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The N-terminus of IFT46 mediates intraflagellar transport of outer arm dynein and its cargo-adaptor ODA16

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ABSTRACT Cilia are assembled via intraflagellar transport (IFT). The IFT machinery is composed of motors and multisubunit particles, termed IFT-A and IFT-B, that carry cargo into the cilium. Knowledge of how the IFT subunits interact with their cargo is of critical importance for understanding how the unique ciliary domain is established. We previously reported a Chlamydomonas mutant, ift46-1, that fails to express the IFT-B protein IFT46, has greatly reduced levels of other IFT-B proteins, and assembles only very short flagella. A spontaneous suppression of ift46-1 restored IFT-B levels and enabled growth of longer flagella, but the flagella lacked outer dynein arms. Here we show that the suppression is due to insertion of the transposon MRC1 into the ift46-1 allele, causing the expression of a fusion protein including the IFT46 C-terminal 240 amino acids. The IFT46 C-terminus can assemble into and stabilize IFT-B but does not support transport of outer arm dynein into flagella. ODA16, a cargo adaptor specific for outer arm dynein, also fails to be imported into the flagella in the absence of the IFT46 N-terminus. We conclude that the IFT46 N-terminus, ODA16, and outer arm dynein interact for IFT of the latter.

INTRODUCTION

Cilia and flagella (terms used interchangeably here) are microtubule-based organelles that extend from the cell surface into the environment. They are important for cell motility, for cells to sense their environment, and for signal transduction. Defects in ciliary structure or signaling cause a large number of human diseases, collectively called ciliopathies (Mitchison and Valente, 2017).

Ciliary assembly and signaling both depend on a highly conserved process known as intraflagellar transport (IFT; Kozminski et al., 1993; Rosenbaum and Witman, 2002; Lechtreck, 2015; Taschner and Lorentzen, 2016). IFT is the movement of large particles, termed IFT trains, from the base to the tip of the flagellum (anterograde IFT) and back to the base (retrograde IFT). Anterograde IFT is powered by kinesin motors (Kozminski et al., 1995; Snow et al., 2004; Verhey et al., 2011), while retrograde IFT is driven by cytoplasmic dynein 2/1b (Pazour et al., 1998, 1999; Porter et al., 1999; Hou and Witman, 2015). The trains are composed primarily of two complexes, IFT-A and IFT-B, the latter of which can be subdivided into IFT-B1 and IFT-B2 (Table 1) (Cole et al., 1998; Lechtreck, 2015; Taschner and Lorentzen, 2016). The trains transport structural components of the axoneme to the tip of the flagellum, where axonemal assembly occurs, and return the products of axonemal turnover to the cell body (Qin et al., 2004; Ishikawa et al., 2014; Lechtreck, 2015). IFT also transports signals and signaling proteins between cilia and the cell body in several pathways, including mating in Chlamydomonas (Wang et al., 2006) and Hedgehog, Wnt, transforming factor β, receptor tyrosine kinase, and Notch signaling in vertebrates (Mourão et al., 2016).

With the exception of tubulin (Bhogaraju et al., 2013; Craft et al., 2015; Kubo et al., 2016), little is known about how IFT particles interact with their cargoes of axonemal precursors. Two highly conserved proteins that are providing a paradigm for such interactions are ODA16 and IFT46.

ODA16, the crystal structure of which was recently determined (Taschner et al., 2017), is proposed to be an IFT cargo adaptor.
transcribing $ift46$-1 with a construct expressing a similarly truncated protein containing only $ift46$ aa 106–344 that also stabilizes IFT-B and supports better flagellar growth under stress. Outer arm dynein in this strain is competent to bind to axonemes, but its transport into the flagellum is largely curtailed. We further find that the N-terminus of IFT46 is crucial for transport of ODA16 into the flagellum. The results establish a model for how an IFT-particle protein links to a major axonomal cargo to establish the unique ciliary protein composition. Finally, we explore the requirement for stress to enable flagellar assembly when IFT-B is defective.

RESULTS

A transposon in the $Sup_{ift46}^1$ allele enables expression of a truncated IFT46 protein that supports flagellar assembly

To determine the genomic basis for the suppression of $ift46$-1, we cloned and sequenced the $ift46$ alleles in $ift46$-1 and $Sup_{ift46}^1$. In $ift46$-1, the NIT1 sequence originally used to create $ift46$-1 by insertional mutagenesis (Hou et al., 2007) was found inserted into the fifth intron of the IFT46 gene (Figure 1A). In $Sup_{ift46}^1$, MRC1 (minature retrotransposon of *Chlamydomonas*) sequence was found inserted into the exogenous NIT1 sequence (Figure 1A). To see whether this change at the genomic level caused the transcription of the 3′ end of the IFT46 gene, we cloned the 5′ end of the transcript by rapid amplification of cDNA ends (RACE). Two cDNAs were identified that differed in their 5′ untranslated regions (UTRs); one clone was 78 base pairs longer than the other. The 5′ UTR and first exon of these cDNAs are from MRC1, and the second, third, and subsequent exons are from the sixth, seventh, and subsequent exons, respectively, of the IFT46 gene (Figure 1, A and B). This result shows that the insertion of the transposon MRC1 into the $ift46$-1 allele in $Sup_{ift46}^1$ causes expression of hybrid RNAs combining the MRC1 and IFT46 sequences.

The complete MRC1 transposon includes a sequence of 420 base pairs bracketed by 600–base pair long terminal repeats (LTRs) (Kim et al., 2006). In $Sup_{ift46}^1$, MRC1 is inserted in the opposite orientation to the IFT46 gene, with one of its LTRs adjacent to the NIT1 and IFT46 genes. Sequence analysis predicted a bacterial promoter and a known bacterial transcription factor RpoD binding site (rpoD15) at the correct positions relative to the identified transcription start point for the longer RNA, and a eukaryotic promoter just upstream of the transcription initiation site for the shorter RNA (Figure 1B).

The hybrid RNAs from $Sup_{ift46}^1$ are predicted to encode a fusion protein (which we term IFT46T1) in which the first 10 amino acids are derived from the MRC1 sequence, followed by 240 amino acids corresponding to IFT46 aa 105–344. The published IFT46 antibody (anti-IFT46N) was raised against the first 19 amino acids of IFT46 and did not detect IFT46 sequence in $Sup_{ift46}^1$ (Figure 1C, left) (Hou et al., 2007). To determine whether IFT46T1 is expressed in $Sup_{ift46}^1$, we generated a guinea pig polyclonal antibody (anti-IFT46C) to IFT46 aa 105–344. In Western blots of whole-cell lysates, the anti-IFT46C antibody reacted strongly with IFT46 at ~48 kDa in wild type and with a band of ~28 kDa, which is the predicted size for IFT46T1, in $Sup_{ift46}^1$ (Figure 1C, center). Therefore IFT46T1 is expressed in $Sup_{ift46}^1$.

IFT46T1 is assembled into IFT-B and stabilizes it

To determine whether IFT46T1 enters flagella, we probed Western blots of isolated flagella with anti-IFT46C (Figure 2A, left). IFT46T1 was detected in flagella of $Sup_{ift46}^1$ at a level similar to that of IFT46 in wild-type flagella.

We previously showed that other IFT-B proteins, which are largely destabilized in $ift46$-1 cell bodies, are restored to near-normal levels...
FIGURE 1: A transposon inserted into the ift46-1 allele allows expression of the IFT46 C-terminus. (A) Diagram of the IFT46 gene in wild-type, ift46-1, and Supift46-1 as revealed by sequencing of genomic DNA and cDNAs. Exons are shown as tall boxes and introns as lines; the polyadenylation site is indicated by a circle. The ift46-1 allele was generated by insertion of the NIT1 gene (green box) into intron 5 of IFT46. The Supift46-1 allele resulted from insertion of the MRC1 transposon (red) into the exogenous NIT1 gene. After transcription and splicing, a hybrid mRNA, and protein levels show that, in Supift46-1 whole-cell lysates (Hou et al., 2007). To determine whether the expression of IFT46T1 resulted in normal levels of other IFT proteins in Supift46-1 flagella, we probed Western blots of isolated flagella with antibodies to several IFT-A and IFT-B proteins (Figure 2A, center). The IFT-B protein IFT72 was elevated relative to wild type. In contrast, the IFT-B protein IFT172 was slightly decreased. IFT72 can function independently of the IFT particle (Pedersen et al. 2005), and its level often trends differently from levels of other IFT-B proteins (Hou et al., 2007; Brown et al., 2015). IFT-A, as represented by IFT139, was elevated relative to wild type. Therefore expression of IFT46T1 results in slight changes in levels of IFT proteins in flagella of Supift46-1.

