty acid desaturase also occurs both in hypoxia and at lower temperature (Nakagawa *et al.*, 2002). Our observations

provide additional evidence that low temperature and hypoxia intersect in cellular signaling. Regardless of the exact mechanism of suppression, our results indicate that Ift52p and its homologues in other organisms, play some role in signaling pathways regulating the assembly of cilia. It seems not to be a mere coincidence that NGD5, the murine homolog of Ift52p, was originally identified as a gene whose expression was down-regulated after exposure to an agonist of the δ -opioid receptor (Wick *et al.*, 1995). It is therefore possible that Ift52p is located at the crossroads of some unknown signaling pathways that regulate IFT.

ACKNOWLEDGMENTS

We are particularly grateful to Douglas Cole (University of Idaho, Moscow, ID) whose suggestion led to the identification of oxygen as a ciliation regulating factor. We thank M. Farmer, J. Shields, and P. Brown (Center for Advanced Ultrastructural Research, University of Georgia, Athens, GA) for assistance with confocal and electron microscopy. We also thank J. Frankel (University of Iowa, Iowa City, IA), D. Pennock (Miami University, Miami, OH), J. Rosenbaum (Yale University, New Haven, CT), G. Witman (University of Massachusetts, Amherst, MA), and J.S. Willis, J. Crim, C. Keith, and H. Ritter (University of Georgia) for helpful suggestions. We thank M. Gorovsky (University of Rochester, Rochester, NY) for providing the SG antiserum and A. Turkewitz (University of Chicago, Chicago, IL) for the GFP construct. This work was supported by an American Cancer Society grant, RPG-99-245-01 to J.G. Contributions from N.A.F. were supported from grant 13347 to Dr. Ronald E. Pearlman from the Canadian Institutes for Health Research.

