***De novo* disease-associated mutations in KIF1A dominant negatively inhibit axonal transport of synaptic vesicle precursors**

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25   **Abstract**

26   KIF1A is a kinesin superfamily molecular motor that transports synaptic vesicle precursors in axons.
 27   Mutations in *Kif1a* lead to a group of neuronal diseases called KIF1A-associated neuronal disorder
 28   (KAND). KIF1A forms a homodimer and KAND mutations are mostly *de novo* and autosomal
 29   dominant; however, it is not known whether the function of wild-type KIF1A is inhibited by
 30   disease-associated KIF1A. No reliable *in vivo* model systems to analyze the molecular and cellular
 31   biology of KAND have been developed; therefore, here, we established *Caenorhabditis elegans*
 32   models for KAND using CRISPR/cas9 technology and analyzed defects in axonal transport. In the *C.*
 33   *elegans* models, heterozygotes and homozygotes exhibited reduced axonal transport phenotypes. In
 34   addition, we developed *in vitro* assays to analyze the motility of single heterodimers composed of
 35   wild-type KIF1A and disease-associated KIF1A. Disease-associated KIF1A significantly inhibited
 36   the motility of wild-type KIF1A when heterodimers were formed. These data indicate the molecular
 37   mechanism underlying the dominant nature of *de novo* KAND mutations.

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 39   Keywords: axonal transport, KAND, neuropathy, kinesin, UNC-104, KIF1A

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49 **Significance Statement**

50 KIF1A is a molecular motor that transports synaptic vesicle precursors in axons. Recent studies have
 51 identified many *KIF1A* mutations in congenital neuropathy patients; however, the molecular
 52 mechanism of pathogenesis remains elusive. This study established a model for KIF1A-associated
 53 neuronal disorder (KAND) in *Caenorhabditis elegans* to analyze the molecular and cell biology of
 54 the disease *in vivo*. This study also established *in vitro* single-molecule assays to quantitatively
 55 analyze the effect of KAND mutations when mutant KIF1A forms heterodimers with wild-type
 56 KIF1A. Our findings provide a foundation for future genetic screening and for drug screening to
 57 search for KAND treatments.

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73 **Introduction**

74   Neuronal function depends on specific intracellular transport called axonal transport (1-3).
 75   Neurons transmit information via synaptic vesicles that accumulate at synapses (4). The constituents
 76   of synaptic vesicles are synthesized and assembled in the cell body and transported down axons to
 77   synapses. The transported organelle is called a synaptic vesicle precursor (5). KIF1A is a kinesin
 78   superfamily protein that transports synaptic vesicle precursors in axons (5, 6). KIF1A has a motor
 79   domain and a cargo-binding tail domain (5). The motor domain, which is conserved among Kinesin
 80   superfamily members, has microtubule-dependent ATPase activity that drives movement on
 81   microtubules (7, 8). The tail domain of KIF1A is composed of a protein-binding stalk domain and a
 82   lipid binding Pleckstrin-homology (PH) domain (6, 9-11).

83   *Caenorhabditis elegans* (*C. elegans*) is a good model animal to study axonal transport
 84   (12-19). UNC-104 is a *C. elegans* orthologue of KIF1A (20, 21). Electron and light microscopy
 85   analyses have shown that synapses as well as synaptic vesicles are mislocalized in *unc-104* mutants
 86   (20). The mechanism of axonal transport is well conserved between *C. elegans* and mammals and
 87   the expression of a human *Kif1a* cDNA can rescue the phenotype of *unc-104* mutant worms (22).

88   Mutations in the motor domain of *KIF1A* can cause congenital neuropathies (23, 24). More
 89   than 60 mutations have been found in the motor domain of *KIF1A* in neuropathy patients. Some
 90   cases are familial, but most are sporadic. For example, KIF1A(R11Q) was found in autism spectrum
 91   disorder and attention-deficit hyperactivity disorder (25), and KIF1A(R254Q) was found in Japanese
 92   spastic paraplegia patients (26). KIF1A(R254) is a hot spot for a broad range of neuropathies, such
 93   as KIF1A(R254W), have been described in non-Japanese patients (24). These neuropathies caused
 94   by KIF1A mutations is called KIF1A-associated neuronal disorder (KAND). Both dominant and
 95   recessive mutations are associated with KAND. KAND is caused by both gain of function and loss
 96   of function mechanisms. We have shown that familial mutations, KIF1A(V8M), KIF1A(A255V) and

97 KIF1A(R350G), over activate KIF1A and axonal transport. Recent *in vitro* studies have shown that
98 *de novo* KAND mutations are loss of function. KIF1A(P305L) reduces the microtubule association
99 rate of the motor (27). KIF1A(R169T) disrupts the microtubule-dependent ATPase activity of the
100 motor domain (28). KIF1A(R254W) reduces the velocity and run length of the motor protein (24) .

101 While these loss of function mutations have been intensively studied using *in vitro* assays,
102 reliable models to study the neuronal cell biology of KAND mutations *in vivo* are awaited. Moreover,
103 previous *in vitro* studies have mostly analyzed homodimers composed of disease-associated KIF1A
104 (24, 27) because it was difficult to purify heterodimers composed of wild-type and
105 disease-associated KIF1A (29). Activated KIF1A forms a homodimer to move on microtubules (30);
106 therefore, wild-type KIF1A is very likely to dimerize with disease-associated KIF1A in patient
107 neurons. However, it is not known whether *de novo* KAND mutations inhibit the function of
108 wild-type KIF1A in a dominant negative fashion.