Importantly, the Supift46-1 flagella contained normal levels of the inner arm dynein intermediate chain ICl38, but antibody failed to detect the α, β, or γ outer arm dynein heavy chains and detected only a trace of the outer arm dynein intermediate chain IC2, even at long exposure times (Figure 2A, center and right). These results confirm our previous conclusions, based on electron microscopy (Hou et al., 2007), that the outer dynein arms are drastically and specifically reduced in Supift46-1 axonomes.

To investigate whether IFT46T1 in the Supift46-1 flagella is associated normally with other IFT-B proteins, we analyzed the flagellar detergent-soluble membrane-plus-matrix fraction by sucrose density gradient centrifugation followed by Western blotting. Under our experimental conditions, proteins of the wild-type IFT-B1 subcomplex remained associated with one another, as indicated by comigration of IFT46 and IFT81, but were separate from IFT172 and IFT139 (Figure 2B). In fractions from Supift46-1 flagella, IFT46T1 similarly comigrated with IFT81 but not with IFT172 or IFT139. Therefore IFT46T1 is incorporated into the IFT-B1 subcomplex.
whether part of the phenotype of Supift461 might be due to abnormal expression of IFT46T1 or to its MRC1-derived amino acids. To clarify this, we cotransformed ift46-1, which is null for IFT46 and is nonmotile, with a drug-resistance gene and a construct designed to express a Met plus IFT46 aa 106–344 (termed IFT46106-344) from the endogenous IFT46 promoter. About 10% of the resulting transformants contained slow-swimming cells when grown in 96-well plates. Western blotting of 11 of the partially motile cell lines revealed that they all expressed a protein of appropriate size that is detected by our anti-IFT46C antibody but is not detected in ift46-1 (Figure 3); this protein thus must be IFT46106-344. Southern blotting of DNA from the partially motile transformants confirmed that they were the products of independent transformation events (Supplemental Figure S1). Thus the C-terminal 239 amino acids of IFT46 are sufficient to partially rescue the ift46-1 phenotype.

To more rigorously examine the ability of IFT46106-344 to carry out IFT46 function, we chose the cell line YH6RT4 (hereafter ift46-1 IFT46Δ105) for detailed analysis, because it expresses IFT46106-344 at a level similar to that of IFT46 in wild type (Figure 3). Like Supift461, these cells have very short flagella when aerated; however, they are much more readily induced to form longer flagella when stressed. Western blotting of whole-cell lysates showed that IFT complex A and B proteins are present above normal levels in these cells (Figure 4A), suggesting that IFT46106-344 is assembled into IFT-B1 and stabilizes the complex. The increased expression of both IFT-A and B proteins may reflect a response by the cell to try to remedy a defect in IFT (see IFT is reduced in ift46-1 IFT46Δ105 flagella).

To confirm that IFT46106-344 is assembled normally into IFT-B1, we checked the distribution of the truncated protein in sucrose gradient fractions of ift46-1 IFT46Δ105 flagellar membrane-plus-matrix and found that IFT46106-344 cosedimented normally with IFT81 (Figure 2B). We also used label-free quantitative mass spectrometry (MS) to determine whether IFT46 and IFT46106-344 were incorporated into IFT-B1 in the same molar ratio in wild type and ift46-1 IFT46Δ105, respectively. The wild-type or ift46-1 IFT46Δ105 flagellar membrane-plus-matrix was fractionated on sucrose gradients, and the peak fractions of IFT46 or IFT46106-344 were concentrated and used for the analysis. The intensities of the top three common precursors in the MS spectra for IFT46/IFT46106-344 and IFT81 (the internal standard) were used to calculate, for each protein, the fold change between the wild-type and ift46-1 IFT46Δ105 samples. The fold change for

Expression of IFT46 aa 106–344 is sufficient for partial rescue of ift46-1

These results indicate that the C-terminal two-thirds of IFT46 is sufficient to stabilize IFT-B and support some flagellar assembly in Supift461. However, because IFT46T1 is expressed from an unusual exogenous promoter and is a fusion protein in which the first 10 amino acids are derived from the MRC1 sequence, it was unclear...
IFT46/IFT46\textsubscript{106-344} was 0.8, while that for IFT81 was 0.6. We conclude that a normal molar ratio of IFT46\textsubscript{106-344} is incorporated into axonemes, which is consistent with the finding that outer arm dynein in the original ift46 mutant is fully preassembled and competent to bind to axonemes.

In our analyses of ift46-1 IFT46\Delta105 flagella, we detected small amounts of IC2 (Figure 4B). To determine whether this represented outer arm dynein bound to the axoneme or simply present in the flagellar matrix, possibly associated with IFT particles, flagellar samples were fractionated by detergent treatment into membrane-plus-matrix and axonemal fractions. The axonemal fraction was further treated with ATP to yield an ATP-soluble fraction and an ATP-insoluble fraction. The former contains residual IFT particles not removed during the demembranation step (Hou et al., 2004) and some outer arm dynein that is solubilized by ATP treatment (Goodenough and Heuser, 1984; Hou et al., 2004). Western blotting of the fractions (Figure 4E) revealed 1) that the outer arm dynein in the ift46-1 IFT46\Delta105 flagella is associated with the axoneme, not the flagellar matrix; and 2) that it has the same distribution in the ATP-treated fractions as does outer arm dynein in the wild-type fractions, indicating that its attachment to the axoneme is normal.

We also considered the possibility that outer dynein arms are transported normally by IFT into the flagella of ift46-1 IFT46\Delta105 but not released from the IFT particle and so are returned directly to the cell body rather than being incorporated into the axoneme. This is unlikely to be the case because the above experiment showed that the small amount of IC2 in the ift46-1 IFT46\Delta105 flagellum is located in the axoneme, not the membrane-plus-matrix fraction, where we would expect it to be if the truncation of IFT46 did not prevent IFT of outer arm dynein but did block release of outer arm dynein from the IFT particle. Nevertheless, to further test this hypothesis, we compared the membrane-plus-matrix fractions from half-length regenerating flagella of wild-type and ift46-1 IFT46\Delta105 cells. We used regenerating flagella because, although the number of IFT particles traversing the flagella is more or less independent of flagellar length (Dentler et al., 2005; Engel et al., 2009), the loading of axonemal precursors (as represented by DRC4, a subunit of the nexin-dynein regulatory complex) onto IFT particles increases ~10-fold in such flagella (Wren et al., 2013). Western blotting revealed that there was much less IC2 in the ift46-1 IFT46\Delta105 membrane-plus-matrix fraction than in the wild-type membrane-plus-matrix fraction (Figure 4F). These results strongly suggest that loading of outer arm dynein onto IFT particles is severely reduced in the absence of the IFT46 N-terminus.

The low level of outer arm dynein in ift46-1 IFT46\Delta105 flagella is due to a defect in IFT

Outer arm dynein is preassembled in the cell cytoplasm before it is transported into the flagellum (Fowkes and Mitchell, 1998), and it must undergo a poorly understood maturation step to be able to attach to its binding site on the outer doublet microtubules (Desai et al., 2015). To investigate whether ift46-1 IFT46\Delta105 outer arm dynein in the cytoplasmic pool is preassembled, mature, and competent to bind to axonemes, we performed an in vitro binding assay. Cell lysates from wild-type and ift46-1 IFT46\Delta105 cells were incubated with ift46-1 IFT46\Delta105 axonemes, the axonemes were then separated from unbound proteins by centrifugation, and binding of outer arm dynein to the axonemes was assessed by Western blotting using an anti-IC2 antibody as a probe (Figure 4D). Similar amounts of IC2 from wild-type and ift46-1 IFT46\Delta105 whole-cell lysates bound to the axonemes, indicating that outer arm dynein in the ift46-1 IFT46\Delta105 cell cytoplasm is fully preassembled and competent to bind to axonemes. This result also shows that the ift46-1 IFT46\Delta105 axoneme is capable of binding outer arm dynein. This is consistent with the previous finding that outer arm dynein in the original ift46 mutant is fully preassembled and competent to bind to axonemes (Ahmed et al., 2008).

Levels of outer arm dynein proteins are normal in the whole cell but greatly reduced in the flagella of ift46-1 IFT46\Delta105

We previously found that IC2 is present in the S\textsubscript{Sup}IFT46-1 cell body at normal levels (Hou et al., 2007); similarly, in this study, we found by Western blot analysis that outer arm dynein heavy and intermediate chains were present at normal levels in whole-cell lysates of ift46-1 IFT46\Delta105 (Figure 4A). Therefore truncation of the IFT46 N-terminus has no effect on the level of expression of outer arm dynein proteins.

