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Cilia loss and dynein assembly defects in planaria lacking an outer arm dynein docking complex subunit

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Abstract

The outer dynein arm docking complex (ODA-DC), which was originally found in the green alga *Chlamydomonas reinhardtii*, is a protein complex that mediates the binding of axonemal dynein and doublet microtubules. To understand the conservation and functional diversity of the ODA-DC, we knocked down a homolog of DC2, a major subunit of the ODA-DC, in the planarian *Schmidtea mediterranea*. Planaria are carnivorous flatworms that move by beating cilia on their ventral surface against a secreted mucus. These organisms have recently been used for cilia research because knockdown-flatworms can be easily obtained by RNAi through feeding with dsRNA. Lack of DC2 in *S. mediterranea* yielded several defects in cilia; low beat frequency, decreased ciliary density, and a reduction in ciliary length. The DC2-lacking *C. reinhardtii* mutant *oda1* shows slow jerky swimming but has two flagella of almost normal length. These data suggest that the function of a DC2 homolog in *S. mediterranea* cilia may be somewhat different from DC2 in *C. reinhardtii* flagella.

Keywords

planaria; RNA interference; cilia; dynein; *Chlamydomonas*

Introduction

Motile cilia are thin organelles that project from the cell and generate force to power cell movement or fluid flow over tissue surfaces. Ciliary beating is generated by the sliding of doublet microtubules within the “9+2” structure of ciliary axonemes. This motion is

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Author contributions

TH, SMK and KW designed research; AK and RSP-K performed experiments and analyzed data. All authors wrote the paper.

Competing Interests

The authors declare they have no competing interests.

powered by the axonemal dynein motors. Axonemal dyneins can be classified into outer arm dyneins and inner arm dyneins, and each dynein has a distinct function in the generation of ciliary bending movement. The motor properties of these dyneins have been well studied using dynein-deficient mutants of the green alga *Chlamydomonas reinhardtii*. Outer arm dyneins are important for producing high beat frequency and propulsive force, while inner arm dyneins are important for generating large bend angles (Brokaw and Kamiya, 1987; Kamiya et al., 1991; Kato-Minoura et al., 1997).

Structure and composition of outer arm dyneins (OADs) are mostly conserved among organisms, but there are differences that reflect varying motor and regulatory requirements. In *C. reinhardtii*, OAD is a large protein complex (~2 MDa) containing three heavy chains, two intermediate chains, and 11 light chains (King, 2018). In metazoans, OADs contain only two heavy chains and also exhibit variation in the number and properties of some intermediate and light chains compared to *C. reinhardtii*.

The mechanism by which OAD docks to microtubules also differs between organisms. In *C. reinhardtii*, OAD binds to the axonemal doublet microtubules *via* the outer dynein arm docking complex (ODA-DC), which contains three subunits, DC1, DC2 and DC3 (Takada and Kamiya, 1994; Koutoulis et al., 1997; Wakabayashi et al., 2001; Takada et al., 2002; Casey et al., 2003). The ODA-DC cooperatively binds along the doublet microtubules and captures OADs *via* several distinct interactions (Ide et al., 2013; Owa et al., 2014). In vertebrates, two DC2 homologues CCDC63 and CCDC114 function in OAD docking with several other proteins including MNS1 and ARMC4 (Zhou et al., 2012; Hjeij et al., 2013; Onoufriadis et al., 2014). In addition, CCDC151, CCDC103 and c15orf26 have also been suggested to be involved in OAD docking (Panizzi et al., 2012; Austin-Tse et al., 2013; Hjeij et al., 2014; King and Patel-King, 2015). When the expression of these ODA-DC-related proteins is reduced in knock-out or knock-down experiments, OAD is not assembled on the doublet microtubules. Most mutations that result in primary ciliary dyskinesia, a human disease caused by dysfunction of motile cilia, lead to defects in the formation or assembly of OADs. Thus, it is important to clarify the molecular mechanism(s) of OAD docking to microtubules and how the ODA-DC proteins function in this process.

