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

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Abstract

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

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

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Significance Statement

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

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Introduction

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

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

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

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

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

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

Results

C. elegans models of *de novo* KAND

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

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

Synaptic vesicles are mislocalized in homozygotes

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

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

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

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

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

Synaptic vesicles are mislocalized in heterozygous worms

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

Reduced Axonal transport in heterozygous worms

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

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

Disease mutant/wild type heterodimers have reduced motor properties

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

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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 ± 0.24 µm/sec and 1.03 ± 0.26 µm /sec, run length: 7.99 ± 6.42 µm and 8.07 ± 6.30 µm, landing rate: 0.011 ± 0.003 $\mu m^{-1}s^{-1}$ and 0.010 ± 0.004 $\mu m^{-1}s^{-1}$ for homodimers and heterodimers respectively. Mean ± 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.

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

Discussion

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

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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

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

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

Methods

Worm experiments

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

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313 *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 μM 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 μM 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 10μg/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

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Statistical analyses and graph preparation

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

Purification of homodimers

Reagents were purchased from Nacarai tesque (Kyoto, Japan), unless described. Plasmids to express recombinant KIF1A are described in ***supplementary table S3***. To purify KIF1A(1–393)::LZ::mScarlet-I::Strep homodimers, BL21(DE3) was transformed and selected on LB agar supplemented with kanamycin at 37°C overnight. Colonies were picked and cultured in 10 ml LB medium supplemented with kanamycin overnight. Next morning, 5 ml of the medium was transferred to 500 ml 2.5× YT (20 g/L Tryptone, 12.5 g/L Yeast Extract, 6.5 g/L NaCl) supplemented with 10 mM phosphate buffer (pH 7.4) and 50 μg/ml kanamycin in a 2 L flask and shaken at 37°C. Two flasks were routinely prepared. When OD_{600} reached 0.6, flasks were cooled in ice-cold water for 30 min. Then, 23.8 mg IPTG was added to each flask. Final concentration of IPTG was 0.2 mM. Flasks were shaken at 18°C overnight.

Next day, bacteria expressing recombinant proteins were pelleted by centrifugation (3000 g, 10 min, 4°C), resuspended in PBS and centrifuged again (3000 g, 10 min, 4°C). Pellets were resuspended in protein buffer (50 mM Hepes, pH 8.0, 150 mM KCH_3COO, 2 mM $MgSO_4$, 1 mM EGTA, 10% glycerol) supplemented with Phenylmethylsulfonyl fluoride (PMSF). Bacteria were lysed using a French Press G-M (Glen Mills, NJ, USA) as described by the manufacturer. Lysate was obtained by centrifugation (75,000 g, 20 min, 4°C). Lysate was loaded on Streptactin-XT resin (IBA Lifesciences, Göttingen, Germany) (bead volume: 2 ml). The resin was washed with 40 ml Strep wash buffer (50 mM Hepes, pH 8.0, 450 mM KCH_3COO, 2 mM $MgSO_4$, 1 mM EGTA, 10% glycerol). Protein was

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eluted with 40 ml Strep elution buffer (50 mM Hepes, pH 8.0, 150 mM KCH_3COO, 2 mM $MgSO_4$, 1 mM EGTA, 10% glycerol, 300 mM biotin). Eluted solution was concentrated using an Amicon Ultra 15 (Merck) and then 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 (Merck). Concentrated proteins were aliquoted and snap frozen in liquid nitrogen.

Purification of heterodimers

BL21(DE3) cells transformed with KIF1A(1–393)::LZ::mScarlet-I::Strep plasmid were cultured in LB supplemented with kanamycin at 37°C. Competent cells were prepared using a Mix&Go kit (Zymogen). The competent cells were further transformed with KIF1A(1–393)::LZ::His plasmid and selected on LB agar supplemented with ampicillin and kanamycin. Colonies were picked and cultured in 10 ml LB medium supplemented with ampicillin and kanamycin overnight. Next morning, 5 ml of the medium was transferred to 500 ml 2.5× YT supplemented with carbenicillin and kanamycin in a 2 L flask and shaken at 37°C. Two flasks were routinely prepared. The procedures for protein expression in bacteria and preparation of bacterial lysate were the same as for the purification of homodimers. Lysate was loaded on Streptactin-XT resin (bead volume: 2 ml). The resin was washed with 40 ml wash buffer. Protein was eluted with 40 ml protein buffer supplemented with 300 mM biotin. Eluted solution was then loaded on TALON resin (Takara Bio Inc., Kusatsu, Japan)(bead volume: 2 ml). The resin was washed with 40 ml His-tag wash buffer (50 mM Hepes, pH 8.0, 450 mM KCH_3COO, 2 mM $MgSO_4$, 10 mM imidazole, 10% glycerol) and eluted with His-tag elution buffer (50 mM Hepes, pH 8.0, 450 mM KCH_3COO, 2 mM $MgSO_4$, 10% glycerol, 500 mM imidazole). Eluted solution was concentrated using an Amicon Ultra 15 and then separated on an NGC chromatography system (Bio-Rad) equipped with a Superdex 200 Increase

