Force generation of KIF1C is impaired by pathogenic mutations

Highlights

- KIF1C stalls at loads of ~3.7 pN, which is intermediate between kinesin-1 and KIF1A
- KIF1C has a propensity to backslip while remaining weakly attached to the microtubule
- HSP-causing mutations in KIF1C retain fast, processive single-molecule motility
- KIF1C mutations are severely impaired under the load of an optical trap or cellular cargo

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In brief

Siddiqui et al. characterize single-molecule force generation by kinesin-3 KIF1C and report the intermediate properties between the strong kinesin-1 and backslip-prone kinesin-3 KIF1A. Two KIF1C mutations causing hereditary spastic paraplegia permit fast and processive single-molecule motility but are severely impaired under load.
Intracellular transport is essential for neuronal function and survival. The most effective plus-end-directed neuronal transporter is the kinesin-3 KIF1C, which transports large secretory vesicles and endosomes.\textsuperscript{1–4} Mutations in KIF1C cause hereditary spastic paraplegia and cerebellar dysfunction in human patients.\textsuperscript{5–8} Here, we establish a baseline for the single-molecule mechanics of Kif1C. We show that full-length KIF1C molecules can processively step against the load of an optical trap and reach average stall forces of 3.7 pN. Compared with kinesin-1, KIF1C has a higher propensity to slip backward under load, which results in a lower maximal single-molecule force. However, KIF1C remains attached to the microtubule while slipping backward and re-engages quickly, consistent with its super processivity. Two pathogenic mutations, P176L and R169W, that cause hereditary spastic paraplegia in humans\textsuperscript{7,8} maintain fast, processive single-molecule motility\textit{in vitro} but with decreased run length and slightly increased unloaded velocity compared with the wild-type motor. Under load in an optical trap, force generation by these mutants is severely reduced. In cells, the same mutants are impaired in producing sufficient force to efficiently relocate organelles. Our results show how its mechanics supports KIF1C’s role as an intracellular transporter and explain how pathogenic mutations at the microtubule-binding interface of KIF1C impair the cellular function of these long-distance transporters and result in neuronal disease.

**RESULTS AND DISCUSSION**

**Force generation properties of KIF1C**

To determine the force output of KIF1C, we used single-bead optical tweezer assays,\textsuperscript{11,12} full-length human KIF1C,\textsuperscript{10} and, for comparison, full-length \textit{Drosophila melanogaster} kinesin heavy chain (KHC).\textsuperscript{13,14} Forces were calculated from bead displacements under conditions in which less than 20% of beads move, thus ensuring that a single kinesin pulls on the bead. Examining the force traces suggested that KIF1C and KHC produced force events of comparable magnitude and duration, although KIF1C events were disrupted twice as frequently by long backward slips (Figures 1A, 1B, and S1). The median duration of force events was about 0.5 s for both motors, but KHC showed a significantly larger fraction of force events lasting longer than 2 s (Figure S1). The average stall force of KHC, i.e., the force that motors held for at least 10 ms before the bead returned to the trap center, was 5.4 pN, in agreement with the literature.\textsuperscript{15–17} KIF1C forces were \textasciitilde30% lower and stalled at 3.7 pN on average. Stall forces were consistent over a range of minimal stall-time criteria, from 10 to 800 ms, with the average stall time being 0.3 s for KIF1C and 0.5 s for KHC (Figures 1C–1E). Free-running beads with a single KIF1C moved at 922 ± 40 nm/s, but their average forward speed rapidly decreased under the load of the trap and reached zero at 7 pN. Although KHC’s unloaded velocity was \textasciitilde20% slower, KHC moved at significantly higher speeds at forces \textasciitilde1 pN, maintaining an average speed of 450 nm/s at forces \textasciitilde2.5 pN before slowing down rapidly, with forward speed reaching zero at 8 pN (Figures 1F and S1). To look at the force-dependence of the motors more closely, we analyzed forward and backward steps. As expected, KIF1C took forward steps of approximately 8 nm in length (Figure S1), consistent with all other kinesins studied so far.\textsuperscript{15,18–22} The force at which the probability of taking a forward step or slipping backward is equal, was 7.3 pN for KHC and 5.7 pN for KIF1C (Figure 1G). These values are consistent with the \textasciitilde98th percentile of stall forces we measured (Figures 1C and 1D). Closer inspection of the step sizes observed at different forces (Figure 1H) revealed that KHC backslips once every 9 detectable forward steps, and the vast majority of KHC backward displacements are 8 nm. Kif1C backslips twice as frequently, and 3 times as many KIF1C backslips are larger than 12 nm, suggesting that  

**SUMMARY**

Intracellular transport is essential for neuronal function and survival. The most effective plus-end-directed neuronal transporter is the kinesin-3 KIF1C, which transports large secretory vesicles and endosomes.\textsuperscript{1–4} Mutations in KIF1C cause hereditary spastic paraplegia and cerebellar dysfunction in human patients.\textsuperscript{5–8} Here, we establish a baseline for the single-molecule mechanics of Kif1C. We show that full-length KIF1C molecules can processively step against the load of an optical trap and reach average stall forces of 3.7 pN. Compared with kinesin-1, KIF1C has a higher propensity to slip backward under load, which results in a lower maximal single-molecule force. However, KIF1C remains attached to the microtubule while slipping backward and re-engages quickly, consistent with its super processivity. Two pathogenic mutations, P176L and R169W, that cause hereditary spastic paraplegia in humans\textsuperscript{7,8} maintain fast, processive single-molecule motility\textit{in vitro} but with decreased run length and slightly increased unloaded velocity compared with the wild-type motor. Under load in an optical trap, force generation by these mutants is severely reduced. In cells, the same mutants are impaired in producing sufficient force to efficiently relocate organelles. Our results show how its mechanics supports KIF1C’s role as an intracellular transporter and explain how pathogenic mutations at the microtubule-binding interface of KIF1C impair the cellular function of these long-distance transporters and result in neuronal disease.
Figure 1. KIF1C single-molecule force generation

(A) Representative traces from single-bead optical trapping experiment for human KIF1C and Drosophila melanogaster kinesin heavy chain (KHC). Dotted lines indicate 8-nm displacement.

