Somatic CRISPR–Cas9-induced mutations reveal roles of embryonically essential dynein chains in Caenorhabditis elegans cilia

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Cilia are microtubule-based structures that play diverse roles in motility, sensory perception, and signaling (Rosenbaum and Witman, 2002; Scholey, 2003; Eggenschwiler and Anderson, 2007; Ishikawa and Marshall, 2011). The assembly and maintenance of cilia require bidirectional intraflagellar transport (IFT), which ferries ciliary precursors bound to the IFT particle protein complex from the base of the ciliary axoneme to the tip (Rosenbaum and Witman, 2002; Scholey, 2003, 2013). Anterograde IFT is powered either by a single heterotrimeric kinesin-2 motor, which was first proposed in Chlamydomonas reinhardtii, or by the concerted action of a heterotrimeric kinesin-2 and a homodimeric OSM-3-kinesin in kinesin-2 family in Caenorhabditis elegans neuronal cilia (Rosenbaum and Witman, 2002; Scholey, 2003, 2013). Retrograde IFT is driven by a special cytoplasmic dynein that is proposed to recycle the anterograde motors, IFT particles, and breakdown products of ciliary turnover back to the cell body (Pazour et al., 1998, 1999; Porter et al., 1999; Signor et al., 1999; Rosenbaum and Witman, 2002; Scholey, 2003).

Cytoplasmic dyneins include cytoplasmic dynein 1 (DYN1) and the IFT-specific cytoplasmic dynein 2 (DYN2; Pfister et al., 2005). DYN1 consists of a heavy chain homodimer, intermediate chains, light intermediate chains, and light chain dimers of the LC8, Tctex1, and Roadblock protein families (Kardon and Vale, 2009). These accessory subunits contribute to the structural integrity, motor regulation, and cargo specificity of the dynein complex. Moreover, cytoplasmic dynein motor activity appears to be governed by the Lis1–NudEL complex and the dynactin complex (Kardon and Vale, 2009; Roberts et al., 2013). In contrast, the composition and regulation of IFT–dynein remain unclear. Biochemical studies in C. reinhardtii revealed that IFT–dynein contains the heavy chain DHC1b, intermediate chains FAP133 and FAP163, light intermediate chain D1bLIC, and light chain LC8 (Pazour et al., 1998, 1999; Porter et al., 1999; Hou et al., 2004; Rempol).
Figure 1. The somatic CRISPR–Cas9 technique generates conditional mutations in IFT components in C. elegans ciliated sensory neurons. (A) Schematic illustrating the use of somatic CRISPR–Cas9 for creating conditional mutants. (B) osm-3 and xbx-1 gene models. Exons are in blue, and arrows indicate sgRNA sequences corresponding to exons (Table S4). The NGG (PAM) sequences are denoted in red. (C) Representative gels showing the T7EI assay results for osm-3 and xbx-1. Indels are indicated at the bottom. (D, left) Schematic of cilia in WT and osm-3 animals. DS, distal segments; MS, middle segments;
et al., 2007; Patel-King et al., 2013). A recent study using a temperature-sensitive allele of DHC1b demonstrated that retrograde IFT regulates flagellar assembly and function but not maintenance (Engel et al., 2012). Genetic research in Caenorhabditis elegans revealed that mutations of che-3 (DHC1b homologue) or xbx-1 (D1bLIC) caused defects in retrograde IFT (Signor et al., 1999; Schaefer et al., 2003).

Identification of IFT–dynein components largely relied on biochemical fractionation and proteomic analysis of flagellar proteins in C. reinhardtii, and this strategy does not differentiate between the integral components and the cargo molecules of IFT–dynein, as axonemal dynein complexes share subunits with IFT and cytoplasmic dyneins (e.g., LC8; Pazour et al., 1998). Moreover, mass spectrometry may not be sensitive enough to detect IFT regulators present at substoichiometric concentrations. The C. elegans neuronal cilia provide a complementary system to study IFT–dynein using genetic and imaging approaches. However, the efficiency of RNAi in knocking down gene activity in C. elegans ciliated neurons has been very limited (Pastrana, 2010). Moreover, the use of RNAi may lead to variable penetrance of the phenotype due to the incompleteness of protein depletion, and may also be accompanied by off-target effects (Pastrana, 2010). Thus, new techniques that are efficient and robust are needed to access the role of the target gene in cilia.