In wild-type Chlamydomonas, ~90% of the total cellular pool of IFT proteins is in the cell body; only ~10% is in the flagella (Ahmed et al., 2008; Lechtreck et al., 2009). In contrast, outer arm dynein proteins are about evenly distributed between the cell body and the flagella (Ahmed et al., 2008). To determine how truncation of the IFT46 N-terminus affects localization of these proteins to the flagella, we compared the protein content of isolated flagella from wild-type and ift46-1 IFT46\Delta105 by Western blotting (Figure 4B). IFT\textsubscript{0} (IFT-8) and IFT139 (IFT-A) protein levels were the same as in wild-type flagella; IFT172 was slightly reduced. However, the amount of the outer arm dynein intermediate chain IC2 was greatly decreased in ift46-1 IFT46\Delta105 flagella. Immunofluorescence labeling of wild-type and ift46-1 IFT46\Delta105 cells with an antibody to the outer arm dynein \(\alpha\) heavy chain confirmed a drastic reduction in the fluorescence signal for outer dynein arms in ift46-1 IFT46\Delta105 flagella (Figure 4C). Therefore, despite being expressed at normal levels in the cytoplasm, outer arm dynein is not properly localized to the flagella of ift46-1 IFT46\Delta105 cells. The loss of outer arm dynein arms undoubtedly accounts for the slow-swimming phenotype of the ift46-1 IFT46\Delta105 cells.

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We also considered the possibility that outer dynein arms are transported normally by IFT into the flagella of ift46-1 IFT46\Delta105 but not released from the IFT particle and so are returned directly to the cell body rather than being incorporated into the axoneme. This is unlikely to be the case because the above experiment showed that the small amount of IC2 in the ift46-1 IFT46\Delta105 flagellum is located in the axoneme, not the membrane-plus-matrix fraction, where we would expect it to be if the truncation of IFT46 did not prevent IFT of outer arm dynein but did block release of outer arm dynein from the IFT particle. Nevertheless, to further test this hypothesis, we compared the membrane-plus-matrix fractions from half-length regenerating flagella of wild-type and ift46-1 IFT46\Delta105 cells. We used regenerating flagella because, although the number of IFT particles traversing the flagella is more or less independent of flagellar length (Dentler et al., 2005; Engel et al., 2009), the loading of axonemal precursors (as represented by DRC4, a subunit of the nexin-dynein regulatory complex) onto IFT particles increases ~10-fold in such flagella (Wren et al., 2013). Western blotting revealed that there was much less IC2 in the ift46-1 IFT46\Delta105 membrane-plus-matrix fraction than in the wild-type membrane-plus-matrix fraction (Figure 4F). These results strongly suggest that loading of outer arm dynein onto IFT particles is severely reduced in the absence of the IFT46 N-terminus.
axoneme fraction was further divided into ATP-soluble (ATP sup) and ATP-insoluble (ATP pellet) membrane-plus-matrix (M+M) and axoneme (Axo) fractions via detergent treatment. The

1/14 that loaded for detection of IC2. (E) Western blots of wild-type and

β in their cytoplasm; because the heavy chain of outer arm dynein but have normal levels of the other outer arm dynein subunits cell lysate served as a control for nonspecific binding to the axonemes. Cells of ift46-1 IFT46Δ105 cells have impaired transport of outer arm dynein into their flagella. (A) Western blots of wild-type and ift46-1 IFT46Δ105 whole-cell lysates indicate that levels of outer arm dynein subunits are normal in ift46-1 IFT46Δ105. Levels of IFT-A (IFT139) and IFT-B (IFT81, IFT57) proteins are above normal in these cells. A separate blot using 1/200 of each sample was analyzed for the tubulin loading control. Here and in the following figures, ift46-1 IFT46Δ105 is abbreviated IFT46Δ105. (B) Western blots of wild-type and ift46-1 IFT46Δ105 flagella revealed that the latter contain greatly reduced amounts of outer arm dynein as represented by IC2. IFT139 and IFT81 levels are normal. For the tubulin control, the amount of protein loaded was 1/100 that loaded for detection of the other proteins. (C) Immunofluorescence images of wild-type and ift46-1 IFT46Δ105 cells doubly labeled with antibodies to α-tubulin and the α heavy chain of outer arm dynein. The outer arm dynein signal in ift46-1 IFT46Δ105 flagella was greatly reduced compared with that in wild-type flagella. In the ift46-1 IFT46Δ105 cell shown, most of the outer arm dynein label was near the flagellar base, but in general, any labeling was distributed evenly along the flagella in ift46-1 IFT46Δ105 cells. Scale bar: 5 µm. (D) Outer arm dynein preassembled in the ift46-1 IFT46Δ105 cell body is competent to bind to axonemes. Lysates from wild-type and ift46-1 IFT46Δ105 cells were incubated with ift46-1 IFT46Δ105 axonemes, and the axonemes were then separated from unbound protein by centrifugation and assayed for bound IC2. The Western blot of the cell lysate (input cell lysate) confirmed that the wild-type and ift46-1 IFT46Δ105 cells contained about the same amount of IC2. The Western blot of the axonemes (pull down) showed that outer arm dynein from wild-type and ift46-1 IFT46Δ105 cytoplasm is equally competent to bind to axonemes. An oda4 cell lysate served as a control for nonspecific binding to the axonemes. Cells of oda4 lack the β heavy chain of outer arm dynein but have normal levels of the other outer arm dynein subunits in their cytoplasm; because the β heavy chain is missing, the dynein cannot bind specifically to the axoneme (Ahmed et al., 2008). For the tubulin control, the amount of protein loaded was 1/14 that loaded for detection of IC2. (E) Western blots of wild-type and ift46-1 IFT46Δ105 flagellar fractions probed with an anti-IC2 antibody. The flagella were fractionated into membrane-plus-matrix (M+M) and axoneme (Axo) fractions via detergent treatment. The axoneme fraction was further divided into ATP-soluble (ATP sup) and ATP-insoluble (ATP pellet) fractions. The trace amount of IC2 in the ift46-1 IFT46Δ105 flagella is associated with the axoneme, not the matrix. IC2 was distributed similarly between the two axonemal fractions in wild-type and ift46-1 IFT46Δ105, suggesting that its attachment to the axoneme is normal in ift46-1 IFT46Δ105. For samples from the same strain, protein from an equal number of flagella was loaded in each lane; more protein was loaded for the ift46-1 IFT46Δ105 samples than for the wild-type samples in order to detect the very small amount of IC2 present in the former. (F) Western blots of membrane-plus-matrix fractions from regenerating flagella of wild-type and ift46-1 IFT46Δ105 cells probed with antibodies to IC2 and IFT81 (loading control). IC2 was greatly reduced in the ift46-1 IFT46Δ105 sample. Cells were dejagged by pH shock and were allowed to regenerate new flagella by bringing the pH back to the pre-deflagellation level. The regenerating flagella were then collected when about half-grown (4.0 ± 1.3 µm for wild-type flagella; 3.8 ± 0.8 µm for ift46-1 IFT46Δ105 flagella; n = 100, p = 0.08).

In summary, ift46-1 IFT46Δ105 cells have normal amounts of outer arm dynein, and the dynein is competent to bind to ift46-1 IFT46Δ105 axonemes in vitro. The few arms that are present in ift46-1 IFT46Δ105 flagella appear to be bound to the axoneme normally. Given that the only known genetic defect in ift46-1 IFT46Δ105 cells is in IFT46, we conclude that the reduction in outer dynein arms in ift46-1 IFT46Δ105 flagella is a result of the cells being unable to move preassembled, assembly-competent outer arm dynein efficiently into the flagella due to truncation of the N-terminus of IFT46.

