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Region-specific Loss of Two-headed Ciliary Dyneins in Ascidian Endostyle

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A mucous secreting organ in ascidians, the endostyle, consists of several epithelial zones with different ciliary length, density, and beating direction. Here we found by transmission electron microscopy that long cilia in endostyle zone 1 showed 9+2 axonemal structures but completely lacked the outer arm dynein. In contrast, cilia in other zones bore both outer and inner dynein arms. Western blotting and immunofluorescence microscopy further revealed that zone 1 appeared to lack not only outer arm dynein but also two-headed inner arm dynein. These results suggest a mechanism for a region-specific gene suppression that causes the limited loss of two-headed axonemal dyneins in the endostyle epithelium. The loss of these dyneins in zone 1 is considered to contribute to the generation of undulating ciliary movement that is essential for a unique circuit of mucus flow in the endostyle.

Key words: cilia, flagella, dynein, ciliogenesis, *Ciona*, endostyle

INTRODUCTION

Cilia and flagella are microtubule-based organelles with conserved internal 9+2 cytoskeletal structures, called the axonemes (Gibbons, 1981; Inaba, 2003; Konno et al., 2012). Motile cilia and flagella drive the generation of extracellular fluid flow, such as that in cell locomotion, epithelial mucociliary clearance, and vertebrate left-right symmetry breaking event. The motility is generated by two projections on the nine outer doublet microtubules, the outer and inner arm dyneins, which are responsible for the sliding of adjacent doublet microtubules in the axonemes. The outer and inner arm dyneins mainly play roles in the increase of beat frequency and in the formation and propagation of basic ciliary or flagellar bend, respectively. Loss of dyneins causes human diseases affecting multiple organs, called primary ciliary dyskinesia (PCD), Kartagener syndrome or immotile ciliary syndrome (Afzelius, 1976; Satir and Christensen, 2008).

Metazoan cilia and flagella show structural diversity. The 9+2 motile cilia are considered to be ancient forms of immotile cilia, which might have lost the structures for motility during ciliary diversifications (Mitchell, 2007; Inaba, 2015). For example, sea urchin swimming blastula possess long cilia on the animal pole, called the apical tuft. These cilia show less motility and appear specialized for mechanical reception with apparently normal 9+2 structures (Jin et al., 2013). In ascidian larvae, the structures of cilia show more diversity than those of sea urchins. They have not only completely motile cilia in the neural tube but also many diverse forms of immotile cilia in the epithelia and sensory vesicle (Konno et al., 2010). Completely immotile cilia lack the dynein arms or the central two singlet microtubules as well in some cases. These cilia are specialized for cellular antennas that sense extracellular stimuli and convert them into an intracellular signaling pathway, as seen in primary cilia or sensory cilia (Marshall and Nonaka, 2006).

The endostyle of adult ascidians is considered to be an ancient organ homologous to the vertebrate thyroid and plays a critical role in the transport of mucus to the branchial basket (Fujita and Nanba, 1971; Thorndyke, 1978; Holley, 1986; Burighel and Cloney, 1997; Sasaki et al., 2003). The ciliary types and their distribution in the endostyle epithelium are well organized in order to drive fluid flow for efficient transport of secreted mucus to the branchial basket (Fujita and Nanba, 1971; Pennachetti, 1984; Holley, 1986; Burighel and Cloney, 1997; Petersen, 2007). Here, we show the structural diversity of ciliary axonemes in endostyle of the ascidian *Ciona intestinalis*. We found that the zone 1 of endostyle epithelia with extremely long cilia lacked both outer and inner arm two-headed dyneins. To our knowledge, this is the first work that reports such cilia in nature.

MATERIALS AND METHODS

Preparation and observation of endostyle

Adults of *Ciona intestinalis* (recently renamed as *Ciona intestinalis* type A or *Ciona robusta*) were collected at Onagawa Bay near the Education and Research Center of Marine Bio-Resources, Tohoku University, and in part supplied by the Department of Zoology, Kyoto University and by Misaki Marine Biological Station, University of Tokyo through National Bio-Resource Project (NBRP), Japan. A closely related species, *Ciona savignyi*, was collected in Otsuchi Bay and used for supplementary use for observation of the endostyle structure. Endostyles were cut out from the

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branchial basket and transferred to a petri dish filled with filtered seawater. They were further trimmed and divided into small pieces for observation under a differential interference contrast microscope (Olympus BX53). Images and movies were recorded using a DF74 (Olympus) or D3 high-speed (DITECT) camera with a 10× or 100× objective for zone 1 or 5 cilia, respectively, and analyzed using ImageJ.