The planarian flatworm *Schmidtea mediterranea* has recently become a useful model organism to study the function of ciliary components and especially ciliary dyneins, because knock-down flatworms can be easily obtained by RNAi through feeding with dsRNA. Rompolas et al. observed the cilia of *S. mediterranea*, and found these flatworms move by beating cilia on their ventral surface against mucus secreted from goblet cells (Rompolas et al., 2010). When OAD subunits IC2 and LC1 were knocked down in this organism, in addition to defects in OAD assembly, metachronal synchrony of ciliary beating was lost. This study clearly showed that OAD functions in the coordination of ciliary beating potentially by a feedback mechanism that maintains hydrodynamic coupling.

In this study, to investigate if the ODA-DC is functionally conserved in *S. mediterranea*, we knocked down a DC2 homolog by RNAi. Intriguingly, in contrast to knocking-down OAD subunits (Rompolas et al., 2010), defects in DC2 expression caused a reduction in cilia number, shorter cilia, as well as lower beat frequency of the remaining organelles. As the

DC2-deficient *C. reinhardtii* mutant *oda1* shows a slow jerky swimming phenotype but has two flagella of almost normal length, these data suggest that the function of the ODA-DC differs significantly among species.

Materials and Methods

Animals and Culture Conditions

The hermaphroditic sexual strain (Zayas et al., 2005) of *S. mediterranea* was used in this study. Planaria were maintained using the method previously described (Rompolas et al., 2010). In brief, planaria were kept in a 1× solution of Montjuïc salts (1.6 mM NaCl, 1.0 mM CaCl₂, 1.0 mM MgSO₄, 0.1 mM MgCl₂, 0.1 mM KCl, 1.2 mM NaHCO₃) (Cebria and Newmark, 2005), in a dark incubator at room temperature. Animals were fed weekly with minced calf liver. The colony was expanded by cutting larger planaria into smaller pieces and allowing them to regenerate. Planaria were starved for one week before experiments.

DNA database and Cloning

Smed-dc2 gene sequence was identified using the *S. mediterranea* genome database (PlanMine: <http://planmine.mpi-cbg.de/planmine/begin.do>) (Rozanski et al., 2018) with the respective homologous *C. reinhardtii* flagellar outer dynein arm-docking complex protein 2 (DC2) sequence as the query. To prepare plasmids for RNAi, total planarian RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) and first-strand cDNA was made using AMV reverse transcriptase (New England Biolabs, Beverly, MA). To synthesize the vectors for RNAi, a fragment encoding 249 base pairs from the *Smed-dc2* (RNAi) cDNA open reading frames was amplified using the following primers: forward, GCGGGATCACCTAGAGAACG; reverse, GTGGCAGCGGATGAGTTTTTC. The cDNA sequence was inserted into the XbaI/XhoI sites of plasmid L4440 (Timmons and Fire, 1998), flanked by two opposing T7 RNA polymerase promoters that mediate the synthesis of dsRNA upon induction.

RNA interference

Inhibition of gene expression *via* double-stranded RNA (dsRNA)-mediated RNA-interference (RNAi) in *S. mediterranea* was carried out by the method described in Newmark et al., 2003. In brief, cDNA fragments of the target genes were inserted into the vector pL4440, and HT115 (DE3) *Escherichia coli* cells that lack RNase III were transformed with the resultant vector (Timmons et al., 2001). Expression of dsRNA in the bacterial culture was induced with 1 mM IPTG at 37°C for 2 h. A pellet of the *E. coli* culture was mixed with liver homogenate and red food dye which allows feeding to be monitored. Animals were allowed to feed on the food mix every 3 days for a total of four feedings. After 15–20 days, animals were sampled to assess mRNA levels of the targeted and control genes.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out by the method described in Rompolas et al., 2009. In brief, total planarian RNA was isolated using Trizol (Invitrogen) and was used to set up first-strand cDNA synthesis reactions with cloned AMV reverse transcriptase (New

England Biolabs). PCR amplification was carried out with essentially the same primers that were used to make the RNAi vectors. Both OAD IC2 and actin were employed as controls.

High-Speed Video Microscopy

High-speed video microscopy was carried out by the method previously described (Rompolas et al., 2010). In brief, the head region of live planaria was captured between a coverslip and microscope slide and observed with an Olympus BX51 microscope (Olympus America) using differential interference contrast optics and a Plan Apo 100×/1.35 NA oil objective lens. Videos were captured with an X-PRI F1 camera at 120 fps and processed using Virtualdub (www.virtualdub.org) and Image J (<http://rsbweb.nih.gov/ij/>; Dentler et al., 2009).