ODA16 is not transported normally into ift46-1 IFT46Δ105 flagella

ODA16, a 49-kDa WD-repeat protein, is required for assembly of a full complement of outer dynein arms into the axoneme (Ahmed and Mitchell, 2005; Ahmed et al., 2008). Its transport into the flagellum is dependent on IFT and occurs even in the absence of outer arm dynein, suggesting that it interacts directly with the IFT machinery, an interaction confirmed by coimmunoprecipitation experiments. Yeast two-hybrid experiments and pull-down assays using recombiantly produced proteins have shown that ODA16 interacts directly with IFT46 in vitro (Ahmed et al., 2008) and that this interaction is mediated by the IFT46 N-terminus (Taschner et al., 2017). To determine whether loss of the N-terminus of IFT46 affects the transport of ODA16 into flagella, we used Western blotting to compare

![Figure 4: ift46-1 IFT46Δ105 cells have impaired transport of outer arm dynein into their flagella. (A) Western blots of wild-type and ift46-1 IFT46Δ105 whole-cell lysates indicate that levels of outer arm dynein subunits are normal in ift46-1 IFT46Δ105. Levels of IFT-A (IFT139) and IFT-B (IFT81, IFT57) proteins are above normal in these cells. A separate blot using 1/200 of each sample was analyzed for the tubulin loading control. Here and in the following figures, ift46-1 IFT46Δ105 is abbreviated IFT46Δ105. (B) Western blots of wild-type and ift46-1 IFT46Δ105 flagella revealed that the latter contain greatly reduced amounts of outer arm dynein as represented by IC2. IFT139 and IFT81 levels are normal. For the tubulin control, the amount of protein loaded was 1/100 that loaded for detection of the other proteins. (C) Immunofluorescence images of wild-type and ift46-1 IFT46Δ105 cells doubly labeled with antibodies to α-tubulin and the α heavy chain of outer arm dynein. The outer arm dynein signal in ift46-1 IFT46Δ105 flagella was greatly reduced compared with that in wild-type flagella. In the ift46-1 IFT46Δ105 cell shown, most of the outer arm dynein label was near the flagellar base, but in general, any labeling was distributed evenly along the flagella in ift46-1 IFT46Δ105 cells. Scale bar: 5 µm. (D) Outer arm dynein preassembled in the ift46-1 IFT46Δ105 cell body is competent to bind to axonemes. Lysates from wild-type and ift46-1 IFT46Δ105 cells were incubated with ift46-1 IFT46Δ105 axonemes, and the axonemes were then separated from unbound protein by centrifugation and assayed for bound IC2. The Western blot of the cell lysate (input cell lysate) confirmed that the wild-type and ift46-1 IFT46Δ105 cells contained about the same amount of IC2. The Western blot of the axonemes (pull down) showed that outer arm dynein from wild-type and ift46-1 IFT46Δ105 cytoplasm is equally competent to bind to axonemes. An oda4 cell lysate served as a control for nonspecific binding to the axonemes. Cells of oda4 lack the β heavy chain of outer arm dynein but have normal levels of the other outer arm dynein subunits in their cytoplasm; because the β heavy chain is missing, the dynein cannot bind specifically to the axoneme (Ahmed et al., 2008). For the tubulin control, the amount of protein loaded was 1/14 that loaded for detection of IC2. (E) Western blots of wild-type and ift46-1 IFT46Δ105 flagellar fractions probed with an anti-IC2 antibody. The flagella were fractionated into membrane-plus-matrix (M+M) and axoneme (Axo) fractions via detergent treatment. The axoneme fraction was further divided into ATP-soluble (ATP sup) and ATP-insoluble (ATP pellet) fractions. The trace amount of IC2 in the ift46-1 IFT46Δ105 flagella is associated with the axoneme, not the matrix. IC2 was distributed similarly between the two axonemal fractions in wild-type and ift46-1 IFT46Δ105, suggesting that its attachment to the axoneme is normal in ift46-1 IFT46Δ105. For samples from the same strain, protein from an equal number of flagella was loaded in each lane; more protein was loaded for the ift46-1 IFT46Δ105 samples than for the wild-type samples in order to detect the very small amount of IC2 present in the former. (F) Western blots of membrane-plus-matrix fractions from regenerating flagella of wild-type and ift46-1 IFT46Δ105 cells probed with antibodies to IC2 and IFT81 (loading control). IC2 was greatly reduced in the ift46-1 IFT46Δ105 sample. Cells were dejagged by pH shock and were allowed to regenerate new flagella by bringing the pH back to the pre-deflagellation level. The regenerating flagella were then collected when about half-grown (4.0 ± 1.3 µm for wild-type flagella; 3.8 ± 0.8 µm for ift46-1 IFT46Δ105 flagella; n = 100, p = 0.08).
Flagellar assembly requires both stress and a threshold level of truncated IFT46

Cells of sup<sup>ift46-1</sup> have abnormal flagellar assembly kinetics

To determine how the absence of the IFT46 N-terminus affects the rate of flagellar assembly, we subjected cells to pH shock, which causes abscission of any flagella present, thus resetting flagellar length to zero. We then centrifuged the cells, resuspended them in fresh medium, and subsequently incubated them in a beaker with light but no aeration. Under these conditions, wild-type cells rapidly formed new flagella with kinetics similar to that typically observed for wild-type strains (Rosenbaum et al., 1969) (Figure 6C). Sup<sup>ift46-1</sup> cells did not noticeably increase as a result of the stress. These results suggest that the ift46-1 IFT46Δ105 cells are more readily induced to assemble flagella because they have cytoplasmic levels of truncated IFT46 above a required threshold; whereas the lower amount of truncated IFT46 in sup<sub>ift46Δ105</sub> cells is below that threshold and inadequate to support flagellar assembly.

In the second regimen, which is the most optimal that we have found for inducing flagellar growth in sup<sub>ift46Δ105</sub> cells, cells were grown to mid-log phase with aeration, and aeration was then stopped overnight. The next morning, ~10% of sup<sub>ift46Δ105</sub> cells and 90% of ift46-1 IFT46Δ105 cells had flagella of approximately normal length and were swimming. Western blot analysis revealed that the level of IFT46Δ106-344 in the ift46-1 IFT46Δ105 cells did not change under the stress conditions (Figure 6B). In contrast, IFT46T1 in the sup<sub>ift46Δ105</sub> cells was significantly increased by these stress conditions, although its level did not reach that of IFT46Δ106-344 (unpublished data). These findings suggest that the level of IFT46T1 in sup<sub>ift46Δ105</sub> cells had to increase above a threshold level for the cells to grow flagella. The results support the hypothesis that, compared with ift46-1 IFT46Δ105 cells, sup<sub>ift46Δ105</sub> cells are less readily induced to grow flagella because they have lower levels of truncated IFT46.

Finally, the experiments shown in Figure 6, A and B, together indicate that a level of IFT46Δ106-344 in ift46-1 IFT46Δ105 cells that is similar to that of IFT46 in wild type and adequate to support flagellar assembly under stress conditions is not sufficient to support flagellar assembly in the absence of stress. In the presence of adequate amounts of truncated IFT46, stress itself enables flagellar assembly through a still unknown mechanism.

ift46-1 IFT46Δ105 cells have abnormal flagellar assembly kinetics

To determine how the absence of the IFT46 N-terminus affects the rate of flagellar assembly, we subjected cells to pH shock, which causes abscission of any flagella present, thus resetting flagellar length to zero. We then centrifuged the cells, resuspended them in fresh medium, and subsequently incubated them in a beaker with light but no aeration. Under these conditions, wild-type cells rapidly formed new flagella with kinetics similar to that typically observed for wild-type strains (Rosenbaum et al., 1969) (Figure 6C). Sup<sup>ift46-1</sup> cells reformed only very short flagella, consistent with the poor short flagella when grown with aeration, but are much more readily induced to form flagella under a variety of stress conditions. To better understand the basis for this phenotypic difference, we compared the levels of truncated IFT46 in these strains under two different treatments for inducing stress.

In the first regimen, mid-log phase cells were concentrated eightfold during the day, and aerations were then stopped. This treatment does not induce flagellar growth in sup<sub>ift46Δ105</sub> cells but does induce slow growth of flagella of uniform length in ift46-1 IFT46Δ105 cells (see Figure 6D). Western blotting showed that, before application of stress, wild-type and ift46-1 IFT46Δ105 cells had similar amounts of IFT46 or IFT46Δ106-344, respectively, whereas the level of IFT46T1 was much less in sup<sub>ift46Δ105</sub> cells (Figure 6A). The level of IFT46 in wild-type cells or of truncated IFT46 in sup<sub>ift46Δ105</sub> or ift46-1 IFT46Δ105 cells did not noticeably increase as a result of the stress. These results suggest that the ift46-1 IFT46Δ105 cells are more readily induced to assemble flagella because they have cytoplasmic levels of truncated IFT46 above a required threshold, whereas the lower amount of truncated IFT46 in sup<sub>ift46Δ105</sub> cells is below that threshold and inadequate to support flagellar assembly.