Transmission electron microscopy (TEM)

The endostyles were dissected out and fixed with the fixative (2.5% glutaraldehyde, 0.45 M sucrose, 0.1 M sodium cacodylate, pH 7.4) for 2 h at 4°C. The subsequent steps were conducted according to the method described previously (Konno et al., 2010).

Analysis of dynein arm structures

To obtain the average images of dyneins on each doublet microtubule, TEM images of cross-sectioned cilia were corrected and transformed by rotation according to the method described by Gadelha et al. (2006).

Preparation of endostyle cilia

Excised endostyles were rinsed several times with filtered sea water and transferred to an ice-cold hypertonic solution containing 1.5 M NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 20 mM Tris-HCl, pH 8.0 (Konno et al., 2015). They were kept for 2 min under gentle agitation for deciliation. After the tissues were removed, the suspension was briefly centrifuged at 500 g for 3 min to precipitate the tissue debris. The supernatant containing endostyle cilia was used for immunostaining. For biochemical analysis, the supernatant was further centrifuged at 8000 g for 10 min at 4°C to recover cilia as a pellet. The pellet was rinsed twice with filtered sea water and used for SDS-PAGE and subsequent immunoblotting.

Preparation of sperm flagella

Sperm were collected from the sperm duct of an adult by dissection. The flagella were isolated by homogenization and centrifugation, according to the method described previously (Padma et al., 2003).

Antibodies

Primary antibodies used in this study were those against outer arm IC2 and IC3 (Hozumi et al., 2006; Padma et al., 2001); outer arm LC1 (LRR) and LC2 (Tctex2) (Hozumi et al., 2006; Padma et al., 2001); PKA-R (Hozumi et al., 2008); calaxin (Mizuno et al., 2009); Ap58 (Ogawa and Inaba, 2006); inner arm IC116 (Hozumi et al., 2008); RSP3 (Padma et al., 2003); HSP40 (Satouh et al., 2005); LRR37 (Padma et al., 2001); MORN40 (Satouh et al., 2005); PF16 (Ci-Spag6; Satouh and Inaba, 2009); inner arm dynein IC105 (ortholog of *Chlamydomonas* f/I1 dynein IC97; Wirschell et al., 2009). All antibodies were prepared according to the method of Padma et al. (2003). Anti-acetylated α-tubulin (Lys40) rabbit monoclonal antibody (RM318) was purchased from Invitrogen Corp. Anti-centrin mouse monoclonal antibody (clone 20H5) was purchased from Sigma-Aldrich Co., Ltd. For secondary antibody, goat anti-mouse IgG (H+L)-HRP (Invitrogen Corp.) was used for western blotting. Alexa 488-conjugated anti-rabbit IgG antibody and Alexa 546-anti-mouse IgG antibody were purchased from Molecular Probes and used as secondary antibodies for immunofluorescent labeling (1:1000 dilution).

Electrophoresis and immunoblotting

SDS-PAGE was performed according to the method described by Laemmli (1970) with 10% polyacrylamide as the separating gel. Proteins were transferred onto a polyvinylidene fluoride membrane and subjected to western blotting according to the method previously described (Padma et al., 2003). The blots were developed using the ECL® (enhanced chemiluminescence)-plus detection system (Amersham Biosciences Inc.).

Immunofluorescence microscopy

Cilia detached from endostyle epithelia were attached on a poly-L-lysine-coated glass slide and fixed with cold methanol at –30°C for 10 min. After air-drying, the samples were rehydrated by PBS and incubated in 10% goat serum in PBS in a moist chamber for 1 hr. Immunostaining was performed using a previously described method (Hozumi et al., 2008) and observed using an Olympus BX53 fluorescent microscope with a ×20 objective.