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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-PEG_2-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 μM unlabeled tubulin, 1.5 μM biotin-labeled tubulin and 1.5 μM 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 μM taxol was added and further incubated for more than 15 min. The solution was loaded on BRB80 supplemented with 300 mM sucrose and 20 μM taxol and ultracentrifuged at 100,000 g for 5 min at 30°C. The pellet was resuspended in BRB80 supplemented with 20 μM 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 KCH_3COO, 2 mM $Mg(CH_3COO)_2$, 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.

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Data Availability

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

Competing interest

The authors declare no competing interest.

Author contribution

S.N. designed research; Y.A., T.K and S.N. performed research; Y.A., T.K and S.N. analyzed data; Y.A., T.K., K.H. and S.N. wrote the paper.

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Figure legends

Figure 1 Establishment of disease model worms by genome editing

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

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corresponding *C. elegans* UNC-104 mutations analyzed in this study are indicated.

(B) Sequence comparison between human KIF1A and *C. elegans* UNC-104.

(C) Macroscopic phenotypes of KAND model homozygotes. Mutant worms are smaller than wild-type worms and do not move well on the bacterial feeder. Bars, 1 mm.

(D) Swim test. The number of body bends in a water drop was counted for 1 min and plotted. Dots represents data points. Green bars represent median values. Kruskal-Wallis test followed by Dunn's multiple comparison test. N = 20 worms for each genotype. **, adjusted P value < 0.01. ****, adjusted P value < 0.0001.

Figure 2 Synaptic vesicle localization in KAND model homozygous worms

(A) Schematic drawing show the morphology of the DA9 neuron. Green dots along the axon show synaptic vesicle distribution. The magenta circle shows the proximal axon.

(B–E) Representative images showing the distribution of synaptic vesicles in the DA9 neuron in *wild type* (B), *unc-104(R9Q)* (C), *unc-104(R251Q)* (D), and *unc-104(P298L)* (E). Synaptic vesicles are visualized by GFP::RAB-3. Arrowheads show mislocalization of synaptic vesicles in the dendrite and proximal axon. Bars, 50 μm.

(F) Dot plots showing the number of puncta in the axon (left panel) and dendrite (right panel) of DA9. Ordinary one-way ANOVA followed by Dunnett's multiple comparison test. Green bars show the mean ± standard deviation (S.D.). N = 60 worms for each genotype. ****, adjusted P value < 0.0001.

(G) Representative kymographs of *wild type* (upper panel) and *unc-104(R251Q)* (lower panel). The axonal transport of synaptic vesicle precursors was visualized by GFP::RAB-3. The proximal axon shown in panel (A) was observed. Vertical and horizontal bars show 10 seconds and 10 μm, respectively.

(H) Dot plots showing the frequency of anterograde axonal transport (left panel) and retrograde axonal transport (right panel). Green bars represent median values. Kruskal-Wallis test followed by Dunn's multiple comparison test. N = 14 *wild type*, 14 *unc-104(R9Q)*, 18 *unc-104(R251Q)* and 16 *unc-104(P298L)* axons. ****, adjusted P Value < 0.0001.

Figure 3 Single molecule behavior of disease-associated KIF1A mutants

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

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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 μm, 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 μm.

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

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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.

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 μm, 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 ± 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 ± 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.

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.

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(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 μm, 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.

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 μm. (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.

