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The CC1-FHA dimer is essential for KIF1A-mediated axonal transport of synaptic vesicles in *C. elegans*

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ABSTRACT

KIF1A, a member of kinesin-3 motors, plays a pivotal role in anterograde axonal transport of synaptic vesicles (SVs). We have shown that the CC1-FHA tandem of KIF1A forms a stable dimer that is crucial for both the dimerization and activation of the motor. However, it remains to be determined whether the CC1-FHA dimer is essential for KIF1A-mediated axonal transport *in vivo*. Here, we use *Caenorhabditis elegans* as the model organism to probe the *in vivo* function of the CC1-FHA dimer. Disruption of the CC1-FHA dimer severely impairs the KIF1A-mediated regulation of the locomotion and pumping behavior of *C. elegans* and exerts a significant impact on KIF1A-mediated axonal SV transport. Thus, together with previous structural and biochemical studies, the *in vivo* data presented in this study firmly establish the essential role of the CC1-FHA dimer for KIF1A-mediated neuronal transport.

1. Introduction

Kinesins are a superfamily of microtubule-based molecular motors responsible for long-range intracellular transport of membrane vesicles/organelles and protein complexes in polarized cells such as neurons [1–3]. Kinesins have been divided into 14 sub-families (kinesin-1 to kinesin-14) [4]. KIF1A, a member of kinesin-3 motors, was identified from a screening of murine brain cDNAs that are essential for neuronal transport [5]. Interestingly, UNC-104, KIF1A homolog in *Caenorhabditis elegans*, was also demonstrated to be responsible for neuronal transport, i.e., anterograde transport of synaptic vesicle (SVs) precursors in axon [6,7], and loss of function *unc-104(e1265)* mutant showed accumulation of SVs in the cell bodies [8]. Based on the previous functional characterizations, KIF1A/UNC-104 was regarded as a kinesin motor dedicated for the fast anterograde axonal transport of SVs in neurons [8].

Compared to the conventional kinesin motors, which can form processive dimers for transport, kinesin-3 KIF1A was surprisingly found to adopt a monomeric form *in vitro* and thus denoted as an unconventional "single-headed" kinesin [6]. However, recent several lines of studies have demonstrated that, similar to the conventional kinesin motors, kinesin-3 KIF1A can exist as processive dimers *in vivo*, and the dimerization of the motor is largely mediated by the neck coil (NC), a short coiled-coil immediately following the motor domain (MD) [9,10]. Moreover, kinesin-73, another kinesin-3 motor from *Drosophila*, can form processive dimers *in vivo* as well [11,12]. Thus, more and more data support the view that kinesin-3 family motors may adopt "two-headed" dimers but not "single-headed" monomers for the processive movement [13].

In KIF1A/UNC-104, a motor regulatory region immediately follows the N-terminal MD and NC, which includes a number of short non-continuous coiled-coil domains (CC1 to CC3) and a FHA domain (Fig. 1A). CC1 can directly sequester NC to prevent the NC-mediated dimerization of the motor, while CC2 can fold back to directly interact with the FHA domain to interfere with the motor activity [14]. Recently, we have found that the CC1-FHA tandem of KIF1A forms a stable dimer, which can promote the formation of motor dimers for the processive movement [15]. The structural studies of the CC1-FHA tandem further revealed that the linker between CC1 and the FHA domain unexpectedly forms a β-finger structure, which integrates the two domains forming a domain-swapped homo-dimer (Fig. 1B). Since both CC1 and the β-finger are essential for the motor inactivation, formation of the CC1-FHA dimer can directly sequester these two domains to prevent the CC1/β-finger-mediated motor inhibition [15]. Thus, in addition to facilitating the dimerization of KIF1A, the CC1-FHA tandem plays a critical role in regulating the motor activation, denoted as a central hub for controlling the dimerization and activation of KIF1A [15]. However, it remains to be determined whether the CC1-FHA dimer biochemically and structurally characterized *in vitro* is essential for the KIF1A-mediated axonal cargo transport *in vivo*.

