PTPN21 and Hook3 relieve KIF1C autoinhibition and activate intracellular transport

Nida Siddiqui, Alexander James Zwetsloot, Alice Bachmann, Daniel Roth, Hamdi Hussain, Jonathan Brandt, Irina Kaverina & Anne Straube

The kinesin-3 KIF1C is a fast organelle transporter implicated in the transport of dense core vesicles in neurons and the delivery of integrins to cell adhesions. Here we report the mechanisms of autoinhibition and release that control the activity of KIF1C. We show that the microtubule binding surface of KIF1C motor domain interacts with its stalk and that these autoinhibitory interactions are released upon binding of protein tyrosine phosphatase PTPN21. The FERM domain of PTPN21 stimulates dense core vesicle transport in primary hippocampal neurons and rescues integrin trafficking in KIF1C-depleted cells. In vitro, human full-length KIF1C is a processive, plus-end directed motor. Its landing rate onto microtubules increases in the presence of either PTPN21 FERM domain or the cargo adapter Hook3 that binds the same region of KIF1C tail. This autoinhibition release mechanism allows cargo-activated transport and might enable motors to participate in bidirectional cargo transport without undertaking a tug-of-war.
I ntracellular transport is essential for cell polarity and function. Long-distance transport of cellular cargo is mediated by microtubule-based motors, dynein and kinesin. While dynein is the main transporter towards the minus end of microtubules, most kinesins walk towards the microtubule plus end. However, many cargoes carry motors of both directionality in order to allow directional switching when they encounter a roadblock and the relative activity of the opposite directionality motors determines the net progress of the cargo towards the cell periphery (where usually most plus ends are located) or towards the cell centre (where microtubule minus ends are abundant). We have previously identified the kinesin-3 KIF1C as the motor responsible for the transport of d5β1-integrins. The delivery of integrins into cellular processes such as the tails of migrating cells allows the maturation of focal adhesion sites. KIF1C is also required for the formation and microtubule-induced turnover of podosomes—protrusive actin-based adhesion structures—in both macrophages and vascular smooth muscle cells. Moreover, KIF1C contributes to MHC class II antigen presentation, Golgi organisation and transport of Rab6-positive secretory vesicles. In neurons, KIF1C transports dense-core vesicles both into axons and dendrites and appears to be the fastest human cargo transporter. Consistently, human patients with missense mutations in KIF1C resulting in the absence of the protein suffer from spastic paraplegia and cerebellar dysfunction. Mutations that result in reduced motor function also cause a form of hereditary spastic paraplegia.

KIF1C-dependent cargo moves bidirectionally even in highly polarised microtubule networks and depletion of KIF1C in the reduction of transport in both directions, suggesting that KIF1C cooperates with dynein as had been suggested for other kinesin-3 motors. To begin to unravel how KIF1C contributes to bidirectional cargo transport, we need to identify the mechanisms that switch KIF1C transport on and off. Most kinesin-3 motors are thought to be activated by a monomer–dimer switch, whereby cargo binding releases inhibitory intramolecular interactions of neck and stalk regions by facilitating the dimerisation of the neck coil and other coiled-coils regions in the tail. The motor thereby transitions from an inactive, diffusive monomer to a processive dimer. In the alternative tail-block model, the motors are stable dimers, but regions of the tail interact with the motor or neck domains and interfere with motor activity until cargo binding occupies the tail region and releases the motor.

Here we show that KIF1C is a stable dimer that is autoinhibited by intramolecular interactions of the stalk domain with the microtubule binding interface of the motor domain. We demonstrate that protein tyrosine phosphatase N21 (PTPN21) activates KIF1C by binding to the stalk region. This function does not require catalytic activity of the phosphatase, and its N-terminal FERM domain alone is sufficient to stimulate the transport of KIF1C cargoes in cells as well as increasing the landing rate of KIF1C on microtubules in vitro. The cargo adapter Hook3 binds KIF1C in the same region and activates KIF1C in a similar way, suggesting that both cargo binding and regulatory proteins might contribute to the directional switching of KIF1C–dynein transport complexes.

