KIF1Bβ mutations detected in hereditary neuropathy impair IGF1R transport and axon growth

Fang Xu1, Hironori Takashahi1, Yosuke Tanaka1, Sotaro Ichinose1, Shinsuke Niwa1, Matthew P. Wicklund2, and Nobutaka Hirokawa1,3

KIF1Bβ is a kinesin-3 family anterograde motor protein essential for neuronal development, viability, and function. KIF1Bβ mutations have previously been reported in a limited number of pedigrees of Charcot-Marie-Tooth disease type 2A (CMT2A) neuropathy. However, the gene responsible for CMT2A is still controversial, and the mechanism of pathogenesis remains elusive. In this study, we show that the receptor tyrosine kinase IGF1R is a new direct binding partner of KIF1Bβ, and its binding and transport is specifically impaired by the Y1087C mutation of KIF1Bβ, which we detected in hereditary neuropathic patients. The axonal outgrowth and IGF-I signaling of Kif1b−/− neurons were significantly impaired, consistent with decreased surface IGF1R expression. The complementary capacity of KIF1Bβ-Y1087C of these phenotypes was significantly impaired, but the binding capacity to synaptic vesicle precursors was not affected. These data have supported the relevance of KIF1Bβ in IGF1R transport, which may give new clue to the neuropathic pathogenesis.

Introduction
Kinesin superfamily proteins (KIFs) largely serve as microtubule molecular motors that transport essential cellular materials to specific destinations, while very little is known regarding how they regulate the cellular signal transduction cascades specifically involved in important cellular functions (Hirokawa et al., 2010; Hirokawa and Tanaka, 2015). KIF1Bβ is a kinesin-3 family member that plays an indispensable role in neuronal survival, morphogenesis, and function (Zhao et al., 2001). Kif1b−/− mice die soon after birth due to apnea. The brains of Kif1b−/− mice are reduced in volume by ~10% compared with the brains of WT littermates. The cellularity, organization, and the development of the brain stem nuclei and commissural fibers are also significantly decreased. The dissociated hippocampal neuron culture shows severe neuronal death by day in vitro (DIV) 3, and KIF1Bβ expression restores neuron number. However, a distinct splicing isoform with a totally different cargo-binding domain, KIF1Bα, cannot restore the neuron number. In contrast, KIF1Bβ and its closely related motor protein KIF1A both transport synaptic vesicle precursors including synaptophysin, SV2, and synaptotagmin down the neuronal axons (Zhao et al., 2001) by binding to the dynamic adaptors Rab3A-GTP and differentially expressed in neoplastic versus normal cells protein (DENN)/MAPK-activating death domain (MADD; Niwa et al., 2008). In oligodendrocytes, KIF1Bβ is essential for Mbp and 36k mRNA localization, and it has a role in the development of myelinated axons in the central and peripheral nervous systems (Lyons et al., 2009).

Interestingly, the phenotypes of Kif1b−/− neurons are much more severe than those deficient of presynaptic proteins or adaptor proteins such as synaptophysin, synaptotagmin, SV2A or 2B, and Rab3A (Geppert et al., 1994a,b; McMahon et al., 1996; Crowder et al., 1999; Janz et al., 1999), suggesting that there may be a novel cargo of KIF1Bβ other than synaptic vesicle precursors that essentially regulates neuronal survival and axonal outgrowth.

Insulin-like growth factors (IGFs) control the growth and differentiation of neural cells, enhancing dendrite extension, axon elongation, synaptogenesis, spine formation, and myelination. IGF1R dominantly mediates the growth-promoting actions of IGF-I and IGF-II (O’Kusky and Ye, 2012). Igf-I–knockout (KO) mice and Igf1r-KO mice tend to die immediately after birth from respiratory failure, showing reduced brain sizes, muscle hypoplasia, reduced neuron numbers, and impaired commissural fiber formation (Liu et al., 1993; Beck et al., 1995). IGF1R is a heterotetrameric glycoprotein with paired disulfide-linked extracellular α- and transmembrane β-subunits (Baron-Van Evercooren et al., 1991). The binding of the α-subunit to IGF-I leads to the activation of the β-subunit kinase domain and subsequently transduces the signal to downstream MAPK and PI3K-Akt pathways (Kuemmerle and Murthy, 2001).

© 2018 Xu et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms/). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 4.0 International license, as described at https://creativecommons.org/licenses/by-nc-sa/4.0/).
IGF-I signaling was reported to play a role in neuronal survival in vivo and in vitro. Significant neuronal loss was reported in IGF-I−/−KO mice (Beck et al., 1995; Camarero et al., 2001) and IGFIR-deficient mice (Liu et al., 2009). In contrast, IGF-I-overexpressing transgenic mice showed a substantial increase in the number of neuron bodies (Dentremont et al., 1999; O’Kusky et al., 2000; Hodge et al., 2005). IGFIR is required for the IGF-I-activated Akt survival cascade in neuronal cells (Dudek et al., 1997; Lu et al., 2008). Furthermore, IGF-I stimulates the axonal outgrowth of motor and sensory neurons (Özdinler and Macklis, 2006; Scolnick et al., 2008). Commissural fibers of IGF-I−/− mouse brains are greatly reduced in area and thickness compared with WT littermates (Beck et al., 1995). The IGFIR-mediated PI3K pathway is essential for the establishment of the polarity of hippocampal neurons (Sosa et al., 2006) and for regulating membrane expansion at the nerve growth cone, which is necessary for axonal outgrowth (Laurino et al., 2005). MAPK signaling has been also reported to promote axonal outgrowth (Forcet et al., 2002). Changes in serum IGF levels were found to be associated with neurodegeneration (Busiguitina et al., 2000). Recent research also showed that treatment with recombinant human IGF-I ameliorates the symptoms of a mouse model of Rett Syndrome with IGF-I deficiency (Castro et al., 2014). Thus, the IGF-I signaling pathway is a potential therapeutic target for neurological disorders.

Charcot-Marie-Tooth (CMT) disease is one of the most common inherited peripheral neuropathies, with a prevalence of 1:2,500 (Skre, 1974) as the result of summing up the mutations in >30 genes (Saporta et al., 2011); this condition is also known as hereditary motor and sensory neuropathy. CMT disease is divided into two main types on the basis of electrophysiological properties and neuropathology: CMT1 and CMT2. Demyelinating CMT1 has a nerve conduction velocity <38 m/s, and axonal CMT2 has nerve conduction velocities >38 m/s. For CMT1, axonal loss occurs largely due to affected Schwann cells, whereas Mitofusin 2 (MFN2) mutations, which impair mitochondrial fusion and ER-mitochondria tethering, are responsible for the most common known axonal form of CMT2A2 (Züchner et al., 2004; Kijima et al., 2005; Lawson et al., 2005; Baloh et al., 2007). Although a KIF1B mutation in the same genomic interval was the first identified in a pedigree with CMT2A, only a few pedigrees with KIF1Bβ mutation for CMT2A1 have been reported until recently. We previously reported one CMT2A1 pedigree with the clinical mutation of human Kif1bβ gene in the patients from two independent pedigrees of hereditary neuropathy suffering from CMT2A symptoms including progressive muscle weakness and slight central nervous system defects. We show cell biological evidence that this Y1087C mutation significantly affects the IGFIR binding capacity of KIF1Bβ and that KIF1Bβ Y1087C is impaired in complementing the defects in IGFIR transport and axonal outgrowth of Kif1b−/− mouse primary hippocampal neurons. These data will newly propose that KIF1Bβ as a responsible gene preventing hereditary axonopathy through the association of its specific binding domain with IGFIR, which enhances the expression of IGFIR and the IGF-I–mediated signaling in the peripheral axon.

Results

KIF1B is essential for axonal outgrowth

We previously reported that the corpus callosum of Kif1b−/− mice was significantly malformed (Zhao et al., 2001), which was further confirmed by histological analyses (Fig. S1, A–D). We hypothesized that this is because of the impairment in axonal outgrowth. To evaluate it, we compared axonal outgrowth in vitro using dissociated hippocampal neurons from 17.5–d postcoitum (dpc) mouse brains. TagRFP was transiently transfected to show the morphology of single neurons at 1 d before the observation. The cells were then fixed and visualized by confocal microscopy using the 568-nm channel. We found that axonal outgrowth was significantly impaired in Kif1b−/− neurons at DIV2–3 (Fig. 1, A and B). In the knockdown experiments using the KIF1Bα- and -β–specific miRNA vectors, we found that the β isoform of KIF1B was specifically responsible for this impairment in axonal outgrowth, which could be rescued by expression of RNAi-resistant KIF1Bβ (Fig. 1, C–E; and Fig. S1, E and F). Using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of dissociated hippocampal neuron culture at DIV3, we found that neurite-bearing Kif1b−/− neurons were not largely apoptotic, excluding the nonspecific effect of apoptosis as the cause of the axonal elongation phenotype (Fig. 1 F). However, the ratio of apoptotic cells in Kif1b−/− neurons was significantly higher than that in Kif1b−/− neurons (Fig. 1 G), consistent with our previous data (Zhao et al., 2001). IGFIR is specifically associated with KIF1Bβ