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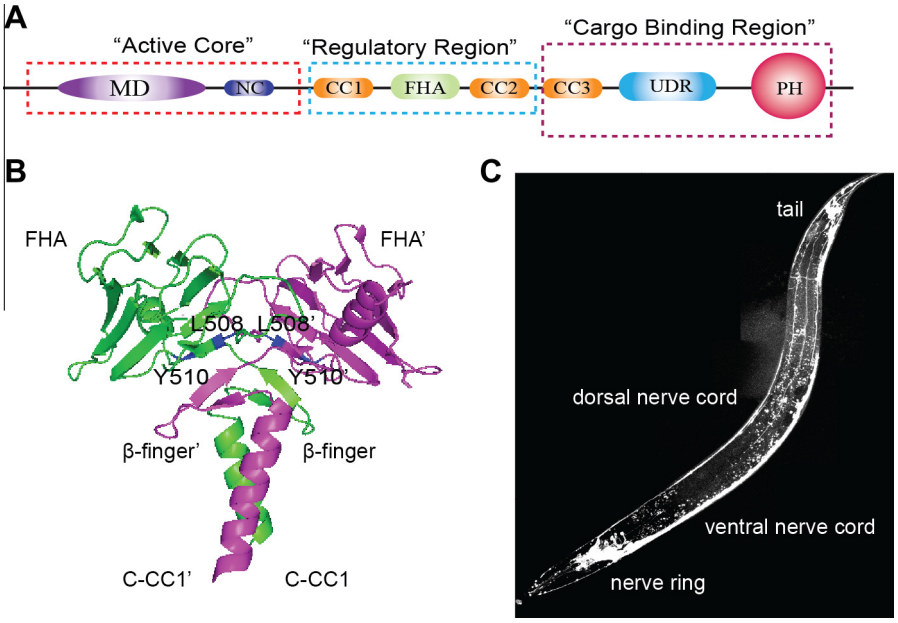

Fig. 1. Schematic diagram of KIF1A and expression patterns under promoter of *unc-104* in *C. elegans*. (A) KIF1A is composed of N-terminal motor domain (MD) and neck coil (NC), following by CC1-FHA-CC2 domain in the middle and a C-terminal PH domain binding cargo. (B) Ribbon diagram of the C-CC1-FHA dimer structure. Two subunits of the C-CC1-FHA dimer are colored in green and magenta, respectively. The mutations (L508 and Y510) are labeled in the blue color. (C) A photomontage of *unc-104(e1265)* expressing KIF1A::GFP under the control of *unc-104* promoter. (For interpretation of color in this figure, the reader is referred to the web version of this book).

In this study, we used *C. elegans* as the model organism to explore the potential role of the CC1-FHA dimer for the KIF1A-mediated axonal transport *in vivo*. Exogenous expression of either KIF1A or the mutant with deletion of the β-finger ([474–486]-KIF1A) could largely rescue the locomotion defect of *unc-104(e1265)* mutant, whereas the mutant with point mutations that dissociate the CC1-FHA dimer (exposing CC1 and the β-finger, (L508Q/Y510Q-KIF1A)) was unable to rescue this locomotion defect. Moreover, dissociation of the CC1-FHA dimer with the mutation exposing CC1 and the β-finger also significantly impaired the axonal transport of SVs leading to the accumulation of SVs in the cell bodies. Together with previous *in vitro* biochemical studies, the *in vivo* data shown here firmly establish the essential role of the CC1-FHA dimer for KIF1A-mediated axonal SV transport.

2. Materials and methods

2.1. C. elegans transformation

mkif1a:: gfp with various mutations were PCR amplifed from pGW1-*mkif1a* plasmid [16] and cloned into pD95.75 vector containing *Punc-104* promoter. Germline transformation was performed by a standard microinjection method [17]. 80 ng/μl [*Punc104::mkif1a::*RFP] plasmid was injected into *unc104(e1265); ceIs62*[*Punc-129:*ANF*::Venus, Punc-129:*RFP::SNB-1, *Pttx-3::*RFP] worms. Multiple transgenic lines for each transgene were examined for fluorescence expression.

2.2. Behavioral assays

All behavioral assays were performed with young adults. The locomotion assay was done as previously described [18]. Briefly, worms moving forward continuously were counted for 20 s at 20 °C on NGM plates spread with a thin layer of freshly grown OP50 bacteria. Pumping rate assay was analyzed using a previous described assay [19]. We counted pharyngeal contraction during 60 s. At least 15 animals were tested per experiment.