(B) Number of force-producing events per microtubule (MT) encounter for KIF1C and KHC. Boxes indicate quartiles and whiskers at 10/90th percentile. Data shown are mean ± SD. n = 350, 1,239 MT encounters analyzed. p = 1 \times 10^{-12} (Kolmogorov-Smirnov test).

(C) Distributions of stall forces held for a minimum of 10 ms by KIF1C and KHC. Average force ± SD of Gaussian fit shown. n = 399, 1,251 stall events analyzed. p = 5 \times 10^{-14} two-sample t test.

(D) Stall force distributions as a function of minimal stall time for KIF1C and KHC. Individual force events overlaid by box plot indicating quartiles and whiskers at 10/90th percentile. For KIF1C: 50 ms: 3.69 ± 0.03 pN, n = 1,244; 100 ms: 3.69 ± 0.03 pN, n = 1,177; 200 ms: 3.77 ± 0.04 pN, n = 715; 400 ms: 3.91 ± 0.06 pN, n = 263; 800 ms: 4.0 ± 0.1 pN, n = 70. For KHC: 50 ms: 5.37 ± 0.05 pN, n = 398; 100 ms: 5.39 ± 0.05 pN, n = 392; 200 ms: 5.53 ± 0.05 pN, n = 326; 400 ms: 5.60 ± 0.07 pN, n = 187; 800 ms: 5.81 ± 0.1 pN, n = 68. Data listed are mean ± SEM.
KIF1C is both more likely to slip backward and to slip further when it does so (Figures 1H and 1I). Indeed, a significant fraction of KIF1C detachment events—defined as backward motion whereby the bead returns to the trap center—might be long backward slips whereby the motor remains (weakly) attached to the microtubule. The time lag between detachments and the start of a new force-generating event follows a triexponential distribution for both motors (Figure S1). The fastest timescale presumably reflects long backslips where the force reduces below the 1.2 pN cutoff. This accounts for 31% of KIF1C detachment events (t½ = 74 ms) and 13% of KHC detachments (t½ = 104 ms) (Figure S1). After reassigning these events, KIF1C has a 3.5-fold increased propensity to backslip and a slightly higher ratio of backslips versus detachments than KHC.

Taken together, single molecules of full-length human KIF1C can generate substantial stall forces, although their force output is about 30% lower than that of conventional kinesin. For comparison, the average stall forces of constitutively active KIF1A and Unc104 were reported as 3.0 and 2.6 pN, respectively. While monomeric truncated and native full-length KIF1A are only weakly processive, with a stall force below 0.3 pN. KIF1C has a 3.5-fold increased probability of backward slipping under load compared with KHC, constitutively active KIF1A shows 50-fold enhanced slipping. Thus, our data suggest that KIF1C has unique force-generating properties that are intermediate between KIF1A and kinesin-1. These intermediate properties might enable KIF1C to be both highly processive and generate substantial forces at low motor numbers, which might explain why KIF1C has been identified as the most effective cellular cargo transporter.

We proposed recently that kinesin-1 backslips arise when phosphate is released before a forward step is completed, which converts the motor into a weakly bound state that is pulled backward under the load of the trap. Backward slipping is stopped when one of the two motor domains in the ADP state re-engages at a binding site and releases nucleotides to start another cycle. However, due to their interaction with microtubules being significantly stronger, kinesin-3s stay attached to the microtubule even in a weakly bound state for long enough to wait for the next step. The ability to slip in a weakly bound state might also help kinesin-3 motors to work efficiently in teams, as it might permit motors to slip forward as well as backward, allowing the team to potentially move faster than a single motor.

Pathogenic mutations in KIF1C increase off rate and unloaded speed

In recent years, disease-causing mutations in KIF1C have been identified in patients presenting with complex forms of hereditary spastic paraplegia or spastic ataxias. Two of these, P176L and R169W, are located in the motor domain of KIF1C at the microtubule-binding interface (Figure 2A), but how these affect the biophysical properties of KIF1C is currently unclear. Thus, we introduced both mutations and purified full-length recombinant human KIF1C-GFP from insect cells (Figure 2B). To investigate the effect of these pathogenic mutations, we first determined in single-molecule assays whether the motors can still bind and move along microtubules. Both, KIF1C R169W-GFP and KIF1C P176L-GFP were motile in the single-molecule motility assays and could still reach the microtubule plus ends (Figures 2C and 2D), suggesting that the mutants are still able to move processively along microtubules. The landing rate of all motors was comparable, suggesting that the mutations do not alter the balance of autoinhibited versus active motors nor the initial binding occurring in the ADP state (Figure 2E). Under the conditions of the assay, we observe slightly more than half of the motors being static or moving very slowly (< 25 nm/s). The fraction of static motors was slightly increased for both mutants. Diffusing motors are observed occasionally for wild-type (WT) KIF1C but were increased more than 2-fold for the mutants. This resulted in a ~30% reduction in motile motors (Figure S2). We also observed a 50% reduction in the fraction of microtubule plus ends decorated by a kinesin for each of the mutants (Figure S2). Detailed analysis of single-molecule motility revealed that the dwell time of both mutants was significantly reduced (Figure 2F). As both mutations are located at the microtubule-binding interface of KIF1C, the increase in diffusive events and faster off rates suggest that microtubule binding of both mutants is reduced. Even though the speed was increased (Figure 2G), the fast off rate resulted in a significantly shorter run length of both mutants compared with WT KIF1C (Figure 2H). KIF1C motors frequently might be a trade-off for being faster motors. Consistent with this, KIF1A was proposed to exist in a one-head-bound or weakly bound state for more than 90% of its mechanochemical cycle. However, due to their interaction with microtubules being significantly stronger, kinesin-3s stay attached to the microtubule even in a weakly bound state for long enough to wait for the next step. The ability to slip in a weakly bound state might also help kinesin-3 motors to work efficiently in teams, as it might permit motors to slip forward as well as backward, allowing the team to potentially move faster than a single motor.
pause during runs (Figure 2D); the duration of run phases was more strongly reduced as a result of the mutations than the duration of pauses (Figure S2), suggesting that motors are more prone to detachment during runs. Thus, our single-molecule analysis of full-length KIF1C WT and P176L and R169W mutants suggests that both pathogenic mutations reduce microtubule-binding affinity, which results in faster motility but a higher off rate and shorter run length.