Considering that these phenotypes were most similar to those of IGFIR-KO mice (Liu et al., 1993, 2009), we sought to investigate whether IGFIR is a novel cargo of KIF1Bβ. Because the nascent IGFIR protein undergoes proteolysis to generate a N-terminal extracellular α-subunit (IGFIRα) that has a ligand-binding domain and a C-terminal transmembrane β-subunit (IGFIRβ) that is extracellularly linked to the α-domain by disulfide bonds and possesses an intracellular catalytic domain (Massagué and Czech, 1982), the intracellular domain (ICD) of IGFIRβ is considered the only portion exposed to the cytoplasm. First, we conducted yeast two-hybrid analysis to explore the direct binding capacity between KIF1Bβ and IGFIR. Using the ICD of IGFIRβ as the prey, blue colonies indicating a positive signal were successfully formed with the indicated bait fragments of

Xu et al.
The KIF1Bβ kinesin transports IGF1R down the axon
the mouse KIF1Bβ stalk domain (Fig. 2 A). The binding domain of KIF1Bβ was thus minimized to 885–1,410 aa. However, mouse KIF1A 836–1,346 aa, which was homologous to mouse KIF1Bβ 885–1,410 aa, did not result in blue colony formation (Fig. S2, A and B), suggesting that IGF1Rβ specifically binds to KIF1Bβ instead of KIF1A. The stalk domain of KIF1Bβ shares a high homology with the corresponding domain of KIF1A, with a sequence identity of 65%. Because we found a nonconserved 20-aa stretch just before the core-binding residue Tyr1087 (Fig. S2 A), it is reasonable to hypothesize that these two motors have different binding specificities.

Second, this binding was confirmed by vesicle immunoprecipitation (IP) using newly established Kif1bβ-EGFP-knock-in mice, of which hippocampal neurons represented axonal transport of fluorescent organelles at ∼1 µm/s (Fig. S2, C–F; Video 1). We immunoprecipitated the whole-brain lysates of adult Kif1bβ-EGFP and Kif1bβ+/− mice using an anti-GFP antibody. The immunoprecipitates were labeled against GFP, KIF1Bβ, and IGF1Rα, and this labeling specifically showed the association between KIF1Bβ-EGFP and IGF1R-associated vesicles (Fig. 2 B).

Third, we conducted a vesicle coflotation assay. Adult WT mouse brains were homogenized in Hepes-sucrose buffer, and the S1 fraction was subjected to Nycodenz density-gradient differential centrifugation (Fig. 2 C). The results showed that IGF1R and synaptophysin cofloted with KIF1Bβ.

To evaluate whether the interaction is direct or not, we next performed a pulldown assay using purified recombinant proteins. FLAG-tagged ICD of IGF1Rβ purified from the HEK293 cell could significantly bind to purified GST-tagged KIF1Bβ 885–1,410 aa compared with the purified GST-only as a negative control (Fig. 2 D).

Finally, we conducted live imaging. KIF1Bβ-EGFP and signal peptide (SP)-IGF1Rβ-RFP expression vectors were cotransfected into dissociated hippocampal neurons at DIV5, and the neurons were observed 2 d later using a spinning-disk confocal microscope to assess the comigration of these two proteins (Fig. 2 E and...
Video 2). Kymograph analyses suggested that the comigration speeds of these vesicles were \sim 1 \mu m/s (Fig. 2 F), comparable with the previously measured velocity of KIF1Bβ (Zhao et al., 2001). To confirm that IGF1R is transported by KIF1Bβ, we transfected Kif1b+/+, Kif1b+/−, and Kif1b−/− hippocampal neurons with SP-IGF1Rβ-RFP and compared the number of anterograde transport of IGF1R. Kif1b+/− neurons showed less transport than Kif1b+/+ neurons (Fig. S2, G–J). Furthermore, little transport was observed in Kif1b−/− neurons. These data indicate that IGF1R requires KIF1Bβ to be transported down the axon. Altogether, these data suggest that the stalk domain of KIF1Bβ is associated with membrane vesicles containing IGF1R through the ICD of IGF1Rβ.

**Decreased surface expression of IGF1R in Kif1b−/− neurons**

To explore the physiological relevance of KIF1Bβ to the IGF1R distribution, we dissected hippocampal neurons from Kif1b−/− mice and compared the number of anterograde transport of IGF1R. Kif1b−/− neurons showed less transport than Kif1b+/+ neurons (Fig. S2, G–J). Furthermore, little transport was observed in Kif1b−/− neurons. These data indicate that IGF1R requires KIF1Bβ to be transported down the axon. Altogether, these data suggest that the stalk domain of KIF1Bβ is associated with membrane vesicles containing IGF1R through the ICD of IGF1Rβ.

**Figure 2. IGF1R is a novel cargo of KIF1Bβ.**

(A) Yeast two-hybrid assays assessing the direct binding between the ICD (961–1,373 aa) of mouse IGF1Rβ and various deletion mutants of mouse KIF1Bβ. Note that the 885–1,410-aa region within the KIF1Bβ stalk domain is the minimal essential region for its binding. PH, Pleckstrin homology. (B) Endogenous vesicle IP. IB of the brain lysates (Lys) of Kif1bβGFP/GFP and Kif1bβ+/+ (WT) mice and their immunoprecipitates using an anti-GFP antibody (IP by anti-GFP) labeled with the indicated antibodies. (C) Nycodenz gradient vesicle flotation assay of adult mouse brain lysates labeled with the indicated antibodies. Note that KIF1Bβ, IGF1Rα, IGF1Rβ, and synaptophysin are detected in the same fractions 14–15. (D) A Coomassie Brilliant Blue–stained gel for a pulldown assay between purified GST–KIF1Bβ885–1,410 and IGF1RβICD–3×FLAG showing their direct interaction. (E and F) Double-color time-lapse fluorescence images of a dissociated hippocampal neuron at DIV7 transfected with KIF1Bβ-EGFP and SP-IGF1Rβ-TagRFP using spinning-disk confocal microscopy equipped with 100×/1.46 Plan Apochromat oil-immersion objective lens (E) and its kymograph (F). The cell body is on the left side. Bars, 10 µm. Note that the speed of the comigrating vesicle (arrows in E) is \sim 1 \mu m/s. See Video 2.
The KIF1Bβ kinesin transports IGF1R down the axon

using cortical neurons of Kif1b+/+ and Kif1b−/− mouse embryos at 17.5 dpc. The neurons were cultured in 10-cm dishes for ~7 d. The membrane proteins were biotinylated, prepared for immunoblotting (IB), and normalized with the β-actin levels of the input. The IGF1R level in the cell surface fraction of Kif1b−/− neurons was significantly lower than that of Kif1b+/+ neurons (Fig. 3 E). The statistical analysis was obtained from eight repetitions of the IB results of three independent experiments (Fig. 3 F). Interestingly, KIF1Bβ was detected in the surface fractions. Considering that another molecular motor cytoplasmic dynein has been reported to be associated with the plasma membrane and tethers microtubules, KIF1Bβ might be recruited by these cortically-tethered microtubules (Hendricks et al., 2012). We also noted that the total amount of IGF1R of Kif1b−/− neurons was significantly decreased compared with that of Kif1b+/+ neurons. Bar, 10 μm. n = 31–42. *, P < 0.05; **, P < 0.01; one-sided unpaired Welch’s t test.

Impaired IGF-I signaling in KIF1Bβ-deficient neurons
To examine the possible changes in IGF1R signal transduction caused by KIF1B deficiency, dissociated hippocampal neurons were cultured for 3 d, starved for 2 h without serum or B27 supplement, and stimulated for 5 min with 10 nM IGF-I in the medium (G) accompanied with the statistical analysis (H). The axons of Kif1b+/+ neurons with 10 nM IGF-I in the culture were significantly longer than the ones without IGF-I. However, the IGF-I stimulation was less effective in Kif1b−/− neurons. Bars, 25 μm. n = 29–40. *, P < 0.05; ns, P > 0.05, one-sided unpaired Welch’s t test.
The responsible isoform of KIF1B was further specified into KIF1Bβ using RNA interference. WT dissociated hippocampal neurons were electroporated with the TurboRFP-conjugated miRNA vector for the control or KIF1Bβ, plated, cultured for 4 d, and then starved for 2 h. The cells were treated with or without 10 nM IGF-I stimulation for 5 min and then fixed and immunostained using the anti-pAkt and anti-pERK antibodies. KIF1Bβ knockdown led to significant reduction in pAkt and pERK levels both in the axon tips and in the cell bodies (Fig. 4, C and D; and Fig. S4, C and D).

We further evaluated whether the impairment in IGF1R/PI3K signal transduction affected the axon outgrowth in Kif1b−/− neurons. Kif1b−/− neurons transfected with constitutively active Ras V12, which is upstream to the PI3K–Akt and ERK signaling (Castellano and Downward, 2011; Tanaka et al., 2016), showed a significant increase in the axon length compared with the Kif1b−/− neurons at DIV3 (Fig. 4, E and F). Interestingly, the effect of Ras V12 was more evident in the Kif1b−/− neurons than Kif1b+/+ neurons. These data indicate that KIF1Bβ is essential for IGF1R-mediated PI3K and ERK signaling and axonal outgrowth.

KIF1Bβ-Y1087C mutation detected in patients affects IGF1R binding capacity

Coincidentally, we identified two hereditary neuropathy pedigrees carrying the same KIF1Bβ mutation Y1087C (Fig. 5, A and B; Table 1) by exome sequencing of 33 CMT2/neuropathy-related genes (Table 2). According to the in silico pathogenicity evaluation programs PP2 and SIFT, this KIF1Bβ mutation was the only one with disease-causing capacity in the single-nucleotide polymorphisms (SNPs) within these 33 genes including MFN2.

