Unusual intragenic suppression of an IFT52 gene disruption links hypoxia to the intraflagellar transport in *Tetrahymena thermophila*.

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Abstract
IFT52 protein is a conserved intraflagellar transport protein (a part of the IFT complex B) that is essential for assembly and maintenance of cilia. *Tetrahymena* null mutants with an insertion of a *neo* gene cassette into the *IFT52* gene undergo frequent suppressions that lead to conditional assembly of cilia only under hypoxic conditions (Brown et al. 2003). Here we show that these conditional suppressions are intragenic and occur by a novel mechanism. First, the non-native (bacterial) portion of the DNA sequence of the *neo* cassette is deleted during the process of genome rearrangement that occurs in the developing macronucleus of conjugating *Tetrahymena*. Next, the residual sequences of the *neo* cassette (of *Tetrahymena* origin) within the *IFT52* mRNA are recognized as multiple introns and undergo splicing, leading to a restoration of the translational frame of *IFT52*. The resulting hypoxia-dependent IFT52 protein contains an insertion of 43 new amino acids that replace 7 original amino acids. Taken together with a study in *Chlamydomonas reinhardtii* showing a hypoxia-dependence of another IFT subunit mutant, IFT46, (Hou et al. 2007), our observations generalize that defective IFT complex subunits can regain functionality under hypoxia.

Results and Discussion
Intraflagellar transport (IFT) is a bidirectional motility of ciliary precursors that occurs inside cilia (Kozminski et al. 1993). Kinesin-2 is the anterograde IFT motor, whereas cytoplasmic dynein1b is responsible for the retrograde IFT (Kozminski et al. 1995; Pazour et al. 1999; Porter et al. 1999). These motors move IFT trains, that are composed
of two protein complexes, A and B (Cole et al. 1998; Piperno and Mead 1997). IFT52 is a complex B protein that is required for the assembly and maintenance of cilia (Brazelton et al. 2001; Brown et al. 2003; Deane et al. 2001).

The ciliate *Tetrahymena thermophila* has two nuclei, a transcriptionally silent micronucleus (Mic) and a transcriptionally active macronucleus (Mac) (reviewed in (Yao and Chao 2005)). Earlier (Brown et al. 2003), *IFT52* was disrupted by insertion of the *neo2* marker within exon 4 (Figure 1D). Heterokaryons were constructed with Macs carrying wild-type *IFT52* alleles and Mics homozygous for the disrupted alleles. Most progeny cells of mating *IFT52* heterokaryons are completely paralyzed due to the lack of cilia and cannot complete cytokinesis since they are unable to rupture the connecting cytoplasmic bridge (rotokinesis). Surprisingly, 3% of the heterokaryon progeny recover partial motility due to spontaneous suppressions. Importantly, the suppressed cells (*IFT52Δsm*) assemble motile cilia when grown at either a lower temperature or in hypoxia. In a single suppressed strain, an additional event produced *IFT52Δmov* cells, which are capable of assembling cilia independently of temperature or hypoxia (Brown et al. 2003).

The high frequency of the *IFT52Δsm* conditional suppressions and the fact that these suppressions occur only during conjugation (Brown et al. 2003), suggested that the mechanism of suppression is based on processes that occur inside the developing new Mac. Conjugating *Tetrahymena* cells undergo a series of nuclear events that culminate in replacement of the parental Mac by a new Mac that develops by differentiation from a zygotic Mic (reviewed in (Coyne et al. 1996)). About 15% of the Mic genome is removed from the new Mac, by a pathway that involves an RNAi-dependent sequence recognition
and degradation (reviewed in (Yao and Chao 2005)). Yao and colleagues showed that a foreign sequence, neo2, inserted into multiple loci, undergoes RNAi-mediated deletion (Yao et al. 2003). Thus, we tested whether neo2 inserted into IFT52 also undergoes deletions that could be a cause of the conditional suppressions.