Results
KIF1C is an autoinhibited dimer. To determine the mechanism of KIF1C regulation, we first aimed to determine its mode of autoinhibition. The monomer–dimer–switch and tail-block models can be distinguished by determining the oligomeric state of the motor. Thus we performed classic hydrodynamic analysis using glycerol gradients and size exclusion chromatography on both purified recombinant full-length human KIF1C-GFP from insect cells and KIF1C-Flag in human cell lysate (Fig. 1a–c, Supplementary Fig. 1). The sedimentation coefficient and Stokes radius were determined in comparison to standard proteins. We find that the apparent molecular weight determined both at physiological levels of salt (150 mM) and in high (500 mM) salt buffer is consistent with KIF1C being a dimer (KIF1C-GFP: apparent MW = 272 ± 57 kDa, expected dimer MW = 308 kDa; KIF1C-Flag: apparent MW = 178 ± 45 kDa, expected dimer MW = 251 kDa, all errors are SEM based on

![Figure 1](image-url) Fig. 1 KIF1C is a dimer. a Fractions from glycerol gradients of KIF1C-GFP at different salt concentrations as indicated. Elution peaks of standard proteins are indicated with orange arrowheads. GOx, glucose oxidase. Errors are SEM. Uncropped gels are provided in Source Data file. b Size exclusion chromatography of KIF1C-GFP at 150 mM NaCl (grey) and 500 mM NaCl (orange). Elution peaks of standard proteins (Tg thyroglobulin, Af apoferritin, Cat catalase, BSA bovine serum albumin) and void volume V0 are indicated by orange arrowheads. Errors are SEM. c Frictional coefficient of KIF1C-GFP at different salt concentrations indicating that KIF1C elongates with increasing ionic strength. d, e Bleach curve of KIF1C-GFP on microtubules showing discrete steps in fluorescent decay in d. Experimentally determined bleach steps are shown in e together with best fit to a mixed binomial model of dimers and tetramers with x being the fraction of tetramer and p the fraction of active GFP molecules. n=108 motors. Data are provided in Source Data file.
errors of calibration curve fit parameters and estimated precision of peak location as 1/2 of fraction size). Consistent with this, the majority of KIF1C-GFP molecules bound to microtubules in the presence of non-hydrolysable AMPPNP show two bleach steps and the distribution of bleach steps found fits best to a simulation of 88% dimers and 12% tetramers (Fig. 1d, e), assuming that about 80% of GFPs are active, which is realistic based on previous findings. Interestingly, KIF1C elonates at increasing salt concentrations from a moderately elongated conformation (frictional coefficient of 1.5) at physiological salt to highly elongated (frictional coefficient = 1.9) at 500 mM (Fig. 1c), suggesting that intramolecular electrostatic interactions might hold the KIF1C dimer in a folded, autoinhibited state.

To determine the interaction surfaces involved in a possible autoinhibited state, we performed crosslink mass spectrometry. Purified KIF1C was treated with the 11 Å crosslinker BS3 or with the zero length crosslinker EDC, digested with trypsin and then subjected to tandem mass spectrometry analysis. Crosslinked peptides were identified using StavroX. Only crosslinked peptides whose identity could be verified by extensive fragmention with none or few unexplained major peaks were retained (Supplementary Figs. 2 and 3). These high-confidence crosslinks were between K273-K591, K273-K645, K464-K645, K640-K645, K464-E606 and E644-K854 (Fig. 2a, Supplementary Figs. 2 and 3), showing that the end of the FHA domain and the third coiled-coil domain contact the motor domain near K273. This residue is within alpha-helix 4 at the centre of the microtubule interaction interface of the motor domain (Fig. 2b). In the presence of 500 mM NaCl, the abundance of the stalk-to-motor domain crosslinks was dramatically reduced consistent with the observed elongation of KIF1C dimer in glycerol gradients (Supplementary Figs. 2e and 2c). These findings suggest that KIF1C adopts a more...
PTPN21 stimulates transport of KIF1C cargoes. We next aimed to identify a regulator that could engage with the stalk domain and thereby activate the motor. Two proteins had previously been shown to interact with the KIF1C stalk domain, non-muscle Myosin IIA and protein tyrosine phosphatase N21 (PTPN21, also known as PTPD1). We reasoned that a KIF1C activator would be required for KIF1C function in cells and thus would phenocopy KIF1C depletion. As a readout of diminished KIF1C activity, we used the reduced podosome number in vascular smooth muscle cells that we reported previously. Myosin IIA inhibition did not phenocopy KIF1C depletion. Instead, inhibition of Myosin IIA with Blebbistatin or indirectly via inhibition of Rho kinase using Y27632 did not result in reduced podosome number, while remaining actin stress fibres were efficiently removed. As depletion of Myosin IIA did also have opposite effects on the stability of cell tails and directional persistence of cell migration we previously reported for KIF1C depletion, we excluded Myosin IIA as being involved in KIF1C activation. In contrast, PTPN21 depletion using siRNA resulted in a dramatic reduction of podosome number (Fig. 3e–h). To confirm specificity of the RNAi we rescued the phenotype with HA-tagged PTPN21. Interestingly, the catalytically inactive mutant PTPN21C1108S (ref. 25) could also fully rescue the PTPN21 depletion phenotype (Fig. 3e, f), suggesting that a scaffold function is required rather than phosphatase activity.