REFERENCES

- Austerberry, C.F., and Yao, M.-C. (1988). Sequence structures of two developmentally regulated, alternative DNA deletion junctions in *Tetrahymena thermophila*. *Mol. Cell. Biol.* 8, 3947–3950.
- Brazelton, W.J., Amundsen, C.D., Silflow, C.D., and Lefebvre, P.A. (2001). The *blt1* mutation identifies the *Chlamydomonas osm-6* homolog as a gene required for flagellar assembly. *Curr. Biol.* 11, 1591–1594.
- Brown, J.M., Hardin, C., and Gaertig, J. (1999a). Rotokinesis, a novel phenomenon of cell locomotion-assisted cytokinesis in the ciliate *Tetrahymena thermophila*. *Int. Cell Biol. Rep.* 23, 841–848.
- Brown, J.M., Marsala, C., Kosoy, R., and Gaertig, J. (1999b). Kinesin-II is preferentially targeted to assembling cilia and is required for ciliogenesis and normal cytokinesis in *Tetrahymena*. *Mol. Biol. Cell* 10, 3081–3096.
- Cassidy-Hanley, D., Bowen, J., Lee, J., Cole, E.S., VerPlank, L.A., Gaertig, J., Gorovsky, M.A., and Bruns, P.J. (1997). Germline and somatic transformation of mating *Tetrahymena thermophila* by particle bombardment. *Genetics* 146, 135–147.
- Chau, M.F., and Orias, E. (1996). Developmentally programmed DNA rearrangement in *Tetrahymena thermophila*: isolation and sequence characterization of three new alternative deletion systems. *Biol. Cell* 86, 11–120.
- Cole, D.G., Diener, D.R., Himelblau, A.L., Beech, P.L., Fuster, J.C., and Rosenbaum, J.L. (1998). *Chlamydomonas* kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in *Caenorhabditis elegans* sensory neurons. *J. Cell Biol.* 18, 993–1008.
- Collet, J., Spike, C.A., Lundquist, E.A., Shaw, J.E., and Herman, R.K. (1998). Analysis of *osm-6*, a gene that affects sensory cilium structure and sensory neuron function in *Caenorhabditis elegans*. *Genetics* 148, 187–200.
- Fillingham, J.S., Chilcoat, N.D., Turkewitz, A.P., Orias, E., Reith, M., and Pearlman, R.E. (2002). Analysis of expressed sequence tags (ESTs) in the ciliated protozoan *Tetrahymena thermophila*. *J. Eukaryot. Microbiol.* 49, 99–107.
- Gaertig, J., Gao, Y., Tishgarten, T., Clark, T.G., and Dickerson, H.W. (1999). Surface display of a parasite antigen in the ciliate *Tetrahymena thermophila*. *Nat. Biotechnol.* 17, 462–465.
- Gaertig, J., Gu, L., Hai, B., and Gorovsky, M.A. (1994). High frequency vector-mediated transformation and gene replacement in *Tetrahymena*. *Nucleic Acids Res.* 22, 5391–5398.
- Guttman, S.D., and Gorovsky, M.A. (1979). Cilia regeneration in starved *Tetrahymena*: an inducible system for studying gene expression and organelle biogenesis. *Cell* 17, 307–317.
- Haddad, A., and Turkewitz, A.P. (1997). Analysis of exocytosis mutants indicates close coupling between regulated secretion and transcription activation in *Tetrahymena*. *Proc. Natl. Acad. Sci. USA* 94, 10675–10680.
- Hai, B., and Gorovsky, M.A. (1997). Germ-line knockout heterokaryons of an essential alpha-tubulin gene enable high-frequency gene replacement and a test of gene transfer from somatic to germ-line nuclei in *Tetrahymena thermophila*. *Proc. Natl. Acad. Sci. USA* 94, 1310–1315.
- Howard, E.A., and Blackburn, E.H. (1985). Reproducible and variable genomic rearrangements occur in the developing somatic nucleus of the ciliate *Tetrahymena thermophila*. *Mol. Cell. Biol.* 5, 2039–2050.
- Kozminski, K.G., Beech, P.L., and Rosenbaum, J.L. (1995). The *Chlamydomonas* kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane. *J. Cell Biol.* 131, 1517–1527.
- Kozminski, K.G., Johnson, K.A., Forscher, P., and Rosenbaum, J.L. (1993). A motility in the eukaryotic flagellum unrelated to flagellar beating. *Proc. Natl. Acad. Sci. USA* 90, 5519–5523.
- Malvin, G.M., and Wood, S.C. (1992). Behavioral hypothermia and survival of hypoxic protozoans *Paramecium caudatum*. *Science* 255, 1423–1425.
- Marszalek, J.R., and Goldstein, L.S. (2000). Understanding the functions of kinesin-II. *Biochem. Biophys. Acta* 1496, 142–150.
- Marszalek, J.R., Liu, X., Roberts, E.A., Chui, D., Marth, J.D., Williams, D.S., and Goldstein, L.S. (2000). Genetic evidence for selective transport of opsin and arrestin by kinesin-II in mammalian photoreceptors. *Cell* 102, 175–187.
- Matsuura, K., Lefebvre, P.A., Kamiya, R., and Hirono, M. (2002). Kinesin-II is not essential for mitosis and cell growth in *Chlamydomonas*. *Cell Motil. Cytoskeleton* 52, 195–201.
- Nakagawa, Y., Sakumoto, N., Kaneko, Y., and Harashima, S. (2002). Mga2p is a putative sensor for low temperature and oxygen to induce OLE1 transcription in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* 291, 703–713.
- Nanney, D.L., and Caughey, P.A. (1953). Mating type differentiation in *Tetrahymena pyriformis*. *Genetics* 39, 1057–1063.
- Nonaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A., Kanai, Y., Kido, M., and Hirokawa, N. (1998). Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell* 95, 829–837.
- Orias, E., and Bruns, P.J. (1976). Induction and isolation of mutants in *Tetrahymena*. In: *Methods in Cell Biology*, ed. D. M. Prescott, vol. 13, New York: Academic Press, 247–282.
- Orias, E., and Rasmussen, L. (1976). Dual capacity for nutrient uptake in *Tetrahymena*. IV. Growth without food vacuoles. *Exp. Cell Res.* 102, 127–137.