Gliding Assays

Gliding motion of planaria was recorded at 15 fps with a DFK 31BU03 color CCD camera (The Imaging Source, Charlotte, NC) using IC-capture version 2.2 software. Video segments were decompiled using Blaze Media Pro. The tracks of individual animals were revealed by overlaying every 50th frame using Photoshop CS4 (Adobe). The distance travelled by each animal was measured directly from the overlay.

Electron Microscopy

For transmission electron microscopy (TEM), planarians were fixed at room temperature in 1% glutaraldehyde in PBS, pH 7.2, for 15 min and then changed into 1% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, for 50 min. Samples were post-fixed with 1% osmium tetroxide, 0.8% KFe(CN)₆ in 50 mM sodium cacodylate at room temperature. Fixed samples were stained *en bloc* with 1% uranyl acetate, dehydrated, and embedded in Epon. Ultrathin sections were examined in a Hitachi H-7650 transmission electron microscope operating at 80 kV. For scanning electron microscopy (SEM), planarians were prefixed at room temperature with relaxant solution (1% HNO₃, 0.83% formaldehyde, 50 mM MgSO₄) for 16 h and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 (EM Science, Gibbstown, NJ) for 24 h at 4°C. Samples were post-fixed at room temperature with 1% osmium tetroxide, for 1 h, in the dark, and dehydrated through a graded ethanol series. Dehydrated flatworms were dried in Autosamdri-815, Series A critical point dryer (Tousimis Research, Rockville, MD) and mounted with carbon tape and colloidal silver paint. Samples were sputter-coated with gold using a Cressington 208 HR sputter coater (Ted Pella, Irvine, CA) before imaging with a JEOL JSM5900 scanning electron microscope.

Results and Discussion

Knock down of a DC2 homolog in *S. mediterranea*

We identified two DC2-related genes in the *S. mediterranea* Genome Database dd_Smed_v6_4191_0_1 (PlanMine; (Rozanski et al., 2018)); (Identity: 29.79%; E-value: 7.75×10^{-35}) and dd_Smed_v6_70204_0_1 (Identity: 28.23%; E-value: 1.63×10^{-20}) (Fig. 1). According to the available sequence data, the latter is much shorter than the other, and has several gaps in the amino acid alignment when compared to *C. reinhardtii* DC2. In addition, amino acid sequence analysis using the COILS program indicates that

dd_Smed_v6_4191_0_1 contains three coiled-coil domains, which is similar to *C. reinhardtii* DC2, and dd_Smed_v6_70204_0_1 contains only two coil-coil domains (Fig. 2). From the expression profile in the PlanMine database, dd_Smed_v6_4191_0_1 is highly expressed in the whole body, while dd_Smed_v6_70204_0_1 is unevenly expressed along the anterior-posterior axis of the body with a quite low level of expression (PlanMine; (Rozanski et al., 2018)). These profiles suggest that dd_Smed_v6_4191_0_1 functions in the cilia for regular motion and dd_Smed_v6_70204_0_1 functions in the reproduction pathway. We therefore predicted that dd_Smed_v6_4191_0_1 is the DC2 orthologue employed in the ventral cilia, and hereinafter it is referred to as *Smed-dc2*. DC3, the third subunit of the ODA-DC in *C. reinhardtii*, is a calmodulin-like protein, that is missing in *S. mediterranea*. Although it has not been shown by direct biochemical analysis, it is likely that DC3 binds to DC2 via the IQ-motif in *C. reinhardtii*. Consistent with this, and unlike *C. reinhardtii* DC2, *Smed-dc2* does not contain an IQ-motif that would allow for interaction with calmodulin-like proteins.

Following the method of Rompolas et al., a sequence of 249 base pairs encoding residues 162–410 of *Smed-dc2* was inserted into the L4440 vector for expression of double-strand (ds) RNA in *E. coli* strain HT115 (DE3) that lacks RNaseIII ((Rompolas et al., 2010); see Materials and Methods for details). Planarians were fed with dsRNA-containing bacteria to knock down *Smed-dc2* or with bacteria containing an empty vector as a negative control. Semi-quantitative RT-PCR of the resulting flatworms showed that the transcript of *Smed-dc2* was significantly reduced (Fig. 3). These data suggest that the expression of *Smed-dc2* was well suppressed in these planaria, and hereinafter they are termed *Smed-dc2(RNAi)* flatworms.