Strikingly, expression of PTPN21C1108S also efficiently compensated for partial depletion of KIF1C and fully rescued podosome formation (Fig. 4a–c). An N-terminal 378 amino acid fragment of PTPN21, which contains a FERM domain, was previously shown to be sufficient to interact with KIF1C24. Therefore we tested this construct, and found that the PTPN21 FERM domain alone was sufficient to rescue the KIF1C depletion phenotype (Fig. 4a–c). This suggests that PTPN21 scaffolding could activate the remaining pool of KIF1C, which is reduced to about 25–30% in cells treated with siKIF1C-2 compared to control cells. Alternatively, PTPN21 could also activate another kinesin to compensate for KIF1C depletion. KIF16B, another kinesin-3, was an obvious candidate for this as its interaction with PTPN21 FERM domain had been shown already. To discriminate between these two possibilities, we depleted KIF1C and KIF16B individually and simultaneously in PTPN21 FERM rescue experiments. KIF16B depletion on its own resulted only in a mild reduction in podosome numbers and expression of FERM domain significantly increased the number of podosomes formed in control cells and in those where either of the kinesins was depleted. However, PTPN21 FERM did not increase podosome numbers in cells that were depleted for both KIF1C and KIF16B (Fig. 4d, Supplementary Fig. 4). These findings suggest that PTPN21 rescue depends on the presence of a sizeable pool of either KIF1C or KIF16B. To explore this activity further, we investigated more directly whether PTPN21 stimulates KIF1C-dependent transport processes. We have shown previously that KIF1C is required for the bidirectional transport of integrin-containing vesicles in migrating RPE1 cells. The depletion of KIF1C results in a reduction of both plus-end and minus-end-directed transport and an increase in stationary vesicles. Expression of either wild-type PTPN21, the catalytically inactive PTPN21C1108S mutant or PTPN21FERM reactivated integrin vesicle transport in KIF1C-depleted cells (Fig. 4e, f). To test whether the ability of PTPN21FERM to activate KIF1C-dependent transport universally applies, we next isolated primary hippocampal neurons and observed the transport of dense-core vesicles labelled with NPY–RFP in the presence of either a control plasmid or pFERM. Consistent with a function to activate KIF1C-dependent transport, we observed a dramatically increased frequency of vesicles moving in anterograde direction through the neurites in cells expressing the FERM domain of PTPN21 (Fig. 4g–i).
previously identified as minimal PTPN21-binding domain (Fig. 6a). First, we assessed whether either or both constructs are hyperactive by localisation of the construct in cells. We had described previously that KIF1C accumulates specifically in tails of migrating RPE1 cells3. Expression of KIF1CΔCC3-GFP and KIF1CΔS-GFP in RPE1 cells resulted in accumulation of the construct in cell tails and also elsewhere at the periphery of the cell (Fig. 6b). As tail accumulation is sensitive to tail state, i.e. whether it is forming or retracting3, we used KIF1C-mCherry as an internal control. In comparison to KIF1C-GFP, which was enriched in tails to a similar extent as KIF1C-mCherry (tail:cytoplasm ratio 6.1 ± 1.3 for KIF1C-mCherry and 7.4 ± 1.2 for KIF1C-GFP, n = 26 cells, errors are SEM), KIF1CΔCC3-GFP was almost threefold more enriched (tail:cytoplasm ratio 3.9 ± 0.6 for KIF1C-mCherry and 13.6 ± 2.1 for KIF1CΔCC3-GFP, n = 32 cells), and KIF1CΔS-GFP was fourfold more enriched (tail:cytoplasm ratio 1.8 ± 0.3 for KIF1C-mCherry and 7.7 ± 0.8 for KIF1CΔS-GFP, n = 39 cells) (Fig. 6c). Secondly, we purified recombinant KIF1CΔCC3-GFP and KIF1CΔS-GFP from insect cells (Fig. 6d) and performed single-molecule assays. KIF1CΔCC3-GFP had a threefold higher landing rate compared to wild-type KIF1C-GFP, and addition of PTPN21 FERM did not further increase the landing rate of KIF1CΔCC3-GFP (Fig. 6e–g, k). KIF1CΔS-GFP had a 20-fold higher landing rate.