Because Tyr1087 is located in the middle of the IGF1R minimal binding sequence (885–1,410 aa) as above, we conducted GST-pulldown assays to investigate whether this Y1087C mutation affected the binding capacity of KIF1Bβ to IGF1R and synaptic vesicle precursors. WT adult mouse brain lysates were pulled down against GST-fused mouse KIF1Bβ 885–1,410 aa with or without the Y1087C mutation. The mutated KIF1Bβ 885–1,410 aa (Y1087C) fragment tended to exhibit a lower binding capacity to the IGF1R subunits than WT KIF1Bβ 885–1,410 aa. However, the binding capacities to the synaptic vesicle precursor pro-
Table 1. Summary of the clinical manifestations

| Patient 1 | Basic information | Major problems | Laboratory tests and diagnosis | Family history |
|-----------|------------------|----------------|--------------------------------|----------------|
|           | Left-handed Caucasian male, 55 yr old (on diagnosis) | Progressive, symmetric, length dependent, sensory motor, predominantly axonal polyneuropathy with intermittent cranial nerve involvement, Bell’s palsy, etc. | Hearing loss; normal MRI of the brain; severe symmetric sensorimotor length-dependent, axonal polyneuropathy with acute and chronic features; mixed axonal and demyelinating neuropathy of the right radial nerve; median nerve conduction velocity 38.9m/s; diagnosed with CMT2 disease | No symptom of three brothers, three sisters and mother; however, father died from an accident at a young age |

| Patient 2 | Basic information | Major problems | Laboratory tests and diagnosis | Family history |
|-----------|------------------|----------------|--------------------------------|----------------|
|           | Left-handed Caucasian male, 40 yr old (on diagnosis) | Mental retardation, bipolar disorder, hypertension, cystic kidney disease, and right basal ganglia lacune | An MRI of the brain showing thinned corpus callosum in the posterior half; electromyography and nerve conduction velocity measurements implying predominantly length-dependent, axonal, sensorimotor polyneuropathy; diagnosed with autosomal-dominant CMT2 disease | Brother having the constellation of mental retardation, psychiatric disease, kidney disease, and axonal CMT disease; mother having a history of CMT disease and mild cognitive dysfunction |

Figure 5. KIF1Bβ-Y1087C protein reduced the affinity to IGF1R. (A) An American pedigree of CMT2A1 patients. The proband (Patient 2 in Table 1) was a 43-yr-old male with KIF1Bβ Y1087C mutation genetically confirmed, and so was his 48-yr-old brother. Their mother was also clinically diagnosed as CMT2 and deceased in her 60s, while their father was asymptomatic and healthy at the age of 72 yr. (B) Amino acid sequence alignment of the human KIF1Bβ stalk region spanning the Y1087C mutation between WT and the new CMT2A pedigree (Mut) together with WT mouse KIF1Bβ. (C and D) The GST-pull-down assay of mouse brain lysates using GST-mouse KIF1Bβ 885–1,410 aa with or without the Y1087C mutation immunoblotted with the indicated antibodies (C) and its quantification (D). The signal intensities were normalized by the respective GST-tagged protein expression levels. Note that the binding capacity to IGF1R but not to DENN/MADD or synaptophysin was significantly decreased by the mutation. CBB, Coomassie Brilliant Blue. n = 3. * P < 0.05, one-sided paired Welch’s t test. (E–G) Superresolution immunocytochemistry of hippocampal neurons at DIV7 against DENN/MADD (green) and IGF1Rα (red) at low (E) and high (F) magnifications, respectively, followed by the quantification (G). Bars: 10 µm (E); 1 µm (F). Note that there are few colocalizing vesicles with ∼17% for DENN/MADD (n = 142) and 8% for IGF1R (n = 299) of the total vesicles, respectively.
The characterization of the CMT2A mutation

| Mutated gene | Position | Amino acid change | Zygosity | Inheritance | 1000 Genomes Minor Allele Frequency |
|--------------|----------|------------------|----------|-------------|-----------------------------------|
| KIF1B        | CHR1:     | Y1087C           | Heterozygote | Autosomal dominant | 0.0395                           |

**Table 2.**

Analyzed genes

- AARS (601065), ATL1 (606439), DNMT2 (602378), DNMT1 (126375), DYNC1H1 (600112), EGR2 (129010), FGD4 (611104), GARS (600287), GDAP1 (606598), GJB1 (304040), GLA (300644), HSFBP1 (602195), HSF8 (608014), KIF1B (605995), LITAF (603795), LAMA1 (150330), LRSAM1 (610933), MED25 (610197), MFN2 (608507), MYB (159440), MTRM2 (603557), NDRG1 (605262), NEFL (162280), PM2 (601097), PRK (605725), RAB7A (602928), SBF2 (607697), SCN9A (603415), SH3TC2 (608206), SPTLC2 (605713), TRPV4 (605427), TTR (176300), and YARS (603623)

The KIF1Bβ kinesin transports IGF1R down the axon

To determine whether IGF1R and synaptic vesicle precursors are transported on the same vesicles, we then conducted fluorescence immunocytochemistry of WT dissociated hippocampal neurons against DENN/MADD and IGF1R and observed them using superresolution structured illumination microscopy (SR-SIM). DENN/MADD and IGF1R scarcely colocalized with each other (Fig. 5, E–G). These data collectively suggest that IGF1R directly binds to a specific domain in KIF1Bβ stalk domain including Tyr1087 and is transported independently from synaptic vesicle precursors. The mutation Y1087C in KIF1Bβ critically affected the IGF1R transport and, accordingly, may affect downstream IGF-I signaling to enhance the neurotropic vesicle precursors. The mutation Y1087C in KIF1Bβ critically and specifically affected the IGF1R transport and, accordingly, may affect downstream IGF-I signaling to enhance the neurotropic symptoms.

**KIF1Bβ-Y1087C is impaired in complementing KO neuron phenotypes**

To further investigate the functional deficits of the KIF1Bβ-Y1087C mutation, we conducted rescue experiments using KIF1Bβ−/− hippocampal neurons. First, to assess axon length, dissociated neurons were transfected using the respective EGFP-tagged expression vectors together with a TagRFP expression vector at DIV1, fixed at DIV3, and analyzed for axon lengths using confocal microscopy observations with the 568-nm excitation. Transfection of mouse WT KIF1Bβ restored axonal outgrowth. However, transfection of KIF1Bβ-Y1087C mutants KIF1Bβ-Q98L, KIF1Bα, and KIF1A significantly failed in this complementation capacity compared with the WT KIF1Bβ, while KIF1Bβ-Y1087C could partially rescue it (Fig. 6 A). The expression of recombinant proteins of the predicted lengths was confirmed by IB (Fig. 6 B), and the statistical analyses of the axon lengths are shown in Fig. 6 C. Knockdown rescue experiments also confirmed the dysfunction of KIF1Bβ-Y1087C (Fig. S1, E and F). Knockdown-resistant KIF1Bβ WT successfully rescued the impairment in axon outgrowth, while the neurons transfected with KIF1Bβ-Y1087C showed shorter axon lengths than those with WT.

To evaluate whether the impairment of axon outgrowth in the neurons expressing KIF1Bβ-Y1087C was caused by the impaired IGFIR/PI3K signal transduction, we next quantified the pAkt levels in the neurons transfected with miRNA and knockdown-resistant KIF1Bβ. Knockdown of KIF1Bβ caused a reduction in the pAkt levels in the axon shaft when stimulated with IGF-I, while KIF1A knockdown as a control showed no significant changes, which was consistent with the results mentioned above (Fig. 7, D and E). Besides, the neurons expressing KIF1Bβ-Y1087C also showed a significant reduction in pAkt levels, whereas KIF1Bβ-WT successfully rescued this reduction. Considering that the total Akt level was not changed in any condition (Fig. S5, A and B), these data suggest that IGFIR-mediated PI3K signal transduction was incomplete in the KIF1Bβ−/− neurons expressing KIF1Bβ-Y1087C and thus, the incomplete signaling cascade might impair the axon outgrowth.

**Y1087C mutation impaired the IGF1R transport**

As we mentioned, the impairment in axon outgrowth and the reduction of pAkt levels in KIF1Bβ−/− neurons may be induced by the decreased transport of IGFIR. Likewise, we hypothesized that KIF1Bβ-Y1087C could not transport IGFIR sufficiently. To compare the activity of KIF1Bβ mutants for the surface presentation of IGFIR, KIF1Bβ−/− hippocampal neurons were cultured at a moderate density, transfected with 3× FLAG-tagged expression vectors together with a TagRFP expression vector at DIV1, fixed at DIV4, and immunostained using an anti-IGFIRα antibody without permeabilization. KIF1Bβ−/− neurons were also stained as a positive control. The axonal surface IGFIR expression levels at ~100 µm distal from the cell body were analyzed. The expression of mouse KIF1Bβ significantly rescued the axonal IGFIR expression level compared with control, while other constructs had lesser capacity for this complementation, similar with the case of axonal outgrowth above (Fig. 7, A and B). The expression of recombinant proteins of the predicted lengths was confirmed by IB (Fig. 7 C), and the statistical analysis of the axonal surface IGFIR levels at 100 µm distal from the cell body is shown in Fig. 7 D. These data indicate that the KIF1Bβ−/− neurons expressing KIF1Bβ-Y1087C could not still sufficiently transport IGFIR.

