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Fasciculation and elongation protein zeta-1 (FEZ1) participates in the polarization of hippocampal neuron by controlling the mitochondrial motility

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Abstract

The fasciculation and elongation protein zeta-1 (FEZ1), a mammalian orthologue of *Caenorhabditis elegans* UNC-76 protein, is a 45-kDa protein with four coiled–coiled domains and efficiently promotes the neurite elongation in the rat phaeochromocytoma PC12 cells. UNC-76 proteins of *C. elegans* and *Drosophila* have been genetically demonstrated to be involved in the axonal guidance. We here show that FEZ1 RNA interference (RNAi) represses the formation of axon in rat embryo hippocampal neurons. An anterograde mitochondrial movement is also retarded in neurites of the RNAi-treated hippocampal neurons. Moreover, the size of mitochondria is considerably elongated by the RNAi treatment. The transport of mitochondria from soma to axon or dendrites is essential for the neuronal differentiation. Therefore, our results strongly suggest that FEZ1 participates in the establishment of neuronal polarity by controlling the mitochondrial motility along axon.

Keywords: FEZ1; Hippocampal neuron; RNA interference; Neuronal polarity; Mitochondrial transport; Neuronal differentiation

The fasciculation and elongation protein zeta-1 (FEZ1) is a brain-specific coiled-coil protein involved in axonal outgrowth [1,2]. FEZ1 is a mammalian orthologue of the *Caenorhabditis elegans* UNC-76 protein. FEZ1 rescues both abnormal neuronal morphology and locomotory defects caused by *unc-76* mutations in the nematode, strongly suggesting that the common function of FEZ1 and UNC-76 in axonal guidance is conserved among species [2]. In adult rats, *FEZ1* mRNA is expressed exclusively in the brain. In mouse embryos, abundant expression of *FEZ1* mRNA starts at 7 days postcoitum (dpc) and ends before 17 dpc [1,3]. *FEZ1* mRNA expression coincides with the period of neurogenesis; soon after 7 dpc the neural plate is formed and by 17 dpc the central nervous system is well generated. Thus, FEZ1 is suggested to participate in the development of the central nervous system during mammalian embryogenesis.

FEZ1 interacts with proteins related to neuronal diseases, DISC1 (Disrupted-in Schizophrenia 1) and necdin. Disrupted *DISC1* gene is associated with the syndrome of schizophrenia [4] and necdin is one of four proteins inactivated in the *Prader–Willi* syndrome [5]. The interaction of DISC1 with FEZ1 promotes neurite outgrowth in rat phaeochromocytoma PC12 cells. The interaction of FEZ1 with necdin protects FEZ1 from proteasomal degradation. In *Drosophila*, the FEZ1 orthologue UNC-76 interacts with the motor protein kinesin, essential for the axonal transport of vesicles and organelles [6]. The loss of UNC-76 function in *Drosophila* causes the defects of axonal transport and locomotion with similar phenotypes as those observed in kinesin mutants.

To elucidate the role of FEZ1 in mammalian neurons, we have studied the expression and localization of the

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endogenous FEZ1 in hippocampal neurons isolated from rat embryo. We have observed that RNAi (RNA interference) targeting to *FEZ1* mRNA inhibits the formation of axon. Moreover, both mitochondrial transport and morphology are affected by the FEZ1 repression. The presence of mitochondria in the distal part of neuron, i.e., dendrites and axon, is crucial to maintain both cell functions and survival [7,8]. In neurons, mitochondria are transported from soma to axon and dendrites with the help of motor proteins along the microtubule rails [7]. Taken together, these results strongly suggest that FEZ1 is essential for the establishment of neuronal polarity by controlling the mitochondrial transport in hippocampal neurons.

Materials and methods

Antibodies. An anti-FEZ1 rabbit polyclonal antibody was prepared as described previously [4]. An anti-tau-1 mouse monoclonal antibody (Clone PC1C6, Chemicon, Temecula, CA) and an anti-MAP2 mouse monoclonal antibody (Clone AP20, Leinco Technologies, Inc., St. Louis, MS) were used for immunohistochemical analyses.

Western blot. Rat whole brains (about 1.5 g) were homogenized in 13 ml of the lysis buffer [1], centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatants were used as brain lysates. The lysates were subjected to Western blotting with the anti-FEZ1 polyclonal antibody (dilution, 1:200).