RESULTS

The endostyle is a gutter-shaped organ running along the whole ventral side of a branchial sac and is composed of nine parallelly running regions or "zones". All regions but zones 7 and 9 are ciliated. The ciliary length, density, or beating directions are different among zones (Fig. 1A). Among them, zone 1 runs in the midline and forms a tuft of quite long cilia with the lengths reaching over 500 μm (448 ± 68 μm, n = 18). Typical cilia of zone 1 in the tuft showed very slow undulating or irregular movement with small amplitudes (less than 10 μm), with beat frequency of 0.86 ± 0.38 Hz (n = 12) (Fig. 1B; see Supplementary Movie S1). Atypical movements with larger amplitudes and shorter wavelengths were occasionally observed in the ciliary tuft when the endostyle was flattened and zone 1 cilia were spread laterally between the glass slide and the coverslip (see Supplementary Movie S2).

On the other hand, the cilia in other zones showed typical ciliary movements with high beat frequency. For example, the middle region of an endostyle, zone 5, possessed short cilia that actively beat (see Supplementary Movie S3). The ciliary length and the beat frequency of zone 5 cilia were estimated as 8.7 ± 0.8 μm (n = 16) and 6.1 ± 1.5 Hz (n = 12), respectively. Although several TEM observations have been done in the endostyle (Burighel and Cloney, 1997), the ciliary ultrastructure has not been reported in detail.

To determine the ciliary structures in different zones of the endostyle in more detail, we examined the structures of zone 1 cilia and compared them with those in zone 5, which showed typical ciliary beating and was the best region to easily prepare TEM samples for analysis. Cilia from both zones were multiciliated (Fig. 1C and 1D). Close observations of the axonemal structures revealed that cilia of zone 1 had 9+2 structures without the outer dynein arms (Fig. 1E), whereas those in zone 5 showed normal motile 9+2 structures with both outer and inner dynein arms (Fig. 1F).

To confirm biochemically if the cilia of zone 1 lacked the outer arm dyneins and other axonemal components, we carried out western blotting of whole endostyle cilia with antibodies against the subunits of outer arm dynein, as well as those against other axonemal components (Fig. 2). We compared the blot patterns of endostyle cilia with those of sperm flagella, which have complete sets of outer and inner arm dyneins (Padma et al., 2001, 2003; Hozumi et al., 2006, 2008; Satouh et al., 2005; Satouh and Inaba, 2009). We expected that the immunoblots reflected the components of zone 1 cilia because their long cilia occupied a substantial portion of whole endostyle cilia. In fact, the ratio of the number of zone 1 long cilia to the sum of short cilia in other zones would be 8:100, based on the schematic distribution of cilia in an endostyle (Burighel and Cloney, 1997). If we suppose