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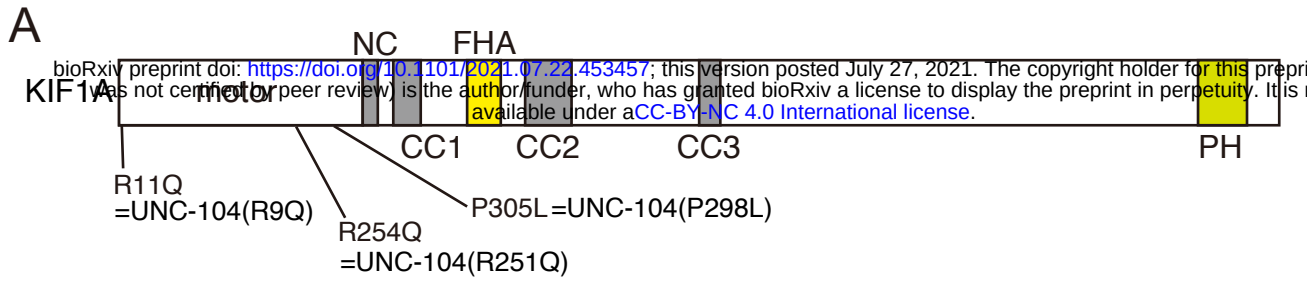