Cell culture. Hippocampal neurons were isolated from rat embryo (E18, embryonic day 18) as described previously [9]. Neurons (2×10^5 cells/well) were plated onto glass cover slides (14 mm diameter; Matsunami, Tokyo, Japan) coated with poly-D-lysine (PDL; Sigma) and laminin (Iwaki, Tokyo, Japan) in a 24-well plate, and cultured at 37 °C in 5% CO_2. After the attachment of neurons, the medium was changed to Neurobasal medium (Invitrogen, Carlsbad, CA) with B-27 supplement (Invitrogen) and 1 mM glutamine.

RNA interference (RNAi). The specific hairpin sequence of rat *FEZ1* gene [1] corresponding to positions 456–476 (FEZ1-RNAi-456) of the coding sequence (5′-ATAGCGGCCGCAAAAAACGACTCTGGTATC AACGAGAAGCTTGAATCGTTGATACCAGAGTCGTCGGTGTT TCGTCCTTTCCACA-3′) was used to amplify the vector carrying the U6 promoter [10]. The PCR fragment was digested using *Bgl*II and *Not*I, and then subcloned into the shuttle vector to construct pEGSH-FEZ1 RNAi-456. The same length of randomized cDNA was used to construct pEGSH-Control RNAi (control RNAi).

Transfection. Hippocampal neurons were transfected with various plasmids using Lipofectamine 2000 (Invitrogen) as described preciously [9]. Neurons were co-transfected at a ratio of 10:1 with pEGSH-FEZ1 RNAi-456 and either pTB701-hGFP (a mammalian expression vector for humanized green fluorescent protein) [1] or pDsREDmito (a gift of Prof. Demaurex, a mammalian expression vector for a red fluorescent protein tagged with the mitochondrial targeting signal derived from the cytochrome *C* oxidase subunit VIII).

Immunohistochemistry. Neurons were fixed on 3 days *in vitro* (DIV3) or DIV4 with 3.7% paraformaldehyde in PBS for 30 min at 37 °C (for an anti-tau1 antibody) or 10 min on ice (for anti-FEZ1 and anti-MAP2 antibodies), followed by treatment with cold methanol for 10 min at −20 °C and 10% normal goat serum (NGS) in PBS for 1 h at room temperature. The neurons were incubated with the anti-FEZ1 polyclonal antibody (dilution, 1:50) at 4 °C for 48 h, the anti-tau1 monoclonal antibody (dilution, 1:1000), or the anti-MAP2 monoclonal antibody (dilution, 1:500) at 4 °C overnight. Subsequently, the cells were incubated with an Alexa 546 (or 488)-conjugated goat anti-rabbit IgG (H + L) (Molecular Probes, Eugene, OR) (dilution, 1:1000) or an Alexa 546-conjugated goat anti-mouse IgG (H + L) (Molecular Probes) (dilution, 1:2000) at room temperature for 1 h. The fluorescence was visualized with a confocal laser scanning microscope Pascal 5 (Carl Zeiss, Jena, Germany) or a fluorescence inverted microscope IX70 (Olympus, Tokyo, Japan).

Analysis of neuronal polarity. Neuronal polarity was assessed with neurons at DIV3 by determining the percentage of neurons with a single long tau1-positive process at least twice as long as the other processes. At least 50 transfected cells from three different preparations were analyzed, and both the mean and standard error of mean (SEM) were calculated.

Image acquisition and quantification of mitochondria. Mitochondrial morphology and movement were analyzed with hippocampal neurons coexpressing DsREDmito and FEZ1 RNAi. Cell images were taken with a cooled charge-coupled device (CCD) Orca-ER camera (Hamamatsu, Shizuoka, Japan) mounted on the Olympus IX70 microscope equipped with a Lambda 10–2 filter wheel (Sutter instrument, Novato, CA) and a 60× oil immersion PlanApo objective lens (N.A. 1.4, Olympus). The cells were illuminated with a 75-W Xe lamp through a 10% neutral density (ND) filter (Omega Optical Inc., Tokyo, Japan). The intensities of DsREDmito-derived fluorescence along the processes of each neuron were measured using a user-defined threshold with MetaMorph software (Universal Imaging, West Chester, PA). The mitochondrial length in neurites was calculated by tracing each mitochondrion. The mitochondrial occupancy was determined as the area of DsREDmito-derived fluorescence/the area of total processes with the MetaMorph software. For analyzing mitochondrial movement, about 2×10^5 hippocampal neurons were plated on a 35-mm glass bottom dish coated with PDL and laminin. After 3 days, the medium was changed with fresh phenol red-free Neurobasal medium with B-27 supplement, 1 mM glutamine, and 10 mM Hepes (pH 7.4). Cells were then maintained at 37 °C in 5% CO_2 incubator (Thermoplate, Tokai Hit, Shizuoka, Japan) on the stage of Olympus IX70 inverted microscope. DsREDmito-derived fluorescent images were captured every 2.5 s for 15 min with a CCD Orca-ER camera. Movement of each mitochondrion was manually analyzed using the track object function of the MetaMorph software.