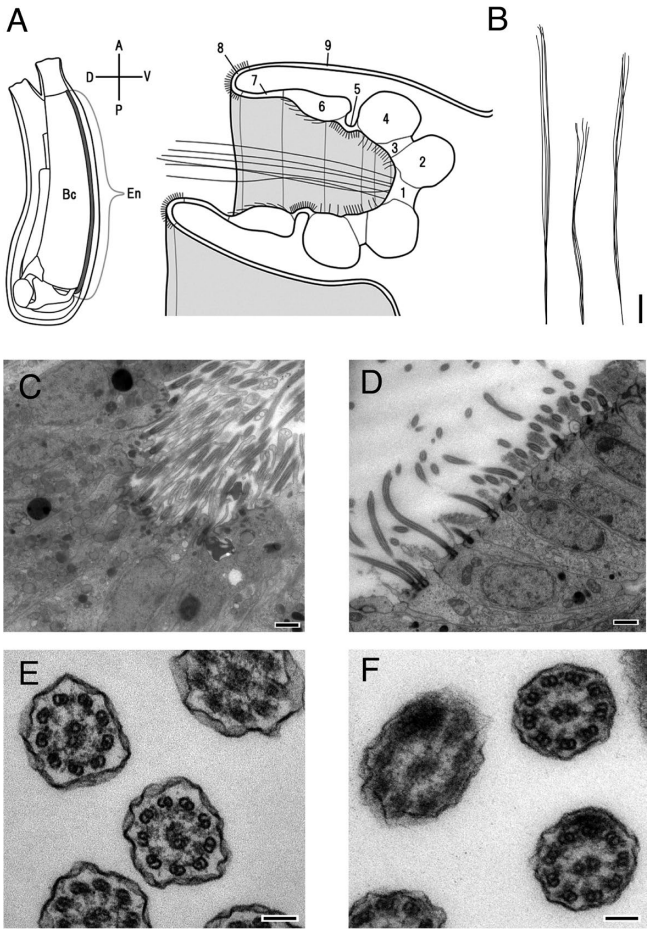

Fig. 1. Cilia in the zone 1 of an endostyle lacking outer arm dyneins. **(A)** Left, a schematic drawing of adult *C. intestinalis*. The body axes are shown; ventral (V), dorsal (D), anterior (A), posterior (P). Traditionally a branchial side is treated as ventral. Endostyle runs along the entire ventral midline of the branchial sac. Bc, branchial sac; En, endostyle. Right, a schematic drawing of a cross section of the endostyle. It is composed of nine distinct zones. **(B)** Movements of typical cilia in zone 1. Five sequential images with 50 msec intervals are overwritten. Scale bar, 50 μm. **(C)** An electron micrograph of zone 1. Lower magnification. Scale bar, 500 nm. **(D)** An electron micrograph of zone 5. Lower magnification. Scale bar, 500 nm. **(E)** A cross section of long cilia found in zone 1, showing 9+2 structure without outer dynein arms. Scale bar, 100 nm. **(F)** A cross section of short cilia found in zone 5, showing 9+2 structure with both dynein arms. Scale bar, 100 nm.

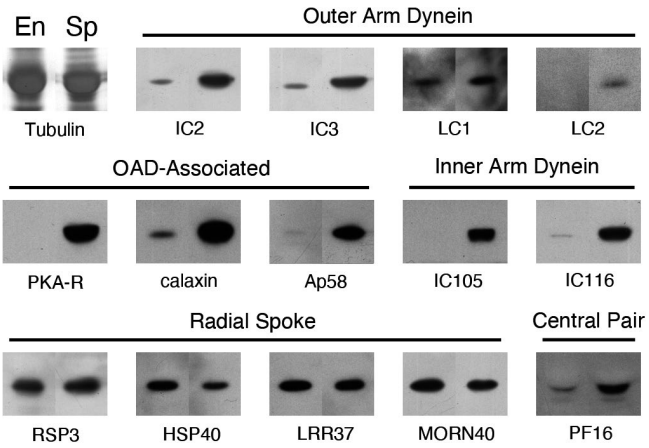

Fig. 2. Western blot analysis of axonemal components in sperm flagella and endostyle cilia. Coomassie Brilliant Blue R250 stained tubulin bands are shown as a loading control. The proteins were detected in endostyle cilia (left lanes) and sperm flagella (right lanes) using primary antibodies as follows: outer arm dynein intermediate chains IC2 and IC3; outer arm dynein light chains LC1 and LC2; the regulatory subunit of cAMP-dependent protein kinase (PKA-R); outer arm dynein associated calcium sensor calaxin; outer arm dynein associated protein Ap58; intermediate chains of two-headed inner arm dynein IC105 and IC116; radial spoke protein RSP3, heat shock protein HSP40, leucine-rich repeat protein LRR37; MORN repeat protein MORN40; central apparatus protein PF16 (Ci-Spag6). En, endostyle cilia; Sp, sperm flagella.

that the ciliary length in zone 1 is 500 μm and that in other zones is 10 μm, the ratio of ciliary amount would be 4000:1000. This demonstrates that zone 1 cilia contribute to the total amount of ciliary protein in the endostyle significantly enough to detect the lack of an axonemal protein by western blotting.

Regarding the components of outer arm dynein, two intermediate chains (IC2 and IC3) and two light chains (a leucine-rich repeat light chain, LC1, and a Tctex2-related light chain, LC2; Inaba, 2011) showed significant decreases in endostyle cilia, compared to those in sperm flagella (Fig. 2). Interestingly, LC1 exceptionally showed signal with similar levels between endostyle cilia and sperm flagella. In addition, three proteins that are known to be associated with the outer arm dynein were also tested: i.e., the regulatory subunit of type II cAMP-dependent protein kinase (PKA-R; Nomura et al., 1999; Itoh et al., 2000; Hozumi et al., 2008), calaxin (Mizuno et al., 2009, 2012), and Ap58 (Ogawa and Inaba, 2006). All of these proteins showed no or only weak signals in endostyle cilia (Fig. 2).