Motility of *Smed-dc2(RNAi)* knock-down planaria

We carried out phenotypic analyses of *Smed-dc2(RNAi)* flatworms. First, the average gliding velocity of *Smed-dc2(RNAi)* flatworms (~0.19 mm/sec) was significantly lower than that of the control (~1.26 mm/sec) (Fig. 4A, B); the residual motility was due to the characteristic muscle-based body contractions observed to occur when cilia can no longer generate sufficient propulsive force to move the animals (Supplementary Movie S1). Interestingly, the gliding velocity of *Smed-dc2(RNAi)* animals was even lower than that of *Smed-ic2(RNAi)* (~0.39 mm/sec) reported in a previous study, in which expression of an OAD intermediate chain IC2 was suppressed and the OAD was almost completely lost from the axoneme (Rompolas et al., 2010). This suggests that loss of DC2 protein in planaria has additional effects on ciliary axonemes and their motility beyond simply acting to dock the OAD.

We then measured the ciliary beating frequency (CBF) by generating kymographs from videos taken at 120 frames per sec using a high-speed camera. The CBF of *Smed-dc2(RNAi)* flatworms was ~13 Hz, whereas that of the control was ~18 Hz (Fig. 4C). Reduced CBF is a typical phenotype of OAD-missing cilia in many organisms including *C. reinhardtii* (Kamiya, 1988) and *S. mediterranea* (Rompolas et al., 2010). In *C. reinhardtii*, the flagella of the DC2-lacking mutant *oda1* lose both the OAD and the ODA-DC, whereas those of the IC2-lacking mutant *oda6* are missing only the OAD while retaining the ODA-

DC (Takada and Kamiya, 1994). Flagella of those two strains beat at almost the same frequency (~20 Hz), which is lower than the wild-type strain (~60 Hz) (Takada and Kamiya, 1994). Similar to *Smed-ic2(RNAi)* planaria missing the OAD IC2 protein, coordination of ciliary beating was lost in *Smed-dc2(RNAi)* flatworms, and the cilia did not form metachronal waves implying that they cannot maintain hydrodynamic coupling (Fig. 4C, Supplementary Movies S2 and S3). However, the CBF of *Sme-dic2(RNAi)* was reported to be ~10 Hz (Rompolas et al., 2010), which is slightly lower than that observed in *Smed-dc2(RNAi)* animals. The lack of a direct correlation between CBF and velocity likely reflects the switch from cilia-driven locomotion to muscle-based body contractions and also effects on ciliary stability (see below).

Loss of OAD in *Smed-dc2* knock-down flatworms

To confirm that the reduced CBF was caused by loss of OAD, ultra-thin sections of cilia were observed by TEM. The OADs that protruded from the doublet microtubules in control cilia were clearly missing in *Smed-dc2* knock-down flatworms (Fig. 5). These data showed that DC2 functions as a mediator of docking of OAD to the doublet microtubules in planarian axonemes similar to what was found in *C. reinhardtii* (Takada and Kamiya, 1994, 1997; Takada et al., 2002).

Loss and shortening of cilia in *Smed-dc2* knock-down flatworms

Loss of either DC2 or IC2 results in the same ciliary phenotypes - the loss of OAD and a reduction in beat frequency. However, *Smed-dc2(RNAi)* animals show slower swimming velocity with slightly higher CBF than observed previously for *Smed-dc2(RNAi)*. We surmised that this difference is caused by the loss of cilia in *Smed-dc2(RNAi)* planarians. SEM observations revealed that the number of cilia was reduced in *Smed-dc2(RNAi)* animals and that overall length of many of the remaining cilia was reduced (Fig. 6). The slower translocation velocity of *Smed-dc2(RNAi)* animals with higher CBF than *Smed-ic2(RNAi)* may be due to the combination of both ciliary loss and shortening of the remaining organelles.