Fig. 3 PTPN21, but not Myosin IIa, is required for podosome formation. a, b A7r5 cells treated with 5 µM PDBu and different concentrations of Blebbistatin or Y27632 for 1 h before staining for actin and cortactin. Scale bars 20 µm. c A7r5 cells transfected with siControl and siPTPN21 and either control plasmid (pFlag) or an RNAi-protected construct of wild-type PTPN21 or a catalytically inactive mutant PTPN21C1108S were treated with 5 µM PDBu for 1 h. Scale bar 20 µm. d, e Quantification of podosome numbers under different experimental conditions as indicated. n = 90 cells pooled from three independent experiments. Box plots show quartiles with 10/90% whiskers and mean indicated by a circle. Statistical significance with p < 0.05 is indicated with asterisks, *** represents p < 0.0005 (Mann-Whitney U-test with Bonferroni). Data are provided in Source Data file. g, h RT-PCR from random-primed cDNA of A7r5 cells treated with siRNA as indicated. For each experiment, duplicates of siPTPN21 and five different concentrations of cDNA from siControl were analysed. Quantification of RT-PCR band intensities for siPTPN21 relative to siControl standard curve from four independent experiments is shown as mean ± SEM with overlaid data points. Uncropped gels and data are provided in Source Data file.
than KIF1C-GFP and also moved at about threefold higher average speed (Fig. 6h–m). Therefore, these experiments confirm that binding of PTPN21 to the stalk domain of KIF1C relieves the autoinhibition of KIF1C and thereby enables the motor to engage with microtubules.

Hook3 binds to KIF1C stalk and also activates KIF1C. Finally, we wondered how universal this mechanism is and whether other proteins binding KIF1C stalk could activate KIF1C in a similar manner. To identify KIF1C stalk interactors we performed a BioID experiment with full-length KIF1C and KIF1CΔ (Fig. 7a). We identified 43 proteins or clusters of proteins isolated with streptavidin-beads upon biotinylation that were significantly enriched in either the KIF1C-BioID2 or KIF1CΔ-BioID2 samples. Eight of those were enriched more than 10-fold in the full-length sample. The most significant hit was Hook3 (Fig. 7b) which was 50-fold enriched in the full-length sample and thus

![Image](https://example.com/image1.png)
Fig. 4 PTPN21 activates intracellular transport. a Primary structure of PTPN21 and N-terminal fragment used in this study. The region identified to interact with kinesins, actin and Src kinase is indicated below. b, c A7r5 cells treated with 5 μM PDBu for 1 h and stained for cortactin as a marker for podosomes. Expression of catalytically inactive PTPN21 or just the FERM domain rescues the podosome formation phenotype of KIF1C-depleted cells. n = 60 cells. ***p < 0.0001 (Mann-Whitney U-test with Bonferroni). Scale bar 20 μm. Data are provided in Source Data file. d Podosome formation in A7r5 cells treated with siKIF1C, siKIF16B or both. Expression of the FERM domain increases podosome formation unless both kinesins are depleted. n = 90 cells pooled from three independent experiments. **p < 0.0001 **p < 0.001 (Mann-Whitney U-test with Bonferroni). See Supplementary Fig. 4 for representative examples. Data are provided in Source Data file. e, f Kymographs of α5-integrin vesicles in the tail of migrating RPE1 cells. KIF1C depletion increases in vesicles moving less than 1.5 μm. Expression of various PTPN21 constructs suppresses the KIF1C depletion phenotype. n = 36–158 cells pooled from 3 to 11 independent experiments. ***p < 0.0001 and n.s. p > 0.5 (ANOVA + Tukey post hoc relative to siControl). Data are provided in Source Data file. g-i Representative kymographs show primary hippocampal neurons isolated from a P2 mouse, transfected with NPY-RFP and either pFlag (as control) or pERM. Number of NPY-positive vesicles passing a location per minute and the average speed of vesicles is shown for anterograde and retrograde movement. Data pooled from three independent experiments (DIVS-6). n = 3760 neurites/197,953,136,480 vesicles. ***p < 0.0001; **p < 0.005; n.s. p > 0.5 (Kolmogorov-Smirnov).