109 Here, we established *C. elegans* models of *de novo* KAND mutations. Heterozygous
110 worms, as well as homozygous worms, show synaptic deficiencies that are caused by axonal
111 transport defects. We also established an *in vitro* single molecule analysis system to measure the
112 motility parameters of a single heterodimer composed of wild-type and disease-associated KIF1A.
113 Heterodimers composed of wild-type KIF1A and disease-associated KIF1A showed reduced motility
114 *in vitro*.

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116 **Results**

117 ***C. elegans* models of *de novo* KAND**

118 To study molecular and cellular deficiencies caused by *de novo* disease-associated KIF1A mutations,
119 we established *C. elegans* models for KAND using CRISPR/cas9 (31). *C.elegans unc-104* gene is an
120 orthologue of human *Kif1a*. We introduced following mutations in *unc-104* gene: *unc-104(R9Q)*,

121 *unc-104(R251Q)* and *unc-104(P298L)* (Figure 1A and B). These *unc-104* residues are conserved in
 122 human KIF1A and these mutations correspond to KIF1A(R11Q), KIF1A(R254Q) and
 123 KIF1A(P305L) mutations, respectively. All of them are causes of *de novo* and autosomal dominant
 124 KAND (25-27). Introduction of mutations was confirmed by Sanger sequencing. Then, we observed
 125 the macroscopic phenotypes of disease model worms. The body size of homozygous worms was
 126 smaller than that of wild-type worms [1.09 ± 0.09 mm, 0.66 ± 0.06 mm, 0.64 ± 0.07 mm, $0.74 \pm$
 127 0.06 mm, respectively for *wild type*, *unc-104(R9Q)*, *unc-104(R241Q)*, and *unc-104(P298L)*] (Figure
 128 1C). Moreover, homozygous worms showed uncoordinated (unc) phenotypes and did not move well
 129 on the culture plate (Figure 1C). To quantitatively analyze the movement of worms, the number of
 130 body bends in a water drop was counted during one minute (Figure 1D). We found homozygous
 131 worms did not move well in the water. These results collectively show that all three mutants
 132 phenocopy a well-established loss-of-function allele of *unc-104*, such as *unc-104(e1265)* (20),
 133 indicating that KIF1A(R11Q), KIF1A(R254Q) and KIF1A(P305L) mutations are loss of function.

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135 **Synaptic vesicles are mislocalized in homozygotes**

136 UNC-104 is an orthologue of human KIF1A and is a molecular motor that determines the
 137 localization of synaptic vesicles in *C. elegans*; therefore, we visualized synaptic vesicles in KAND
 138 model worms. The DA9 neuron in *C. elegans* is highly polarized and forms *en passant* synapses
 139 along the dorsal side of the axon (32) (Figure 2A). The characteristic morphology of DA9 is suitable
 140 for analyzing axonal transport and synaptic localization (33). We expressed a synaptic vesicle
 141 marker GFP::RAB-3 in the DA9 neuron using the *itr-1* promoter to visualize DA9 synapses (Figure
 142 2B). In KAND models, GFP::RAB-3 signals were reduced in the axon and misaccumulated in the
 143 dendrite (Figure 2C–F). Only a trace amount of GFP::RAB-3 signal was observed in the DA9 axon
 144 in KAND models.

145 We then observed axonal transport of synaptic vesicle precursors in the proximal region of the DA9
146 axon (33) (Figure 2A, magenta circle). We used GFP::RAB-3 as a representative marker for axonal
147 transport of synaptic vesicle precursors because previous studies have shown that GFP::RAB-3
148 co-migrates with other synaptic vesicle and pre-synaptic proteins in the axon and is, therefore, a
149 good marker to visualize axonal transport (6, 19, 33). In the wild-type worms, both anterograde and
150 retrograde transport were observed in the axon (Figure 2G and H). In contrast, the frequency of both
151 anterograde and retrograde events was significantly reduced in all three mutant strains (Figure 2G
152 and H). In more than 70% of mutant worms, no vesicular movement was detected in the 30 sec time
153 window. These data indicate that axonal transport of synaptic vesicles is strongly affected in
154 *unc-104(R9Q)*, *unc-104(R251Q)* and *unc-104(P298L)* strains.

155

KAND mutations disrupt the motility of motor proteins *in vitro*

157 To study the effect of KAND mutations *in vitro*, we observed the motility of purified
158 human KIF1A protein using total internal reflection fluorescence (TIRF) microscopy (34, 35). To
159 directly study motility parameters, regulatory domains and cargo binding domains were removed
160 (Figure 3A). The neck coiled-coil domain of mammalian KIF1A does not form stable dimers (36);
161 therefore, we stabilized human KIF1A dimers using a leucine zipper domain as described previously
162 (24, 27). A red fluorescent protein, mScarlet-I, was added to the C-terminus of the protein to observe
163 movement (Figure 3A). Resultant KIF1A homodimers [KIF1A(1–393)::LZ::mSca] were purified by
164 Strep tag and gel filtration (Figure 3B). This recombinant protein was then used to analyze the
165 motility of single KIF1A dimers on microtubules (Figure 3C–J). The motility of
166 KIF1A(1–393)::LZ::mSca dimers was observed at 10 pM (Figure 3C).
167 KIF1A(1–393)(R11Q)::LZ::mSca did not move well on microtubules even at 100 pM (Figure 3D),
168 while KIF1A(1–393)::LZ::mSca was saturated on microtubules under the same conditions (Figure

169 3G). KIF1A(1–393)(R254Q)::LZ::mSca moved on microtubules at 10 pM (Figure 3E). We observed
170 frequent binding of KIF1A(1–393)(R254Q)::LZ::mSca with microtubules (Figure 3I) but the
171 velocity was lower and the run length was shorter than for wild type (Figure 3H and J). The landing
172 rate of KIF1A(1–393)(P305L)::LZ::mSca was significantly lower than wild type (Figure 3I),
173 consistent with previous studies (24, 27). Although affected parameters were different depending on
174 the mutated residues, these data are consistent with the reduced axonal transport phenotypes
175 observed in KAND model worms.