The two characterized mutations are both located in loop8/strand-b5, which was shown to make additional connections to the microtubule surface in kinesin-3 motors compared with kinesin-1.29 When two mutations were simultaneously introduced into this region in KIF1A (R167S and H171D), the run length was 10-fold and the speed 2-fold reduced, matching kinesin-1 motility properties.26 Thus, our findings that the two pathogenic mutations in KIF1C retain motility but with reduced run length is consistent with this. Pathogenic mutations in other regions of the microtubule-binding interface of KIF1A23,30 also showed reduced run lengths. However, we found that the unloaded velocity of the motors was not negatively affected and even slightly increased, while mutations across the microtubule-binding interface of KIF1A resulted in lower single-molecule velocities.23,26,30 Alanine scanning mutagenesis in kinesin-1 also identified two mutations (E220A and L317A) with a slightly higher velocity but reduced microtubule affinity.31 Thus, the different outcome of reducing microtubule affinity on unloaded speed across kinesin-3 motors could be due to the biophysical properties of KIF1C being intermediate between kinesin-1 and KIF1A.

Pathogenic mutations in KIF1C severely reduce force generation
Molecular motors haul cargoes against the viscous drag of the cytoplasm and various intracellular structures. Thus, to understand how the reduced microtubule affinity affects the ability to step under load, we analyzed both KIF1C\textsubscript{P176L}-GFP and KIF1C\textsubscript{R169W}-GFP using the optical trap. At the trap stiffness we used to analyze KIF1C WT motors (Figure 1), we did not observe
significant bead displacements for the mutants. To confirm that motors were present on the beads, we positioned beads on the microtubule and then switched the trap off to let the motor run without load. This confirmed the presence of functional motors (Figure 3A), but these were too weak to generate forces comparable to the WT motor. We then collected bead displacement data at reduced trap stiffness (see STAR Methods for details) and detected short runs for KIF1C<sub>P176L</sub>-GFP with an average peak force of 1.3 pN, compared with 3.5 pN for the WT motor (Figures 3B and 3C). KIF1C<sub>R169W</sub>-GFP moved the bead only small distances when the trap was switched off and could not generate forces above 1 pN (Figures 3B and 3C). These data suggest that the moderate reduction in processivity of unloaded motors observed in single-molecule assays are exacerbated once the motors generate forces against a load.

Thus, P176L and R169W affect KIF1C force generation much more dramatically than their unloaded motility. This seems not to be the case for the recently characterized P305L mutation in the 3<sub>10</sub>-helix of KIF1A, which showed a roughly 5-fold reduced landing rate, a 2-fold reduced speed and run length, and a 2-fold reduced force. It will require detailed analysis of mutations in different regions of the microtubule-binding interface to understand whether these differences are due to a specific function of the loop8/strand-b5 region in limiting the velocity and enhancing force generation of KIF1C or whether the relationship of microtubule affinity and force generation differs between motors from the kinesin-3 family.

Pathogenic mutations impair cellular cargo transport

In cells, motors could work in teams and thereby compensate for reduced single-molecule force. To test this, we first analyzed the ability of KIF1C with pathogenic mutations to localize to the cell periphery, which would indicate their ability to move through the cytoplasm.
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KIF1C-GFP accumulates in the tails of migrating RPE1 cells as previously reported.4,10 KIF1C\textsubscript{P176L}-GFP and KIF1C\textsubscript{R169W}-GFP were also found in cell tails, but comparable tail accumulation was only reached at higher cytoplasmic levels (Figure 4A). Quantifying the ratio of tail to perinuclear intensity revealed that tail accumulation of KIF1C\textsubscript{R169W}-GFP was 2-fold reduced compared with WT KIF1C-GFP, while KIF1C\textsubscript{P176L}-GFP accumulation was only very slightly reduced (Figure 4B). This suggests that single-molecule force generation above 2 pN is not required for motor relocation, but it is not clear from this assay whether tip-localized motors actively transported cargo.

To more directly test force generation in cells, we recruited WT and mutated motors to mitochondria as a large cargo expected to generate a significant amount of viscous drag but also to allow large teams of motors to work together. To do this, we co-expressed FKBP-GFP-myc-tagged monoamine oxidase (MAO) with either KIF1C\textsubscript{mCherry-FRB}, KIF1C\textsubscript{P176L}-mCherry-FRB, or KIF1C\textsubscript{R169W}-mCherry-FRB. GFP-tagged MAO indicated normal mitochondrial morphology and distribution before recruiting the full-length motors to the outer mitochondrial membrane (Figure 4C). Recruitment of WT KIF1C caused dramatic changes in mitochondrial morphology within seconds of adding rapamycin. The pulling forces fragmented mitochondria into thin outer membrane tubules and beads (Figure 4D). Both tubules and beads were transported toward the cell edges efficiently, resulting in a near complete accumulation of mitochondria to the cell tips within the 20 min of the experiment (Figures 4E and 4F). KIF1C\textsubscript{P176L}-mCherry-FRB caused the same mitochondrial beading effect, albeit more slowly, and transported mitochondria to the cell periphery less efficiently than the WT motor (Figures 4E and 4F). KIF1C\textsubscript{R169W}-mCherry-FRB was recruited very efficiently to mitochondrial membranes and accumulated on the periphery-facing tip of the membrane. Beading was only observed in cells expressing a relatively large amount of KIF1C\textsubscript{R169W} and then the motor was unable to efficiently move the large (700- to 1000-nm diameter) cargo. In most cases, normal tubular mitochondrial morphology was retained and a subset of these was transported toward the cell periphery (Figures 4E and 4F). At the beginning of the experiment, the fluorescence signal for the mitochondrial marker near the cell periphery was about 25% lower than in the perinuclear area and equal among all three conditions (Figure 4G). Immediately after adding rapamycin, the signal shifted toward the periphery, albeit at a significantly higher rate in cells expressing WT KIF1C-mCherry-FRB or KIF1C\textsubscript{P176L}-mCherry-FRB than the R169W mutant. 20 min after adding rapamycin, the ratio of peripheral versus centrally located mitochondria was clearly lower compared with WT KIF1C (Figure 4G) (WT: 1.58 ± 0.09; P176L: 1.31 ± 0.05, p = 0.01; R169W: 0.94 ± 0.04, p = 3 × 10\textsuperscript{−11} [mean ± SEM, t tests with Bonferroni correction]). Thus, pathogenic mutations in KIF1C resulting in reduced single-molecule force also have a reduced ability to relocate cargoes in cells, even when working in teams.