The clustered regularly interspersed short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system has been recently demonstrated as a powerful tool for genome editing (for review see Hsu et al., 2014). The specificity of Cas9 nuclease is determined by a synthetic guide RNA (sgRNA) that base-pairs with a G(N)19NGG “protospacer” DNA sequence in the genome. Cas9 generates double-strand breaks, which are often inaccurately repaired by the nonhomologous end joining pathway, and may also be accompanied by off-target effects (Pastrana, 2010). Thus, new techniques that are efficient and robust are needed to access the role of the target gene in cilia.

Results and discussion

Generation of conditional mutants in ciliated neurons of C. elegans

We generated transgenic animals that specifically express Cas9 in ciliated neurons of C. elegans (Shen et al., 2014). Here, we first demonstrate that somatic CRISPR–Cas9 efficiently creates conditional mutations in ciliated neurons. Using this platform, we bypass the embryonic requirement of dynein-associated chains and investigate their roles in the construction of C. elegans neuronal cilia.
of osm-3 and xbx-1. By comparing mutation efficiencies and the penetrance of ciliary phenotypes between osm-3 and xbx-1, we noticed that the higher indel frequency (28% for osm-3, 10% for xbx-1) may correlate with the higher dye filling defect (15% for osm-3, 9% xbx-1; Fig. 1, C and F).

Next, we examined the defects in cilium structure and IFT in osm-3-sg and xbx-1-sg animals using the OSM-6::IFT52::GFP reporter, which undergoes biphasic IFT along the entire cilium (Fig. 1 D). The osm-3-sg animals lost their ciliary distal segments, and the cilium length was reduced from 8 µm to 4–5 µm (Fig. 1 D), phenocopying osm-3(p802) null mutants (Snow et al., 2004). We further performed live imaging analysis of IFT in osm-3-sg mutants and found that the IFT velocity was reduced from 0.77 ± 0.02 µm/s (mean ± SE, n = 213) in the WT animals to 0.58 ± 0.01 µm/s (n = 323) in the remaining middle segments, which is also comparable to the IFT speed in osm-3(p802) mutants (Fig. 1 G). In xbx-1-sg conditional mutants, OSM-6::GFP formed aggregates around the transition zone and no IFT was detected in the residual cilia (Fig. 1 D), which are similar to those observed in xbx-1(ok279) deletion mutants (Schafer et al., 2003). To extend our initial successes to other IFT components, we conditionally mutated the IFT–dynein heavy chain (che-3), IFT particle A (che-11), and IFT particle B (osm-1) components by using the heat-shock strategy (Signor et al., 1999; Scholey, 2003). We showed that these conditional mutants reproduced the defects in cilium structure caused by their corresponding null alleles, and that the Dyf penetrance ranged from 10% to 25% in four phasmid ciliated neurons (Fig. 1, F and H). Collectively, somatic CRISPR–Cas9 can be used to generate conditional mutations of IFT motors (osm-3, xbx-1, and che-3) and IFT particles (che-11 and osm-1).

The functions of cytoplasmic dynein components in ciliogenesis

The C. elegans genome encodes 14 dynein-associated chains (Hao et al., 2011), five of which are essential for embryonic development (WormBase), including the intermediate chain (DYCI-1), light intermediate chain (DLI-1), light chain LC8 (DLC-1), Tctex-type light chain (DYLT-3), and roadblock-type light chain (DYRB-1; Fig. 2 A). We first generated transgenic animals that expressed Cas9 under the control of the Phsp promoter and ubiquitously expressed sgRNAs that target one site for dylt-3 and dyrb-1 or two sites for dycl-1, dlc-1, and dli-1 (Fig. 2 A). Our T7EI assays detected the expected molecular lesions (Fig. 2 B). Although the heat-shock treatment did not alter the embryonic viability of WT embryos, the conditional mutant embryos exhibited embryonic lethality with penetrances ranging from 40% to 55% after heat shock (Fig. 2 C). These data indicated that we generated conditional mutants of embryonically essential dynein subunits.