The IFT52 knockout was done by inserting the neo2 disruption cassette into exon 4 (Figure 1D). neo2 consists of the bacterial neomycin phosphotransferase (neo) coding region placed between DNA fragments of Tetrahymena origin; the HHF4 promoter and the BTU2 transcription terminator (Gaertig et al. 1994; Kahn et al. 1993). We isolated total genomic DNA from wild-type, IFT52Δ, IFT52Δsm and IFT52Δmov cells and amplified the IFT52 locus across the neo2 insertion site (Figure 1D). Amplification of genomic DNA of wild-type cells produced a fragment of expected size (1.3kb). The same primers used with IFT52Δ (non-suppressed) DNA produced a larger fragment (~2.7kb) consistent with presence of an intact neo2 cassette (Figure 1A). Strikingly, the same primers amplified a smaller fragment (~1.9kb) from the genomic DNA of both conditional and non-conditional suppressors (IFT52Δsm and IFT52Δmov). This suggested that the suppressions are associated with deletions around the neo2 insertion site. Sequencing of fragments amplified from multiple independent suppressor strains showed deletions of a portion of neo (~0.8kb) with deletion junctions at exactly the same positions, while the flanking sequences of neo2 (of Tetrahymena origin) remained largely intact (Figure 1D). Specifically, all deletions analyzed were between the nucleotide at position +45 in the neo coding sequence and the fifth nucleotide downstream of the stop codon within the BTU2 segment (Figure S1). These observations are consistent with
earlier reports on deletions of neo2 sequences during macronuclear development (Liu et al. 2005; Yao et al. 2003).

The observed neo sequence deletions, do not explain the mechanism of suppression because the sequence of the neo2 cassette remnant has stop codons in all forward translational frames. An Ift52p translated from the predicted mRNA containing the neo2 remnant would be severely truncated; lacking 5 out of 7 exons, all containing conserved sequences (Cole 2003) (Figure S1). Nevertheless, the suppressions correlate with deletions of the neo coding sequence.

To establish whether the suppressed IFT52 locus with the residual neo2 is sufficient to restore partial motility, we introduced the rearranged fragment of the IFT52Δmov genomic DNA into IFT52Δ cells by biolistic bombardment (Figure 1D). As a control we mock-transformed the same number of IFT52Δ cells (9x10^6). After 7-9 days of incubation at room temperature, we obtained 2 clones that regained motility in the population bombarded with the rearranged (IFT52Δmov) fragment and none in the mock-transformed IFT52Δ cells. We confirmed that the targeting fragment replaced the corresponding region of the fully disrupted IFT52 locus by PCR (results not shown). The rescued cells showed the conditional suppression phenotype, a cell density (pericellular hypoxia)-dependent ciliary motility (Brown et al. 2003) (Figure 1C and results not shown). These data indicate that the IFT52Δmov cells underwent an additional, unknown genetic or epigenetic change that resulted in a non-conditional suppression.

The rearranged IFT52Δsm/mov gene contains a residual neo2 sequence that somehow provides a partially functional Ift52p. Either an extremely truncated Ift52p is sufficient for conditional ciliary assembly or an additional mechanism restores the
translational frame across the residual neo2. To determine the sequence of the translated Ift52p in IFT52Δsm cells, we used RT-PCR to amplify the IFT52 cDNA obtained from mRNA of IFT52Δsm cells (Figure 1D). For a spliced wild-type IFT52 mRNA, the amplified fragment was expected to be ~0.3kb. For the IFT52Δsm mRNA with residual neo2 cassette, the cDNA fragment was expected to be ~0.9kb. However, the size of the amplified product from the IFT52Δsm cDNA was ~0.4kb, indicating that an additional splicing event occurs in the IFT52Δsm mRNA (Figure 1B). The sequencing of a cloned IFT52Δsm cDNA revealed that ~0.8kb of the residual neo2 was absent. Most of the residual neo2 sequence, mainly comprising of the HHF4 and BTU2 sequences, was removed from the mRNA as 3 (artificial) introns. The artificial intron junctions have sequences consistent with the native intron junctions observed in ciliates such as Paramecium and Tetrahymena (Figure S1) (Jaillon et al. 2008). The processing of the residual neo2 as a set of artificial introns restores the translational frame across the site of neo2 insertion (Figure S2). Hence, the predicted suppressor Ift52p has 43 additional amino acids but lacks 7 original amino acids as a result of the neo2 cloning procedure (Figures 1D and S2). Either the presence of these extra amino acids or the absence of the 7 endogenous amino acids in Ift52p-sm (or both) results in the intragenic conditional suppression.