To obtain further confirmation, we next observed the IGFIR transport in the KIF1Bβ−/− hippocampal neurons expressing KIF1Bβ WT or Y1087C with live imaging. Because of the reduction in endogenous KIF1Bβ and IGFIR levels, the KIF1Bβ−/− neurons provided a very suitable system for live imaging of transfected proteins (Fig. 8, A–C). As a result, the number of transported IGFIR-containing vesicles was not rescued in the neurons expressing KIF1Bβ-Y1087C, whereas it was rescued in the neurons expressing WT (Fig. 8 D). To obtain further insight of the mutant, we analyzed the transport quantitatively. The ratio of anterograde over retrograde transports of IGFIR was not significantly altered, suggesting that the retrograde transport is not impaired.
transport was proportionally impaired because of the reduction of IGF1R levels in the distal part of the axon in the neurons expressing the Y1087C mutant (Fig. 8 E). The velocity of KIF1Bβ was not significantly different either between WT and Y1087C or between the KIF1Bβ vesicles with and without IGF1R (Fig. 8 F). These data indicate that neither Y1087C mutation or the IGF1R interaction affected the motor activity. However, the ratio of comigrating over total KIF1Bβ-containing vesicles was significantly reduced in the neurons expressing Y1087C (Fig. 8 G). These results suggested that Y1087C could not gain the sufficient affinity to IGF1R. Thus, KIF1Bβ-Y1087C failed in transporting IGF1R to the axon. These mutation analyses in vivo confirmed that the IGF1R is a novel cargo of KIF1Bβ, and a disorder of this transport inhibited the IGF-I signaling and proper axon outgrowth. Because KIF1Bβ-Y1087C mutation in the hereditary neuropathy patients partially but significantly affected these capacities, it probably enhances the pathogenesis of neuropathic symptoms in those patients.

Discussion

KIF1Bβ has been reported to transport synaptic vesicle precursors including synaptophysin, SV2, and synaptotagmin through the regulatory adaptors Rab3-GTP and DENN/MADD (Niwa et al., 2008). KIF1Bβ also transports dendritically localized mRNPs and is recruited to synapses in an activity-dependent manner (Charalambous et al., 2013). In oligodendrocytes, KIF1Bβ is involved in the localization of Mbp and 36k mRNA and is essential
Xu et al.
The KIF1Bβ kinesin transports IGF1R down the axon

for the development of myelinated axons (Lyons et al., 2009). However, transport defects in these cargos cannot explain the phenotypes of Kif1b−/− neurons, i.e., severe apoptosis and impaired axonal outgrowth; therefore, the existence of an unknown cargo promoting cell viability and axonal outgrowth has long been expected.

In this study, we first identified the association of IGF1R with KIF1Bβ using yeast two-hybrid, vesicle IP, cofloation, pulldown assay, and comigration experiments, and we also detected impairments in the surface presentation of IGF1R in Kif1b−/− neuronal axons, suggesting that IGF1R is a novel cargo of KIF1Bβ. These data clarify some of the missing information. IGF1R specifically bound to KIF1Bβ 885–1,410 aa, and coincidentally, we identified human pedigrees of CMT2A carrying the Y1087C mutation of KIF1Bβ, which turned out to specifically impair its binding capacity to IGF1R.

We have established a mechanistic link between KIF1Bβ-mediated axonal outgrowth and the IGF-I signaling pathway and propose the hypothesis that KIF1Bβ transports IGF1R in axons to stimulate axonal outgrowth. Because cell density can also affect cell outgrowth, in our research, a higher cell density was applied to compensate for apoptosis and its effect on axonal outgrowth.
due to the lowered cell density (Fig. 1F). Because dissociated hippocampal culture contains growth factor–secreting cells such as astrocytes, a higher density might provide a sufficient concentration of growth factors for the survival of Kif1b−/− neurons. Furthermore, a low transfection efficiency of hippocampal neurons and evidence for impaired responsiveness to exogenous IGF-I (Fig. 4) suggested that the rescue of axonal outgrowth was primarily caused in a cell-autonomous way to increase the responsiveness to IGF-I.

CMT2 is defined as axonal hereditary neuropathy with a normal range of axon conduction speeds but exhibits severe axonal degeneration (Bucci et al., 2012). There are several pedigrees of CMT2A with MNF2 point mutations (Züchner et al., 2004; Kijima et al., 2005; Lawson et al., 2005; Baloh et al., 2007); however, MNF2 as well as >30 related genes were intact in our newly identified CMT2A pedigrees. Both of these pedigrees coincidentally carried the KIF1Bβ mutation of Y1087C, which strengthens the previously known mechanistic link between CMT2A and KIF1Bβ (Zhao et al., 2001; Drew et al., 2015). We present in vitro evidence that this mutation functionally impairs the capacity of KIF1Bβ for specific binding and transport of IGF1R down the axons to affect IGF-I/IGF1R signaling, in contrast with previous negative predictions (Ho et al., 2017). Our in vitro evidence suggested that this mutation is at least responsible for increasing susceptibility to neuropathies by reducing the IGF-I signal transduction.

IGF-mediated MAPK and PI3K–Akt signal transduction has long been known to be essential for neuronal survival and axonal development (Beck et al., 1995; Dudek et al., 1997; Camarero et al., 2001; Özdinler and Macklis, 2006; Scolnick et al., 2008; Liu et al., 2009). Besides, changes in serum IGF levels were found to be associated with neurodegeneration (Busiguina et al., 2000). We have shown in this study that the IGF-mediated signaling is significantly affected by KIF1B deficiency (Figs. 4 and S4), and overexpression of Ras V12 that directly stimulates MAPK and PI3K signaling pathways (Kuemmerle and Murthy, 2001; Castellano and Downward, 2011) could rescue the axonal outgrowth phenotype (Fig. 3). Thus, deficiency in IGF1R signaling could provide a better explanation of the cause of neurodegeneration and impaired axonal elongation in Kif1b−/− mice. These data will also stimulate future translational research into the activation
of the KIF1Bβ/IGF1R cascade to protect neurons against hereditary neuropathies.

Interestingly, IGF1R did not bind to the corresponding region of KIF1A (Fig. S2). This finding was consistent with the fact that KIF1A expression did not restore either axonal outgrowth or axonal surface IGF1R expression in Kif1b−/− neurons (Figs. 6 and 7). Although KIF1A is very similar to KIF1Bβ and synergistically transports synaptic vesicle precursors (Niwa et al., 2008), we recently proposed that instead of KIF1Bβ, only KIF1A transports the nerve growth factor (NGF) receptor TrkA through the Rab3-GTP adaptor (Tanaka et al., 2016). As we have identified in this study that only KIF1Bβ transports the IGF-1 receptor IGF1R by the direct binding of the stalk domain, KIF1A and KIF1Bβ may complementarily transport different receptor tyrosine kinases down the axons to respectively augment the NGF and IGF signaling pathways. Although both kinesin-3 motors are highly expressed in adult brains, dorsal root ganglion neurons express KIF1Bβ only in the juvenile or regenerating stages and solely express KIF1A in the mature stage (Takemura et al., 1996; Gumy et al., 2011; Tanaka et al., 2016). Furthermore, we previously identified that another kinesin-3 motor, KIF16B, transports fibroblast growth factor receptor for stem cell differentiation and proliferation (Ueno et al., 2011). Because FGF-, IGF-, and NGF-mediated transmembrane receptor tyrosine kinase pathways have been reported to sequentially and cooperatively enhance neuronal differentiation and survival through modality-specific adaptor proteins (Recio-Pinto et al., 1986; Nakafuku et al., 1992; Jones et al., 2003), the down-regulation of KIF1Bβ in adult peripheral nervous system may provide a hint to the answers of long-standing questions such as why the symptoms of CMT disease are peripheral dominant even though the responsible genes are ubiquitously expressed (Sullivan et al., 2008; Buccigrossi et al., 2012) and why axonal regeneration and axonal outgrowth are generally suppressed in the adult central nervous system (Raivich and Makwana, 2007; Wu et al., 2007; Huebner and Strittmatter, 2009; Luigetti et al., 2016). Among these three kinesin-3 motors, the direct interaction of KIF1Bβ with the cargo was first identified in this study, and it is unique compared with the other two kinesin-3 motors, suggesting divergence in the transport regulation mechanism.

Materials and methods
Mice
Kif1b-KO mice have been previously described (Zhao et al., 2001). Mouse brain histology was performed using paraffin sections as previously described (Zhao et al., 2001). Kif1b−/−EGFP knock-in mice were generated by using the gene-targeting vector (kindly provided by K. Sakimura, which was electroporated in C57BL/6N strain mouse–derived mice were generated by using the gene-targeting vector (Fig. S2), (a Kif1b genomic fragment 130,394–131,090 nt; RefSeq expression no. NC_000070.6) according to methods previously described (Mishina and Sakimura, 2007). C57BL/6J, C57BL/6N, and ICR mice were purchased from CLEA Japan. The KO and knock-in mice were maintained in a specific pathogen-free environment under a 14/10-h light/dark cycle. All animal experiments were conducted under the University of Tokyo’s restrictions and permissions regarding animal experimentation (notification numbers I22T124, I22T126, M-P15-118, and M-P15-119).