Results

FEZ1 is important for the establishment of neuronal polarity

In mammals, *FEZ1* mRNA is preferentially expressed in the olfactory bulb, cortical and hippocampal neurons but not in oligodendrites or astrocytes, as shown previously by *in situ* hybridization [3,4]. Therefore, we focused on the FEZ1 function during neuronal differentiation in hippocampal neurons isolated from rat embryonic brain. Western blot experiment using an anti-FEZ1 antibody [4] showed that FEZ1 is expressed as a 45-kDa protein in rat brain (Fig. 1A). FEZ1 was detected in the hippocampal neurons at DIV3 by immunofluorescence using the same anti-FEZ1 antibody (Fig. 1B). FEZ1 was detected in both cell body and extending processes. Abundant presence of FEZ1 was found in the cell body and neurite tips (see arrow heads). Double staining for FEZ1 and either axon-specific microtubule-associated protein (tau1) or a dendrite-specific microtubule-associated protein (MAP2) has shown that FEZ1 is localized in all extending neurites, both axon and dendrites.

To study the role of FEZ1 during neuronal differentiation, we used the RNAi approach by expressing a single hairpin RNA specifically targeting *FEZ1* mRNA (FEZ1-RNAi-456). Measurement by quantitative PCR showed the FEZ1-RNAi-456 and control RNAi repressed $60 \pm 10\%$ ($n = 4$) and $0 \pm 12\%$ ($n = 4$) of the *FEZ1* mRNA expression in PC12 cells, respectively. Hippocampal neurons were co-transfected with the gene for mitochondria