Next, we examined two components of a two-headed inner arm dynein (f/I1 dynein), IC105 (ortholog of *Chlamydomonas* f/I1 dynein IC97; Wirschell et al., 2009) and IC116 (ortholog of *Chlamydomonas* IC140; Hozumi et al., 2008); four components of radial spokes, RSP3 (Padma et al., 2003), HSP40 (Satouh et al., 2005), LRR37 (Padma et al., 2003), and MORN40 (Satouh et al., 2005), and a central apparatus component, PF16 (Satouh and Inaba, 2009) (Fig. 2). All four components of radial spokes showed similar signals in western blotting between endostyle cilia and sperm flagella. However, the signal of PF16 was significantly decreased in endostyle cilia. We particularly focused on the fact that two components of f/I1 dynein, IC105 and IC116, were included in the endostyle cilia at significantly lower levels. This suggests that f/I1 dynein, as well as the outer arm dynein, is missing in endostyle zone 1.

To examine the structure of the inner arm dyneins in zone 1, we further analyzed TEM images of dynein by averaging (Gardner et al., 1994; Gadelha et al., 2006). The averaged images clearly showed the lack of outer arm dynein in zone 1 but also the difference in the structure of inner arm dyneins (Fig. 3A and 3B). In zone 5 cilia, the inner arm dynein was observed as two projections toward the adjacent B-tubule Fig. 3C), as reported previously (Kamiya et al., 1992). The axoneme of zone 1 cilia lacked the outer part of the projections (Fig. 3D; dotted circle in Fig. 3C), which cor-

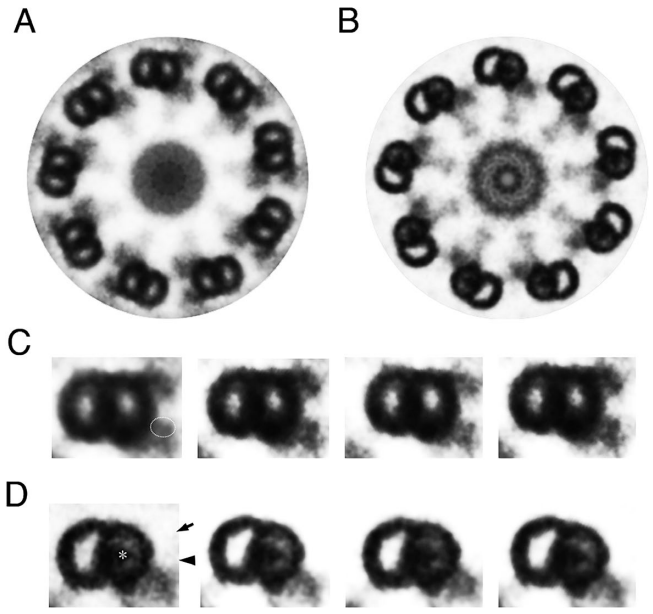

Fig. 3. Nine-fold averaged images of axonemal structures in zones 1 and 5. The images were obtained by rotational averaging of two high-contrast, cross-sectioned TEM images of zone 1 and zone 5 cilia. **(A)** An averaged image of zone 5 cilia. **(B)** An averaged image of zone 1 cilia. **(C)** Magnified four images of a doublet microtubule in zone 5. **(D)** Magnified four images of a doublet microtubule in zone 1. The zone 1 cilia lack the outer dynein arm (arrow) and the outer part of inner dynein arm (arrowhead). A dashed circle in **(C)** (left panel) indicates the outer part of inner dynein arm in zone 5 cilia that is missing in zone 1. The asterisk in **(D)** (left panel) indicates the electron-dense structure in the inside lumen of A-tubule.