Genotyping PCR
For genotyping PCR of Kif1b-KO mice, tail biopsy lyses were subjected to PCR as previously described (Zhao et al., 2001). For that of Kif1b−/−EGFP knock-in mice, the lyses were subjected to PCR using Amplitaq DNA polymerase (PerkinElmer) on a thermal cycler (GeneAmp PCR system 9700; PerkinElmer). For amplifying the knock-in allele, the samples were cycled at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s for 30 cycles using the following primer sets: EGFP-F, 5′-CCATGTCGAGCAAGCCGAGGGTGTTTCA-3′, and EGFP-R, 5′-CATGTCGAGCCGAGGGTGTTTCA-3′, to reveal a 240-bp product. For the WT allele, they were cycled at 94°C for 30 s, 56.4°C for 30 s, and 72°C for 30 s for 30 cycles using the following primer sets: KIF1Bβ-F, 5′-TGGTTTATGAGCTGAGTGG-3′, and KIF1Bβ-R, 5′-TTGTAGAAGATGCTGAG-3′, to reveal a 386-bp product.

Expression and knockdown vectors
To construct mouse IGF1R deletion expression vectors, mouse IGF1R cDNA (IMAGE: 8861891; Open Biosystems) was amplified by PCRs using KOD polymerase (Toyobo) and KAPA HiFi DNA polymerase (KAPA Biosystems). For conformation experiments, SP (the first 30 residues of IGF1R) and Igf1rβ were amplified with PCR and ligated into the pTagRFP-N (Evrogen) vector using the BglII and HindIII sites or HindIII and EcoRI sites, respectively. Mouse Kif1bβ cDNA (Zhao et al., 2001), mouse Kif1a cDNA (Niwa et al., 2008), and human KIF1Bα cDNA (Niwa et al., 2008) were subcloned into the pEGFP-N1 vector (Takara Bio Inc.) and a 3× FLAG (DYKDDDDK)-Ni vector (modified from pEGFP-N1 vector) using the Nhel and SmaI sites or Nhel and HindIII sites, respectively. Mouse Kif1bβ cDNA was mutated using the QuickChange II XL kit (200522; Agilent Technologies) respectively with the following primer sets: 5′-CCACCGGGATCCCTCTCAGAGTGTGCGACACTCT-3′ (sense) and 5′-AGATGTCCTCACTGTCAGAAGGATCCCCGCTGG-3′ (antisense) for introducing a Y1087C mutation in a specific pathogen-free environment under a 14/10-h light/dark cycle. All animal experiments were conducted under The University of Tokyo’s restrictions and permissions regarding animal experimentation (notification numbers I22T124, I22T126, M-P15-118, and M-P15-119).

Expression and knockdown vectors
To construct mouse IGF1R deletion expression vectors, mouse IGF1R cDNA (IMAGE: 8861891; Open Biosystems) was amplified by PCRs using KOD polymerase (Toyobo) and KAPA HiFi DNA polymerase (KAPA Biosystems). For conformation experiments, SP (the first 30 residues of IGF1R) and Igf1rβ were amplified with PCR and ligated into the pTagRFP-N (Evrogen) vector using the BglII and HindIII sites or HindIII and EcoRI sites, respectively. Mouse Kif1bβ cDNA was mutated using the QuickChange II XL kit (200522; Agilent Technologies) respectively with the following primer sets: 5′-CCACCGGGATCCCTCTCAGAGTGTGCGACACTCT-3′ (sense) and 5′-AGATGTCCTCACTGTCAGAAGGATCCCCGCTGG-3′ (antisense) for introducing a Y1087C mutation in a specific pathogen-free environment under a 14/10-h light/dark cycle. All animal experiments were conducted under The University of Tokyo’s restrictions and permissions regarding animal experimentation (notification numbers I22T124, I22T126, M-P15-118, and M-P15-119).

Genotyping PCR
For genotyping PCR of Kif1b-KO mice, tail biopsy lyses were subjected to PCR as previously described (Zhao et al., 2001). For that of Kif1b−/−EGFP knock-in mice, the lyses were subjected to PCR using Amplitaq DNA polymerase (PerkinElmer) on a thermal cycler (GeneAmp PCR system 9700; PerkinElmer). For amplifying the knock-in allele, the samples were cycled at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s for 30 cycles using the following primer sets: EGFP-F, 5′-CCATGTCGAGCAAGCCGAGGGTGTTTCA-3′, and EGFP-R, 5′-CATGTCGAGCCGAGGGTGTTTCA-3′, to reveal a 240-bp product. For the WT allele, they were cycled at 94°C for 30 s, 56.4°C for 30 s, and 72°C for 30 s for 30 cycles using the following primer sets: KIF1Bβ-F, 5′-TGGTTTATGAGCTGAGTGG-3′, and KIF1Bβ-R, 5′-TTGTAGAAGATGCTGAG-3′, to reveal a 386-bp product.

Expression and knockdown vectors
To construct mouse IGF1R deletion expression vectors, mouse IGF1R cDNA (IMAGE: 8861891; Open Biosystems) was amplified by PCRs using KOD polymerase (Toyobo) and KAPA HiFi DNA polymerase (KAPA Biosystems). For conformation experiments, SP (the first 30 residues of IGF1R) and Igf1rβ were amplified with PCR and ligated into the pTagRFP-N (Evrogen) vector using the BglII and HindIII sites or HindIII and EcoRI sites, respectively. Mouse Kif1bβ cDNA was mutated using the QuickChange II XL kit (200522; Agilent Technologies) respectively with the following primer sets: 5′-CCACCGGGATCCCTCTCAGAGTGTGCGACACTCT-3′ (sense) and 5′-AGATGTCCTCACTGTCAGAAGGATCCCCGCTGG-3′ (antisense) for introducing a Y1087C mutation in a specific pathogen-free environment under a 14/10-h light/dark cycle. All animal experiments were conducted under The University of Tokyo’s restrictions and permissions regarding animal experimentation (notification numbers I22T124, I22T126, M-P15-118, and M-P15-119).
TGTATAGTGAGAGCTAAGGGCTTGGCCACCTGACCTGACGC 
CTTACCTCCTTACCTA-3′ (KIF1Bβ-miRNA), in which the underlined nucleotides indicate the antisense target sequences. The TurboRFP CDNA (Evrogen) was inserted before the 5’ miR-flanking region as a marker of transfection. The specific knockdown capacities were verified using IB. The target and TurboRFP coding sequences were subcloned into the pCAGEN vector (a kind gift from Y. Gotoh, The University of Tokyo, Tokyo, Japan) for in vitro electroporation.

**Antibodies**

The following antibodies were purchased from commercial sources: anti-IGF1Rα mouse monoclonal antibody (RRID: AB_10650003; sc-271606; Santa Cruz Biotechnology) diluted 1:500 for IB or 1:100 for immunofluorescence (IF); anti-IGF1Rβ rabbit polyclonal antibody (RRID: AB_2122378; 3027; Cell Signaling Technology) diluted 1:1,000 for IB; an anti-β tubulin III mouse monoclonal antibody (RRID: AB_532291; T5076; Sigma-Aldrich) diluted 1:10,000 for IB or 1:500 for IF; anti-α- tubulin mouse monoclonal antibody DMA (RRID: AB_477593; T9026; Sigma-Aldrich) diluted 1:1,000 for IB; anti-GFP rabbit polyclonal antibody (RRID: AB_221569; A1122; Thermo Fisher Scientific) diluted 1:1,000 for IB or 2 µg/ml for IF; anti-DENN/MADD rabbit polyclonal antibody (RRID: AB_2629447; 13038; Cell Signaling Technology) diluted 1:500 for IB; anti-KIF1Bβ rabbit polyclonal antibody (RRID: AB_94947; MAB368; EMD Millipore) diluted 1:500 for IB; anti–DDD DK-tag mouse monoclonal antibody (RRID: AB_11122930; M185-3L; MBL) diluted 1:1,000 for IB; anti-Akt rabbit polyclonal antibody (RRID: AB_329827; 9272; Cell Signaling Technology) diluted 1:1,000 for IF; anti–phospho-Akt (Thr308; D25E) rabbit monoclonal antibody (RRID: AB_2629447; 13038; Cell Signaling Technology) diluted 1:500 for IF; anti-IGF1Rβ mouse monoclonal antibody (RRID: AB_2571746, Zhao et al., 2001) and was diluted 1:500 for IF. Anti-KIF1Bβ rabbit polyclonal antibody was previously described (RRID: AB_2571746; Zhao et al., 2001) and was diluted 1:500 for IB. An anti-KIF1Bβ rabbit polyclonal antibody was previously described (RRID: AB_2571746; Zhao et al., 2001) and was diluted 1:500 for IB.