176

Synaptic vesicles are mislocalized in heterozygous worms

178 KAND mutations, including KIF1A(R11Q), KIF1A(R254Q) and KIF1A(P305L) studied
179 here, are *de novo* and cause neuropathies in an autosomal dominant manner. Moreover, KAND is a
180 progressive disease. We therefore analyzed neuronal phenotypes of heterozygous worms in late adult
181 stages (Figure 4A–F). DA9 synapses were analyzed in heterozygotes at 3 and 6 days after the final
182 molt. The morphology of wild-type synapses was mostly maintained in 3 and 6-day-old adults
183 (Figure 4A, C and E). More than 70% of wild-type worms did not show misaccumulation of
184 GFP::RAB-3 in the proximal axon and dendrite at 3 and 6 days. However, 45 to 70% of
185 *unc-104(R9Q)/+*, *unc-104(R251Q)/+* and *unc-104(P298L)/+* animals showed misaccumulation of
186 GFP::RAB-3 in the proximal axon or dendrite (Figure 4B, D and E). To analyze the movement of
187 heterozygous worms, the number of body bends in a water drop was counted for one minute at day 3,
188 6 and 9 (Figure 4F). The motility defects were not strong but there was a tendency for heterozygous
189 worms to show reduced motility compared with wild-type worms.

190

Reduced Axonal transport in heterozygous worms

192 The DA9 axon and dendrite have plus-end out and minus-end out microtubules, respectively (37).

193 Thus, the mislocalization of synaptic vesicles in the proximal axon and dendrite indicate that
194 anterograde transport is reduced in *unc-104(R9Q)/+*, *unc-104(R251Q)/+* and *unc-104(P298L)/+*
195 worms. We therefore analyzed axonal transport of synaptic vesicle precursors visualized using
196 GFP::RAB-3 in the DA9 axon as described above (Figure 2A). In *wild type*, *unc-104(R9Q)/+*,
197 *unc-104(R251Q)/+* and *unc-104(P298L)/+* worms, both anterograde and retrograde movement of
198 synaptic vesicle precursors was observed in the DA9 axon (Figure 5A–D). Vesicular movement in
199 heterozygous worms was much better than that in homozygous worms (Figure 2). However, in
200 mutant heterozygotes, the velocity of anterograde axonal transport was reduced. In contrast,
201 retrograde transport, which depends on dynein motors, was not significantly changed in mutant
202 heterozygotes. The frequency of both anterograde and retrograde axonal transport was reduced in
203 mutant heterozygotes compared with that in wild type (Figure 5E–H).

204

205 Disease mutant/wild type heterodimers have reduced motor properties

206 The KIF1A motor forms a homodimer for efficient anterograde axonal transport (30). In
207 heterozygotes, half of the motor complex in the neuron is predicted to be heterodimers composed of
208 wild-type KIF1A and disease-associated KIF1A. But the behavior of heterodimers on microtubules
209 remains largely unanalyzed. To analyze the motility of heterodimers at a single-molecule resolution,
210 we purified heterodimers composed of wild-type KIF1A and disease-associated KIF1A. Wild-type
211 KIF1A fused with leucine zipper and mScarlet-I [KIF1A(1–393)::LZ::mSca] and disease-associated
212 KIF1A without fluorescent tag [KIF1A(1–393)::LZ] were co-expressed in bacteria (Figure 6A). The
213 two constructs were respectively fused with a Strep tag and His tag for purification. Tandem affinity
214 purification using His tag and Strep tag followed by gel filtration was performed to purify
215 heterodimers. From a single peak, heterodimers composed of KIF1A(1–393)::LZ::mSca and
216 KIF1A(1–393)::LZ were recovered (Figure 6B). The ratio between the two subunits was calculated

from band intensities and the molecular weights were about 1:1, indicating heterodimers.

As a positive control, we compared the motility of KIF1A(1–393)::LZ::mSca/KIF1A(1–393)::LZ heterodimers with KIF1A(1–393)::LZ::mSca homodimers (Figure 3C and 6C). Velocity, landing rate and run length of wild-type homodimers and heterodimers were statistically the same (velocity: $1.03 \pm 0.24 \mu\text{m}/\text{sec}$ and $1.03 \pm 0.26 \mu\text{m}/\text{sec}$, run length: $7.99 \pm 6.42 \mu\text{m}$ and $8.07 \pm 6.30 \mu\text{m}$, landing rate: $0.011 \pm 0.003 \mu\text{m}^{-1}\text{s}^{-1}$ and $0.010 \pm 0.004 \mu\text{m}^{-1}\text{s}^{-1}$ for homodimers and heterodimers respectively. Mean $\pm$ standard deviation). In contrast, heterodimers composed of wild-type KIF1A and disease-associated KIF1A showed reduced motility (Figure 6C–J). The velocity of KIF1A(1–393)::LZ::mSca/KIF1A(1–393)(R11Q)::LZ, KIF1A(1–393)::LZ::mSca/KIF1A(1–393)(R254Q)::LZ and KIF1A(1–393)::LZ::mSca/KIF1A(1–393)(P305L)::LZ heterodimers was lower than that of wild-type KIF1A (Figure 6H). The landing event of KIF1A(1–393)::LZ::mSca/KIF1A(1–393)(R11Q)::LZ and KIF1A(1–393)::LZ::mSca/KIF1A(1–393)(P305L)::LZ heterodimers on microtubules could not be observed at 10 pM (Figure 6I). At 100 pM, in which wild-type KIF1A homodimers were saturated on microtubules (Figure 6G), the motility of KIF1A(1–393)::LZ::mSca/KIF1A(1–393)(R11Q)::LZ and KIF1A(1–393)::LZ::mSca/KIF1A(1–393)(P305L)::LZ dimers was observed (Figure 6D, F and I) but the run lengths of these wild-type/mutant dimers were much shorter compared with that of wild-type dimers (Figure 6J). The landing rate of KIF1A(1–393)::LZ::mSca/KIF1A(1–393)(R254Q)::LZ heterodimers was higher than that of wild-type dimers (Figure 6I). However, run length of KIF1A(1–393)LZ-mSca/KIF1A(1–393)(R254Q)LZ heterodimers was shorter than that of wild-type dimers (Figure 6J). These results show that KAND mutations strongly affect the landing rate and motility parameters in heterodimers with wild-type KIF1A.