To conclude, we reveal a novel mechanism for intragenic suppression in Tetrahymena that consists of two steps: 1) foreign DNA within the inserted disruption cassette is deleted during macronuclear development, and 2) the remaining AT-rich Tetrahymena native sequences of the disruption marker are processed as introns during
mRNA splicing. The first step almost certainly occurs via the RNAi-mediated developmental genome rearrangement pathway (Mochizuki et al. 2002; Mochizuki and Gorovsky 2004; Yao et al. 2003). This form of genomic DNA deletion is thought to have evolved as a means of genome surveillance to eliminate transposon DNA from the transcriptionally active Mac (reviewed in (Yao and Chao 2005)). In 4 independent suppressor clones we detected a genomic deletion at precisely identical positions. Previous studies showed a variability in the deletion sites (Liu et al. 2005; Yao et al. 2003). It is likely that other deletions occur in the disrupted IFT52 locus but they do not create potential splice junctions that restore the translational frame. When IFT52 heterokaryons undergo conjugation, the majority (97%) of the progeny has a non-suppressed phenotype. In these cells, the deletions of neo2 either do not occur, or occur on an insufficient number of macronuclear chromosomes to achieve a phenotypic threshold for suppression (there are 45 copies of each chromosome in the G1 macronucleus).

*Chlamydomonas* cells carrying an insertional mutation in IFT46 (encoding another complex B protein), also underwent a spontaneous intragenic mutation that led to a hypoxia-dependent cilia assembly (Hou et al. 2007). Both studies taken together ((Hou et al. 2007) and this work), allow for a generalization; that hypoxic conditions can restore the functionality of mutated IFT complex B components. Hou and colleagues observed the assembly of complex B in the flagella of suppressed *Chlamydomonas* IFT46 mutants. It is likely that the suppressed IFT52Δsm *Tetrahymena* cells also assemble complex B. Hou and colleagues proposed that the IFT complex B subunits are folded by a chaperone whose levels increase under hypoxia. Thus, a partly damaged IFT component may still
fold properly when the chaperone activity is increased. Another possibility is that the IFT complex B assembly is regulated directly by an oxygen-dependent post-translational modification of one or more subunits. Regardless of the exact mechanism, both studies indicate that a hypoxia-dependent modulation of the activity of IFT complex B subunits is a conserved mechanism.

**Materials and Methods**

*Cells, cultures and media*

For the maintenance, IFT52Δ, IFT52Δsm and IFT52Δmov cells were grown at the room temperature in MEPP medium (Orias and Rasmussen 1976) with an antibiotic-antimycotic mixture (Invitrogen, Carlsbad, CA).

*DNA preparation and cloning*

Isolation of total genomic DNA was done as described (Dave et al. 2009). The genomic region across exons 3 and 4 was amplified using the following primers: 5’-ATGCCCTCAAATAAT-3’ and 5’-TAGAGTTGGTTTAGATTT-3’. The resulting fragments were cloned into pGEM-T-vector (Promega Corp, Madison, WI) and sequenced.

*Biolistic transformation of Tetrahymena*

To determine whether a genomic fragment of IFT52 of suppressor origin is sufficient to confer suppression of the IFT52Δ phenotype (lack of cilia), IFT52Δ cells were
biolistically bombarded with a genomic fragment of IFT52Δmov origin, that was earlier separated from the pGEM-T-vector plasmid with NcoI and SalI digestion. Bombarded cells were grown at the room temperature and transformants were identified based on recovery of cell motility (Cassidy-Hanley et al. 1997).