**Histology**

Mouse brain histology was performed as previously described (Zhao et al., 2001). Briefly, the brains of mouse embryos at 18.5 dpc were fixed with FEA (5% neutral buffered formalin, 5% glacial acetic acid, and 90% of 80% ethanol [vol/vol]) for 3 d. This process was followed by dehydration with ethanol, clearance with xylene, embedding in Paraplast (Sigma-Aldrich), and sectioning with a Microm HM 355 microtome (speed = 15 mm/s, CONT V = 15, thickness = 7–10 µm, temperature = 42°C, and water flow = ~4). The sections were deparaffinized by xylene, rehydrated, and then stained using Mayer’s hematoxylin for 10 min to discern the nuclei. The sections were washed with deionized water, stained with 0.3% eosin solution (water-soluble eosin) for 5 min, dehydrated, processed with xylene, and mounted. The sections were observed using a DM3000 upright microscope equipped with 1.6×/0.05 and 10×/0.30 HCX Plan S-Apochromat objectives (Leica Microsystems). Leica Application Suite (version 3.4.1) software was used for imaging.

**Primary culture of neurons**

Hippocampal neurons were collected, dissociated, and cultured as previously described (Niwa et al., 2008). Briefly, the hippocampi were dissected from the brains of 16.5–17.5 dpc mouse embryos, digested with 0.25% trypsin for 15 min at 37°C, and plated in a 35-mm dish with a round hole (D11130H; Matsunami), LabTek chambered coverglass (Thermo Fisher Scientific), and a combined device of cover glass (633153; Carolina) and microfluidic chambers (Xona Microfluidics; described below in detail), which were precoated with polyethylenimine (Sigma-Aldrich) and poly-L-lysine (Sigma-Aldrich). The neurons were cultured in MEM (Gibco) containing 33.3 mM glucose, 1 mM sodium pyruvate, 2 mM GlutaMAX-I supplement (Thermo Fisher Scientific), and 10% horse serum (Thermo Fisher Scientific) for 3–4 h in a humid atmosphere with 5% CO2 at 37°C. The medium was changed to a medium with the same ingredients but with B27 supplement (Thermo Fisher Scientific) instead of horse serum. To measure neuronal outgrowth with surface IF microscopy, neurons were plated at 8.6 × 10^4 cells/cm^2 and assayed at DIV2–3 to circumvent apoptosis (Niwa et al., 2008). To measure IGF-I signaling, neurons were plated at 2.6–4.3 × 10^4 cells/cm^2 and assayed at DIV4. Mouse cortical neuron culture was performed as previously described (Ichinose et al., 2015).

**Microfluidic chamber culture**

Microfluidic chamber culture was performed basically as previously described (Zhou et al., 2012). The coverslips (24 × 50 mm; thickness 0.13–0.17 mm; 633153; Carolina) were washed by sonication for 30 min, rinsed with 70% ethanol and double-distilled water (three times), and dried in a 50°C oven overnight. The samples were further coated with poly-L-lysine overnight, which was followed by a double-distilled water wash for 1 h three times, and the samples were finally dried for ~15 min. During washing, the microfluidic chambers were sterilized with 70% ethanol and dried for >1 h. Then, the dried cover glasses and microfluidic chambers were combined and precubated with MEM containing 33.3 mM glucose, 1 mM sodium pyruvate, 2 mM GlutaMAX-I supplement, and 10% horse serum. Dissociated hippocampal neurons were added to the left main channels of the chamber as drops of 10 µl for two to three times at a concentration of ~6 × 10^4 cells/ml. The medium was changed to one containing B27 supplement 30 min after the cells were loaded. The medium was then changed every 2 d.

**Plasmid transfection**

The dissociated cultured hippocampal neurons were transfected using an improved calcium phosphate protocol at DIV1–6 (Jiang and Chen, 2006) or electroporation before plating using a Neon transfection system (Thermo Fisher Scientific) with the indicated constructs following to the manufacturer’s protocol.

---

*Xu et al.*

The KIF1Bβ kinesin transports IGF1R down the axon

---

*Journal of Cell Biology*  https://doi.org/10.1083/jcb.201801085
Briefly, 1 µg DNA was mixed with 4–8 × 10⁴ neurons in 10 µl buffer and inserted in a 10 µl Neon Tip. Then, the electroporation was performed with the following conditions: pulse voltage = 1,400 V, pulse width = 20 ms, and pulse number = 1.

Yeast two-hybrid assays
Yeast two-hybrid assays were performed using the MatchMaker version 3 kit (Takara Bio Inc.) as previously described (Zhou et al., 2009). Five fragments of mouse Kif1bβ cDNA corresponding to aa 764–1,603, 1,611–1,603, 1,411–1,603, 885–1,100, and 1,655–1,754 or mouse Kif1a 836–1,346 aa cDNA were ligated to the bait vector pGBK7 (Takara Bio Inc.). The ICD of mouse Igf1r (961–1,373 aa) was ligated with the prey vector pGADT7 (Takara Bio Inc.). Yeast cells were cotransformed with each pair of bait and prey vectors, and the interaction between the fragments was assessed using α-galactosidase staining (Takara Bio Inc.) according to the Yeast Protocol Handbook (Takara Bio Inc.).

Vesicle IP and flotation assays
Vesicle IP and flotation assays were performed as previously described (Zhao et al., 2001; Tanaka et al., 2016). WT and Kif1bβGFP/GFP adult mouse brains were homogenized with ~5 ml Hapes-sucrose buffer (10 mM Hapes, pH 7.4, 320 mM sucrose, 5 mM MgSO₄, 1 mM EGTA, and protease inhibitors [Complete mini EDTA-free inhibitor; Roche]) and cleared by centrifugation twice at 1,000 g for 10 min at 4°C. For IP, the supernatant (S1) was mixed with 50 µl magnetic beads (μMACS Protein A; 130-042-601; Miltenyi Biotec) and 2 µg anti-GFP antibody for 2 h at 4°C. The beads were washed, eluted, and sampled for IB. For the flotation assay, the supernatant was diluted in 60% Nycodenz at a volume ratio of 1:5 and subjected to step-gradient ultracentrifugation with Nycodenz (0, 10, 20, 30, 40, 50, and 60%; Progen Biotechnik) in OptiSeal tubes (11.2 ml; 362181; Beckman Coulter) in Hepes-sucrose buffer (10 mM Hepes, pH 7.4, 320 mM sucrose, 5 mM MgSO₄, 1 mM EGTA, and protease inhibitors [Complete mini EDTA-free inhibitor; Roche]) and cleared by centrifugation twice at 1,000 g for 10 min at 4°C. For IP, the supernatant (S1) was mixed with 50 µl magnetic beads (μMACS Protein A; 130-042-601; Miltenyi Biotec) and 2 µg anti-GFP antibody for 2 h at 4°C. The beads were washed, eluted, and sampled for IB. For the flotation assay, the supernatant was diluted in 60% Nycodenz at a volume ratio of 1:5 and subjected to step-gradient ultracentrifugation with Nycodenz (0, 10, 20, 30, 40, 50, and 60%; Progen Biotechnik) in OptiSeal tubes (11.2 ml; 362181; Beckman Coulter) and 2 µg anti-GFP antibody for 2 h at 4°C. The beads were washed, eluted, and sampled for IB. For the flotation assay, the supernatant was diluted in 60% Nycodenz at a volume ratio of 1:5 and subjected to step-gradient ultracentrifugation with Nycodenz (0, 10, 20, 30, 40, 50, and 60%; Progen Biotechnik) in OptiSeal tubes (11.2 ml; 362181; Beckman Coulter) and 2 µg anti-GFP antibody for 2 h at 4°C. The beads were washed, eluted, and sampled for IB. For the flotation assay, the supernatant was diluted in 60% Nycodenz at a volume ratio of 1:5 and subjected to step-gradient ultracentrifugation with Nycodenz (0, 10, 20, 30, 40, 50, and 60%; Progen Biotechnik) in OptiSeal tubes (11.2 ml; 362181; Beckman Coulter) and 2 µg anti-GFP antibody for 2 h at 4°C. The beads were washed, eluted, and sampled for IB. For the flotation assay, the supernatant was diluted in 60% Nycodenz at a volume ratio of 1:5 and subjected to step-gradient ultracentrifugation with Nycodenz (0, 10, 20, 30, 40, 50, and 60%; Progen Biotechnik) in OptiSeal tubes (11.2 ml; 362181; Beckman Coulter) and 2 µg anti-GFP antibody for 2 h at 4°C. The beads were washed, eluted, and sampled for IB. For the flotation assay, the supernatant was diluted in 60% Nycodenz at a volume ratio of 1:5 and subjected to step-gradient ultracentrifugation with Nycodenz (0, 10, 20, 30, 40, 50, and 60%; Progen Biotechnik) in OptiSeal tubes (11.2 ml; 362181; Beckman Coulter) and 2 µg anti-GFP antibody for 2 h at 4°C. The beads were washed, eluted, and sampled for IB.
IF microscopy
Immunocytochemistry was performed as previously described (Tanaka et al., 2016). Mouse hippocampal neurons at DIV2–4 were fixed in 4% PFA/PBS at 37°C for 10 min and directly blocked (for surface anti-IGF1Rα staining), or they were permeabilized first with 0.1% Triton X-100 or 1% saponin (for anti-IGF1Rα staining) in PBS, blocked using 1% normal goat serum/PBS, and incubated with primary antibodies overnight at 4°C in the blocking buffer (in the case of saponin, the detergent was added throughout the procedure). This was followed by PBS washes for 5 min each, thrice. They were then incubated with Alexa Fluor–labeled secondary antibodies (Thermo Fisher Scientific) diluted at 1:500 for 1 h at room temperature. They were washed three times with PBS for 5 min each and observed with confocal laser-scanning microscopy (LSM510, LSM5-Duo, LSM710, or LSM780 with Airyscan; ZEISS) equipped with the objectives (40×/1.4 Plan Apochromat oil immersion, 40×/1.3 Plan Apochromat oil immersion, 20×/0.8 Plan Apochromat, or 40×/1.2 C-Apochromat) or SR-SIM (model ELYRA S1; ZEISS) equipped with an α Plan Apochromat objective (100×/1.46 oil immersion) at room temperature. The data acquisition was conducted by using Zen software (ZEISS). Fluorescent quantification was conducted using either ImageJ (National Institutes of Health) or MetaMorph (Molecular Devices) software.