In the second regimen, which is the most optimal that we have found for inducing flagellar growth in sup<sub>ift46Δ105</sub> cells, cells were grown to mid-log phase with aeration, and aeration was then stopped overnight. The next morning, ~10% of sup<sub>ift46Δ105</sub> cells and 90% of ift46-1 IFT46Δ105 cells had flagella of approximately normal length and were swimming. Western blot analysis revealed that the level of IFT46Δ106-344 in the ift46-1 IFT46Δ105 cells did not change under the stress conditions (Figure 6B). In contrast, IFT46T1 in the sup<sub>ift46Δ105</sub> cells was significantly increased by these stress conditions, although its level did not reach that of IFT46Δ106-344 (unpublished data). These findings suggest that the level of IFT46T1 in sup<sub>ift46Δ105</sub> cells had to increase above a threshold level for the cells to grow flagella. The results support the hypothesis that, compared with ift46-1 IFT46Δ105 cells, sup<sub>ift46Δ105</sub> cells are less readily induced to grow flagella because they have lower levels of truncated IFT46.
IFT46 and of truncated IFT46106-344 in sup1Δ cells; there was little or no change in these amounts at the end of the 3-h stress period. A separate blot using 1/5 of the same samples was probed with an anti-tubulin antibody as a loading control. (B) Western blot comparing the levels of IFT46T1 in sup1Δ cells and of IFT46106-344 in ift46-1 IFT46Δ105 cells after cultures were kept overnight with aeration (control) or without aeration (Stressed); the stress condition induced flagellar formation in ∼10% of sup1Δ cells and 90% of ift46-1 IFT46Δ105 cells. The lack of aeration caused a substantial increase in the level of truncated IFT46 in sup1Δ but not in ift46-1 IFT46Δ105. For the tubulin controls, the sample loadings were 1/20th of those used for the detection of truncated IFT46. (C) Flagellar regeneration curves after pH shock. Wild-type and oda9 cells regenerated full-length flagella within 2 h, ift46-1 IFT46Δ105 cells assembled flagella more slowly, and sup1Δ cells very slowly formed only very short flagella. (D) Flagellar assembly curves for ift46-1 IFT46Δ105 cells induced to form flagella by concentration followed by maintenance without aeration in the presence or absence of 10 µg/ml cycloheximide (CH). Control cells were not concentrated and were aerated without interruption in the absence of cycloheximide. For C and D, one flagellum from each of 50 cells was measured and the mean value was calculated for each data point; nonflagellated cells were not included. Error bars show SDs.

FIGURE 6: Truncated IFT46 levels correlate with ability to assemble flagella in sup1Δ and ift46-1 IFT46Δ105 cells, and flagellar assembly is slow in ift46-1 IFT46Δ105 cells. (A) Western blot showing that the levels of IFT46 and truncated IFT46 did not change when the cells were stressed by concentration during the day and then maintained for 3 h without aeration. Under these conditions, ift46-1 IFT46Δ105 cells grew longer flagella, but sup1Δ cells did not. Before stress, the amount of IFT46T1 in sup1Δ cells was much less than the amounts of IFT46 in wild-type cells or of IFT46106-344 in ift46-1 IFT46Δ105 cells; there was little or no change in these amounts at the end of the 3-h stress period. A separate blot using 1/5 of the same samples was probed with an anti-tubulin antibody as a loading control. (B) Western blot comparing the levels of IFT46T1 in sup1Δ cells and of IFT46106-344 in ift46-1 IFT46Δ105 cells after cultures were kept overnight with aeration (control) or without aeration (Stressed); the stress condition induced flagellar formation in ∼10% of sup1Δ cells and 90% of ift46-1 IFT46Δ105 cells. The lack of aeration caused a substantial increase in the level of truncated IFT46 in sup1Δ but not in ift46-1 IFT46Δ105. For the tubulin controls, the sample loadings were 1/20th of those used for the detection of truncated IFT46. (C) Flagellar regeneration curves after pH shock. Wild-type and oda9 cells regenerated full-length flagella within 2 h, ift46-1 IFT46Δ105 cells assembled flagella more slowly, and sup1Δ cells very slowly formed only very short flagella. (D) Flagellar assembly curves for ift46-1 IFT46Δ105 cells induced to form flagella by concentration followed by maintenance without aeration in the presence or absence of 10 µg/ml cycloheximide (CH). Control cells were not concentrated and were aerated without interruption in the absence of cycloheximide. For C and D, one flagellum from each of 50 cells was measured and the mean value was calculated for each data point; nonflagellated cells were not included. Error bars show SDs.

ability of these cells to assemble flagella under most conditions. Cells of ift46-1 IFT46Δ105 formed new flagella, but the initial rapid rate of growth was ∼63% that of wild type. The flagella then transitioned to a slow elongation phase similar to that of wild type (Rosenbaum et al., 1969; Wood et al., 2012), but this transition occurred at a shorter flagellar length than in wild type, and the flagella never reached an average length greater than about two-thirds that of wild-type flagella. Because ift46-1 cells rescued with the wild-type IFT46 gene have normal flagellar regeneration kinetics, and strain YH6RT10 generated by rescue of ift46-1 in an independent transfor- mation event with the construct expressing IFT46106,344 has regeneration kinetics similar to that of ift46-1 IFT46Δ105 (Supplemental Figure S2), the ift46-1 IFT46Δ105 flagellar regeneration phenotype is not due to some other mutation. Its slow initial rate of flagellar assembly also is not due to the absence of outer dynein arms on the ift46-1 IFT46Δ105 doublet microtubules or on IFT particles: cells of oda9, which lack the outer dynein arm inter- mediate chain IC1 (Kamiya, 1988; Wilkerson et al., 1995) and thus do not preassemble outer arms in the cell body (Fowkes and Mitchell, 1998), do not transport them into their flagella as IFT cargo during flagellar regeneration (Supplemental Figure S3), and do not incorporate them into their axonemes (Kamiya, 1988; Wilkerson et al., 1995), had initial rapid regeneration kinetics similar to that of wild-type cells (Figure 6C). Therefore ift46-1 IFT46Δ105 cells, despite having a level of IFT46106,344 similar to the levels of IFT46 in wild type (Figure 6A), have abnormally slow flagellar growth, even after induction of flagellar assembly.

IFT is reduced in ift46-1 IFT46Δ105 flagella

The slower flagellar regeneration kinetics in ift46-1 IFT46Δ105 cells could be the result of a defect in IFT caused by the absence of the IFT46 N-terminus. To investigate this possibility, we used differential interference contrast (DIC) microscopy to compare IFT velocity and frequency in wild-type versus ift46-1 IFT46Δ105 cells. Because shorter flagella exhibit slower IFT speeds (Engel et al., 2009), our measurements were carried out on growing wild-type or ift46-1 IFT46Δ105 flagella that were ∼8 µm long. Anterograde velocity and frequency in ift46-1 IFT46Δ105 were ∼70% of their values in wild type; retrograde velocity and frequency were respectively ∼60% and 70% of their wild-type values (Figure 7A). Figure 7, B and C, shows the distributions of anterograde and retrograde IFT velocities for wild-type and ift46-1 IFT46Δ105 cells. The distributions of anterograde velocities fell into similarly sharp peaks in both strains, whereas the retrograde velocities were much more widely distributed, suggesting that retrograde trains are more heterogeneous in size or number of motors than are anterograde trains; similar differences between the distributions of anterograde and retrograde velocities have been reported previously (Dentler, 2005). The decrease in anterograde velocity coupled with the decrease in anterograde frequency in ift46-1 IFT46Δ105 could account for the reduction in the initial rate of flagellar assembly that we observed in this strain (Figure 6C).

Stress-induced flagella formation does not require new protein synthesis

To try to understand the mechanism by which stress induces flagellar assembly in ift46-1 IFT46Δ105 cells, we investigated whether the induction requires new protein synthesis. Cells were grown to log phase with aeration and then concentrated 8- to 10-fold and thereafter kept in the light without aeration. Immediately after concentration, the cells were divided into two aliquots, and cyclohexi- mide, which in Chlamydomonas blocks protein synthesis within 1–2 min (Rosenbaum et al., 1969), was added to one of the aliquots.
Samples were then taken at 1-h intervals for measurement of flagella analyzed.