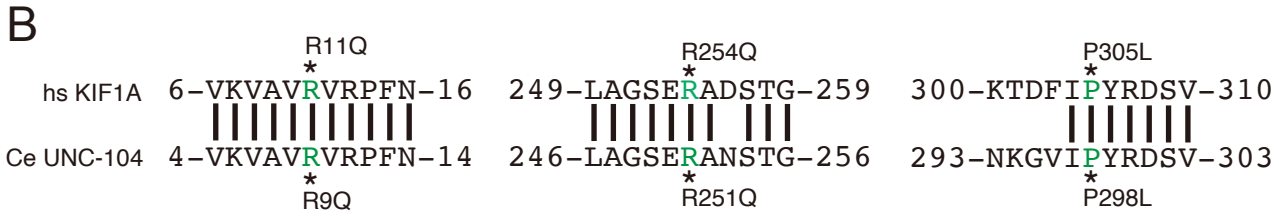

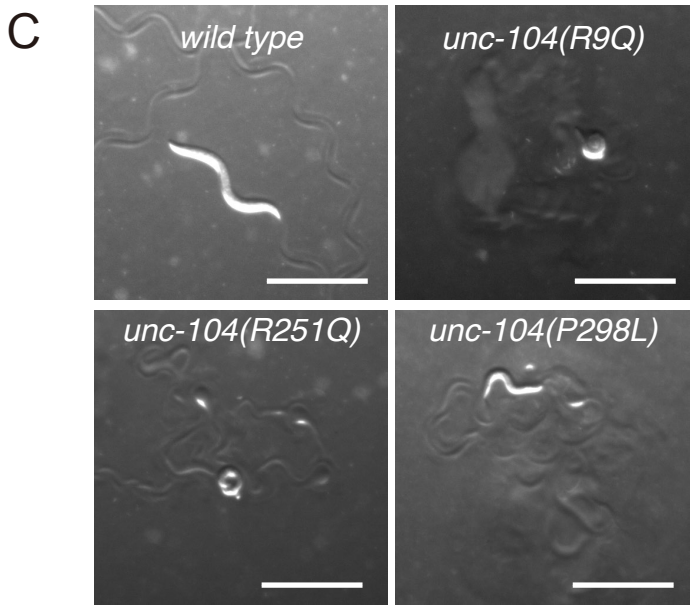

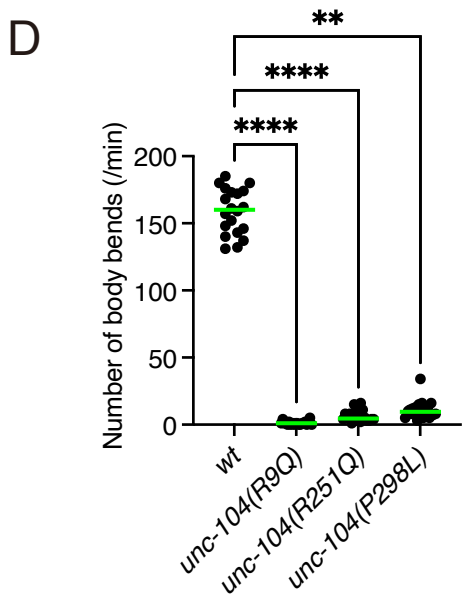

Figure 1

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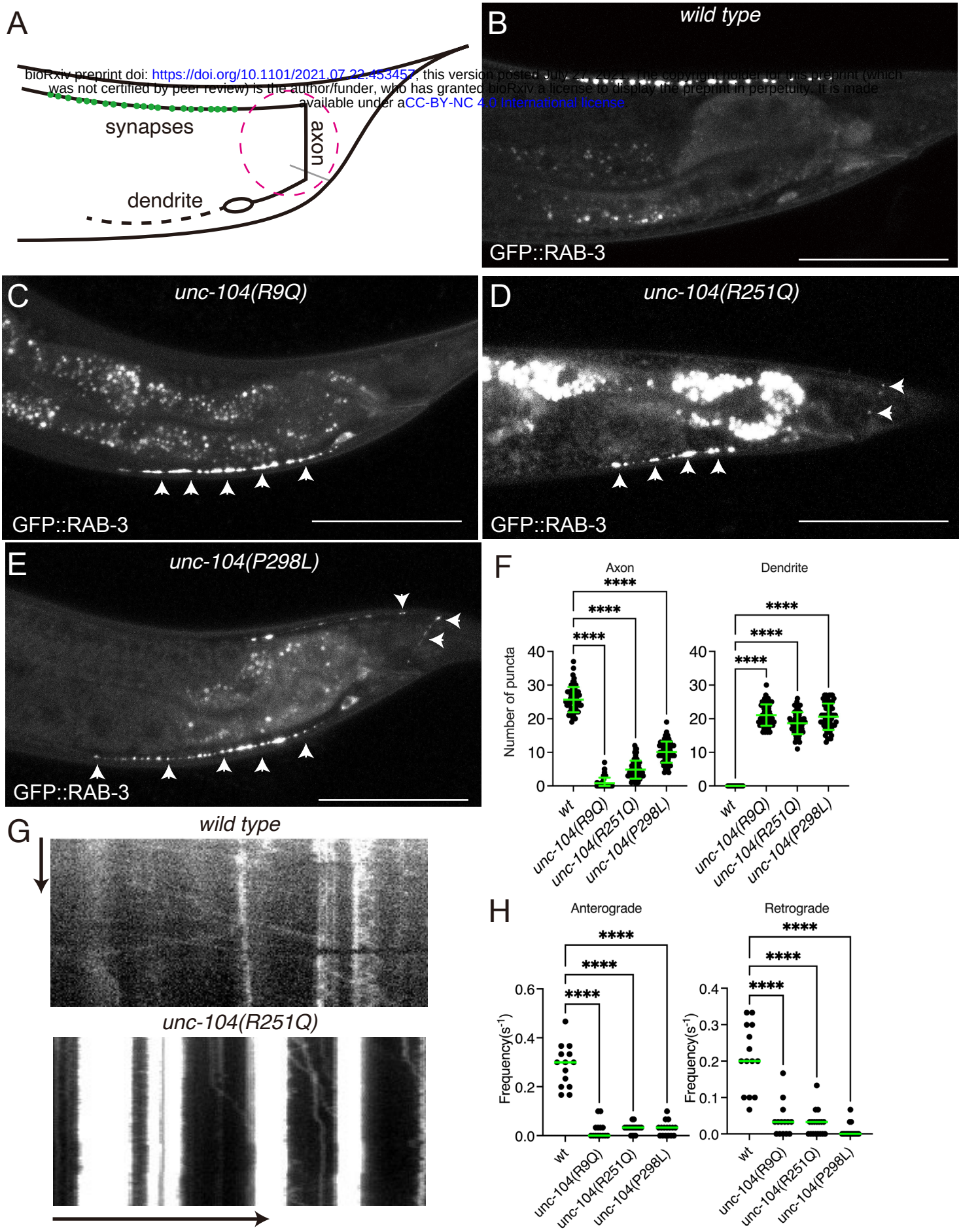