**cDNA preparation**

Cells were grown to a concentration of $2 \times 10^5$ cells/ml in MEPP medium (Gorovsky et al. 1975), washed with 10 mM Tris-HCl buffer pH 7.5 and used for total RNA extraction with TRI-reagent (MRC Inc, Cincinnati, OH) according to manufacturer’s instructions. Total cDNA was prepared using the SMART IV-forward and CDS III-reverse primers from the RT-PCR kit (Clontech Inc, Mountainview, CA).

**Acknowledgements**

This work was supported by the National Science Foundation grant MCB-063994 to JG.

**References**


Figure legends

Figure 1: Two subsequent sequence deletions lead to intragenic suppression of an insertional IFT52 mutation. A. Results of PCR amplifications of the genomic region of IFT52 locus with primers corresponding to sequences in exons 3 and 4 in wildtype (IFT52), gene knockout (IFT52Δ) and the suppressor (IFT52Δsm/mov) cells. Amplified fragments were separated on an agarose gel. An asterisk marks an apparent non-specific amplification product. B. PCR amplifications of total cDNA obtained from mRNA using wildtype (IFT52), knockout (IFT52Δ) and suppressors (IFT52Δsm/mov) cells using the same primers as in panel A. The amplification products were separated on an agarose gel. C. IFT52Δ cells rescued with IFT52Δsm/mov DNA and wild-type cells both at a concentration of 3x10^5 were diluted down to different concentrations (1x to 100x) on a...
96-well microtiter plate, and incubated at 30°C. After 12 hours of incubation, the number of motile cells (cells showing detectable displacement and lacking cytokinesis defects) was determined. D. A schematic diagram detailing the IFT52 locus in the wild-type (IFT52), knockout (IFT52Δ) and the suppressors (IFT52Δsm/mov) cells and the cDNA in the suppressors (IFT52Δsm/mov processed mRNA) cells. F and R represent primers used for the various PCR reactions.
Figure 1

A. Genomic DNA

B. Size marker, IFT52Δsm/mov cDNA, IFT52Δsm/mov cDNA, IFT52 CDNA, IFT52 genomic DNA

C. Motile cells (%) vs. Dilution

D. Schematic representation of IFT52 and IFT52Δsm/mov

IFT52

IFT52Δ

IFT52Δsm/mov

IFT52Δsm/mov processed mRNA
Supplemental data

Figure S1: Sequence of genomic DNA reconstructed from 4 independent IFT52Δsm/mov suppressor strains shows a deletion within the neo2 cassette. The sequence shown corresponds to the region between exons 3 and 4 of the suppressed IFT52 genomic DNA. The three segments of the neo2 cassette are marked in blue (BTU2), pink (neo) and green (HHF4). Natural intron junctions are marked as grey boxes. Artificial intron junctions are marked as open boxes. Note: Within the residual neo cassette left after genomic deletion, there are stop codons (red *) in every translational frame.

Figure S2: The cDNA sequence of IFT52Δsm/mov has 43 extra codons from residual neo2 cassette. The residual neo2 cassette consists of bacterial and Tetrahymena (BTU2 and HHF4) sequences after being processed as artificial introns. The sequence shown corresponds to the region between exons 3 and 4 of the suppressed IFT52 cDNA.
**IFT52Δsm/mov cDNA sequence (470 codons)**

**IFT52 cDNA (black)**

ATG AGT GGA GAA TAG AAA ATT ATT GTC TCC AAC SCT GCT TCA AAG AAG GAG GCT GGT AAC CCT AGT ACA AAT ATT AAA AAG ATT ATC AAG AAA


TAT AAA GAA ACA TAT AAG TGC GGC AGA AAT AAA GAA GAT ATC ACA TAT GAT AGA CTA AAG ATG GCT TCT TGG CTA ATA TTT TTG TGC CCC

Y K E T Y K C G R N K E D I T Y D R L K M A S L V I F F C P

AAA GAA ATG TTT ACC AAG AGG GAA TTC GAT GCC CTC AAA TAA TAC TTA GAA AGT GGT GGT GCT GTC TTA GGT AGC TCT GAA GGT GGT

K E M P T K E E F D A L K Q Y G S G R V L V L S S E G G

**GTG CAT AAG AAT CGC ACA AAT ATA AAT TCC TTT TGG GAA TAA Y T A T G T C T G T A G T G C T G T C T G T A G T A G T C T G T C T G T A G T G C T G T C T G T A G T C T G T