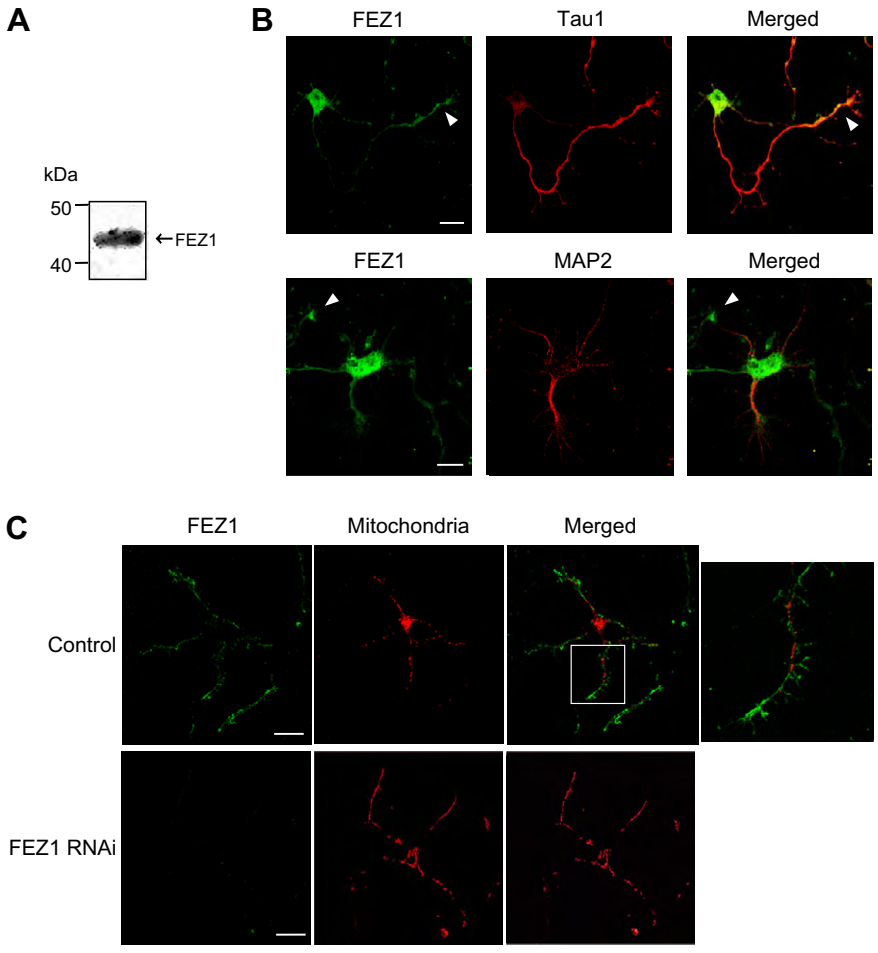

Fig. 1. Localization of FEZ1 in hippocampal neurons. (A) Western blot analysis of rat brain lysate using anti-FEZ1 antibody. (B) Hippocampal neurons (DIV3) were immunostained with anti-FEZ1, anti-tau1 (axonal marker), and anti-MAP2 (dendrite marker) antibodies. White arrowheads indicate the axonal tip. (C) Neurons (DIV0) were co-transfected with DsREDmito and either FEZ1-RNAi-456 (FEZ1 RNAi) or control RNAi at a 1:10 ratio. Three days after the transfection, the cells were immunostained with anti-FEZ1 antibody. The enlarged image of the neurite is shown. Scale bar = 20 μm.

marker DsRED (DsREDmito) and either FEZ1-RNAi-456 or control RNAi at a 1:10 ratio, cultured for 3 days, and then stained with an anti-FEZ1 antibody. The expression of FEZ1 protein was efficiently repressed in the neurons on DIV3 (Fig. 1C).

Neurons co-transfected with both GFP and either FEZ1-RNAi-456 or control RNAi at a 1:10 ratio were cultured for 3–4 days, fixed and stained for neurite markers, tau1 or MAP2 (Fig. S-1). Tau1 was detected in the longest process of control neurons, indicating the presence of axon, whereas other processes did not show any expression of tau1 (Fig. S-1A, left). Thus, control neurons were found to display normal polarity (percentage of polarized neurons: $88.1 \pm 6.0\%$, $n = 55$). In FEZ1-RNAi-456-expressing neurons, despite the presence of several extending processes, the expression of tau1 was unobserved in any process of more than 60% GFP-expressing cells (Fig. S-1A, right). More than 60% of FEZ1-RNAi-456 expressing neurons showed marked defects in polarity (percentage of polarized neurons: $35.3 \pm 8.9\%$, $n = 35$). On the other hand, the expression of MAP2, a dendrite marker, was observed in both of the control and FEZ1-RNAi-456-expressing neurons (Fig. S-1B). These results strongly suggest that FEZ1 plays an important role in the establishment of neuronal polarity and the growth of axon.

FEZ1 controls the morphology and occupancy of mitochondria

UNC-76, a FEZ1-orthologue of *Drosophila*, has been shown to participate in the axonal transport of cargos by interacting with the tail domain of kinesin heavy chain [6]. Recently, we have found that FEZ1 also interacts with α- and β-tubulin in PC12 cells (Fujita et al., unpublished). We therefore studied the involvement of FEZ1 in the transport of mitochondria (as one of the cargos) in hippocampal neurons expressing DsREDmito and either FEZ1-RNAi-456 or control RNAi on DIV3. Although FEZ1 is scarcely co-localized with mitochondria (Fig. 1C), the morphology of mitochondria was considerably different from control cells and their size appeared to be more elongated (Fig. 2A and B). The length of mitochondria localized in neurites of both control RNAi- and FEZ1-RNAi-456-treated neurons is 2.30 ± 0.16 μm ($n = 69$) and