In *C. reinhardtii*, mutants missing either the OAD alone or the OAD plus the ODA-DC show almost the same phenotype - slow and jerky swimming caused by low flagellar beat frequency (Takada and Kamiya, 1994). The only obvious difference between these two mutant classes is the presence of the ODA-DC projections on the doublet microtubules of the OAD subunit mutants. Thus, lack of the ODA-DC does not affect either flagellar length or number of flagella in *C. reinhardtii*. Similarly, in human, respiratory epithelium cilia from patients of primary ciliary dyskinesia with a mutation in CCDC114, one of two DC2 homologs in human, have defects in OAD docking to the doublets, but the ciliary length or number does not seem to be affected (Onoufriadis et al., 2013). In contrast, the flagellar length of the mice spermatozoa in which CCDC63 (the testis-specific DC2 homolog in mice) is knocked out is shortened (Young et al., 2015). Interestingly, OAD is intact in the CCDC63-KO spermatozoa, probably because compensated by overexpression of CCDC114 (Young et al., 2015). From these results, whether ciliary/flagellar length or number is affected by the lack of an ODA-DC subunit may depend on the presence or absence of

compensation system to stabilize cilia/flagella in organisms/organs. In the case of planaria, dd_Smed_v6_70204_0_1 or other proteins cannot compensate the loss of *Smed-dc2*.

Loss of cilia could be caused by the loss of basal bodies, but DC2 and the other ODA-DC subunits are not listed in the basal body proteome database in *C. reinhardtii* (POBdb: <http://pobdb.vital-it.ch/#/welcome>) (Hamel et al., 2017), and are not likely to contribute the stability of the basal body. Indeed, when peptidylglycine α -amidating monooxygenase, an enzyme required for generating amidated bioactive signaling peptides, is knocked down in planaria, the ventral cilia are lost, but the number of basal bodies is not obviously reduced (Kumar et al., 2017). This suggests that the loss of cilia is not necessarily caused by the loss of basal bodies.

What is the mechanism underlying the loss of cilia in *Smed-dc2(RNAi)* planaria? Binding of dyneins along the ciliary length is known to have stabilizing effects on the structure. Indeed, in *C. reinhardtii*, double mutants lacking both the OAD and a subset of inner arm dyneins exhibit a short flagella phenotype that can be rescued by mutation in a tubulin polyglutamylase which leads to the stabilization of axonemal microtubules (Kubo et al., 2015). Thus, one reasonable interpretation of our results is that the stability of planarian axonemal doublet microtubules is more dependent on binding of the ODA-DC than is observed in *C. reinhardtii*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.

Prediction of coiled-coil domains in *C. reinhardtii* DC2, dd_Smed_v6_4191_0_1 and dd_Smed_v6_70204_0_1. *C. reinhardtii* DC2 and dd_Smed_v6_4191_0_1 are suggested to contain three major coiled-coil domains which exhibit similar distributions within the full-length sequences. dd_Smed_v6_70204_0_1 is predicted to contain two short coiled-coil domains.

Figure 3.

Semi-quantitative RT-PCR of wild-type, control (fed with control bacteria containing the L4440 plasmid) and *Smed-dc2*(RNAi). Transcripts for DC2 are significantly reduced in *Smed-dc2*(RNAi) animals whereas actin and IC2 levels remain unchanged.

Figure 4.

(A) Gliding tracks of control (L4440) and *Smed-dc2(RNAi)* planaria over 30 sec. Movies were taken at 15 fps and the composite images prepared by overlaying every 50th frame. Time interval between images = ~3.3 sec.

(B) Gliding velocity of control and *Smed-dc2(RNAi)* animals (n=5). (*p<0.01; Student's *t*-test)

(C) Kymographs of ciliary beating in control (top) and *Smed-dc2(RNAi)* (bottom) planaria prepared from the high-speed recordings in supplemental movie S2. Ciliary beat frequency (CBF) is shown in the bottom right corner of each image (n=20). CBF was significantly reduced in *Smed-dc2(RNAi)* animals (p<0.01; Student's *t*-test).

Figure 5. (Top) Transmission electron micrographs of ultrathin sections of cilia from control and *Smed-dc2(RNAi)*. (Bottom) Superimposed images of nine doublets in the top panels. Outer arm dynein projections are missing in *Smed-dc2(RNAi)* planaria. Bars = 50 nm

Figure 6. Scanning electron micrographs of the ventral surface of control and *Smed-dc2*(RNAi) planaria. Control animals have a dense array of cilia. In contrast, ciliary density on *Smed-dc2*(RNAi) animals was much more uneven, and in many places was very sparse. In addition, many of the remaining cilia appeared much shorter than normal. Bars = 10 μ m.