FIGURE 7: IFT is impaired in ift46-1 IFT46Δ105 cells. (A) ift46-1 IFT46Δ105 cells have lower IFT velocities and frequencies than wild-type cells. In both strains, measurements were carried out on flagella that were ~8 µm long, and the mean values were plotted. Error bars show SDs. (B, C) Distributions of anterograde (B) and retrograde (C) IFT velocities in wild-type and ift46-1 IFT46Δ105 flagella. In flagella of both strains, anterograde velocities were formed at a rate that initially was identical to that of the cells without cycloheximide, and growth continued for several hours before leveling off as the flagella reached a length of ~6 µm. Growth of flagella of wild-type cells in cycloheximide similarly levels off at ~6 µm (Rosenbaum et al., 1969); this is believed to reflect exhaustion of the cellular pool of flagellar precursors. Therefore, consistent with our results for outer arm dynein (Figure 4A), ift46-1 IFT46Δ105 cells likely have a cellular pool of flagellar precursors similar to that of wild-type cells. We conclude that new protein synthesis is not required for the induction of flagellar growth by stress in ift46-1 IFT46Δ105 cells.

DISCUSSION

Genome remodeling by transposon MRC1 under environmental stress and selective pressure restored evolutionary fitness

Our data show that the spontaneous suppression of the ift46-1 mutant was caused by transposition of the mobile element MRC1 (Kim et al., 2006). The original suppressor was identified in an ift46-1 culture (Hou et al., 2007) that had been grown to stationary phase with aeraton and then left for more than 1 wk without aeration, during which time the nonmotile cells sank to the bottom of the flask and likely experienced hypoxia, especially during the dark cycle. It is possible that the stress activated the transposition (Wheeler, 2013); it certainly would have resulted in a strong selection for cells that could swim toward the air:liquid interface.

Although it is known that insertion of transposable elements can activate downstream genes by initiating transcription within the transposon (Charlier et al., 1982; Ciampi et al., 1982), this is the first time that MRC1 was shown to activate gene expression via a similar mechanism. In this case, the insertion of the MRC1 sequence enabled flagellar assembly by providing both gene regulatory elements and a protein-coding sequence that resulted in partial restoration of IFT. Such transposon-mediated restructuring of the genome has been proposed to play an important role in the evolution of plants and algae in response to environmental change (Kim et al., 2006). The current study provides an excellent laboratory example of this.

The level of truncated IFT46 transcript in Supift461 cells is much lower than that of the full-length transcript in wild-type cells (Figure 8 in Hou et al., 2007), suggesting that transcription initiated by the MRC1 LTR sequence is much weaker than that initiated by the endogenous IFT46 promoter. Consistent with this, the level of IFT46T1 protein in Supift461 cells is much less than the level of IFT46 in wild-type cells or of IFT46Δ105 cells. A promoter is predicted upstream of each of the two transcription start sites that we identified in the MRC1 LTR sequence. The promoter that gives rise to the shorter transcript is a eukaryotic polymerase II promoter. A predicted binding site for the transcription factor snail is upstream of this promoter region; however, there is no Chlamydomonas orthologue of snail. The promoter that gives rise to the longer transcript has bacterial promoter elements at positions −31 and −9 and a predicted RpoD binding site at −7, all correctly positioned relative to the transcription initiation site. RpoD is a member of the sigma 70 family of proteins that target RNA polymerase to the transcription start site in prokaryotes and plastids (Paget and Helmann, 2003). Chlamydomonas has only one sigma 70 protein, RPOD (Carter et al., 2004; Bohne et al. 2006; Kawazoe et al., 2012), which is encoded by a nuclear gene but believed to function in the chloroplast. It would be of interest to determine whether RPOD is involved in the initiation of transcription of the ift46 allele in the Supift461 nucleus.
The C-terminus of IFT46 stabilizes IFT-B, while the N-terminus mediates IFT of outer arm dynein

Expression of IFT46 (IFT46Δ344) in ift46-1 cells recapitulated the suppression observed in SupΔ461 cells, confirming that the suppression in the latter is due to expression of an N-terminally truncated IFT46. IFT46 (IFT46Δ344) stabilizes the other IFT-B proteins and is incorporated at the normal molar ratio into IFT81; as a result, ift46-1 IFT46Δ105 cells are able to form flagella. This is consistent with previous findings that the C-terminus of IFT46 interacts with the C-terminus of IFT52 and that the assembly of an IFT46/IFT52 subcomplex is a prerequisite for the assembly of IFT81 (Lucker et al., 2010; Taschner et al., 2014).

However, cells expressing IFT46Δ344, like SupΔ461 cells, still have greatly reduced numbers of outer dynein arms in their flagella. We determined that this is due to reduced ability of IFT to transport outer dynein arms into the flagella. This transport is mediated by ODA16 acting as a cargo adaptor (Ahmed and Mitchell, 2005, Ahmed et al., 2008). Indeed, we found that the level of ODA16 is greatly reduced in ift46-1 IFT46Δ105 flagella, which is consistent with recent studies demonstrating that the N-terminal 147 amino acids of IFT46 interact with ODA16 in vitro and may contain the entire ODA16-binding domain (Taschner et al., 2017). Thus, loss of ODA16 binding by IFT46 explains the reduction in outer arm dynein in flagella of ift46-1 IFT46Δ105. Interestingly, ift46-1 IFT46Δ105 flagella contain even less outer arm dynein than oda16-1 flagella, raising the possibility that the N-terminus of IFT46 is involved in limited outer arm dynein transport in an ODA16-independent manner.

How the very small amount of outer arm dynein present even in ift46-1 IFT46Δ105 flagella gets there is still unknown, but there are several possibilities. It may be carried in by the small amount of ODA16 that still enters the flagellum, perhaps by binding with low affinity to sites between IFT46 aa 106–147. It may diffuse in; although preassembled outer arm dynein is larger than the apparent size limit for diffusion through the flagellar transition zone (Kee et al., 2012; Breslow et al., 2013; Awata et al., 2014), the barrier may not be absolutely impermeable to large molecules (Lin et al., 2013). Finally, a small amount of dynein may enter the flagellum through a pathway that bypasses IFT and the transition zone barrier—for example, through the basal body lumen.

**Stress enables flagellar assembly in IFT mutants with partially repaired IFT-B complexes**

Although IFT46Δ344 stabilizes IFT-B in the cytoplasm, and cells expressing this truncated IFT46 can assemble longer flagella than SupΔ461 cells, they assemble longer flagella only under stress (hypoxic) conditions. When recombinant His_{5}_tagged full-length IFT46 or IFT46 lacking the first 25 amino acids were electroporated into ift46-2 cells, the cells’ flagellar assembly defect was rescued, but similar electroporation of recombinant His_{5}_tagged IFT46 lacking the first 50 or 100 amino acids did not rescue the cells (Lucker et al., 2010). In these experiments, the cells were agitated every few minutes following electroporation, so they probably were not stressed sufficiently to support rescue by the more severely truncated proteins.

In addition to our initial report on stress-induced suppression of ift46-1 (Hou et al., 2007) and the findings reported here, there have been other reports of cells with defects in IFT-B proteins and impaired ciliary assembly that is partially corrected under stress conditions. In Chlamydomonas, the IFT74 null mutant ift74-2 has greatly reduced levels of IFT-B proteins in its cytoplasm and severely impaired flagellar assembly, even in the absence of aeration (Brown et al., 2015). However, in the mutant ift74-1, expression of a truncated form of IFT74 lacking the first 196 amino acids supports IFT-B assembly, but the cells still have severely impaired flagellar assembly unless they are grown without aeration, in which case the cells form half-length flagella and become motile.

In *Tetrahymena*, an IFT25 knockout mutant lacking cilia was observed to undergo frequent spontaneous partial suppression resulting in sparse short cilia usually lacking the central pair of microtubules; these cilia grew longer and acquired a “9+2” axoneme under hypoxic conditions (Brown et al., 2003). Further analysis revealed that the conditional suppression involved gene reorganization and alternative splicing leading to expression of a modified IFT25 that lacks seven of the original amino acids but has 43 additional amino acids (Dave et al., 2016). In *Chlamydomonas*, the lack of IFT25 destabilizes IFT-B (Richey and Qin, 2012), so it is possible that the original *Tetrahymena* IFT25 knockout mutant had a defect in IFT-B assembly or stability that was repaired by expression of the modified IFT25.