Superresolution microscopy
For organelle colocalization assay in Fig. 5, the immunostained samples were subjected to a SR-SIM (ELYRA PS.1) equipped with an oil-immersed α Plan Apochromat lens (100×/1.46) using Zen software and analyzed using ImageJ and MetaMorph software.

Statistics
Statistical analysis was performed in Prism 6 (GraphPad Software), Excel (Microsoft), and R software. Statistical tests, number of samples, and experiments are indicated in the figure legends. No statistical method was used to predetermine the sample size. For t tests, the normal distribution was not assumed according to Welch’s method. Before the ANOVA tests, the normal distribution was tested using Kolmogorov–Smirnov test.

Online supplemental material
In Fig. S1, histological data and in vitro data are presented, suggesting that KIF1B deficiency or the KIF1Bβ Y1087C mutation impairs axon growth. In Fig. S2, the functional difference between KIF1Bβ and KIF1A, generation of KIF1BβGFP/GFP mice, and quantification of IGF1R transport on KIF1B deficiency are described. In Fig. S3, the effect of KIF1B deficiency on surface IGF1Rα levels is described. In Fig. S4, the effect of KIF1B deficiency or KIF1Bβ mutation on IGF-I–mediated ERK signaling is described. In Fig. S5, unchanged total Akt levels in RNAi experiments are shown. In Video 1, live imaging of a KIF1Bβ–EGFP–knock-in neuron is shown. In Video 2, colocalization of KIF1Bβ and IGF1R is shown.

Acknowledgments
We thank Yukiko Gotoh (The University of Tokyo, Tokyo, Japan) for providing the pCAGEN vector and valuable suggestions on receptor tyrosine kinase biology; Kenji Sakimura and his colleagues (Niigata University, Niigata, Japan) for providing REN KA embryonic stem cells and indispensable technical supports; Akio Sekigawa and Kayoko Suenaga (ZEISS) for their help with SR-SIM and Airyscan microscopy; and Ruyun Zhou, Atsushi Odagaki, Fumiyoshi Ishidate, Hiromi Sato, Nobuhisa Onouchi, Takeshi Akamatsu, Haruyo Fukuda, and all the members of the N. Hirokawa laboratory for their technical help and valuable discussions.

This study was supported by the Japan Society for the Promotion of Science KAKENHI grants JP23000013 and JP16H06372 to N. Hirokawa. F. Xu was supported by a Honjo International Scholarship Foundation scholarship from Itoen Company and served as a research assistant at The University of Tokyo during the four years of PhD candidate period.
The authors declare no competing financial interests.

Author contributions: N. Hirokawa conceived of and directed the project. N. Hirokawa and Y. Tanaka conceptualized and supervised the project. F. Xu and Y. Tanaka generated the mice. F. Xu, H. Takahashi, Y. Tanaka, S. Niwa, S. Ichinose, and N. Hirokawa designed and conducted the experiments. M.P. Wicklund contributed the clinical data. All authors discussed the data and wrote the paper.

Submitted: 14 January 2018
Revised: 31 May 2018
Accepted: 5 July 2018

References

Balogh, R.H., R.E. Schmidt, A. Petronk, and J. Millbrandt. 2007. Altered axonal mitochondrial transport in the pathogenesis of Charcot-Marie-Tooth disease from mitofusin 2 mutations. J. Neurosci. 27:422–430. https://doi.org/10.1523/JNEUROSCI.4798-06.2007

Baron-Van Evercooren, A., C. Olichon-Berthe, A. Kowalski, G. Visciano, and E. Van Obberghen. 1991. Expression of IGF-I and insulin receptor genes in the rat central nervous system: a developmental, regional, and cellular analysis. J. Neurosci. Res. 28:244–253. https://doi.org/10.1002/jne.490280212

Beck, K.D., L. Powell-Braxton, H.R. Widmer, J. Valverde, and F. Hefti. 1995. Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. Neuron. 14:717–730. https://doi.org/10.1016/0896-6273(95)90216-3

Bucci, C., O. Bakke, and C. Progida. 2012. Charcot-Marie-Tooth disease and intracellular traffic. Prog. Neurobiol. 99:191–225. https://doi.org/10.1016/j.pneurobio.2012.03.002

Butsugina, S., A.M. Fernandez, V. Barrios, R. Clark, D.L. Tolbert, J. Berciano, and J. Torres-Aleman. 2000. Neuregeneration is associated to changes in serum insulin-like growth factors. Neurobiol. Dis. 7(6, 6 P B):657–665. https://doi.org/10.1006/nbd.2000.0311

Camarero, G., C. Avendano, C. Fernandez-Moreno, A. Villar, J. Conterras, F. de Pablo, J.G. Pichel, and I. Varela-Nieto. 2001. Delayed inner ear maturation and neuronal loss in postnatal Igf1-deficient mice. J. Neurosci. 21:7630–7641. https://doi.org/10.1523/JNEUROSCI.21-19-07630-2001

Castellano, E., and J. Downward. 2011. RAS interaction with PI3K: more than oncogenic effects of NGF and IGF-1 on neurite growth in adult sensory neurons. J. Neurosci. 31:191–225. https://doi.org/10.1523/JNEUROSCI.4798-06.2007

Castronovo, V., Y. Corset, F. Llambi, and S. Marchetto. 2010. Mitochondrial transport mechanisms and roles in brain function, development, and disease. Neuron. 68:610–638. https://doi.org/10.1016/j.neuron.2010.09.039

Hirokawa, N., S. Niwa, and Y. Tanaka. 2010. Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. Exp. Cell Res. 334:16–25. https://doi.org/10.1016/j.yexcr.2015.02.016

Segal, D.R. Kaplan, and M.E. Greenberg. 1997. Regulation of neuronal survival by the serine-threonine protein kinase Akt. J. Neurosci. 17:85–98. https://doi.org/10.1523/JNEUROSCI.4798-06.2007

Kuemmerle, J.F., and K.S. Murthy. 2001. Coupling of the insulin-like growth factor-1 receptor tyrosine kinase to G i2 in human intestinal smooth muscle: G βγ-dependent mitogen-activated protein kinase activation and growth. J. Biol. Chem. 276:7187–7194. https://doi.org/10.1074/jbc.M011145200

Kuemmerle, J.F., and K.S. Murthy. 2001. Coupling of the insulin-like growth factor-1 receptor tyrosine kinase to G αi2 in human intestinal smooth muscle: G βγ-dependent mitogen-activated protein kinase activation and growth. J. Biol. Chem. 276:7187–7194. https://doi.org/10.1074/jbc.M011145200

Laurino, L., X.X. Wang, B.A. de la Houssaye, L. Sosa, D. Dupraz, A. Caceres, K.H. Penninger, and S. Quiroga. 2005. PI3K activation by IGF-1 is essential for the regulation of membrane expansion at the nerve growth cone. J. Cell Sci. 118:3653–3662. https://doi.org/10.1242/jcs.029490

Forcet, C., E. Stein, L. Pays, V. Corset, F. Lambli, M. Tessier-Lavigne, and P. Melchion. 2002. Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer–dependent MAPK activation. Nature. 417:443–447. https://doi.org/10.1038/nature748

Hirokawa, N., and Y. Tanaka. 2015. Kinesin superfamily proteins (KIFs): Various functions and their relevance for important phenomena in life and diseases. Exp. Cell Res. 334:16–25. https://doi.org/10.1016/j.yexcr.2015.02.016

Janz, R., Y. Goda, M. Geppert, and T.C. Sudhof. 1999. SV2A and SV2B function as redundant Ca2+ regulators in neurotransmitter release. Neuron. 24:1003–1016. https://doi.org/10.1016/S0896-6273(00)80416-4

Jiang, M., and G. Chen. 2006. High Ca2+-phosphate transfection efficiency in low-density neuronal cultures. Nat. Protoc. 1:695–700. https://doi.org/10.1038/nprot.2006.86

Jones, D.M., B.A. Tucker, M. Rahimtula, and K.M. Mearow. 2003. The synergetic effects of NGF and IGF-1 on neurite growth in adult sensory neurons: convergence on the PI 3-kinase signaling pathway. J. Neurochem. 86:1116–1128. https://doi.org/10.1046/j.1471-4159.2003.01925.x

Kanai, Y., N. Dohmae, and N. Hirokawa. 2004. Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. Neuron. 43:513–523. https://doi.org/10.1016/j.neuron.2004.07.022

Kijima, K., C. Numakura, H. Izumino, K. Umetsu, A. Nezu, T. Shiiki, M. Ogawa, Y. Ishizaki, T. Kitamura, Y. Shozawa, and K. Hayasaka. 2005. Mitochondrial transport in the pathogenesis of Charcot-Marie-Tooth disease from mitofusin 2 mutations. J. Neurosci. 25:85–98. https://doi.org/10.1523/JNEUROSCI.4798-06.2007