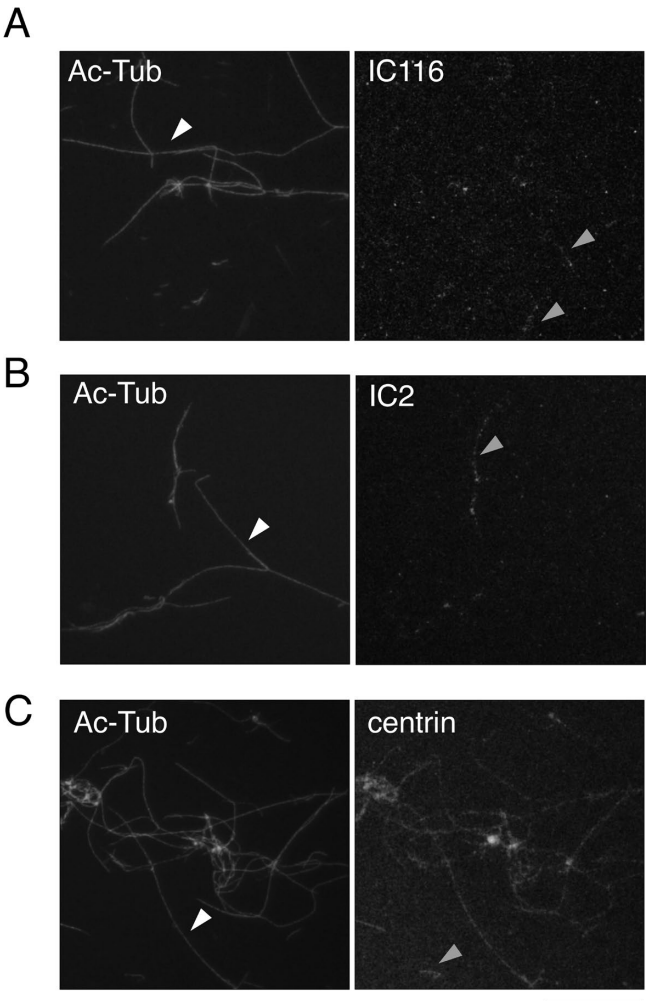

Fig. 4. Immunofluorescence microscopy of isolated cilia. **(A)** Double staining by antibodies against acetylated α-tubulin and two-headed inner arm dynein IC116. **(B)** Double staining by antibodies against acetylated α-tubulin and outer arm dynein IC2. **(C)** Double staining by antibodies against acetylated α-tubulin and centrin, a component of single-headed inner arm dyneins. White and gray arrowheads show the long cilia stained by acetylated α-tubulin antibody and the short cilia stained by antibodies against dynein components, respectively. Scale bar, 50 μm.

responds to the two-headed f/I1 dynein (Kamiya et al., 1992). This result supports the data of western blotting that suggest the absence of f/I1 in zone 1 cilia (Fig. 2). In addition, an electron dense structure was seen inside the lumen of the A-tubule in zone 1 cilia (white asterisk in Fig. 3D). Immunofluorescence microscopy further showed that an intermediate chain of f/I1 inner arm dynein, IC116, was only detected in short cilia but not in long cilia (Fig. 4A). Outer arm dynein IC2 was not also detected in long cilia, supporting the idea that both outer and inner arm two-headed dyneins are missing in the zone 1 long cilia (Fig. 4B). In contrast, centrin, a component of some single-headed inner arm dyneins, was detected in both long and short cilia from the endostyle (Fig. 4C).

DISCUSSION

The most intriguing finding in this study is that zone 1 cilia lack both outer and inner arm two-headed dyneins. This unique property in the axonemal structure is thought to be linked to the production of undulating motility by these long cilia. The outer arm dynein is known to contribute to the increase of beat frequency (Gibbons and Gibbons, 1979; Brokaw and Kamiya, 1987), whereas the f/I1 inner arm dynein is essential for beating in the axonemes lacking outer arm dynein or single-headed inner arm dyneins (Kamiya and Yagi, 2014). In *Chlamydomonas*, double mutants lacking two of three types of dyneins, i.e., outer arm, inner arm, and single-headed inner arm dyneins, are completely non-motile under physiological conditions (Kamiya, 1995; Kamiya and Yagi, 2014).

However, zone 1 cilia in *Ciona* endostyle show undulating motility, though they lack both outer and inner arm two-headed dyneins and appear to possess only single-headed inner arm dyneins. It is known that apparent non-motile double mutants in *Chlamydomonas* become motile under non-physiological conditions, such as high concentrations of salt or organic compounds (Yagi and Kamiya, 2000). The motility of zone 1 cilia would therefore be induced by some non-physiological factors, most likely the mechanical constraints by tight association in the tuft of zone 1 cilia. This might be supported by the fact that a part of cilia in the tuft change their motility when they are mechanically spread (Supplementary Movie S2).