241 Finally, to show that a KAND mutation dominant negatively inhibits axonal transport *in*
 242 *vivo*, an *unc-104(R9Q)* cDNA corresponding to the KIF1A(R11Q) mutant, was overexpressed in the
 243 DA9 neuron (Figure 7A–C). In 70% of UNC-104(R9Q)-overexpressed animals, synaptic vesicles
 244 misaccumulated in the proximal region of the DA9 axon (Figure 7B and C). This phenotype is
 245 similar to a weak loss-of-function allele of *unc-104* (38), indicating that KAND mutations inhibit
 246 axonal transport by counteracting wild-type motor activity.

247

248 Discussion

249 Axonal transport motors form homodimers that move processively on microtubules (39).
 250 When a mutation in a motor protein gene is dominant, and if the mutation does not affect the
 251 stability or expression of the protein, half of the motor dimers in the cell are predicted to be
 252 heterodimers composed of wild-type motor and disease-associated motor. Many disease-associated
 253 mutations in motor proteins are caused by autosomal dominant mutations; however, little attention
 254 has been paid to the properties of heterodimers in motor-associated diseases. Previous studies have
 255 mainly analyzed properties of disease-associated homodimers but not heterodimers (23, 24, 40, 41).
 256 We show here that disease-associated KIF1A perturbs the function of wild-type KIF1A by forming
 257 dimers. Interestingly, properties of wild-type/mutant heterodimers are different from those of
 258 wild-type homodimers and mutant homodimers (Figure 3 and 6). Even though KIF1A(R11Q)
 259 homodimers do not show any processive movement, KIF1A(R11Q)/KIF1A(WT) heterodimers
 260 showed processive motility (Fig 3D and 6D). These suggest an importance of analyzing the
 261 properties of heterodimers. A previous study has analyzed the effect of heterodimerization in a
 262 semi-*in vitro* reconstitution in which motor proteins in COS-7 cell lysates, but not purified motor
 263 proteins, are observed (29). Some important phenomena, such as landing motor proteins on
 264 microtubules, cannot be quantitatively analyzed in the semi-*in vitro* reconstitution system because it

is difficult to precisely control the concentration of motors. In our study, using purified heterodimers, we demonstrate that the landing rate of KIF1A(wt)/KIF1A(R11Q) and KIF1A(wt)/KIF1A(P305L) is significantly lower than that of wild-type KIF1A. As KIF1A(R11Q) and KIF1A(P305L) homodimers also have a much lower landing rate than wild-type KIF1A, these mutant subunits inhibit the landing rate of wild-type subunits. KIF1A(R254Q) has a high landing rate and therefore inhibits wild-type KIF1A by a different mechanism, as described below. Mutations in other axonal transport motors, such as KIF5A and cytoplasmic dynein heavy chain 1 genes, are causes of autosomal dominant neuropathies (40-42). Similar phenomena to those observed here may underlie the pathogenesis of these neuropathies and it would be interesting to analyze heterodimers composed of wild-type and disease-associated motors in these cases. In the axon, multiple motors bind to and cooperatively transport a vesicle (43). Disease-associated KIF5A homodimers, which have lower motor activity than wild-type homodimers, inhibit the activity of wild-type KIF5A homodimers when mixed in the microtubule-gliding assay, which mimics cooperative transport (44). Considering this, both mutant KIF1A homodimers and wild-type/mutant KIF1A heterodimers, which both have lower motor activities (Figure 3 and 6), should dominant negatively perturb wild-type homodimers and axonal transport of cargo vesicles (Figure 7D). Consistent with this idea, overexpression of UNC-104(R9Q), mimicking KIF1A(R11Q), causes mislocalization of synaptic vesicles in wild-type neurons (Figure 7A–C). Reconstitution using a chassis composed of recombinant kinesins and DNA origami would help quantify how mutant homodimers and wild-type/mutant heterodimers inhibit axonal transport in KAND (45, 46).

Our worm models and single molecule analyses indicate that the velocity of axonal transport is affected by *de novo* autosomal dominant KAND mutations. The frequency of vesicle transport is reduced in disease worm models. Previous studies have suggested that the landing rate of KIF1A is a significant parameter in the pathogenesis of KAND (22, 27). KIF1A(V8M) and

289 KIF1A(P305L) are gain of function and loss of function mutants with elevated and reduced landing
 290 rate, respectively. Our result of KIF1A(R11Q) having a significantly lower landing rate is consistent
 291 with these findings. Model worms expressing KIF1A(R11Q) showed reduced axonal transport. In
 292 contrast, KIF1A(R254Q) showed an elevated landing rate but its velocity and run length were
 293 reduced. A previous study has shown that KIF1A dimers are faster than and have a much longer run
 294 length than another axonal transporter, KIF5 (47). Interestingly, the velocity of KIF1A(R254Q)
 295 shown here is comparable to that of KIF5 and the run length of KIF1A(R254Q) is still longer than
 296 that of KIF5. It is possible that the high velocity and/or the extremely long run length of KIF1A is
 297 fundamental to axonal transport of synaptic vesicle precursors, although the involvement of other
 298 parameters has not been completely excluded.