Kuemmerle, J.F., and K.S. Murthy. 2001. Coupling of the insulin-like growth factor-1 receptor tyrosine kinase to G αi2 in human intestinal smooth muscle: G βγ-dependent mitogen-activated protein kinase activation and growth. J. Biol. Chem. 276:7187–7194. https://doi.org/10.1074/jbc.M011145200

Kuemmerle, J.F., and K.S. Murthy. 2001. Coupling of the insulin-like growth factor-1 receptor tyrosine kinase to G αi2 in human intestinal smooth muscle: G βγ-dependent mitogen-activated protein kinase activation and growth. J. Biol. Chem. 276:7187–7194. https://doi.org/10.1074/jbc.M011145200

Laurino, L., X.X. Wang, B.A. de la Houssaye, L. Sosa, D. Dupraz, A. Caceres, K.H. Penninger, and S. Quiroga. 2005. PI3K activation by IGF-1 is essential for the regulation of membrane expansion at the nerve growth cone. J. Cell Sci. 118:3653–3662. https://doi.org/10.1242/jcs.029490

Xu et al. Journal of Cell Biology 2018;180:1083–1098
O’Kusky, J.R., P. Ye, and A.J. D’Ercole. 2000. Insulin-like growth factor-I and type I IGF receptor (Igfr). Cell. 75:59–72.

Lu, W., P. Ye, J.R. O’Kusky, and A.J. D’Ercole. 2009. Type I insulin-like growth factor receptor signaling is essential for the development of the hippocampal formation and dentate gyrus. J. Neurosci. Res. 87:2821–2832. https://doi.org/10.1002/jnr.22129

Lu, X., F. Kambe, X. Cao, M. Yamauchi, and H. Seo. 2008. Insulin-like growth factor-1 activation of Akt survival cascade in neuronal cells requires the presence of its cognate receptor in caveolae. Exp. Cell Res. 314:342–351. https://doi.org/10.1016/j.yexcr.2007.10.012

Luigetti, M., G.M. Fabrizi, G. Bisogni, A. Romano, F. Taioli, M. Ferrarini, D. B ernardo, P.M. Rossini, and M. Sabatelli. 2016. Charcot-Marie-Tooth type 2 and distal hereditary motor neuropathy: Clinical, neurophysiological and genetic findings from a single-centre experience. Clin. Neuro. Neurours. 144:67–71. https://doi.org/10.1016/j.clinneu.2016.03.007

Lyons, D.A., S.G. Naylor, A. Scholze, and W.S. Talbot. 2009. Kifib is essential for mRNA localization in oligodendrocytes and development of myelinated axons. Nat. Genet. 41:854–858. https://doi.org/10.1038/ng.376

Massagué, J., and M.P. Czech. 1982. The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. J. Biol. Chem. 257:5038–5045.

McMahon, H.T., V.Y. Bolshakov, R. Janz, R.E. Hammer, S.A. Siegelbaum, and T.C. Südhof. 1996. Synaptophysin, a major synaptic vesicle protein, is not essential for neurotransmitter release. Proc. Natl. Acad. Sci. USA. 93:4760–4764. https://doi.org/10.1073/pnas.93.10.4760

Mishina, M., and K. Sakimura. 2007. Conditional gene targeting on the pure C57BL/6 genetic background. Neurosci. Res. 58:105–112. https://doi.org/10.1016/j.neures.2007.01.004

Nakahukou, M., T. Satoh, and Y. Kaziro. 1992. Differentiation factors, including nerve growth factor, fibroblast growth factor, and interleukin-6, induce an accumulation of an active RasGTP complex in rat pheochromocytoma PC12 cells. J. Biol. Chem. 267:19448–19454.

Niwa, S., Y. Tanaka, and N. Hirokawa. 2008. KIF1Bβeta and KIF1A-mediated axonal transport of presynaptic regulator Rab3 occurs in a GTP-dependent manner through DENN/MADD. Nat. Cell. Biol. 10:1269–1279. https://doi.org/10.1038/ncl1785

O’Kusky, J., and P. Ye. 2012. Neurodevelopmental effects of insulin-like growth factor signaling. Front. Neuroendocrinol. 33:230–251. https://doi.org/10.1016/j.yfnef.2012.06.002

O’Kusky, J.R., P. Ye, and A.J. D’Ercole. 2000. Insulin-like growth factor-I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. J. Neurosci. 20:8435–8442. https://doi.org/10.1523/JNEUROSCI.20-22-08435.2000

Özdinler, P.H., and J.D. Macklis. 2006. IGF-I specifically enhances axon outgrowth of corticospinal motor neurons. Nat. Neurosci. 9:1371–1381. https://doi.org/10.1038/nn1789

P. Ye, J.R. O’Kusky, and A.J. D’Ercole. 2009. Type I insulin-like growth factor receptor signaling is essential for the development of the hippocampal formation and dentate gyrus. J. Neurosci. Res. 87:2821–2832. https://doi.org/10.1002/jnr.22129

Scolnick, J.A., K. Cui, C.D. Duggan, S. Xuan, X.B. Yuan, A. Efstratiadis, and A.D. Chisholm. 2007. Expression of KIF1Bβ in CMT2A causes Charcot-Marie-Tooth neuropathy type 2A. J. Neurosci. Res. 87:2821–2832. https://doi.org/10.1002/jnr.22129

Spreafico, F., and M. Sabatelli. 2016. Charcot-Marie-Tooth disease subtypes and genetic testing strategies. Ann. Neurol. 69:22–33. https://doi.org/10.1002/ana.22166

Skre, H. 1974. Genetic and clinical aspects of Charcot-Marie-Tooth’s disease. Clin. Genet. 6:98–118. https://doi.org/10.1111/j.1399-0004.1974.tb00638.x

Sosa, L., S. Dupraz, L. Laurino, F. Bollati, M. Biscal, A. Cáceres, K.H. Pfenniger, and S. Quiroga. 2006. IGF-I receptor is essential for the establishment of hippocampal neuronal polarity. Nat. Neurosci. 9:999–995. https://doi.org/10.1038/nn1742

Sullivan, K.A., B. Kim, and E.L. Feldman. 2008. Insulin-like growth factors in the peripheral nervous system. Endocrinology. 149:5963–5971. https://doi.org/10.1210/en.2008-1020

Takemura, T., N. Nakata, Y. Okada, H. Yamazaki, Z. Zhang, and N. Hirokawa. 1996. mRNA expression of KIF1A, KIF1B, KIF2A, KIF3A, KIF3B, KIF4, KIF5, and cytoplasmic dynein during axonal regeneration. J. Neurosci. 16:31–35. https://doi.org/10.1523/JNEUROSCI.16-01-00031.1996

Tanaka, Y., S. Niwa, M. Dong, A. Farkhondeh, L. Wang, R. Zhou, and N. Hirokawa. 2016. The Molecular Motor KIF1A Transports the TrkA Neurotrophin Receptor and Is Essential for Sensory Neuron Survival and Function. Neuron. 90:1215–1229. https://doi.org/10.1016/j.neuron.2016.05.002

Ueno, H., X. Huang, Y. Tanaka, and N. Hirokawa. 2011. KIF1Bβ/Rab14 molecular motor complex is critical for early embryonic development by transporting FGF receptor. Dev. Cell. 20:60–71. https://doi.org/10.1016/j.devcel.2010.11.008

Verbeek, J.J., L. Ku, and P. Delafontaine. 1993. Regulation of insulin-like growth factor I receptors on vascular smooth muscle cells by growth factors and phorbol esters. Circ. Res. 72:1285–1292. https://doi.org/10.1161/01.RES.72.6.1285

Wu, Z., A. Ghosh-Roy, M.F. Yanik, J.Z. Zhang, Y. Jin, and A.D. Chisholm. 2007. Caenorhabditis elegans neuronal regeneration is influenced by life stage, ephrin signaling, and synaptic branching. Proc. Natl. Acad. Sci. USA. 104:15132–15137. https://doi.org/10.1073/pnas.0707011010

Zhao, C., J. Takita, Y. Tanaka, M. Setou, T. Nakagawa, S. Takeda, H.W. Yang, S. Terada, T. Nakata, Y. Takei, et al. 2001. Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bβeta. Cell. 105:587–597. https://doi.org/10.1016/S0092-8674(01)00363-4

Zhou, B., Q. Cai, Y. Xie, and Z.H. Sheng. 2012. Snapin recruits dynactin to BD-NF-Trkβ signaling endosomes for retrograde axonal transport and is essential for dendrite growth of cortical neurons. Cell Reports. 2:42–51. https://doi.org/10.1016/j.celrep.2012.06.010

Zhou, R., S. Niwa, N. Homma, Y. Takei, and N. Hirokawa. 2009. KIF26A is an unconventional kinesin and regulates GDNF-Ret signaling in enteric neuronal development. Cell. 139:802–813. https://doi.org/10.1016/j.cell.2009.10.023

Züchner, S., I.V. Mersiyanova, M. Muglia, N. Bissar-Tadmouri, J. Rochelle, E.L. Dadali, M. Zappia, E. Nels, A. Pattucchi, J. Sander, and et al. 2004. Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nat. Genet. 36:449–451. https://doi.org/10.1038/ng1341