In none of these cases is the mechanism by which hypoxia promotes ciliary assembly clear. However, in at least two of the cases, there was expression of a partially functional IFT-B protein that stabilizes the IFT-B complex in the cytoplasm but does not restore full function to the complex. Stress then enables flagellar assembly to proceed. This is not due to a mechanism that bypasses IFT, because it does not occur in null mutants lacking IFT-B. Rather, it likely involves further repair or stabilization of the partially restored IFT-B. This does not involve new protein synthesis, because we found that stress can induce ift46-1 IFT46Δ105 cells to assemble half-length flagella in the presence of cycloheximide. The mechanism could involve postranslational modification of IFT-B itself or of a chaperone that then interacts with IFT-B, repairing IFT-B’s ability to assemble into IFT trains or bind cargo. It also could involve the way IFT particles interact with the flagellar transition zone. When a *Chlamydomonas* mutant null for the transition zone protein CEP290 is grown with aeration, most cells have extremely short flagella, but when grown in the absence of aeration, many cells form longer flagella, indicating a connection between stress and transition zone function (Craigie et al., 2010).

A similar mechanism to restore IFT may be operating in *Caenorhabditis elegans*. The cilia of hypomorphic IFT mutants, which are severely truncated in young adults, become longer as the worms age. This partial restoration of ciliary length is dependent upon IFT and requires the heat shock factor HSF-1 and probably the HSF1-regulated chaperone Hsp90 (Cornils et al., 2016).

**IFT defects likely explain the abnormal flagellar assembly kinetics of ift46-1 IFT46Δ105 cells**

Although stressed ift46-1 IFT46Δ105 cells assemble new flagella following deflagellation, the initial rapid rate of assembly is slower than normal. This is not due to the lack of incorporation of outer dynein arms into the axoneme, the absence of outer arm dynein as cargo on IFT trains, a mutation at a second site, or a lack of flagellar precursors. It appears to be due to a residual defect in IFT. Anterograde IFT frequency is slower in ift46-1 IFT46Δ105 cells than in wild-type cells; retrograde IFT frequency is slower by about the same amount, likely reflecting the reduced frequency with which anterograde trains reach the flagellar tip. Anterograde and retrograde velocity also are reduced; this might occur if the lower frequency of entry into the flagellum results in larger IFT trains, as trains with larger apparent size move more slowly (Lomini et al., 2001; Schmidts, Hou, et al., 2015). The combination of lower IFT frequency and velocity would be expected to slow delivery of axonemal precursors to the flagellar tip, resulting in a reduction in the flagellar assembly rate.

It is not clear why the frequency with which IFT trains enter the flagellum is reduced in ift46-1 IFT46Δ105 cells. The IFT frequency presumably is not reduced as a result of the inability to load outer arm dynein onto IFT trains, because oda9 cells do not load outer arm dynein onto their IFT trains yet regenerate flagella at nearly normal initial assembly rates. The reduction in IFT frequency may be due to an impairment in the way the partially restored IFT-B1 assemblies with IFT-B2, IFT-A, or the IFT motors; the way it interacts with IFT cargo; the way it docks on the transition fibers during IFT train assembly (Deane et al., 2001; Brown et al., 2015); or the way it interacts with the IFT quality control machinery that regulates entry of trains into the flagellum (Dentler, 2005; Craige et al., 2010). Future studies should be able to distinguish between these possibilities.

**MATERIALS AND METHODS**

**Cells and culture conditions**

*Chlamydomonas reinhardtii* strain 137c (nit1, nit2, mt+) from the Chlamydomonas Resource Center (University of Minnesota, St. Paul, MN) was used as a wild-type control. Additional strains used included oda4 (Okagaki and Kamiya, 1986); oda9 (Kamiya, 1988); oda16-1 (Ahmed and Mitchell, 2005); and ift46-1, 6R29, and Supift461 (Hou et al., 2007). Partially rescued strains were generated by cotransferring ift46-1 cells with linearized plasmid pSP124S (Lumbreras et al., 1998) and the truncated ift46 gene plasmid (see *Creation of DNA constructs for Chlamydomonas transformation*); one of these strains (YH6RT4) was renamed ift46-1 IFT46Δ105.

Cells were grown on a 14:10 h light:dark cycle in M (Sager and Granick, 1953) medium I altered to have 0.0022 M KH₂PO₄ and Δift46-1 IFT46.

Creation of DNA constructs for *Chlamydomonas* transformation

For cloning the ift46 allele from ift46-1, ift46-1 genomic DNA was cut by PstI and self-ligated. Primer pair IFT46-3 and IFT46-10 was used to amplify the region where exogeneous DNA was integrated during the insertional mutagenesis that created ift46-1. The PCR product was cloned and sequenced.

To clone the ift46 allele from Supift461, we used the Vectorette PCR system (Sigma-Aldrich, St. Louis, MO). Supift461 genomic DNA was cut by BamHI. Primer IFT46-34 was used in the first-round PCR, and primer IFT46-10 was used for the nested PCR. The nested PCR product was cloned and sequenced.

For cloning the 5’ end of the ift46 transcripts in Supift461, total RNA was extracted from Supift461 cells using TRIzol LS Reagent (Thermo Fisher Scientific, Waltham, MA). The 5’ RACE then was carried out on the total RNA according to the standard protocol (Anonymous, 2005) with some modifications. Specifically, the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA) was used to produce total cDNA from total RNA. Poly A was added to the end of the cDNAs. Primer IFT46-4 was then used together with the (dT)₁₇-adaptor primer to amplify the ift46 cDNA by PCR. The ift46 cDNA was further amplified by nested PCR using primers IFT46-26 and the adaptor primer. The PCR product was cloned and sequenced.

**Sequence analysis**

The bacterial promoter elements and the bacterial transcription factor binding site rpoD15 were identified using BPROM (prediction of bacterial promoters: http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gllndb). The eukaryotic polymerase II promoter was identified using the Neural Network Promoter Prediction tool (www.fruitfly.org/seq_tools/promoter.html). Putative transcription factor binding sites for snail were identified by means of the sequence analysis tool at http://consite.genereg.net/cgi-bin/consite.

**Creation of DNA constructs for *Chlamydomonas* transformation**

For generation of the truncated IFT46 gene for rescue experiments, a 1.4-kb NcoI fragment that contains the 5’ end of the IFT46 gene was removed from the plasmid that contains the 4.8-kb full-length IFT46 gene (Hou et al., 2007) and cloned into the NcoI site of the pGEM-T Easy Vector (Promega, Madison, WI). To mutate the 1.4-kb NcoI fragment, we used primers IFT46-30 (which encodes the first amino acid from the IFT46 gene and the neighboring upstream vector sequence) and IFT46-33 (which starts with sequence encoding IFT46 amino acid 106 and the following amino acids) to amplify the vector sequence and a portion of the 5’ end of the IFT46 gene that lacks the sequence encoding aa 2–105. The ends of the PCR products were polished by T4 polymerase and T4 kinase. The modified PCR product was self-ligated to yield circular plasmids and sequenced to verify that no additional mutations had been introduced into the coding region by the PCR. The truncated NcoI fragment (0.4 kb) was then inserted back into the IFT46 gene from which the 1.4-kb NcoI fragment had been removed to yield a construct that can express IFT46 aa 1 and 106–344.

**Transformation**

IFT46-1 cells were cotransformed with linearized plasmid pSP124S (obtained from the Chlamydomonas Resource Center: http://chlamycolletion.org/plasmid/psp124s-ble-cassette) and the construct that contains the truncated IFT46 gene using the glass-bead
method of Kindle (1990). The cells were then mixed in TAP medium (Gorman and Levine, 1965) containing 0.5% agar and layered over TAP-1.5% agar containing 10 μg/ml zeocin. Zeocin-resistant transfectants were picked and grown in 96-well plates in M medium.

Antibodies

The antibodies used are listed in Supplemental Table S2. For generation of an antibody to the IFT46 C-terminus, PCR primer pair IFT46-29 and IFT46-14 was used to amplify a 0.7-kb sequence encoding IFT46 aa 105–344 from a cloned IFT46 cDNA (Hou et al., 2007). The PCR product was digested with BamHI and inserted into the BamHI site of pMAL-cR1 (New England Biolabs, Beverly, MA). Expression of this construct in Escherichia coli produced a protein in which IFT46 aa 105–344 were fused to maltose-binding protein. The fusion protein was purified by amylose affinity chromatography, and antibodies were raised against the fusion protein in guinea pigs (Covance Research Products, Denver, PA).