2.3. Automated worm behavior tracking system

We used a WAT-902H2 camera (Automatic System, Inc.) to capture high-contrast dark-field images of our assay plates. The behavior movies were taped at 1× magnification of a ZEISS V8 microscopy at 25 Hz. These data were analyzed automatically by a home-written object-tracking plugin in ImageJ (NIH). Locomotion speed and the 30 s crawling trail were achieved by our ImageJ plugin.

2.4. Imaging and data analysis

Fluorescence images were obtained using Olympus FV500 Laser Scanning Confocal Microscopy with 60 × (NA = 1.40) oil objective. Confocal settings used for image capture were held constant in comparison experiments. For *C. elegans* imaging, young adult stage worms were scored. The images were analyzed by ImageJ (NIH). Data analysis was conducted using IGOR Pro (Wavemetrics) or EXCEL (Microsoft) software.

3. Results

3.1. KIF1A/UNC-104 is broadly expressed in neurons of C. elegans

Given the short life cycle, compact genome, hermaphroditic reproduction and accessibility to genetic manipulation, *C. elegans* is an ideal model system for biological research in terms of genetic, cellular and molecular dissection of organism behavior [20,21]. We set out to use this model system to explore the *in vivo* function of the CC1-FHA dimer for KIF1A-mediated neuronal activities. Based on the crystal structure of the CC1-FHA dimer (Fig. 1B), we selected two types of mutants for the following studies: one is the mutant with disruption of the CC1-FHA dimer and removal of the inhibition of CC1 and the β-finger (such as deletion of the β-finger ([474–486]-KIF1A)); the other is the mutant with disruption of the CC1-FHA dimer but retaining the CC1/β-finger-mediated inhibition (such as the substitution of hydrophobic residues with

hydrophilic ones in the CC1-FHA dimer interface of the FHA domain (L508Q/Y510Q-KIF1A)). By using the *C. elegans* model system, we first exogenously expressed the wild-type *Mus musculus* KIF1A and a series of its mutant forms ([474–486]-KIF1A and L508Q/Y510Q-KIF1A) under the promoter of *unc-104* and checked the *in vivo* distribution of these various forms of KIF1A in *C. elegans*. Consistent with the reported data [22], when we expressed the *Punc-104::kif1a::*GFP in *unc-104(e1265)* mutant, KIF1A was observed in multiple neurons, including the nerve ring, tail neurons, dorsal and ventral nerve cord (Fig. 1C).

3.2. Dissociation of the CC1-FHA dimer affects C. elegans behavior

Many conserved neurotransmitters and neuomodulators exist in neurons of *C. elegans*, which can communicate with each other to modulate multi-behavior of the worms, such as locomotion and pumping [23]. Given that the SVs cannot smoothly transport from the cell body to axon and largely accumulate in the cell body, the *unc-104(e1265)* mutant exhibits multiple locomotion defects induced by deficiency of KIF1A/UNC-104, such as slower body bends and motility [24,25] (Figs. 2A and 3). To explore whether the CC1-FHA dimer is essential for the KIF1A-mediated regulation of the locomotion behavior, we next measured the moving velocity of the worms and evaluated the changes of locomotion. As expected, exogenous expression of KIF1A and [474–486]-KIF1A (the active form of KIF1A) in *unc-104(e1265)* could mostly rescue the locomotion defect induced by defective KIF1A/UNC-104 (Figs. 2A and 3), while over-expression of L508Q/Y510Q-KIF1A (the inactive form of KIF1A) could not and the worms still showed slow movement and body bends (Figs. 2A and 3).

Pumping of *C. elegans* is tightly regulated by 14 types of pharyngeal neurons [26]. When the transport of neurotransmitters is abnormal in *unc-104(e1265)* mutant with defective KIF1A/UNC-104, the nematode also exhibits pumping rate deficiency (Fig. 2B). To further probe the potential role of the CC1-FHA dimer on the KIF1A-mediated regulation of the pumping behavior of *C. elegans*, we next measured the pharyngeal pumping rate of worms with expression of KIF1A, [474–486]-KIF1A and L508Q/Y510Q-KIF1A, respectively. Consistent with the above locomotion results, the pharyngeal pumping rate of the *unc-104(e1265)* mutant and the L508Q/Y510Q-KIF1A expressed strains were strikingly reduced compared with the wild type, while expression of KIF1A and [474–486]-KIF1A largely rescued this pumping defect induced by the *unc-104(e1265)* mutant (Fig. 2B). Taken together, all the above physiological behavior results indicate that the CC1-FHA dimer is essential for KIF1A-mediated regulation of the moving and pumping behavior of *C. elegans in vivo*.