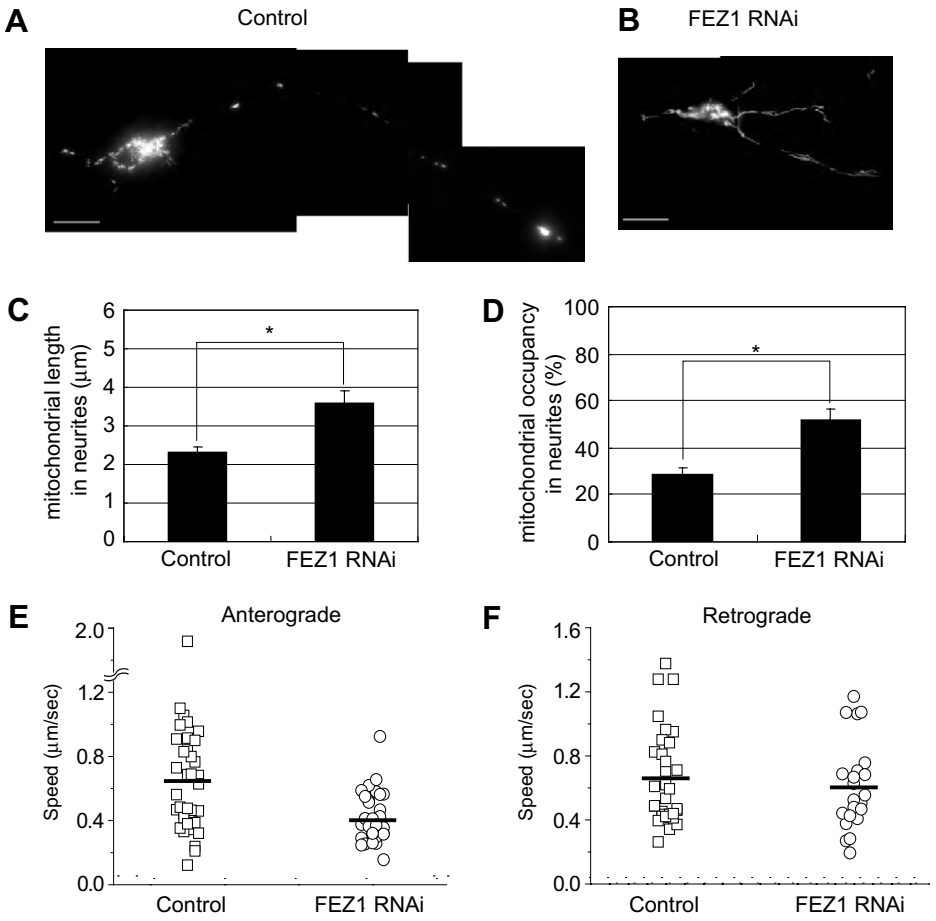

Fig. 2. Effect of FEZ1-RNAi on the length, occupancy, and movement of mitochondria in neurites. Neurons (DIV0) were co-transfected with DsREDmito and either control RNAi (A, Control) or FEZ1-RNAi-456 (B, FEZ1 RNAi) at a 1:10 ratio, and observed on DIV3. (C) The length of mitochondria in neurites. (D) The occupancy of mitochondria in neurites. Values are shown as means ± SEM. Asterisks indicate statistical significance (Student's *t* test) for (C and D) is $P = 0.0002$ and 0.0001, respectively. Movements of DsREDmito-labeled mitochondria in living neurons (DIV3) co-transfected with either control RNAi (Control) or FEZ1-RNAi-456 (FEZ1 RNAi). Images of DsREDmito fluorescence were taken every 2.5 s for 15 min. The anterograde (E) and retrograde (F) velocities of individual mitochondria are represented as open squares (Control) and open circles (FEZ1 RNAi). The means of the speed are indicated by bars.

3.60 ± 0.29 μm ($n = 61$), respectively (Fig. 2C), indicating that mitochondria were elongated by 1.6-folds in FEZ1-RNAi-456-treated neurons. Moreover, the occupancy of mitochondria in all processes increased noticeably [control: $28.8 \pm 2.7\%$ ($n = 10$); FEZ1-RNAi-456: $51.5 \pm 4.8\%$ ($n = 10$); Fig. 2D]. These results show that FEZ1 is able to control the morphology and occupancy of mitochondria in hippocampal neurons, even without co-localization with mitochondria.

FEZ1 is involved in the anterograde movement of mitochondria

By analogy to the involvement of *Drosophila* UNC-76 in the axonal transport of cargos [6], we examined if FEZ1 is also involved in the mitochondrial transport in hippocampal neuron. Movements of DsREDmito-labeled mitochondria in either anterograde or retrograde direction were measured and analyzed as described in 'Materials and methods' (Fig. 2E and F; see Fig. S-2). The anterograde movements of mitochondria were significantly faster ($P < 0.0001$) in control neurons (0.65 ± 0.06 μm/s, $n = 36$) than in FEZ1-RNAi-456-treated neurons (0.40 ± 0.02 μm/s, $n = 37$). However, the retrograde movements were nearly the same between control and FEZ1-RNAi-456-treated neurons [0.66 ± 0.05 μm/s ($n = 32$) and 0.60 ± 0.06 μm/s ($n = 23$), respectively]. As mitochondria morphology was changed dramatically upon FEZ1 repression in neurons, we considered the possibility that the change of size of mitochondria might have affected their movements. However, there could be observed no correlation between the size of mitochondria and the speed of their movements (data not shown). Taken together, these results demonstrate that FEZ1 is involved in the mitochondrial transport during differentiation and polarization of hippocampal neurons and repression of FEZ1 expression results in the significant changes in the anterograde movement, morphology, and occupancy of mitochondria.