Figure 2

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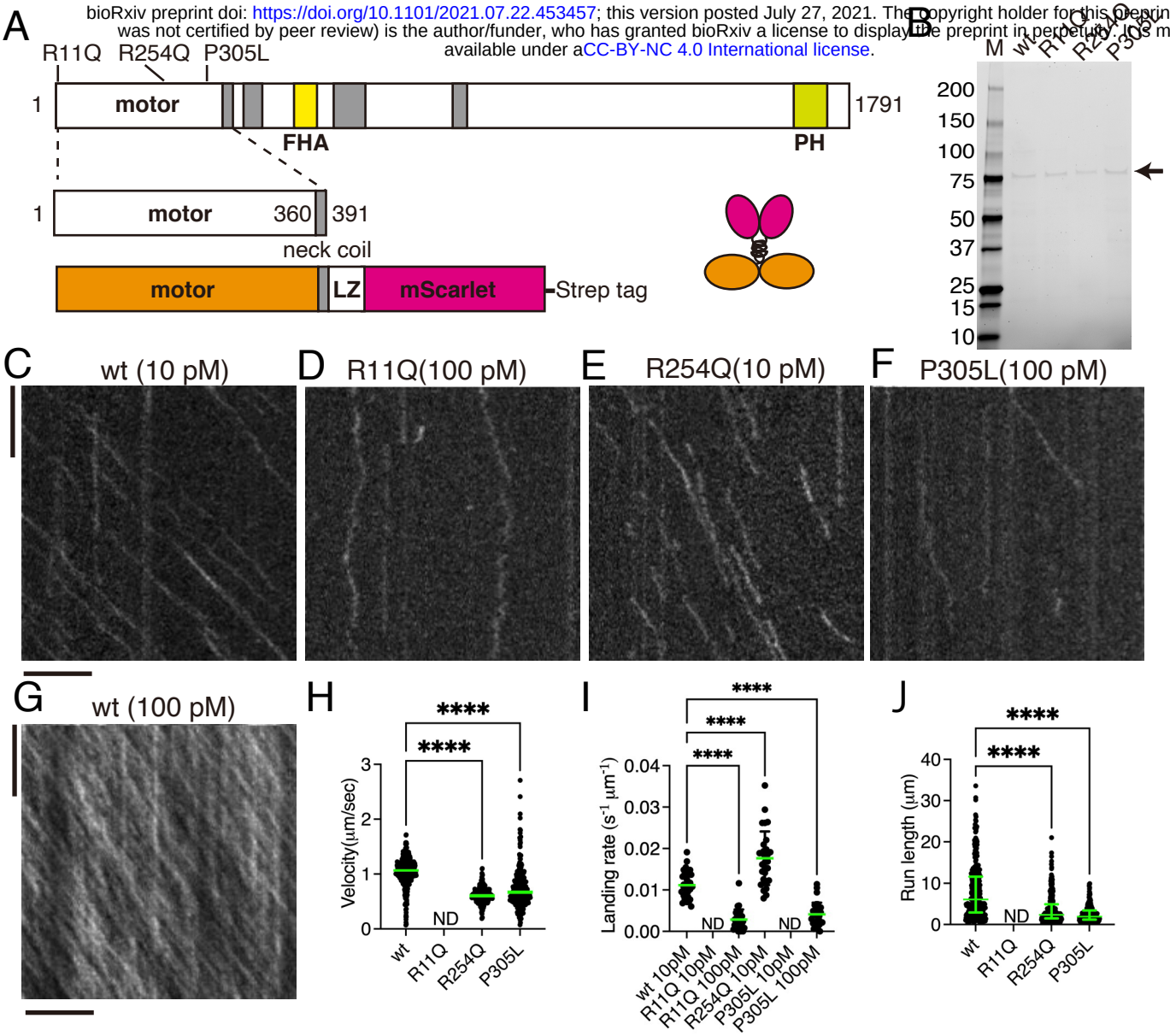