Our work suggests that KIF1C has biophysical properties that are intermediate between kinesin-1 and KIF1A with regard to the maximal force generated, stall times, and the frequency of backward slips. Further, analysis of two pathogenic mutations suggests that the reduced microtubule binding of KIF1C does not impair unloaded speed, in contrast to KIF1A mutations. However, force generation is severely impaired in these mutations, suggesting that KIF1C binds microtubules more strongly than KIF1A to generate higher forces and be less prone to backward slipping. When microtubule interactions are weakened, the motor can still move fast, as does KIF1A, but will not be able to generate substantial forces against a backward load. Our work sets a baseline to correlate changes in the biophysical properties of disease mutations in KIF1C with specific defects in neuronal cargo delivery and organelle distribution, defects in neuronal function and survival, time of onset, and the severity of the disease presentation in patients. This would lead to a detailed understanding of the disease etiology and offer a starting point to develop treatment strategies that delay or, indeed, reverse the symptoms.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- **METHOD DETAILS**
  - Protein expression and purification
  - Force measurements

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**Figure 4.** Pathogenic mutations of KIF1C impair intracellular cargo transport

(A) RPE1 cells treated with KIF1C siRNA and expressing either RNAi-protected wild-type KIF1C-GFP, KIF1C\textsubscript{P176L}-GFP, or KIF1C\textsubscript{R169W}-GFP. Motors accumulate in the tip of cell tails.

(B) Superplot of four cell tail accumulation experiments showing ratio of intensity in cell tails versus perinuclear cytoplasm. Small circles represent one cell and large circles the average of the experiment. Box plots show 10 and 90\textsuperscript{th} percentile whiskers of pooled data. n = 172, 133, and 186 cells, respectively. ***p < 0.0001 (Kruskal-Wallis test, Conover’s test post hoc with Holm correction).

(C) Cellular transport assay based on rapamycin-induced heterodimerization of FKBP and FRB. Upon drug addition, FRB and mCherry-tagged KIF1C motors are recruited to mitochondria labeled with FKBP and GFP through fusion with the outer mitochondrial membrane protein monoamine oxidase (MAO).

(D) Mitochondrial morphology changes upon KIF1C recruitment in RPE1 cell expressing KIF1C\textsubscript{mCherry-FRB} and FKBP-GFP-MAO. GFP channel shown, indicated times relative to rapamycin addition. Orange box indicates location of zoomed region. Orange arrows indicate membrane tubules pulled out, and blue arrowheads indicate beads formed.

(E) RPE1 cell co-expressing FKBP-GFP-MAO and either wild-type KIF1C\textsubscript{mCherry-FRB}, KIF1C\textsubscript{P176L}-mCherry-FRB, or KIF1C\textsubscript{R169W}-mCherry-FRB. Left panel shows KIF1C signal, other panels MAO-labeled mitochondria. Time from rapamycin addition indicated in minutes. Scale bars, 20 μm.

(F) Averaged linescans showing mitochondrial redistribution upon recruitment of wild-type KIF1C, P176L, or R169W mutant. Cell position normalized to 0 in cell center and 1 at cell periphery. Errors show SEM. n = 78–110 cells, as indicated. Time color-coded as indicated on top of panel.

(G) Ratio of GFP-MAO intensity at the cell periphery versus perinuclear area over time. Errors show SEM. n as indicated in (F).
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.07.029.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| X-rhodamine-labelled tubulin | Cytoskeleton Inc. | TL6290M |
| HiLyte Fluor 647-labelled tubulin | Cytoskeleton Inc. | TL670M |
| Biotin-tubulin | Cytoskeleton Inc. | T333P |
| PLL(20)-g[3.5]-PEG(2)/PEG(3.4)-Biotin (50%) | Susos AG | PLL(20)-g[3.5]-PEG(2)/PEGbi |
| streptavidin | Sigma-Aldrich / Merck | S4762 |
| k-casein | Sigma-Aldrich / Merck | C0406 |
| phosphocreatine | Sigma-Aldrich / Merck | P7936 |
| creatine phosphokinase | Sigma-Aldrich / Merck | C3755 |
| catalase | Sigma-Aldrich / Merck | C9322 |
| glucose oxidase | Sigma-Aldrich / Merck | G7141 |
| Acrylamide/Bis-acrylamide, 30% solution | Sigma-Aldrich / Merck | A3699 |
| Ex-Cell 420 serum-free media | Sigma-Aldrich / Merck | 14420C |
| Penicillin-Streptomycin | Sigma-Aldrich / Merck | P0781 |
| L-glutamine | Sigma-Aldrich / Merck | L7513 |
| Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham | Sigma-Aldrich / Merck | D6421 |
| Sodium bicarbonate solution | Sigma-Aldrich / Merck | S8761 |
| Trypsin-EDTA solution | Sigma-Aldrich / Merck | T3924 |
| Glycerol | Thermofisher | 15514-011 |
| Kanamycin | Sigma-Aldrich / Merck | K4000 |
| Tetracycline | Sigma-Aldrich / Merck | T3258 |
| Gentamicin | Sigma-Aldrich / Merck | G1272 |
| IPTG | Melford | MB1008 |
| X-Gal | Melford | MB1001 |
| Escort-IV | Sigma-Aldrich / Merck | L-3287 |
| Imidazole | Sigma-Aldrich / Merck | I2399 |
| Ni NTA Agarose | Qiagen | 30230 |
| DTT | Sigma-Aldrich / Merck | D9779 |
| Pipes | Sigma-Aldrich / Merck | RES0703P-A103X |
| ATP | Roche | 10127531001 |
| **Experimental models: Cell lines** | | |
| *Spodoptera frugiperda* Sf9 cells | VWR | EM71104-3 |
| hTERT RPE1 (human retinal pigment epithelia) | ATCC | CRL-4000 |