Endostyle secretes the mucus that is essential for filter-

ing the sea water and trapping particles such as plankton. The mucus is transported from the endostyle through branchial epithelium to the dorsal lamina (Burighel and Cloney, 1997). The ciliary activity of endostyle epithelia is considered to be important not only for sending mucus out to the branchial basket but also for the formation of the flow circuit for mucus (Holley, 1986). The zone 1 epithelium spreads out long cilia along the midline. However, the cilia show less motility with occasional undulating movement and appear not to exert active fluid flow (Supplementary Movie S1). This poor motility of zone 1 cilia appears to make sense, because the cilia appear to create a fence that separates the mucus flow pathway in two ways along either side of the branchial basket (see Fig. 1B). The occasional undulation of the cilia may facilitate the mucus transport without disturbing the flow along the long cilia.

The radial spokes and central apparatus are involved in the activation of a subset of dynein to generate planar ciliary waves (Porter and Sale, 2000). Lack of these structures results in helical movements, as seen in the flagella of eel sperm (Gibbons et al., 1985) and Asian horseshoe crab (Ishijima et al., 1988). It is interesting that zone 1 cilia still keep the radial spokes and central apparatus, despite the fact that they only show occasional undulating movement. Further waveform analysis of zone 1 cilia might give a clue to know how single-headed inner arm dyneins are regulated by radial spokes and central apparatus and drive ciliary motility.

We found a structure in the inside lumen of the A-tubule in zone 1 cilia (Fig. 3). This structure appears to be one of the lumen proteins of doublet microtubules, called MIPs. A recent study suggested that MIPs directly affect the stability of doublet microtubules (Ichikawa et al., 2019). Although it is unknown why the structure is present in zone 1 cilia but not distinctly in zone 5 cilia, it is possible that the structure might stabilize the long axonemes or reinforce the mechanical weakness of doublet microtubules due to the lack of two-headed dyneins.

The loss of both the outer arm dynein and the two-headed inner arm dynein (IADs) in zone 1 cilia suggests that a common component is involved in the assembly of both dyneins. Dynein subunits, as well as other axonemal components, are assembled in the cytoplasm and transported into cilia by intraflagellar transport (Fowkes and Mitchell, 1998; Inaba and Mizuno, 2016). Several factors for cytoplasmic assembly of axonemal dyneins have been reported so far, including DNAAF1 (LRRC50/ODA7) (Duquesnoy et al., 2009), DNAAF2 (Ktu/PF13) (Omran et al., 2008) and DNAAF3 (PF22) (Loges et al., 2009; Mitchison et al., 2012), DYX1C1/DNAAF4 (Tarkar et al., 2013; Yamamoto et al., 2017), DNAAF5 (HEATR2) (Horani et al., 2012), LRRC6 and its associated assembly factor ZMYND10 (Zariwala et al., 2013), and SPAG1 (Knowles et al., 2010). In *Chlamydomonas*, two assembly factors for IADs are known. MOT48 (IDA10) contains a PIH domain and is involved in the assembly of IAD species b, c and d (Yamamoto et al., 2010). The other is the MIA complex, which is located at the distal part of IAD f/I1 and is involved in the assembly of this dynein (Yamamoto et al., 2013, 2017). However, none of these factors has been shown to be specifically involved in the assembly of two-headed dyneins. Thus, studies on the mechanism of how two-headed dyneins are lost in endostyle zone 1 would give a key clue for finding novel cytoplasmic assembly factors for two-headed axonemal dynein and for determining the mechanism of the region-specific gene regulation of these factors.

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COMPETING INTERESTS

The authors declare they have no competing interests.

AUTHOR CONTRIBUTIONS

All authors designed the research; AK performed electron microscopy and western blot analysis; KI performed video observation, immunofluorescent staining, TEM image analysis and western blotting; All authors performed data analysis and wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online. (URL: https://doi.org/10.2108/zs200095)

Supplementary Movie S1. Motility of zone 1 long cilia. The movie plays at real speed. Scale bar, 100 μm.

Supplementary Movie S2. Some cilia in zone 1 showing movement with larger amplitudes and shorter wavelengths. The movie plays at a speed of ×0.6. Scale bar, 50 μm.

Supplementary Movie S3. Motility of zone 5 cilia. The movie plays at real speed. Scale bar, 20 μm.

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