299 While homozygous disease model worms show strong synaptic phenotypes, heterozygous
 300 model worms have clear but mild phenotypes (Figures 1 and 4). In the case of human, autosomal
 301 dominant KAND mutations cause severe neuropathies. These differences would arise from the fact
 302 that human has complicated neuronal networks and highly developed brain functions. Nevertheless,
 303 we think our worm model is useful. Currently, there is no good strategy for KAND treatment. *C.*
 304 *elegans* is a powerful tool to perform suppressor screening. We suggest that the worm models
 305 established here can be a foundation for genetic and drug screening to search for therapies to treat
 306 KAND.

307

308 **Methods**

309 ***Worm experiments***

310 *C. elegans* strains were maintained as described previously (48). N2 wild-type worms and OP50
 311 feeder bacteria were obtained from the *C. elegans* genetic center (CGC)(Minneapolis, MN, USA).
 312 Nematode growth medium agar plates were prepared as described (48). Transformation of *C.*

313 *C. elegans* was performed by DNA injection as described (49). The swim test was performed as
314 described previously (50).

315

316 **Genome editing**

317 Target sequences for cas9 and repair templates used to make *unc-104* mutants are described in
318 **supplementary table S1**. Target sequences were inserted into pRB1017 (a gift from Andrew Fire,
319 Stanford University, addgene #59936). pDD162 (a gift from Bob Goldstein, UNC Chapel Hill,
320 addgene #47549) was used to express Cas9. These vectors and oligonucleotides were injected into
321 young adult worms as described with a slight modification (31). For *unc-104(R251Q)*, 50 ng of
322 PDD162, 50 ng of *unc-104(A252V)#4* and 0.6 μ M of repair template were mixed and injected.
323 Worms with a strong *unc* phenotype in the next generation were directly picked and genotyped by
324 PCR. For *unc-104(R9Q)* and *unc-104(P298L)*, 50 ng of pDD162, 50 ng of sgRNA expression
325 plasmid for *unc-104*, 50 ng of sgRNA expression plasmid for *ben-1*, and 0.6 μ M of repair template
326 for *unc-104* were mixed and injected. Injected worms were put on nematode growth medium plates
327 with OP50 feeder bacteria supplemented with 10 μ g/ml benzoimidazole. The next generation was
328 scored and benzoimidazole-resistant worms were picked and genotyped by PCR.

329

330 **Strains**

331 Strains used in this study are described in **supplementary table S2**. Male worms carrying *wyIs85* and
332 *wyIs251* were generated by a heat shock procedure. Heterozygotes were generated by crossing
333 *unc-104* homozygotes with *wyIs85* or *wyIs251* males. Homozygotes show strong *unc* phenotypes,
334 while heterozygotes do not. F1 worms showing non-*unc* phenotypes at the L4 stage were picked and
335 transferred to new plates.

336

337 ***Statistical analyses and graph preparation***

338 Statistical analyses were performed using Graph Pad Prism version 9. Statistical methods are
339 described in the figure legends. Graphs were prepared using Graph Pad Prism version 9, exported in
340 the TIFF format and aligned by Adobe Illustrator 2021.

342 ***Purification of homodimers***

343 Reagents were purchased from Nacalai tesque (Kyoto, Japan), unless described. Plasmids to express
344 recombinant KIF1A are described in *supplementary table S3*. To purify
345 KIF1A(1–393)::LZ::mScarlet-I::Strep homodimers, BL21(DE3) was transformed and selected on
346 LB agar supplemented with kanamycin at 37°C overnight. Colonies were picked and cultured in 10
347 ml LB medium supplemented with kanamycin overnight. Next morning, 5 ml of the medium was
348 transferred to 500 ml 2.5× YT (20 g/L Tryptone, 12.5 g/L Yeast Extract, 6.5 g/L NaCl) supplemented
349 with 10 mM phosphate buffer (pH 7.4) and 50 µg/ml kanamycin in a 2 L flask and shaken at 37°C.
350 Two flasks were routinely prepared. When OD₆₀₀ reached 0.6, flasks were cooled in ice-cold water
351 for 30 min. Then, 23.8 mg IPTG was added to each flask. Final concentration of IPTG was 0.2 mM.
352 Flasks were shaken at 18°C overnight.

353 Next day, bacteria expressing recombinant proteins were pelleted by centrifugation (3000 g, 10 min,
354 4°C), resuspended in PBS and centrifuged again (3000 g, 10 min, 4°C). Pellets were resuspended in
355 protein buffer (50 mM Hepes, pH 8.0, 150 mM KCH₃COO, 2 mM MgSO₄, 1 mM EGTA, 10%
356 glycerol) supplemented with Phenylmethylsulfonyl fluoride (PMSF). Bacteria were lysed using a
357 French Press G-M (Glen Mills, NJ, USA) as described by the manufacturer. Lysate was obtained by
358 centrifugation (75,000 g, 20 min, 4°C). Lysate was loaded on Streptactin-XT resin (IBA Lifesciences,
359 Göttingen, Germany) (bead volume: 2 ml). The resin was washed with 40 ml Strep wash buffer (50
360 mM Hepes, pH 8.0, 450 mM KCH₃COO, 2 mM MgSO₄, 1 mM EGTA, 10% glycerol). Protein was

361 eluted with 40 ml Strep elution buffer (50 mM Hepes, pH 8.0, 150 mM KCH₃COO, 2 mM MgSO₄, 1
362 mM EGTA, 10% glycerol, 300 mM biotin). Eluted solution was concentrated using an Amicon Ultra
363 15 (Merck) and then separated on an NGC chromatography system (Bio-Rad) equipped with a
364 Superdex 200 Increase 10/300 GL column (Cytiva). Peak fractions were collected and concentrated
365 using an Amicon Ultra 4 (Merck). Concentrated proteins were aliquoted and snap frozen in liquid
366 nitrogen.