## Oligonucleotides

| Oligonucleotides | Source | Identifier |
|-----------------|--------|------------|
| pfBS (5'-GATTAGCATATCCCAACGACC-3') | This study | N/A |
| AS358 (5'-GAGGGATCCACAGTCTCCCCCATCTCT-3') | This study | N/A |
| AS359 (5'-GGGGATCCCTCCTCCCCGGC-3') | This study | N/A |
| AS376 (5'-TGCTCCTGGTAGGTGACCC-3') | This study | N/A |
| AS358 (5'-GAGGGATCCACAGTCTCCCCCATCTCT-3') | This study | N/A |
| U01 (5'-GAAAATCTGGAGCTATGCTGG-3') | This study | N/A |
| U10170 (5'-ACTGACCTTCTCCGAGTCC-3') | This study | N/A |
| siRNA targeting sequence KIF1C #2 GUGAGCUUAU | Theisen et al. | N/A |
| GGAGACAU-dA[=dC] | | |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anne Straube (anne@mechanochemistry.org).

**Materials availability**
All plasmids generated in this study have been deposited to Addgene under ID numbers 185978–185984.

**Data and code availability**
All data reported in this paper will be shared by the lead contact upon request.
All original code is available in this paper’s supplemental information.
Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

Recombinant protein expression was performed in Sf9 insect cells (VWR). Sf9 cells were maintained in Ex-Cell 420 serum-free media (Sigma, 14420C) at a pre-log density of 0.5–0.75 \( \times 10^6 \) cells/ml at 29 °C.

Cell biology experiments were performed in hTERT RPE1 (ATCC) cells. hTERT RPE1 cells were grown in DMEM/Nutrient F-12 Ham (Sigma, D6421) supplemented with 10% FBS (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml penicillin (Sigma), 100 \( \mu \)g/ml streptomycin (Sigma) and 2.3 g/l sodium bicarbonate (#S8761 Sigma) in a humidified incubator at 37 °C and 8% CO₂.
METHOD DETAILS

Protein expression and purification
pFastBac-M13-6His-KIF1C-GFP was described previously10 and is available from Addgene (130975). The human patient mutations P176L7 and R169W8 were introduced into this plasmid as follows.

For purification, pellets were thawed on ice, and cells were homogenized by sonication in buffer A (50 mM phosphate buffer pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM MgCl2, 0.1 mM ATP, and 40 mM sodium phosphate pH 7.5, 150 mM NaCl, 20 mM Imidazole, 0.1% Tween 20, 1.5 mM MgCl2) per gram of cell pellet, supplemented with 1 mM ATP and cOmplete protease inhibitor cocktail (#05056489001, Roche) and lysed using a douncer (#885301, Kontes) with 20 strokes. Lysates were then cleared by centrifugation at 38,000 g in a SLA-3000 rotor (Thermo Scientific) for 20 min. The pellet was resuspended in 4 ml of SF9 lysis buffer (50 mM Sodium phosphate pH 7.5, 150 mM NaCl, 20 mM Imidazole, 0.1% Tween 20, 1.5 mM MgCl2) per gram of cell pellet, supplemented with 0.1 mM ATP and cComplete protease inhibitor cocktail (#05056489001, Roche) and lysed using a douncer (#885301, Kontes) with 20 strokes. Lysates were then cleared by centrifugation at 38,000 × g in a SS-34 rotor (Sorvall) for 30 min, or 200,000 × g for 40 min in a T865 rotor (Sorvall). SP Sepharose beads (#17-0729-01, GE Healthcare) were equilibrated with the lysis buffer and the cleared lysate obtained is mixed with the beads and batch bound for 1 h. Next, the beads were loaded onto a 5 ml disposable polypropylene gravity column (#29922, Thermo Scientific) and washed with at least 10 CV SP wash buffer (50 mM sodium phosphate pH 7.5, 150 mM NaCl) and eluted with SP elution buffer (50 mM sodium phosphate pH 7.5, 300 mM NaCl). The peak fractions obtained were pooled and diluted with Ni-NTA lysis buffer (50 mM sodium phosphate pH 7.5, 150 mM NaCl, 20 mM Imidazole, 10% glycerol) and batch bound to Ni-NTA beads (#30230, Qiagen) for 1 h. The beads were loaded onto a gravity column and washed with at least 10 CV of Ni-NTA wash buffer (50 mM sodium phosphate pH 7.5, 150 mM NaCl, 50 mM imidazole and 10% glycerol) and eluted with Ni-NTA elution buffer (50 mM sodium phosphate pH 7.5, 150 mM NaCl, 150 mM Imidazole, 0.1 mM ATP and 10% glycerol). The peak fractions were run on an SDS-PAGE gel for visualisation and protein was aliquoted, flash frozen and stored in liquid nitrogen.

Recombinant Kinesin Heavy Chain from Drosophila melanogaster was purified as described previously.16 Briefly, BL21 DE3 (Invitrogen) were transformed with plasmid pPK113-6H-DHx (accession # AF053733), and the cells were grown at 37 °C until OD600nm reached 0.5. Expression was induced with 0.4 mM Isopropyl β-D-1-thiogalactopyranoside at 15 °C overnight. Cells were harvested by centrifugation (3000 × g, 15 min, RT). Cell pellets were stored at -80 °C. For purification, pellets were thawed on ice, and cells were lysed by sonication in buffer A (50 mM sodium phosphate pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM MgCl2, 0.1 mM ATP, and 40 mM Imidazole). The lysate was clarified by centrifugation (100,000 × g, T865 rotor, 30 min, 4 °C) and the supernatant was then loaded onto a 1 ml HisTrap HP column (Qiagen) at 4 °C. Unbound protein was washed with buffer A containing 90 mM imidazole. Finally, the protein was eluted over 300 ml imidazole gradient (10 column volumes). The peak fractions were run on an SDS-PAGE gel for visualisation and protein was aliquoted, flash frozen and stored in liquid nitrogen.