- Pazour, G.J., Baker, S.A., Deane, J.A., Cole, D.G., Dickert, B.L., Rosenbaum, J.L., Witman, G.B., and Besharse, J.C. (2002). The intraflagellar transport protein, IFT88, is essential for vertebrate photoreceptor assembly and maintenance. *J. Cell Biol.* 157, 103–113.
- Pazour, G.J., Dickert, B.L., Vucica, Y., Seeley, E.S., Rosenbaum, J.L., Witman, G.B., and Cole, D.G. (2000). *Chlamydomonas* IFT88 and its mouse homologue, polycystic kidney disease gene Tg737, are required for assembly of cilia and flagella. *J. Cell Biol.* 151, 709–718.
- Pazour, G.J., Dickert, B.L., and Witman, G.B. (1999). The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagellar assembly. *J. Cell Biol.* 144, 473–481.
- Pazour, G.J., Wilkerson, C.G., and Witman, G.B. (1998). A dynein light chain is essential for the retrograde particle movement of intraflagellar transport (IFT). *J. Cell Biol.* 141, 979–992.
- Piperno, G., and Mead, K. (1997). Transport of a novel complex in the cytoplasmic matrix of *Chlamydomonas* flagella. *Proc. Natl. Acad. Sci. USA* 94, 4457–4462.
- Rasmussen, L., and Orias, E. (1975). *Tetrahymena*: growth without phagocytosis. *Science* 190, 464–465.
- Rosenbaum, J.L., Cole, D.G., and Diener, D.R. (1999). Intraflagellar transport: the eyes have it. *J. Cell Biol.* 144, 385–388.
- Semenza, G. (2000). HIF-1 and human disease: one highly involved factor. *Genes Dev.* 14, 1983–1991.
- Shakir, M.A., Fukushige, T., Yasuda, H., Miwa, J., and Siddiqui, S.S. (1993). *C. elegans* *osm-3* gene mediating osmotic avoidance behaviour encodes a kinesin-like protein. *Mol. Neurosci.* 4, 891–894.
- Shang, Y., Song, X., Bowen, J., Castanje, R., Gao, Y., Gaertig, J., and Gorovsky, M.A. (2002). A robust inducible-repressible promoter greatly facilitates somatic and germline gene knockouts, conditional expression and overexpression of homologous and heterologous genes in *Tetrahymena thermophila*. *Proc. Natl. Acad. Sci. USA* 99, 3734–3739.
- Sheta, E.A., Trout, H., Gildea, J.J., Harding, M.A., and Theodorescu, D. (2001). Cell density mediated pericellular hypoxia leads to induction of HIF-1 α via nitric oxide and Ras/MAP kinase mediated signaling pathways. *Oncogene* 20, 7624–7634.
- Signor, D., Wedaman, K.P., Orozco, J.T., Dwyer, N.D., Bargmann, C.I., Rose, L.S., and Scholey, J.M. (1999). Role of a class DHC1b dynein in retrograde transport of IFT motors and IFT raft particles along cilia but not dendrites, in chemosensory neurons of living *Caenorhabditis elegans*. *J. Cell Biol.* 147, 519–530.
- Sloboda, R.D. (2002). A healthy understanding of intraflagellar transport. *Cell Motil. Cytoskeleton* 52, 1–8.
- van Hoek, A.H., van Alen, T.A., Sprakel, V.S., Leunissen, J.A., Brigge, T., Vogels, G.D., and Hackstein, J.H. (2000). Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. *Mol. Biol. Evol.* 17, 251–258.
- Walther, Z., Vashistha, M., and Hall, J.L. (1994). The *Chlamydomonas* *FLA10* gene encodes a novel kinesin-homologous protein. *J. Cell Biol.* 126, 175–188.
- Wick, M.J., David, K.A., and Loh, H.H. (1995). Molecular cloning of a novel protein regulated by opioid treatment of NG108–15 cells. *Mol. Brain Res.* 32, 171–175.