Next, we used these conditional mutants to study the contribution of the dynein components to cilium formation. We first showed that 29% (dycl-1), 19% (dlc-1), and 18% (dli-1) of the conditional mutants failed to take up Dil, whereas <4% of dylt-3 or none of dyrb-1 conditional mutants developed the Dyf phenotype (Fig. 3 A). Consistently, we found that the cilium length was significantly reduced from 8.3 µm in WT animals to 4.4 µm (dycl-1), 4.9 µm (dlc-1), and 4.8 µm (dli-1) in the conditional mutants but was not altered in dylt-3-sg or dyrb-1-sg animals (Fig. 3, A and B). IFT cargo includes IFT particles and ciliary receptors such as a transient receptor potential vanilloid channel OSM-9 (Qin et al., 2005). We did not find that OSM-6::GFP or OSM-9::GFP moved in the remaining cilia of the dycl-1-sg and dlc-1-sg conditional mutants (Figs. 3 B and S3 A). These ciliary defects are similar to those detected in che-3– and xbx-1–null mutants. To further test whether DYCI-1 and DLC-1 function with IFT–dynein, we determined the cellular localization of GFP-tagged DYCI-1 and DLC-1. We showed that they were distributed along the cilia and underwent the same biphasic IFT as XBX-1 (Fig. 3 C and see Fig. 5 B). Furthermore, we genetically introduced XBX-1::YFP into the dycl-1-sg conditional mutant and GFP::DYCI-1 into the xbx-1(ok279) mutant. We found that XBX-1::YFP and GFP::DYCI-1 formed aggregates around the transitional zone and were not motile in cilia (Fig. 3, D and E). The interdependence of XBX-1 and DYCI-1 in IFT further indicated that they may function together with IFT–dynein.

We studied how DLI-1 contributes to ciliogenesis. We did not find that OSM-6::GFP formed aggregates within the cilia of dli-1-sg conditional mutants (Fig. 3 B), nor did we detect the ciliary localization of DLI-1::GFP in transgenic animals expressing the Pdli-1::dli-1::GFP or Pdyf-1::gfp::dli-1 reporter (Figs. 3 C and S2 A). Instead, we found that DLI-1::GFP was distributed along the dendrite in these neurons (Fig. S2 B) and that OSM-6::GFP formed aggregates in the dendrites of dli-1-sg conditional mutants (Fig. S2 C). To further examine the function of DLI-1 in the retrograde dendritic transport, we compared the transport velocities of OSM-6::GFP in the dendrites of WT and dli-1 conditional mutants. Consistent with the previous measurements (Signor et al., 1999), OSM-6::GFP moved along the dendrite at the anterograde speed of 0.75 ± 0.05 µm/s (n = 33) and at 0.90 ± 0.04 µm/s (n = 30) in WT animals. However, we did not detect the retrograde movement of OSM-6::GFP in the dendrites of the dli-1 conditional mutants, whereas we found that OSM-6::GFP moved at the anterograde speed of 0.68 ± 0.02 µm/s (n = 33) in these dendrites. These results indicate that DLI-1 indirectly contributes to cilium formation by regulating the retrograde dendritic transport.

Dynein subunits undergo diverse IFT

To understand the transport behavior of IFT–dynein subunits, we generated kymographs from time-lapse movies of IFT of GFP-tagged dynein subunits (Videos 1–4). We first quantified the transport frequency as the number of transport events per minute. OSM-6::GFP and XBX-1::YFP both showed a frequency of 16 events/min; however, DYCI-1, DLC-1, and DYLT-3 displayed frequencies of ~3, 9, and 6 events/min, respectively (Fig. 4 A). We next examined their turnaround sites. The majority of OSM-6 and XBX-1 turned around at the distal tip, whereas DYCI-1, DLC-1, and DYLT-3 returned at the middle and distal segments (Fig. 4, B and C). 76% of the GFP::DYCI-1 puncta returned along the distal segment and very few reached the distal tip, and 24% of DYCI-1 turned around at the tip of the middle segment. 60% of the DLC-1::GFP puncta returned at the distal segment, whereas 40% of DLC-1 returned at the middle tip. Finally, 69% of the DYLT-3::GFP puncta returned...
in the middle segment, not even reaching the middle tip (Fig. 4, B and C). These data suggest that multiple retrograde IFT pathways may operate within a single cilium.