3.3. The CC1-FHA dimer is essential for the KIF1A-mediated axonal transport

Since the CC1-FHA dimer is critical for the KIF1A-mediated regulation of *C. elegans* behavior and KIF1A is dedicated for the anterograde axonal transport of SVs in neurons, we predicted that the CC1-FHA dimer is also essential for the KIF1A-mediated axonal transport, which is the key reason to cause the abnormal behavior of *unc-104(e1265)*. To test this hypothesis, we next checked the axonal SV transport in neurons of *C. elegans*. To directly visualize the SV transport in the axons, we expressed the synaptic vesicle transmembrane protein SNB-1 tagged with RFP under the *unc-129* promoter, which can drive the specific expression of genes in the subset of DA and DB motor neurons. Given that DA/DB motor neurons possess morphologically distinct axon in the dorsal nerve cord and dendrite in the ventral nerve cord (Fig. 4A) [23], we can further quantify fluorescence intensity (FI) of RFP::SNB-1 in the dorsal nerve cord of worms to check the

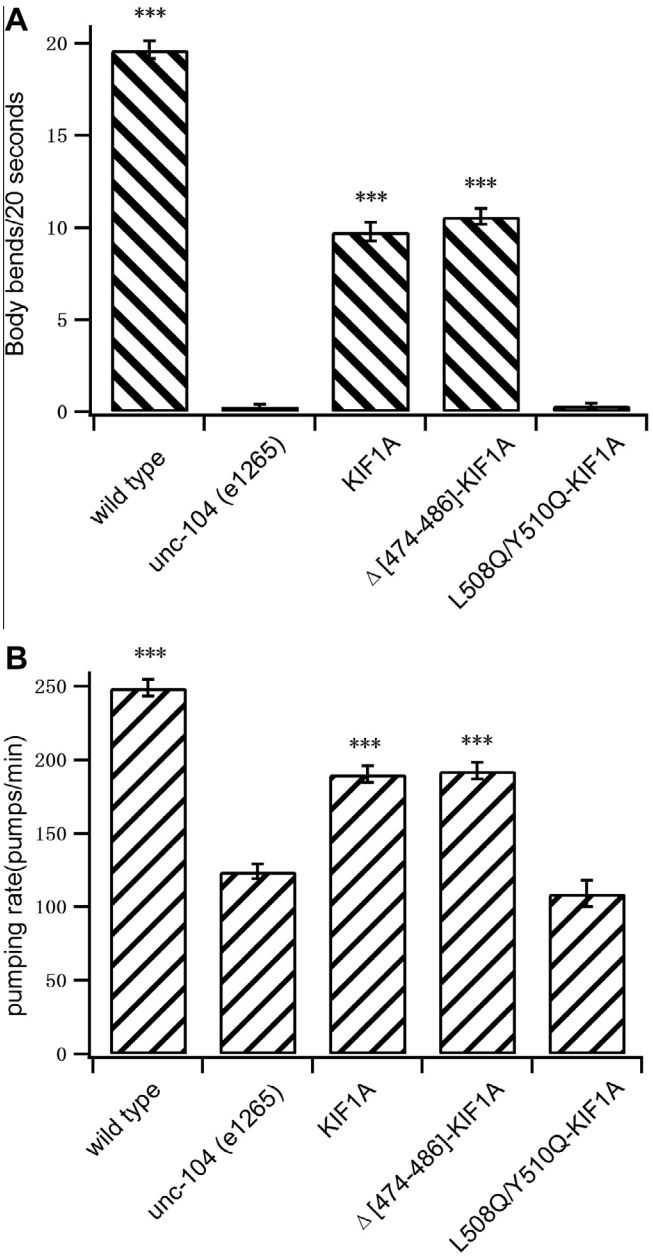

Fig. 2. CC1-FHA dimer is essential for KIF1A-mediated regulation of *C. elegans* behavior. (A) Quantification of body bends for wild type, *unc-104(e1265)*, *unc-104(e1265)* expressing KIF1A, Δ[474–486]-KIF1A and L508Q/Y510Q-KIF1A. (B) Quantification of pumping rate of wild type, *unc-104(e1265)*, *unc-104(e1265)* expressing KIF1A, Δ[474–486]-KIF1A and L508Q/Y510Q-KIF1A. Error bars represent the standard error of the mean (SEM). Asterisks indicate a significant difference from *unc-104(e1265)* (***$p < 0.001$, $n \geqslant 15$).