Figure 3

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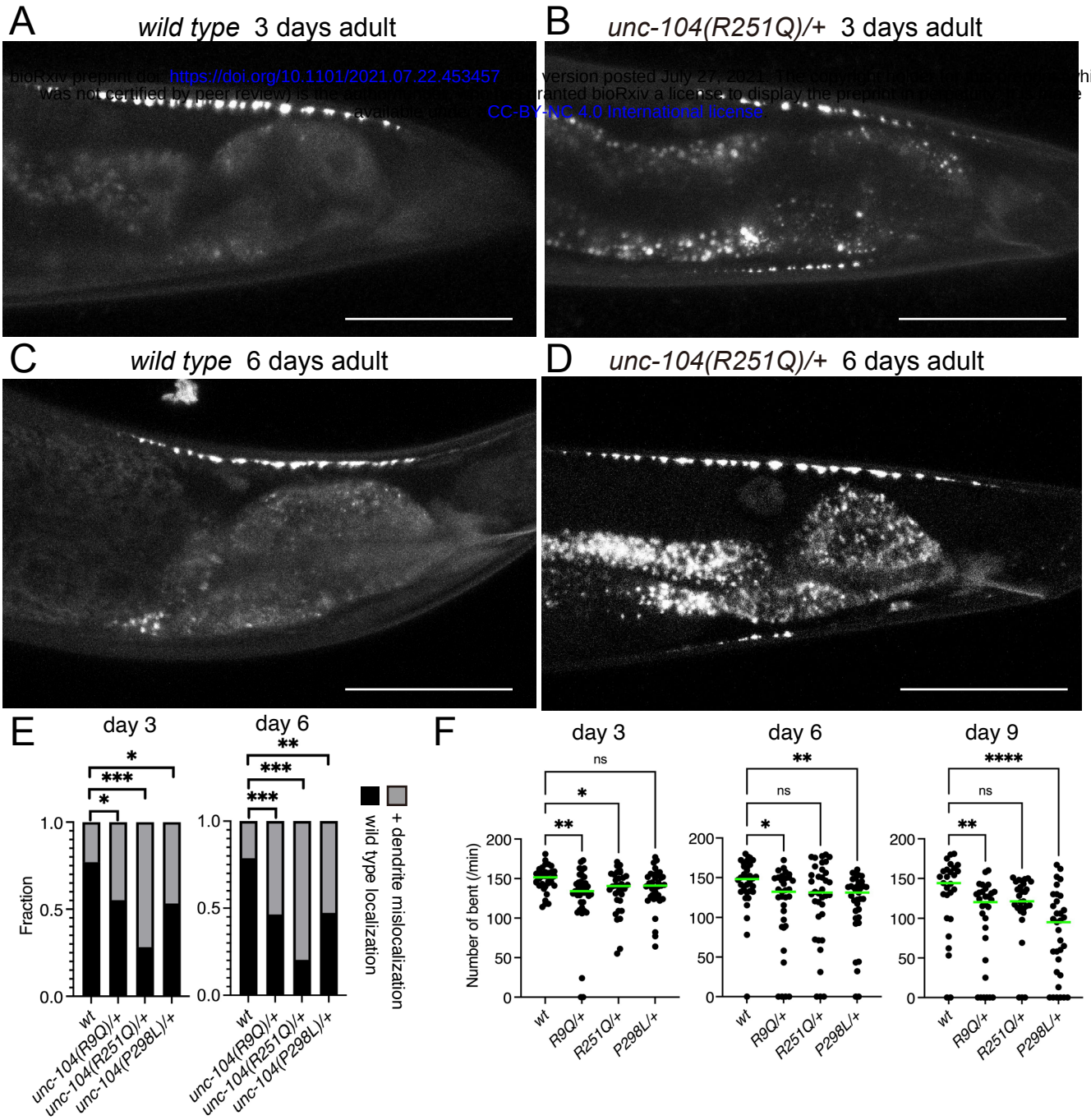

Figure 4

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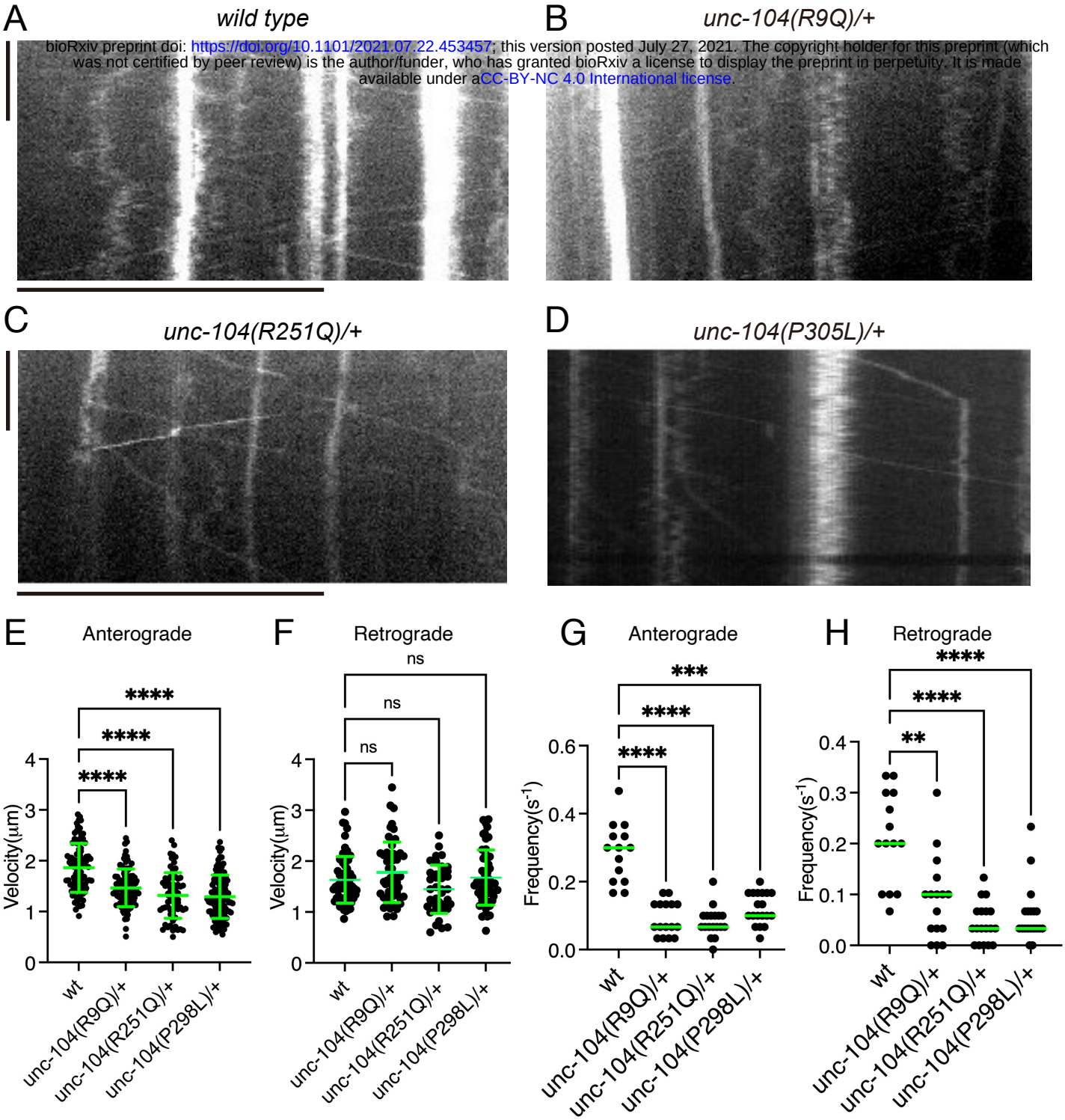