H K N R T N I N F F L E Q Y G I S I N N D C V V R T A F W

**BTU2 cDNA (blue) neo cDNA (pink)**

**HHF4 cDNA (green)**

gct gca att ttc cac ggc cgg aga acc tgc tgg caa tcc atc cca gct tgt cag ttt tgg cag aaa aat tat cca ato aga ato agt ctt

A A P F H H R T C V Q S I Q A C H F S P K N Y P I R I S L

**IFT52 cDNA (black)**

tct aga gat tca gtt gat cct cta ata aga tag GAG ACT ATT GTT GCT TGT ATT TTA AAT GAA GAA GTA ACA AGA GTA GCA AAT

S R D S D F D A S I R Q E T Y V H S G I L N E E V T R V A N

**GTG TTA CCT AAA GAA ACA AAG AGA CCC CAA AAT ACA TCT TTG TAA AAG GCT ATT GAA AAG GAT GAT GAA GAA GAT GAA GAT TAT TAA AAG AAA

G L P K E T K R P Q N T F L Q N V I G K D D D E E D E Y Q K E

**CAA TCT AGA GGT GTT GTA GAT TTT GTT TAT GCC TTG GTT GCT ACC TTG ACT GCT TTA TAA CCT GCA CAC GCT ATT TTA GGT TCT GCT GCT CCT

Q S R V G L D F V Y A F G A T L T Q V Q P A R A I L G S G P

**CTT TCT TAC CCT TCT AAT AGA CCA GTT TCT GCT ATC GTC TAA ATT AAA AAT AAT GGT AGT GCA ATT GCT ATT TTA GTC TCC GAA ATG TTT

L S Y P S N R P V S A I V Q T K N N G R L A V I G S F E M F

**ACA GAT GAA TAT TTT GAC AAT GAA GAT AAC TCC AAG ATT TTT GAT TCC TTT ATA AAA TAT TTG CTC ACA AAT GAG TGC GAA TTA GAA TTT

T D E Y F D N E D N S K I F D F F I K Y L L T N E C B F E F

**AGT CCT AAA GAA CCT GAT GTT GAA TAC TTC AAG GCT ATT GCT GAA TTA GCT GAT AAG CTC AAG ATG TGC TTA CAA GAA AGT GAC

S P R E P D V F Y E F K V P D I A E L A D N L K S C L Q E S D

**CCA TTA CCA TTT GAT AGC AAG CAA TTA TAT ATG GAC TTG TTT TAT AAG GAT GAA GAC TTA GCT CCA GGC GCT GTA AAA TTG TAT GAA

P L P F D S K Q L F M T D L D F K Y D V D L V D P E A V K L Y E

**ACT TCT GGA GTA AAG CAC GAT CTT CCT CTT GTA CCT TAA TTC GAA ACT CCA GTC CTT GTA GTC TCC GCT CCA CCT

T L G V K H D P L A L I V F Q F E T P L L G L V S A V F P P

**ATT TTA AAA GAA TTA GCT CCT CCA AGT TTA GAA TTG TTT GAT TTA GAT GAA TTT GCT TCA GAA AAA GTA AGA CTG GCC TAA CCT ACA

I L K R L A P S L E L F D L D D E F A S E K V R L A Q L T

**AAT AAA TGC AAC AAC AAG TTA GAT TAT TAC ATT AAA GAA TCA GGT GAT ATC TTG GGT GTA ACA GAT AAA GTT AAG AAC AAA CAT GAT

N K C N N N N D L D Y Y I K E S G D I L G V T D K V K N K H D

**GCC AAA GCT ATT TTA AGA TAT GTT TTA GAA GAA TTA ATA AAT TTC AAG AAG CTC AAT AAC TGA

A K A I L R Y V L E L I N F K K L N N •