Protein biochemistry

Preparation of Chlamydomonas whole-cell extracts, PAGE, and Western blotting were performed as described by Pazour et al. (1999). The signals on blots were detected using x-ray film or a FluorChem Q from Cell Biosciences (Santa Clara, CA). Image contrast was adjusted using Adobe Photoshop (Adobe Systems, San Jose, CA). Preparation of Chlamydomonas flagella and the further fractionation of the flagella into membrane-plus-matrix and axonemes were performed as described by Witman (1986), except that HMDEK buffer (30 mM HEPES, pH 7.4, 5 mM MgSO4, 1 mM dithiothreitol, 0.5 mM EGTA, 25 mM KCl) was used instead of HMDEKP buffer. The demembranated axonemal fraction was incubated with HMDEK buffer containing 10 mM ATP magnesium salt for 10 min on ice and then further fractionated into an ATP-soluble fraction and an ATP-insoluble axoneme fraction using the same cen trifugation condition as was used to prepare the whole axoneme fraction (Witman, 1986). Sucrose gradient analysis was as described by Hou et al. (2004). The outer dynein arm binding assay was performed as described by Ahmed et al. (2008).

Flagellar regeneration experiments

After deflagellation by pH shock (Lefebvre, 1995), cells were concentrated and resuspended in the original volume of fresh M medium without aeration. The concentration step and/or subsequent incubation without aeration are necessary to induce flagellar growth in ift46-1 IFT46Δ105 cells; pH shock alone does not accomplish this (unpublished data). Aliquots were removed at various times after pH shock and mixed with an equal volume of 2% glutaraldehyde to fix the cells. Cells were then imaged by phase-contrast optics and photographed using the microscope system described in the next subsection. ImageJ (http://rsweb.nih.gov/ij/) was used to measure 50 flagella per sample. One flagellum per cell was measured.

For determination of whether stress-induced flagellar assembly required protein synthesis, cycloheximide (final concentration 10 μg/ml) was added to the sample either immediately before or after cell concentration.

Immunofluorescence microscopy

Cells were fixed and stained by the alternate protocol of Cole et al. (1998) using Alexa Fluor 488– or 568–conjugated secondary antibodies (Molecular Probes, Eugene, OR); images were acquired with an AxioCam camera, AxioVision 3.1 software, and an Axioskop 2 plus microscope (Zeiss, Thornwood, NY). DIC imaging of IFT and the subsequent analysis of IFT were done as described by Craige et al. (2010).

Label-free quantitation by MS

Flagellar membrane-plus-matrix fractions from wild-type and ift46-1 IFT46Δ105 cells were further fractionated on 5–20% sucrose gradients (Hou et al., 2004). The peak fractions for IFT46/IFT46 Δ105 and IFT46 Δ105 were determined by Western blotting using anti-IFT46C antibody. The peak fractions were combined and concentrated using Amicon Ultra-4 centrifugal filters with 10,000 molecular weight cutoff (Millipore Corporation, Billerica, MA). Proteins were loaded on an SDS–polyacrylamide gel and run briefly for 3–5 min until the dye front was 2–3 mm into the gel. The gel regions containing protein were excised and analyzed three times by MS (Proteomics and Mass Spectrometry Facility, University of Massachusetts Medical School). The MS data were analyzed by Scaffold 3.6.2 software (Proteome Software, Portland, OR). Fold changes were calculated using the top three common precursor intensities with normalization.

ACKNOWLEDGMENTS

We thank D. Cole (University of Idaho), H. Qin (Texas A&M University), W. Sale (Emory University School of Medicine), M. Wirschell (University of Mississippi Medical Center), D. Mitchell (SUNY Upstate Medical University), and J. Rosenbaum (Yale University) for antibodies; B. Craige (University of Massachusetts Medical School) and J. Brown (Salem State University) for help with DIC microscopy; and J. Leszek at the University of Massachusetts Medical School Proteomics and Mass Spectrometry Facility for his expert assistance with MS. This work was supported by National Institutes of Health grants R37 GM030626 and R35 GM122574 to G.B.W. and by the Robert W. Booth Endowment at the University of Massachusetts Medical School (G.B.W.).

REFERENCES

Boldface names denote co–first authors.


Molecular Biology of the Cell

Supplemental Materials
Molecular Biology of the Cell

Hou and Witman
**Supplemental Materials**

**Table S1: List of primers**

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Table S2: List of antibodies

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M: mouse monoclonal; P: rabbit polyclonal; PG: guinea pig polyclonal
Figure S1: Transformants shown in Figure 3 are the products of independent transformation events. (A) Schematic drawings of IFT46 alleles in the indicated strains. Exons are shown as tall boxes. IFT46 exons (grey) are numbered and the polyadenylation site is shown as a circle. The exogenous NIT1 gene used to create ift46-1 is green, and the MRC1 sequence identified in
the Supift461 strain is red. Dashed lines indicate regions not sequenced. PstI sites and the resulting fragment sizes (if known) are marked; note that the start site for the cloned truncated ifi46 construct (marked by asterisks on both the wild-type and truncated IFT46 alleles) is downstream of the first PstI site in the wild-type allele. The positions of the primers used for confirmation by PCR (see panel C) are indicated by > and <. (B) Southern blot of the transformants. Genomic DNA was cut by PstI. Wild-type DNA yielded the predicted products of 1.5 kb and 1.9 kb (arrows). DNA of ifi46-1 yielded products of 2.3 kb and 4 kb (arrowheads) due to the NIT1 insertion. Supift461 has a band pattern different from either wild-type or ifi46-1 due to the MRC1 insertion. The genomic DNA for YH6RT6 was not cut, but DNA from all other rescued transformants yielded the 2.3- and 4-kb bands specific for ifi46-1. In addition, each yielded different unique bands, indicating that they were generated by different transformation events. (C) PCR confirmation that YH6RT4 contains the truncated IFT46 transgene. Upper panel shows that a 1.8-kb PCR product was obtained from wild-type DNA. Due to the insertions of the NIT1 gene and MRC1, no PCR product was obtained for ifi46-1 or Supift461. YH6RT4 DNA yielded only a band of 0.7 kb. All of these results were as predicted from the maps in panel A. No PCR product was obtained for the control (no DNA) sample. Lower panel shows that DNA was present in the wild-type, ifi46-1, Supift461, and YH6RT4 samples using primers to the mating-type plus specific gene FUS.
Figure S2: Independently generated strains expressing IFT46Δ106-344 have similar slow regeneration kinetics. \( \text{ift46-1 IFT46} \Delta 105 \) and YH6RT10 (Supplemental Figure S1B) were created by rescuing \( \text{ift46-1} \) cells with the truncated \( \text{IFT46} \) gene. 6R29 is a strain that was created by rescuing \( \text{ift46-1} \) cells with the full-length \( \text{IFT46} \) gene (Hou et al., 2007). Cells of these three strains and wild type (137c) were deflagellated by pH shock and then returned to normal pH to assemble flagella. Flagella lengths were determined at the indicated time points. Wild-type and 6R29 cells assemble flagella to full length within two hours whereas \( \text{ift46-1 IFT46} \Delta 105 \) and YH6RT10 cells assemble flagella much more slowly. One flagellum from each of 50 cells was measured for every data point. Error bars are standard deviations.
Figure S3: Regenerating and non-regenerating flagella of \textit{oda9} cells have similarly low levels of outer arm dynein. Figure 6C showed that cells of \textit{oda9}, a strain lacking outer dynein arms in its axonemes, has normal flagellar regeneration kinetics. However, to our knowledge, it has not previously been demonstrated that regenerating flagella of \textit{oda9} cells are free of outer dynein arm precursors that, although incompetent to bind to axonemes, might still attach to IFT trains as cargo. To investigate this, we examined the level of outer arm dynein in non-regenerating flagella of wild type (WT) and \textit{oda9} (\textit{oda9} non-regen), and in regenerating flagella of \textit{oda9} (\textit{oda9} regen). All samples contained an approximately equal number of flagella, as indicated by the tubulin control (right panel). The level of the dynein heavy chain in the non-regenerating \textit{oda9} flagella was less than 1/600 of that in the non-regenerating wild-type flagella, and was not increased during flagellar regeneration (left panel). This indicates that the IFT particles of the \textit{oda9} regenerating flagella are not loaded with large amounts of incompletely assembled outer arm dynein as a result of the regulatory mechanism that increases the amount of cargo bound to IFT particles during flagellar regeneration (Wren \textit{et al.}, 2013). Inasmuch as \textit{oda9} lacks the outer arm dynein intermediate chain IC1, the latter result also suggests that the intermediate chains mediate the attachment of outer arm dynein to the IFT-B—ODA16 complex, a possibility not further explored here. To compare the levels of dynein heavy chain in the samples, the wild-type sample was loaded with 1/600 the amount loaded for the \textit{oda9} samples (left panel only). For tubulin blotting, all samples were diluted 1/100.
References