polarized axonal transport of SVs. Compared to the wild type, *unc-104(e1265)* mutant completely abolished axonal transport of SVs from soma to axons in motor neurons (Fig. 4B and C). As expected, exogenous KIF1A largely rescued axonal transport defects to ~65% (Fig. 4B and C), while the L508Q/Y510Q-KIF1A mutant could not rescue SV transport defects induced by the *unc-104(e1265)* mutant (Fig. 4B and C). Moreover, over-expression of the active form of KIF1A, [474–486]-KIF1A, could also restore the RFP::SNB-1 level to ~70% (Fig. 4B and C). Taken together, all the above *in vivo* transport data demonstrated that CC1-FHA dimer is indeed essential for the KIF1A-mediated axonal SV transport.

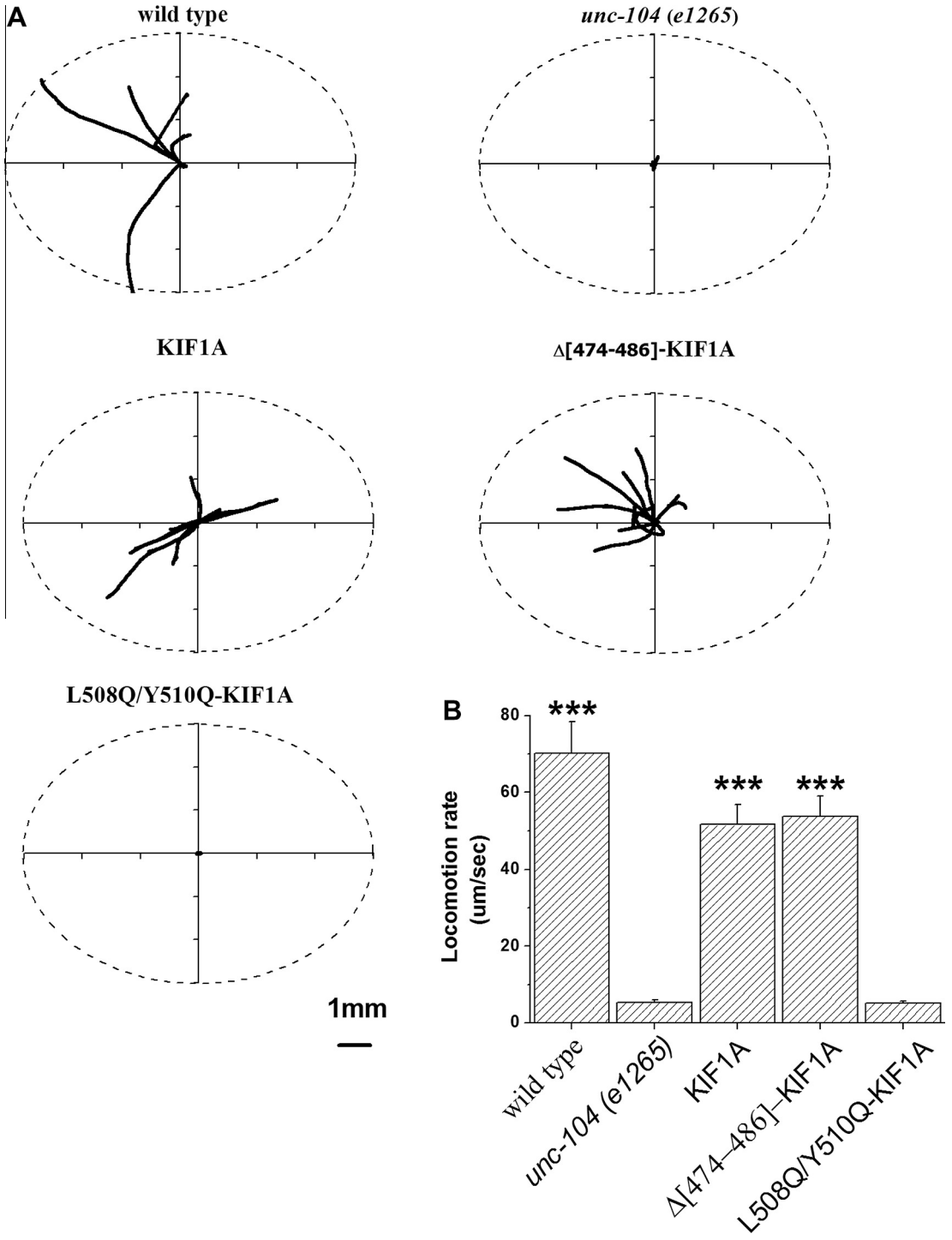

Fig. 3. CC1-FHA dimer regulates KIF1A-mediated locomotion behavior of *C. elegans*. (A) The representative 30 s locomotion trajectories of wild type, *unc-104(e1265)* and *unc-104(e1265)* expressing KIF1A, Δ[474–486]-KIF1A and L508Q/Y510Q-KIF1A are shown. The starting points for each trajectory of each genotype are at the center of the graph. $n > 5$ for each genotype. Scale bar: 1 mm. (B) Locomotion rates are compared for indicated strains. Error bars represent the standard error of the mean (SEM). Asterisks indicate a significant difference from *unc-104(e1265)* ($^{***}p < 0.001$, $n \geqslant 10$).

4. Discussion

Kinesin-3 KIF1A/UNC-104 is one of the key kinesin motors for the axonal transport in neurons, and it is debatable about its monomeric or dimeric conformation for the processive movement on microtubule tracks. We have demonstrated that the CC1-FHA tandem of KIF1A can form a stable dimer in solution, which can facilitate the dimerizaiton of the motor *in vitro* and thus support the view that kinesin-3 KIF1A is a dimeric processive motor. More intriguingly, besides the motor dimerization, the CC1-FHA tandem-mediated dimerization can further activate KIF1A by releasing the CC1/-finger-mediated inhibition. Thus, the CC1-FHA tandem acts as a central regulatory hub for the dimerization and activation of KIF1A. However, it remains unclear whether the CC1-FHA tandem is essential for the KIF1A-mediated neuronal transport and the subsequent biological activities. In this study, we used the *C. elegans* as the model system to extensively investigate the essential role of the CC1-FHA dimer for KIF1A-mediated axonal transport.