Figure 5

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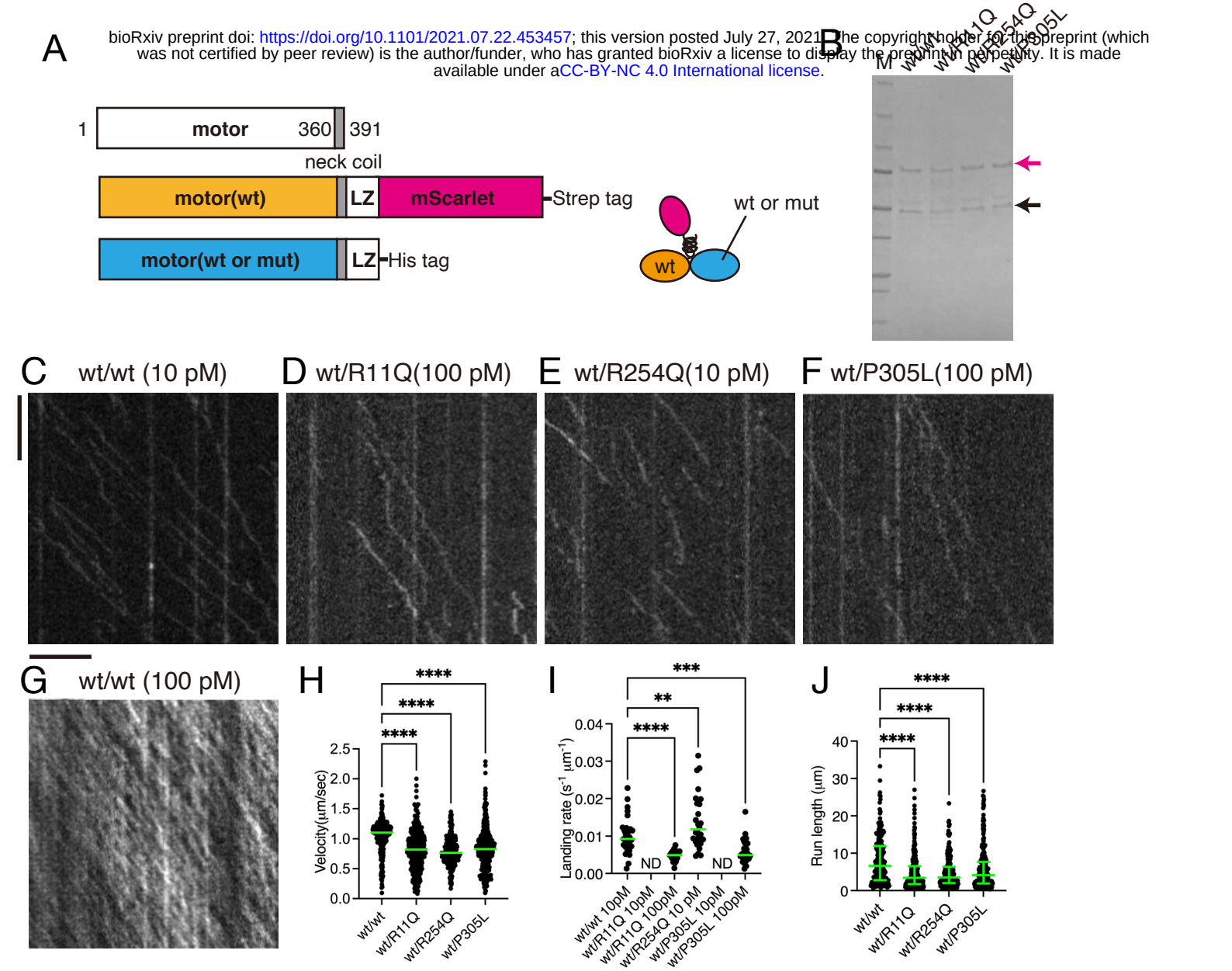