Lissencephaly-1 regulates retrograde IFT

We studied the function of lissencephaly-1 in cilium formation. Lis1 is thought to function as a “clutch” to suppress cytoplasmic dynein motility and to cause the dynein to tightly bind microtubules (Huang et al., 2012). We generated lis-1-sg conditional mutants that exhibited the expected molecular lesions and embryonic lethality (Fig. 2, B and C). In lis-1-sg animals, we found that 22% of worms failed to take up DiI and reduced the cilium length, and that OSM-6::GFP did not move but rather aggregated along the residual cilia (Fig. 3, A and B), which indicates that the function of LIS-1 is similar to that of CHE-3 and XBX-1 in retrograde IFT. We then visualized GFP-tagged LIS-1 in ciliated neurons and showed that GFP::LIS-1 entered the middle segment of cilia and occasionally moved into the distal segment (Fig. 5, A and B). We detected sparse and biphasic IFT of GFP::LIS-1 puncta (Fig. 5, A and B). Thus, LIS-1 undergoes IFT and regulates retrograde IFT.
DYRB-1 are dispensable for ciliogenesis (Fig. 5, A and B). These findings suggest that cytoplasmic dynein and IFT–dynein have unique compositions but share essential components and regulatory mechanisms.

In conclusion, this study demonstrated that embryonically essential dynein-associated chains behave distinctly in *C. elegans* neuronal cilia: the intermediate chain DYCI-1, the light chain LC8/DLC-1, and the dynein regulator LIS-1 undergo bi-phasic IFT and are essential for retrograde IFT; the light intermediate chain DLI-1 regulates the dendritic transport of IFT particles; the light chain Tctex-type DYLT-3 and roadblock-type DYT-1 are dispensable for ciliogenesis (Fig. 5, A and B). These findings suggest that cytoplasmic dynein and IFT–dynein have unique compositions but share essential components and regulatory mechanisms.

To visualize IFT of dynein subunits, this work relied on the GFP fusion technique, a standard protocol that has generated reliable results including ones related to IFT–dynein.
rescue experiments to examine whether the overexpression of GFP-tagged dynein subunits could rescue ciliary phenotypes in the corresponding conditional mutants. We studied *dyci-1* and *dlc-1* as they regulate ciliogenesis. In these experiments, we first made all the possible synonymous mutations in the target sites of transgenes such that they would not be cleaved by CRISPR–Cas9 (Fig. S3 B). We transformed GFP-tagged *mdyci-1* (m for synonymous mutation) and *mdlc-1* into conditional mutants, and we did not detect any Dyf phenotype after (Mello et al., 1991; Signor et al., 1999; Schafer et al., 2003). Despite the fact that we injected DNA with a low concentration (~10 ng/µl), below which the GFP fluorescence is invisible, and that we did not notice any ciliary phenotype in these animals, this method can cause the overexpression of the transgene. Dynein is a holoenzyme complex with a strict stoichiometry, and the overexpression of subunits or GFP tagging could result in artifacts because some complexes would be disrupted or not fully assembled. We thus performed the

\[ \text{Frequency} = \frac{\text{Number of turns}}{\text{Total length}} \]

\[ \text{Turnaround} = \frac{\text{Number of turnaround events}}{\text{Total number of turns}} \]

**Figure 4.** Transport behaviors of dynein subunits in cilia. (A) IFT frequencies of IFT particle and dynein subunits. \( n = 64–212; \) mean ± SE (error bars). ***, \( P \leq 0.001. \) (B) Kymographs (left) of DYCI-1, DLC-1, DYLT-3, and XBX-1 in the middle (M) or distal segments (D). \( M' \) and \( D' \) indicate turnaround events. M and D (right) are 4x enlarged images of turnarounds in the green boxes on the left. Horizontal bar, 2 µm; vertical bar, 5 s. (C) Distribution of turnaround events along the cilia. The y axis indicates the percentage of turnaround events at the specific region of cilia among the total turnaround events. \( n = 58–140. \) The broken line shows the junction between the middle and distal segments.
subunits can be specific (Fig. 3 C). It remains unknown why DL1-1 only functions within the dendrites but does not enter cilia. The emerging evidence suggests that a selective ciliary gate may regulate protein trafficking between cilia and the general cytoplasm (Ishikawa and Marshall, 2011), and DL1-1 may not be able to pass such a gate at the base of cilia.

Our results with IFT–dynein are consistent with biochemical data from other organisms, and also provide new insights. For example, LC8/DLC-1 was proposed as an essential component for retrograde IFT from *C. reinhardtii* (Pazour et al., 1998). However, LC8 can be the subunit or cargo—or both—as it is a known component in axonemal dyneins. Our study from nonmotile cilia enables us to conclude that LC8 can be an IFT–dynein subunit. Similarly, a recent study used the biochemical approach to study IFT–dynein composition in vertebrates, and their data are also largely complementary to ours.