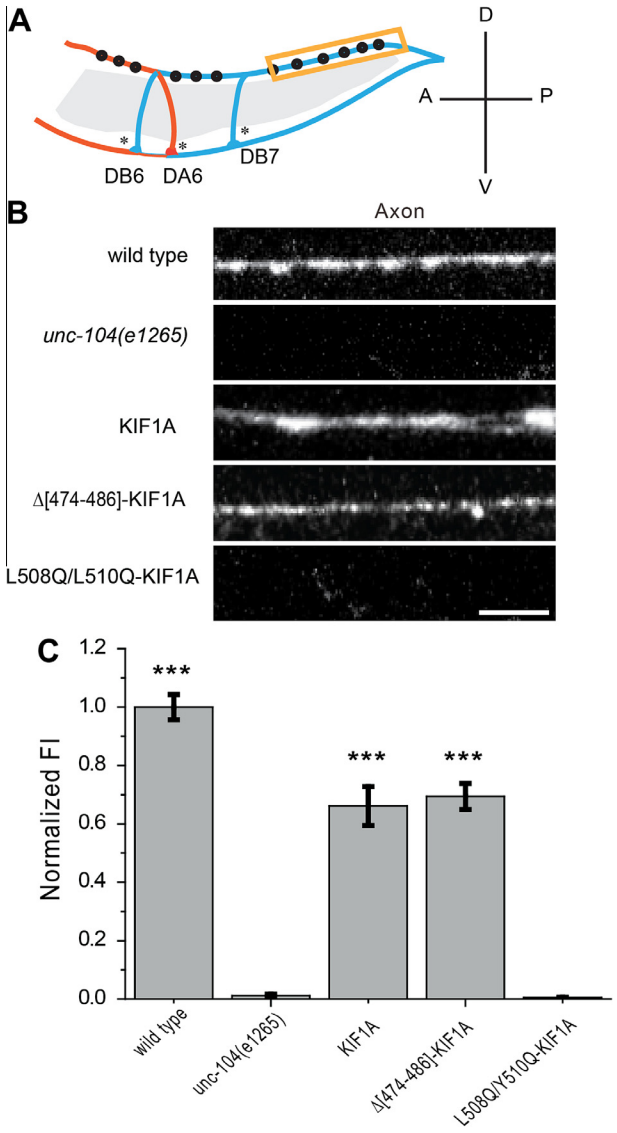

Fig. 4. The CC1-FHA tandem-mediated dimer is essential for KIF1A-mediated axonal transport activity. (A) Schematic diagram of DA/DB motor neuron in the tail of worm under the *unc-129* promoter used in this study. DA neurons (red) process toward anterior, while DB neurons (blue) process toward posterior. The boxed region denotes the axon was imaged and analyzed in (B) and (C). A, anterior; D, dorsal; P, posterior; V, ventral; asterisk, cell body. (B) KIF1A-mediated RFP::SNB-1 distribution in the axon of motor neuron. The figure shows representative images of RFP::SNB-1 distribution in axons from animals of the wild type, *unc-104(e1265)*, *unc-104(e1265)* expressing KIF1A, Δ[474–486]-KIF1A and L508Q/Y510Q-KIF1A. Scale bar: 20 μm. (C) Normalized maximum fluorescence intensity (FI) of RFP::SNB-1 in the indicated strains. Normalized FI is defined as the ratio of the fluorescence intensity of each transgene/allele to that of the wild type in axons. Error bars represent the standard error of the mean (SEM). Asterisks indicate a significant difference from *unc-104(e1265)* ($^{***}p < 0.001$, $n \geqslant 15$). (For interpretation of color in this figure, the reader is referred to the web version of this book).

We demonstrated that disruption of the CC1-FHA dimer has the significant impacts on the KIF1A-mediated axonal transport and the subsequent regulation of *C. elegans* behavior such as moving and pumping. Together with the previous *in vitro* biochemical and structural data, the *in vivo* data shown here establish the role of the CC1-FHA dimer for the KIF1A-mediated neuronal transport and the related biological activities.

Animals integrate various environment stimulus within the nervous system to generate the relevant responses [27]. Defects in the transmission between the neurons will lead to abnormal behavior. As KIF1A/UNC-104 is critical for axonal SV transport, loss of function mutant *unc-104(e1265)* worms exhibit uncoordinated locomotion and sluggish pumping