Figure 6

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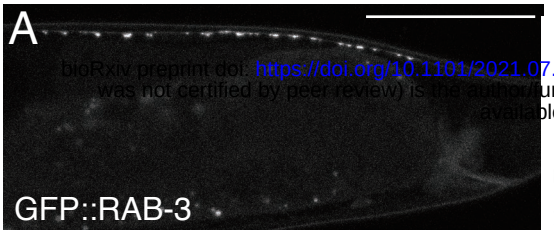

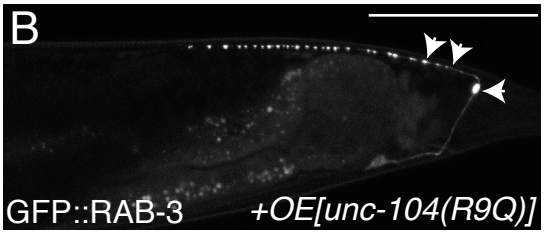

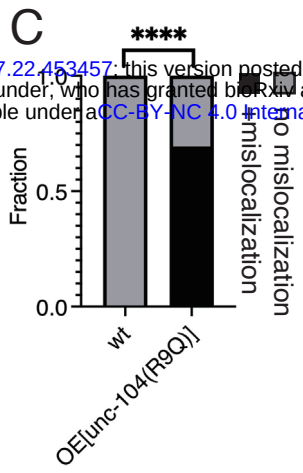

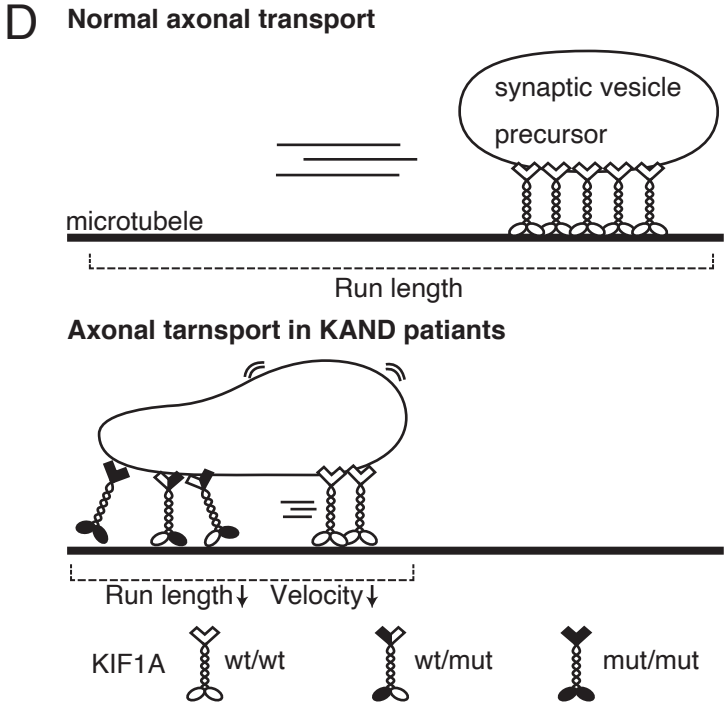

Figure 7