Force measurements
560 nm Polystyrene beads (Polysciences) and motor protein were incubated together in 80 mM PIPES pH 7, 2 mM MgSO4, 1 mM EGTA, 1 mM DTT, 3 mg/ml D-Glucose, 0.2 mg/ml casein and 1 mM ATP. The concentration of motor was decreased such that only 20-30% of the beads moved. Flow cell coverslips were plasma-cleaned and functionalized with APTES silane. Two coverslips were stuck together with Dow Corning High Vacuum Grease. Two lines of grease were applied to the base coverslip (22 × 50 mm) using a syringe, the top coverslip (22 × 22 mm) was placed, forming a flow cell of approximately 10 μl capacity. For covalent attachment of microtubules, glutaraldehyde (8%) was added to the flow cell and incubated for 30 min. The flow cell was washed with MilliQ water. The microtubules were diluted to the required concentration and introduced to the flow cell and allowed to adsorb onto the surface. The microtubules were incubated for an hour to allow for covalent attachment. Next, 0.2 μl of bead-motor protein solution was diluted in 20 μl of assay buffer composed of BRB80, 1 mM ATP, 0.4 mg/ml casein, 10 μM taxol and 0.4 μl oxygen scavenger mix (1 mg/ml catalase, 5 mg/ml Glucose oxidase, 50% glycerol). The beads were then flown to the cell and microtubules were visualised using in-built differential interference contrast on the Optical Trap setup19 at room temperature of 22 °C. The trap was steered to...
position the bead above the microtubule and the image was projected onto the quadrant photodiode detector. Bead positions were acquired at 20 kHz for 180 s, trap stiffness 0.065 to 0.075 pN nm\(^{-1}\) for KHC, 0.06 to 0.067 pN nm\(^{-1}\) for wildtype KIF1C-GFP, 0.01 to 0.052 pN nm\(^{-1}\) for KIF1C\(_{P176L}\)-GFP and 0.014 pN nm\(^{-1}\) for KIF1C\(_{R169W}\)-GFP. A calibration for trap stiffness was done each day before commencement of measurements. Measurements were done under conditions when 20% or less beads were running on microtubules. According to a Poisson distribution, about 12% of moving beads could have more than one motor capable of engaging with the microtubule. The rare traces that showed unusual high force events that suggested more than one motor is engaged were excluded from the analysis. For wildtype KIF1C-GFP, data was pooled from 38 recordings of 7 motile beads from 3 independent experiments. For KIF1C\(_{P176L}\)-GFP, data pooled from 21 recordings of 6 motile beads from 4 independent experiments. For KIF1C\(_{R169W}\)-GFP data pooled from 3 recordings of 2 motile beads from 1 experiment. KHC data are a subset of a previously published dataset. The analysis only included data that were acquired at a comparable trap stiffness to KIF1C data (8 recordings from 8 different beads, 8 microtubules acquired on three different experiment days). In addition, data from superstall conditions were included for the analysis of fore/backstep ratios.

Data analysis was carried out on baseline-corrected traces. From these traces, the number of force events per MT encounter was determined by counting the number of times the motor moved forwards and slipped back for at least 24 nm before detachment. Events where the bead returned to the trap centre but restarted immediately were considered as long backslips rather than detachments and thus considered as the same MT encounter in this analysis.

An automatic run-event analysis using custom code written in R (see Methods S1) was used to determine the peak force, duration of force-generation events, the force-velocity curve and the restart times between events. Data was smoothed by 2000-point averaging (100 ms) and events above 0.5 pN force, lasting more than 200 ms, were included. The end of the run was registered when the event returned below the 0.5 pN threshold. Force-velocity relationship was determined from linear fits to the displacement versus time data smoothed by 200 points (10 ms) over 0.5 pN force bins. Unloaded velocities were determined from DIC videos of beads moving along microtubules after the trapping laser was switched off. DIC videos were converted to digital timelapse movies at 5 frames per second and speeds were obtained from kymographs using a custom macro in ImageJ (see Methods S1). Stall force was determined manually from each identified force event as the average force that was maintained with fluctuations of no more than one (8 nm) step before the bead returned to the trap center or slipped backwards for at least 24 nm. Only stalls of at least 10 ms duration were included in the analysis.

For the step analysis, a moving window t-test algorithm\(^1\)\(^2\) was used with the following parameters: t-test score threshold=30, minimum step size=5 nm, minimum force=1 pN, moving average, n=20 (1 ms) (see Methods S1). The size of the window and the threshold were varied to determine accurate steps. When the bead returned to within 1.2 pN of the centre of the trap, we designated this as a detachment. Other movements towards the trap centre were designated as backslips. To determine the stall force defined as the force at which the motor undertakes as many forward steps as backslips, we only considered backslips of up to 12 nm for the fore/back ratio as their probability of detection is comparable to forward steps.

**Single molecule motility assays**

Microtubules were assembled from 5 \(\mu\)l of 18 mg/ml unlabelled pig tubulin, 0.2 \(\mu\)l of 1 mg/ml HiLyte Fluor 647 or X-rhodamine-labelled tubulin (#TL670M, #TL6290M, Cytoskeleton) and 0.5 \(\mu\)l of 0.5 mg/ml biotin tubulin (#T333P, Cytoskeleton) in 15 \(\mu\)l MRB80 (80 mM PIPES pH 6.8, 4 mM MgCl\(_2\), 1 mM EGTA, 1 mM DTT) supplemented with 4 mM GTP. The mixture was incubated at 37 °C for 90 min before diluting in 80 \(\mu\)l MRB80 supplemented with 20 \(\mu\)M Taxol. Unincorporated tubulin was removed by pelleting microtubules through a glycerol cushion (30% glycerol in MRB80) at 20,238 \(\times\) g for 12 min at room temperature. The microtubule pellet was resuspended in 80 \(\mu\)l of MRB80 with 20 \(\mu\)M Taxol and stored at 28 °C covered from light for use on the same day.