rate (Figs. 2 and 3). We have demonstrated that the CC1-FHA tandem is the regulatory center for the dimerization and activation of KIF1A [15]. Although both the [474–486] and L508Q/Y510Q mutations can dissociate the CC1-FHA dimer, the rescue efficiency of these two mutants is significantly different. Based on the CC1-FHA dimer structure (Fig. 1B), the [474–486] mutation results in an active motor after removal of the -finger-mediated inhibition, while the L508Q/Y510Q mutation exposes the CC1–finger to keep the motor in an inactive conformation. Thus, in line with the previous cellular studies [15], the [474–486]-KIF1A can effectively rescue the transport defects and abnormal behavior induced by the loss of function mutant, while the L508Q/Y510Q-KIF1A cannot (Figs. 2 and 3).

Considering that the sequence similarity between KIF1A and UNC-104 is only ~55.8%, KIF1A might not fully replace the function of UNC-104 in *C. elegans*. Consistent with this assumption, although the exogenously expressed KIF1A can rescue the axonal transport defects of *unc-104(e1265)*, the rescue efficiency is not 100% but merely up to 70%. Moreover, although [474–486]-KIF1A are more active than KIF1A in the precious cellular studies using N2A cells [15], the rescue efficiency of [474–486]-KIF1A was similar to that of KIF1A (Fig. 4), suggesting that the regulatory mechanism of the KIF1A-mediated axonal transport *in vivo* may be more complex than predicted at the cellular level. Again consistent with this, *unc-104(e1265)* expressing the exogenous [474–486]-KIF1A exhibits the similar locomotional behavior and pumping rate with that expressing KIF1A (Figs. 2 and 3).

In summary, this work establish that the CC1-FHA tandem-mediated dimerization of KIF1A is essential for the KIF1A-mediated axonal SV transport *in vivo* and the regulation of *C. elegans* behavior such as moving and pumping, which may direct the further investigation of the KIF1A-mediated intracellular transport in neurons.

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