367

368 ***Purification of heterodimers***

369 BL21(DE3) cells transformed with KIF1A(1–393)::LZ::mScarlet-I::Strep plasmid were cultured in
370 LB supplemented with kanamycin at 37°C. Competent cells were prepared using a Mix&Go kit
371 (Zymogen). The competent cells were further transformed with KIF1A(1–393)::LZ::His plasmid and
372 selected on LB agar supplemented with ampicillin and kanamycin. Colonies were picked and
373 cultured in 10 ml LB medium supplemented with ampicillin and kanamycin overnight. Next
374 morning, 5 ml of the medium was transferred to 500 ml 2.5× YT supplemented with carbenicillin
375 and kanamycin in a 2 L flask and shaken at 37°C. Two flasks were routinely prepared. The
376 procedures for protein expression in bacteria and preparation of bacterial lysate were the same as for
377 the purification of homodimers. Lysate was loaded on Streptactin-XT resin (bead volume: 2 ml). The
378 resin was washed with 40 ml wash buffer. Protein was eluted with 40 ml protein buffer
379 supplemented with 300 mM biotin. Eluted solution was then loaded on TALON resin (Takara Bio
380 Inc., Kusatsu, Japan)(bead volume: 2 ml). The resin was washed with 40 ml His-tag wash buffer (50
381 mM Hepes, pH 8.0, 450 mM KCH₃COO, 2 mM MgSO₄, 10 mM imidazole, 10% glycerol) and
382 eluted with His-tag elution buffer (50 mM Hepes, pH 8.0, 450 mM KCH₃COO, 2 mM MgSO₄, 10%
383 glycerol, 500 mM imidazole). Eluted solution was concentrated using an Amicon Ultra 15 and then
384 separated on an NGC chromatography system (Bio-Rad) equipped with a Superdex 200 Increase

10/300 GL column (Cytiva). Peak fractions were collected and concentrated using an Amicon Ultra
4. Concentrated proteins were aliquoted and snap frozen in liquid nitrogen.

TIRF single-molecule motility assays

TIRF assays were performed as described (22). Tubulin was purified from porcine brain as described
(51). Tubulin was labeled with Biotin-PEG₂-NHS ester (Tokyo Chemical Industry, Tokyo, Japan)
and AZDye647 NHS ester (Fluoroprobes, Scottsdale, AZ, USA) as described (52). To polymerize
Taxol-stabilized microtubules labeled with biotin and AZDye647, 30 μ M unlabeled tubulin, 1.5 μ M
biotin-labeled tubulin and 1.5 μ M AZDye647-labeled tubulin were mixed in BRB80 buffer
supplemented with 1 mM GTP and incubated for 15 min at 37°C. Then, an equal amount of BRB80
supplemented with 40 μ M taxol was added and further incubated for more than 15 min. The solution
was loaded on BRB80 supplemented with 300 mM sucrose and 20 μ M taxol and ultracentrifuged at
100,000 g for 5 min at 30°C. The pellet was resuspended in BRB80 supplemented with 20 μ M taxol.
Glass chambers were prepared by acid washing as previously described (35). Polymerized
microtubules were flowed into streptavidin adsorbed flow chambers and allowed to adhere for 5–10
min. Unbound microtubules were washed away using assay buffer [30 mM Hepes pH 7.4, 50 mM
KCH₃COO, 2 mM Mg(CH₃COO)₂, 1 mM EGTA, 10% glycerol, 0.1 mg/ml biotin-BSA, 0.2 mg/ml
kappa-casein, 0.5% Pluronic F127, 1 mM ATP, and an oxygen scavenging system composed of
PCA/PCD/Trolox. Purified motor protein was diluted to indicated concentrations in the assay buffer.
Then, the solution was flowed into the glass chamber. An ECLIPSE Ti2-E microscope equipped with
a CFI Apochromat TIRF 100XC Oil objective lens, an Andor iXion life 897 camera and a Ti2-LAPP
illumination system (Nikon, Tokyo, Japan) was used to observe single molecule motility.
NIS-Elements AR software ver. 5.2 (Nikon) was used to control the system.

409 **Data Availability**

410 All study data are included in the article and/or supporting information.

411

412 **Competing interest**

413 The authors declare no competing interest.

414

415 **Author contribution**

416 S.N. designed research; Y.A., T.K and S.N. performed research; Y.A., T.K and S.N. analyzed data;

417 Y.A., T.K., K.H. and S.N. wrote the paper.

418

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426

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537

538 **Figure legends**

539 **Figure 1 Establishment of disease model worms by genome editing**

540 (A) Schematic drawing of the domain organization of KIF1A motor protein. NC, neck coiled-coil
541 domain. CC1, Coiled-coil 1 domain. FHA, Forkhead-associated domain. CC2, Coiled-coil 2 domain.
542 CC3, Coiled-coil 3 domain. PH, Pleckstrin-homology domain. The three KAND mutations and

543 corresponding *C. elegans* UNC-104 mutations analyzed in this study are indicated.
 544 (B) Sequence comparison between human KIF1A and *C. elegans* UNC-104.
 545 (C) Macroscopic phenotypes of KAND model homozygotes. Mutant worms are smaller than
 546 wild-type worms and do not move well on the bacterial feeder. Bars, 1 mm.
 547 (D) Swim test. The number of body bends in a water drop was counted for 1 min and plotted. Dots
 548 represents data points. Green bars represent median values. Kruskal-Wallis test followed by Dunn's
 549 multiple comparison test. N = 20 worms for each genotype. **, adjusted P value < 0.01. ****,
 550 adjusted P value < 0.0001.