The GFP-tagged dynein subunits undergo IFT at the same speeds as other IFT motors (Fig. 5 B), which suggests that they are probably associating with the dynein complex. We did not detect DL1-1::GFP in the cilia using the same expression strategy, suggesting that the ciliary location of GFP-tagged dynein subunits can be specific (Fig. 3 C). It remains unknown why DL1-1 only functions within the dendrites but does not enter cilia. The emerging evidence suggests that a selective ciliary gate may regulate protein trafficking between cilia and the general cytoplasm (Ishikawa and Marshall, 2011), and DL1-1 may not be able to pass such a gate at the base of cilia.

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components, and these conditional mutants are also valuable focuses on the ciliary roles of embryonically essential dynein. The present study change the direction of motion, and single molecule biophysics in the functional analysis of embryonically essential dynein subunits.

In summary, the somatic CRISPR–Cas9 technique enables the functional analysis of embryonically essential dynein subunits in *C. elegans* ciliated neurons. By increasing the length of the 5′ UTR regulatory sequence, we detected the expression of *dyci-1* in *C. elegans* ciliated neurons (Hao et al., 2011). By increasing the length of the 5′ UTR regulatory sequence, we detected the expression of *Dyci-1::gfp* in ciliated neurons (Fig. 3 C). Consistently, biochemical studies identified FAP133/DYCI-1 as an IFT–dynein component (Rompolas et al., 2007), and RNAi experiments in *Trypanosoma brucei* showed that DIC5/DYCI-1 regulates retrograde IFT (Blisnick et al., 2014). Moreover, we showed that DLYT-3 and DYRB-1 do not regulate ciliogenesis (Fig. 3, A and B). Dynein-associated chains may complement each other in cilium formation, and the generation of double or multiple conditional mutations by somatic CRISPR–Cas9 may dissect their redundant roles in ciliogenesis. Interestingly, the single or double genetic deletions of *C. elegans* Tetex-type light chain DLYT-1 or DLYT-2 do not cause defects in cilium assembly, even though they undergo biphasic IFT (Hao et al., 2011). We cannot exclude the possibility that these dynein light chains regulate ciliogenesis, but they may also likely modulate currently unidentified physiological events in cilia.

Lis1 has been found to induce slow or nonmoving dynein (Huang et al., 2012). We showed that GFP::LIS-1 is distributed in the middle segment (Fig. 5 A), where it may inactivate IFT–dynein during anterograde IFT. We observed biphasic but infrequent IFT of LIS-1 in *C. elegans* (Fig. 5, A and B), which is consistent with the brief motility of Lis1 in *Aspergillus nidulans* (Egan et al., 2012). Because limited GFP::LIS-1 enters the ciliary distal segment where retrograde IFT commences, IFT–dynein motility may also require the detachment of LIS-1, and a substoichiometric amount of LIS-1 at the distal tip may initiate IFT. In Lis1 mutants, cytoplasmic dynein and its cargo move at normal speeds with reduced frequencies in *A. nidulans* (Egan et al., 2012; Huang et al., 2012). However, we did not observe retrograde IFT in the *lis-1*-*sg* conditional mutants, which suggests that LIS-1 may be more critical for the motility of IFT–dynein. Studies from mammalian motile cilia and *C. reinhardtii* flagella reported that Lis1 regulates the activity of the outer dynein arm (Pedersen et al., 2007), indicating that Lis1 plays manifold roles in the regulation of cytoplasmic dynein, IFT–dynein, and axonemal dynein.

In summary, the somatic CRISPR–Cas9 technique enables the functional analysis of embryonically essential dynein subunits in *C. elegans* ciliogenesis. These dynein-associated molecules may regulate the motor processivity or function as a switch to change the direction of motion, and single molecule biophysics studies should be pursued in the future to better understand the function of these dynein-associated chains. The present study focuses on the ciliary roles of embryonically essential dynein components, and these conditional mutants are also valuable resources for the study of other dynein-based processes. This method is also amenable to investigations of the ciliary function of other essential embryonic genes as well as the formation and function of other organelles.
lists the target sequence of each gene. Table S5 gives the primers for molecular analysis. Videos 1–4 (related to Fig. 4) show the IFT movements of embryonically essential dynein subunits in cilia (GFP::DYCI-1, DLC-1::GFP, DYL1-3::GFP, and GFP::LIS-1). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201411041/DC1.

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