Coverslips (22 \(\times\) 22) were cleaned by incubating in 2.3 M hydrochloric acid overnight at 60 °C. The next day, coverslips were washed with Millipore water and sonicated at 60 °C for 5 min. The wash cycle was repeated five times. The coverslips were dried using a Spin Clean (Technical video) and plasma cleaned using Henniker plasma clean (HPT-200) for 3 min. Flow chambers were made using clean glass slides (Menzel Gläser Superfrost Plus, Thermo Scientific) and double-sided sticky tape (Scotch 3 M) by placing the cleaned coverslip on the sticky tape creating a 100 \(\mu\)m deep flow chamber. The surface was coated with (0.2 mg/ml) PLL(20)-g[3.5]-PEG(2)/PEG(3.4)-Biotin (50%) (#PLL(20)-g[3.5]-PEG(2)/PEGbi, Susos AG). Biotin-647-microtubules were attached to this surface with streptavidin (0.625 mg/ml) (#S4762 Sigma) and the surface was blocked with \(\kappa\)-casein (1 mg/ml) (#C0406 Sigma). KIF1C-GFP, KIF1C\(_{P176L}\)-GFP and KIF1C\(_{R169W}\)-GFP were diluted in TIRF assay buffer (25 mM HEPES-KOH pH 7.2, 5 mM MgSO\(_4\), 1 mM EGTA, 1 mM DTT, 10 \(\mu\)M Taxol) supplemented with 0.05% Tween-20, 25 mM KCl and 200ng/ml \(\kappa\)-casein, spun at 100,000 \(\times\) g for 10 min in an Airfuge (Beckman Coulter) and fluorescence at 507 nm measured using a NanoDrop\(^{TM}\) 3300 Fluorospectrometer to verify concentrations used in the assay and determine landing rates. Proteins were then added to the motility mix (TIRF Assay Buffer supplemented with 5 mM ATP, 5 mM phosphocreatine (#P7936, Sigma), 7 U/ml creatine phosphokinase (#C3755 Sigma), 0.2 mg/ml catalase, 0.4 mg/ml glucose oxidase, 4 mM DTT, 50 mM glucose (#G8270, Sigma), 25 mM KCl, 10 \(\mu\)M taxol, 0.2 mg/ml \(\kappa\)-casein) and flown into the chamber.

Chambers were observed on an Olympus TIRF system using a x100 NA 1.49 objective, 488 and 640 nm laser lines, an ImageEM emCCD camera (Hamamatsu Photonics) under the control of xCellence software (Olympus), an environmental chamber maintained at 25 °C (Okolab, Ottaviano, Italy). Images were acquired at 5 fps for 180 seconds using 2x2 binning, thus the resulting images have 162 nm pixels and 200 ms temporal resolution.
The fraction of microtubules that contain one or more kinesin motors at the plus end was determined by counting all microtubules for which both ends were visible in the first frame of the second movie taken from each chamber, i.e. about 3 minutes after start of the experiment, and scoring for the presence / absence of a GFP signal at one of the ends.

Motility was analysed by tracing microtubules and generating maximum intensity kymographs from 7 pixel wide lines using the ImageJ Kymograph plugin from Arne Seitz (EPFL Lausanne). Motor tracks from kymographs were traced by hand and recorded as ImageJ ROIs. These paths were analysed in a custom-built python analysis script. Individual phases of tracks were segmented from the ROI, and motors were said to be translocating towards the plus-end or minus-end of the microtubule in each phase if their speed towards that end of the microtubule was greater than 25 nm/s. When the motor’s speed was less than 25 nm/s, it was annotated as paused. Motors with an average speed of less than 25 nm/s or a total run length of less than 500 nm were considered as static. Non-static motors that underwent bidirectional motion of at least 324 nm or just moved towards the minus end where classified as diffusing. Only the remaining, plus end directed motors were analysed for run parameters. Dwells times were calculated as the total time the motor spent on the microtubule until it reaches the plus end or the end of the recording. Run length is the total distance covered by the motor. The pause-corrected average speed was calculated by dividing the run length by the dwell time minus any time when the motor was paused. Superplots show the mean speed, run-length, or dwell time for a given experimental day, with smaller colour-coded spots showing the individual measured values of motors. On each of the three experiment days, one to four chambers were prepared for each protein. Statistics were calculated at the per-motor level between experimental groups. The data were tested for normality using D’Agostino and Pearson’s test, and as they were not normally distributed, a Kruskal-Wallis test was used to determine if experimental groups differed significantly. Where a statistically significant difference was indicated, pairwise interactions were tested using Conover’s post-hoc test and p-values were corrected for multiple comparisons using the Holm-Bonferroni method.

To test homogeneity of the motors in the motility assay, the intensity of motors was determined using the ImageJ plugin GaussFit_OnSpot for each line traced in the kymograph. The normalised intensity value output by the function for the first 10 time points of each track were averaged to obtain the fluorescent intensity for the motor. To pool intensity measurements from different experiments, data were divided by the average intensity of all wildtype KIF1C detections and then grouped into highly processive motors with a run length of more than 1 μm and motors that moved less than 1 μm. While a small fraction of motor clusters were observed in the assays, the clusters did not primarily contribute to the highly processive runs (Figure S2). Data were plotted in MATLAB using the plotSpread function from Jonas Dorn.