551

552 **Figure 2 Synaptic vesicle localization in KAND model homozygous worms**

553 (A) Schematic drawing show the morphology of the DA9 neuron. Green dots along the axon show
 554 synaptic vesicle distribution. The magenta circle shows the proximal axon.
 555 (B–E) Representative images showing the distribution of synaptic vesicles in the DA9 neuron in *wild*
 556 *type* (B), *unc-104(R9Q)* (C), *unc-104(R251Q)* (D), and *unc-104(P298L)* (E). Synaptic vesicles are
 557 visualized by GFP::RAB-3. Arrowheads show mislocalization of synaptic vesicles in the dendrite
 558 and proximal axon. Bars, 50 μ m.
 559 (F) Dot plots showing the number of puncta in the axon (left panel) and dendrite (right panel) of
 560 DA9. Ordinary one-way ANOVA followed by Dunnett's multiple comparison test. Green bars show
 561 the mean $\pm$ standard deviation (S.D.). N = 60 worms for each genotype. ****, adjusted P value <
 562 0.0001.
 563 (G) Representative kymographs of *wild type* (upper panel) and *unc-104(R251Q)* (lower panel). The
 564 axonal transport of synaptic vesicle precursors was visualized by GFP::RAB-3. The proximal axon
 565 shown in panel (A) was observed. Vertical and horizontal bars show 10 seconds and 10 μ m,
 566 respectively.
 567 (H) Dot plots showing the frequency of anterograde axonal transport (left panel) and retrograde
 568 axonal transport (right panel). Green bars represent median values. Kruskal-Wallis test followed by
 569 Dunn's multiple comparison test. N = 14 *wild type*, 14 *unc-104(R9Q)*, 18 *unc-104(R251Q)* and 16
 570 *unc-104(P298L)* axons. ****, adjusted P Value < 0.0001.

571

572 **Figure 3 Single molecule behavior of disease-associated KIF1A mutants**

573 (A) Schematic drawing of the domain organization of KIF1A motor protein and the recombinant

574 protein analyzed in Figure 3.

575 (B) Purified KIF1A(1–393)::LZ::mScarlet and its mutants were separated by SDS-PAGE and
576 detected by trichloroethanol staining. M represents a marker lane. Numbers on the left indicate
577 molecular weight (kDa). Arrow indicates KIF1A(1–393)::LZ::mScarlet.

578 (C–G) Representative kymographs showing the motility of 10 pM KIF1A (wt) (C), 100 pM
579 KIF1A(R11Q) (D), 10 pM KIF1A(R254Q) (E), 100 pM KIF1A(P305L) and 100 pM KIF1A (wt)
580 (G). Vertical and horizontal bars represent 5 sec and 5 μ m, respectively.

581 (H) Dot plots showing the velocity of KIF1A. Each dot indicates one datum. Green bars represent
582 median values. Kruskal-Wallis test followed by Dunn's multiple comparison test. n = 433 (wt), 325
583 (R254Q) and 498 (P305L). ****, adjusted P Value < 0.0001. Note that no processive movement was
584 detected for KIF1A(R11Q).

585 (I) Dot plots showing the landing rate of KIF1A. The number of KIF1A that bound to microtubules
586 was counted and adjusted by the time window and microtubule length. Each dot shows one datum.
587 Green bars represent median values. Kruskal-Wallis test followed by Dunn's multiple comparison
588 test. n = 30 (10 pM wt), 28 (100 pM R11Q), 29 (10 pM R254Q) and 30 (100 pM P305L) movies.
589 ****, adjusted P Value < 0.0001. Note that no landing event was detected in 10 pM KIF1A(R11Q)
590 and KIF1A(P305L) experiments.

591 (J) Dot plots showing the run length of KIF1A. Each dot shows one datum. Green bars represent
592 median values with interquartile ranges. Kruskal-Wallis test followed by Dunn's multiple
593 comparison test. n = 312 (wt), 241 (R254Q) and 243 (P305L) homodimers. ****, adjusted P Value <
594 0.0001.

595

596 **Figure 4 Synaptic vesicle localization of heterozygotes**

597 (A–D) Representative images showing synaptic vesicle distribution in 3 day-day-old *wild-type* adult
598 (A), 3 day-day-old *unc-104(R251Q)/+* adult (B), 6 day-day-old *wild-type* adult (C), and 6
599 day-day-old *unc-104(R251Q)/+* adult (D). Synaptic vesicles are visualized by GFP::RAB-3. Bars,
600 50 μ m.

601 (E) The ratios of worms with dendritic mislocalization of synaptic vesicles to worms showing
602 wild-type localization of synaptic vesicles are shown. Chi-square test adjusted by Bonferroni
603 correction. *, adjusted P Value < 0.05. **, adjusted P Value < 0.01. ****, adjusted P Value < 0.0001.

604 (F) Dot plots showing swim test results at 3, 6 and 9 days-old. Each dot shows one datum. Green

bars represent median values. Kruskal-Wallis test followed by Dunn's multiple comparison test. N = 34 (wt), 38 (R9Q/+), 32 (R251Q/+) and 35 (P298L/+) (3-day-old adult worms); 36 (wt), 33 (R9Q/+), 36 (R251Q/+) and 35 (P298L/+) (6-day-old adult worms); 27 (wt), 30 (R9Q/+), 29 (R251Q/+) and 33 (P298L/+) (9-day-old adult worms). ns, adjusted P Value > 0.05 and statistically not significant. *, adjusted P Value < 0.05. **, adjusted P Value < 0.01. ****, adjusted P Value < 0.0001.