Discussion

Several studies have shown that FEZ1 is an essential protein for neuronal outgrowth in PC12 cells [1,4,11]. In rat brain, FEZ1 is strongly expressed in hippocampus [3]. Thus, we then focused on the role of FEZ1 in mammalian neurons. Our present results show that FEZ1 is important for the polarization of hippocampal neurons. Moreover, the alterations of mitochondrial morphology and retardation of their movement were observed by introduction of FEZ1-specific RNAi. Therefore, as demonstrated with the FEZ1 orthologue, UNC-76, in invertebrate *C. elegans* and *Drosophila* [2,6], FEZ1 is strongly suggested to be involved in the axonal outgrowth and cargo transport in mammalian neurons.

How could FEZ1 contribute to the establishment of the neuronal polarity in hippocampal neurons? FEZ1 interacts with and is phosphorylated by an atypical protein kinase C zeta (PKCζ) [1]. In mammalian neurons, the association of PKCζ with Par3 and Par6 is essential for the neuronal polarization through the regulation of the microtubule elongation in growing axons [12–14]. Microtubules are the major component of axons, where they are implicated in transport and neurotransmission [15]. By yeast two-hybrid and GST pull-down assays, FEZ1 has been shown to interact with CLASP2 [16], which is involved in the regulation of microtubules dynamics [17]. Also, we have recently found that FEZ1 interacts directly with α- and β-tubulin, the monomers of microtubules filaments (Fujita et al., unpublished). In the present study, the endogenous FEZ1 is localized in the cell body and both of axon and dendrites of hippocampal neurons (see Fig. 1B). These data suggest that FEZ1 is involved in the microtubule formation through association with tubulin, microtubule-associated proteins (e.g., CLASP2), and/or PKCζ. Interestingly, overexpressed FEZ1-GFP in hippocampal neuron is co-localized with the microtubule-associated protein tau1 (see Fig. S-3). During axonal outgrowth, the phosphorylation of tau1 is necessary for the microtubule formation in axon [18]. To our knowledge, it has been unsettled if PKCζ can phosphorylate tau1 directly or not. It is likely that the FEZ1/PKCζ complex indirectly but positively regulates the phosphorylation of tau1 to determine the neuronal polarity.

Repression of FEZ1 by RNAi retarded the anterograde movement of mitochondria in the neurites of hippocampal neurons (see Fig. 2E). The transport of mitochondria along the microtubules requires motor proteins (e.g., kinesin and dynein) and regulatory proteins (e.g., microtubules-associated proteins) [15,19,20]. Tau1 inhibits kinesin-dependent fast axonal transport of cargos in cell culture models [21,22]. Phosphorylation of tau1 antagonizes the inhibition of axonal transport [23]. UNC-76, a *Drosophila* orthologue of FEZ1, is involved in the axonal transport of cargos through interaction with the tail domain of kinesin heavy chain [6]. Although FEZ1 is not co-localized with mitochondria (see Fig. 1C), the FEZ1/PKCζ complex may upregulate the anterograde mitochondrial transport by acting on the microtubule rails, possibly by promoting the release of tau1 from microtubule, and/or the motor protein kinesin.

On the other hand, in FEZ1-RNAi-456-treated neurons, mitochondria were present in all the neurites processes, the speed of mitochondrial movement was significantly reduced, and the length of mitochondria was longer than that of the control neurons. Although mature cortical neurons show rather uniform morphologies of mitochondria [24], it is unclear how FEZ1 could participate in determination of the mitochondrial morphology. The reduced mobility of mitochondria in FEZ1-RNAi-456-treated neurons may result in the morphological change of mitochondria to more elongated shape by promoting their fusion.

In conclusion, our present study demonstrates that FEZ1 is important for the establishment of neuronal polarity, presumably controlling the mitochondrial transport along axon. Mitochondria are essential for the axonal growth, the regulation of synaptic plasticity, and the establishment of neuronal polarity [8,25,26]. Thus, the axonal guidance machinery involving FEZ1, microtubules, microtubules-associated proteins, and motor proteins should be studied also by taking into account of mitochondria. Clarifying the role of FEZ1 in the regulation of microtubule dynamics and mitochondrial transport in neurons would be expected to shed light on the molecular mechanism of the development of brain and certain brain pathologies, such as schizophrenia [27].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.142.

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