KIF1C localisation and mitochondria transport assays

pKIF1C_P176L-GFP and pKIF1C_R169W-GFP were based on pKIF1C_RIP2-GFP (available from Addgene 130977).\(^\text{32}\) R169W was introduced using a mutagenesis PCR with upstream primer UT01 (5’-GGAATTCTGGAGCTATGGCTGGTG-3’), mutagenesis primer AS376 (5’-CTGGGGGCTGGGAGGACC-3’) and downstream primer UT170 (5’-ACTGACCTTCTCGGAGTCC-3’). The fragment containing the mutation was replaced in pKIF1C_RIP2-GFP using EcoRI and BsWl. P176L was introduced in a mutagenesis PCR with upstream primer UT01, mutagenesis primer AS258 (5’-CTGCCGATACGGCCAGGATG-3’) and downstream primer AS359 (5’-GGGATCCCTCTGGTCCGTTC-3’). The fragment containing the mutation was replaced in pKIF1C_RIP2-GFP using EcoRI and BspEI. The RIP2 mutation contained in all these constructs are a series of silent mutations providing RNAi resistance for siRNA KIF1C #2 GUGAGCUAUAGGAGAUCU-[dA]-[dC].

pKIF1C-mCherry-FRB, pKIF1C_P176L-mCherry-FRB and pKIF1C_R169W-mCherry-FRB plasmids were based on plactin-KIF1C_MD-FRB-GFP, a kind gift from Caspar Hoogenraad.\(^\text{1}\) The KIF1C motor domain was replaced with full length human KIF1C fused to mCherry from pKIF1C-mCherry (available from Addgene 130978) using Nhel and BsrGI. The mutations were introduced from pKIF1C_P176L-GFP and pKIF1C_R169W-GFP using Nhel and BamHI. FKBP-GFP-Myc-MAO was a kind gift from Sean Munro (MRC-LMB, Cambridge).\(^\text{33}\)

For tail localisation experiments, RPE1 cells were seeded into fibronectin-coated glass-bottom dishes were transfected with validated KIF1C siRNA #2 using Oligofectamine (Invitrogen), transfected 24 hours later with either with KIF1C-GFP, pKIF1C_P176L-GFP or pKIF1C_R169W-GFP using Fugene 6 (Promega) and imaged a further 24 hours later on an Olympus DeltaVision microscope (Applied Precision, LLC) equipped with eGFP filter sets and a CoolSNAP HQ2 camera (Roper Scientific) under the control of SoftWorx (Applied Precision). 37°C and 5% CO₂ were maintained in a TOKAI Hit stage incubator. Images from migrating cells with a tail at the rear expressing moderate levels of the GFP constructs were acquired. To determine tail accumulation, average intensity measurements from a tip of cell tails, the perinuclear area and background outside of the cell were taken. After background subtraction, the ratio of tail versus cytoplasm intensity was calculated. 4 experiments with a total of 133-186 cells were analysed for each condition. Statistical analysis was performed on the per cell level between experimental groups. A Kruskal-Wallis test was used to determine if experimental groups differed significantly. Where a statistically significant difference was indicated, pairwise interactions were tested using Conover’s post-hoc test and p-values were corrected for multiple comparisons using the Holm-Bonferroni method.

For mitochondria transport experiments, RPE1 cells were co-transfected with 1 μg KIF1C-mCherry-FRB and 0.5 μg FKBP-GFP-Myc-MAO using Fugene 6 (Promega) in fibronectin-coated glass-bottom dishes and imaged 24 hours later on an Olympus DeltaVision microscope (Applied Precision, LLC) equipped with eGFP, mCherry filter sets and a CoolSNAP HQ2 camera (Roper Scientific) under the control of SoftWorx (Applied Precision). Live cells were imaged using an Olympus 40x UPlanFL N Oil NA1.3 objective in imaging medium (Leibovitz’s L-15 medium (Gibco™ 21083027) without phenol red, supplemented with 10% FBS (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma)) at 37°C. Cells expressing moderate levels of...
both KIF1C and the mitochondrial marker were identified and their stage position labelled. Images in both channels were acquired from each stage position at 2-minute intervals for 22 minutes to determine organelle redistribution. After the first image from each cell, rapamycin was added to the imaging dish at a final concentration of 200 μM. To observe mitochondria morphology change and motility, images from a single cell were acquired at 2 s intervals for 10 minutes and rapamycin added after 30 seconds.

To quantify organelle redistribution, 33 pixel wide line profiles were acquired from the cell centre to the cell tips at each timepoint. The average intensity of the KIF1C signal along the entire line was used to include only cells with comparable expression level across the wildtype and mutant constructs. Positions along line profiles were normalised so that the cell centre was set to 0 and the cell tip to 1. This normalised distance was binned into 100 equal-width bins and intensity values of MAO for each cell were sorted into these bins. The centre of each bin was plotted against the average MAO intensity +/- the standard error of the binned values. To quantify mitochondria redistribution over time, periphery GFP-MAO signal (0.8 to 1.0) was divided by perinuclear GFP-MAO signal (0.0 to 0.2).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The number of experimental repeats, number of beads/cells/microtubules analysed is provided in the method details section. Information on the number of observations shown in each graph is provided in the figure legend. Error bars show either SD or SEM as indicated in the figure legend. Superplots show individual data points and mean values colour-coded for each experiment. Box plots show Median, quartiles and whiskers at 10th and 90th percentile or min/max values as indicated in the figure legend. Histograms and cumulative frequency plots show data pooled from all experiments.

For single molecule experiments, statistics were calculated using python packages scipy and scikit at the per-motor level between experimental groups. The data were tested for normality using D’Agostino and Pearson’s test, and as they were not normally distributed, a Kruskal-Wallis test was used to determine if experimental groups differed significantly. Where a statistically significant difference was indicated, pairwise interactions were tested using Conover’s post-hoc test and p-values were corrected for multiple comparisons using the Holm-Bonferroni method.

For tail localisation data, statistics were calculated using python packages scipy and scikit at the per-cell level between experimental groups. A Kruskal-Wallis test was used to determine if experimental groups differed significantly. Then, pairwise interactions were tested using Conover’s post-hoc test and p-values were corrected for multiple comparisons using the Holm-Bonferroni method.

Cumulative frequency plots were generated using MATLAB and whether distributions are significantly different was tested using Kolmogorov-Smirnov tests. To compare two normal distributions, two sample t-tests were performed.

Data were considered significantly different if p<0.05.