610

Figure 5 Axonal transport in KAND model heterozygotes

(A–D) Representative kymographs showing axonal transport of synaptic vesicle precursors in *wild type* (A), *unc-104(R9Q)/+* (B), *unc-104(R251Q)/+* (C) and *unc-104(P305L)/+* (D). GFP::RAB-3 was used as a marker. Vertical and horizontal bars indicate 10 seconds and 10 μ m, respectively.

(E and F) The velocity of axonal transport. The velocity of anterograde transport (E) and retrograde transport (F) are shown as dot plots. (E) Ordinary one-way ANOVA followed by Dunnett's multiple comparison test. Green bars show the mean $\pm$ S.D.. n = 94 (wild-type), 90 (R9Q/+), 66 (R251Q/+) and 117 (P298L/+) vesicles from at least five independent worms. ****, adjusted P Value < 0.0001.

(F) Ordinary one-way ANOVA followed by Dunnett's multiple comparison test. Green bars show the mean $\pm$ S.D.. n = 63 (wild-type), 54 (R9Q/+), 38 (R251Q/+) and 53 (P298L/+) vesicles from at least five independent worms. ns, adjusted P Value > 0.05 and no significant statistical difference.

(G and H) Frequency of axonal transport. The frequency of anterograde transport (G) and retrograde transport (H) are shown as dot plots. (G) Kruskal-Wallis test followed by Dunn's multiple comparison test. Each dot represents data from each worm. Green bars represent median values. N = 14 (wt), 16 (R9Q/+), 18 (R251Q/+) and 19 (P298L/+) independent worms. ****, adjusted P Value < 0.0001. (H) Kruskal-Wallis test followed by Dunn's multiple comparison test. Each dot represents data from each worm. Green bars represent median values. N = 14 (wt), 16 (R9Q/+), 18 (R251Q/+) and 19 (P298L/+) independent worms. **, adjusted P Value < 0.01, ****, adjusted P Value < 0.0001.

629

Figure 6 The single molecule behavior of wild type/mutant KIF1A heterodimers

(A) Schematic drawing of the recombinant KIF1A heterodimer analyzed in Figure 6.

(B) Purified KIF1A(1–393)::LZ::mScarlet/KIF1A(1–393)::LZ heterodimers were separated by SDS-PAGE and detected by Coomassie brilliant blue staining. M represents marker. Numbers on the left indicate the molecular weight (kDa). Magenta and black arrows indicate KIF1A(1–393)::LZ::mScarlet and KIF1A(1–393)::LZ, respectively.

(C–G) Representative kymographs showing the motility of 10 pM KIF1A (wt) (C), 100 pM KIF1A(R11Q) (D), 10 pM KIF1A(R254Q) (E), 100 pM KIF1A(P305L) and 100 pM KIF1A (wt) (G). Vertical and horizontal bars represent 5 sec and 5 μ m, respectively.

(H) Dot plots showing the velocity of KIF1A. Each dot shows one datum. Green bars represent median values. Kruskal-Wallis test followed by Dunn's multiple comparison test. n = 308 (wt/wt), 315 (wt/R11Q), 294 (wt/R254Q) and 414 (wt/P305L) heterodimers. ****, adjusted P Value < 0.0001.

(I) Dot plots showing the landing rate of KIF1A. The number of KIF1A molecules that bind to microtubules was counted and adjusted by the time window and microtubule length. Each dot shows one datum. Green bars represent median values. Kruskal-Wallis test followed by Dunn's multiple comparison test. n = 29 (10 pM wt/wt), 29 (100 pM wt/R11Q), 28 (10 pM wt/R254Q) and 38 (100 pM wt/P305L) independent observations. **, adjusted P Value < 0.01, ***, adjusted P Value < 0.001, ****, adjusted P Value < 0.0001.

(J) Dot plots showing the run length of KIF1A. Each dot shows one datum. Green bars represent median values and interquartile ranges. Kruskal-Wallis test followed by Dunn's multiple comparison test. n = 215 (wt/wt), 241 (wt/R11Q), 195 (wt/R254Q) and 266 (wt/P305L) heterodimers. ****, adjusted P Value < 0.0001.

653

Figure 7 KAND mutations inhibit axonal transport in a dominant negative fashion

(A–C) UNC-104(R9Q) was overexpressed in the *wild-type* background and the localization of synaptic vesicles was observed. (A and B) Representative images showing the localization of synaptic vesicles in *wild-type* (A) and UNC-104(R9Q)-overexpressing worms (B). Arrow heads show synaptic-vesicle accumulated puncta that are mislocalized in the proximal region of the axon. Bars, 50 μ m. (C) Bar graphs showing the ratio of affected animals. Chi-square test. N = 51 (wt) and 49 [unc-104(R9Q)-overexpressing worm]. ****, p < 0.0001.

(D) Schematic drawing showing how vesicular transport is suppressed in KAND patient axons. Not only mutant homodimers but also wild-type/mutant heterodimers inhibit axonal transport of synaptic vesicle precursors.

664

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

bioRxiv preprint doi: <https://doi.org/10.1101/2021.07.22.453457>; this version posted July 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC 4.0 International license.

B

C

wt/wt (10 pM)

D wt/R11Q(100 pM)

E wt/R254Q(10 pM)

F wt/P305L(100 pM)

G

wt/wt (100 pM)

H

I

J

Figure 6

Figure 7