appeared as a possible KIF1C stalk interactor. We confirmed that both Hook3 and PTPN21 bind to KIF1C in a stalk-dependent manner using co-immunoprecipitation from HEK293 cells (Fig. 7c, Supplementary Fig. 6). Hook3 has previously been identified as an activator of dynein/dynactin—an activity requiring its N-terminal globular domain and all three coiled-coil domains27–30. In contrast, the C-terminus of Hook3 was shown to interact with KIF1C31. To test whether Hook3 could also activate KIF1C, we purified recombinant human Hook3 with a C-terminal SNAP-tag from insect cells (Fig. 7d), labelled it with Alexa647 and performed single-molecule assays with KIF1C. In the presence of Hook3, the landing rate of KIF1C increased twofold (Fig. 7e, f), thus confirming that Hook3 functions as an activator similarly to PTPN21 by binding to the KIF1C stalk region adjacent to the site engaged in intramolecular interactions of KIF1C during autoinhibition. As we could observe that Hook3 was co-transported with KIF1C (Fig. 7e), we asked whether PTPN21 also maintained contact with KIF1C after activating it. We purified and labelled FERM-SNAPf with Alexa647 similarly to our Hook3 construct and performed two-colour TIRF assays both with KIF1C-GFP and KIF1C-GFP. Both activators were co-transported with full-length KIF1C, but not with KIF1CAS (Fig. 8a–d). This suggests that both PTPN21 and Hook3 act as scaffolds to activate KIF1C by engaging with its stalk region (Fig. 8e, f).

Discussion

Our findings support a model whereby KIF1C is a stable dimer that is held in an autoinhibited conformation by interaction of its stalk region including the third coiled-coil domain with the microtubule binding surface of the motor domain (Fig. 8e). Autoinhibition is relieved upon binding of PTPN21 FERM domain or Hook3, allowing the motor domain to engage with microtubules (Fig. 8f). While the model agrees with the findings that KIF1C motors are dimeric in cells32, this is in contrast to the mode of autoinhibition described for other kinesin-3 motors, KIF1A, Unc104, KIF16B, KIF13A and KIF13B that all undergo a monomer–dimer transition14–17. However, interactions of the stalk or tail region with the motor domain have also been described for KIF13B and KIF16B19,20. These might act as a second layer of activity control once dimers are formed or stabilise the inhibited monomer conformation. Our data suggest that KIF1C autoinhibition acts by steric blockage of the microtubule binding. In contrast, kinesin-1, which is also autoregulated by a tail-block mechanism, is inhibited by crosslinking of the motor domains that prevent movement required for neck linker undocking33,34.

We identify the phosphatase PTPN21 as a KIF1C activator. The first 378 amino acids containing the FERM domain are sufficient to activate KIF1C in vitro and in cells and does neither require the catalytic activity nor the phosphatase domain of PTPN21. Even though a scaffold activity is sufficient, it is still possible that PTPN21-mediated dephosphorylation of KIF1C24 can further modulate the activity of KIF1C, modify KIF1C cargo, cargo adapters or the activity of adjacent motors, a possibility that will be interesting to explore in a future study. PTPN21 has been shown to dynamically localise to focal adhesions35 or EGF receptor recycling sites36. It is possible that KIF1C-mediated transport facilitates the efficient turnover of PTPN21 at these sites. Thus the phosphatase could be both a cargo and a regulator of KIF1C. We have implicated KIF1C previously in the transport of integrins required for the maturation of trailing focal adhesions3. It is possible that additionally, KIF1C transport facilitates PTPN21-mediated regulation of Src tyrosine kinase and FAK signalling that promote cell adhesion and migration35.

PTPN21 FERM was able to compensate for depletion of KIF1C by activating KIF16B, a highly processive early endosome transporter that has been shown to also interact with PTPN21 FERM15,26,37. The expression of the FERM domain efficiently stimulated dense-core vesicle transport in primary neurons. PTPN21 has been associated with schizophrenia in a genome-wide association study38 and shown to promote neuronal survival and growth39,40. While the latter is thought to occur via NRG3 or ERK1/2 signalling, neuronal function and morphology is compromised when microtubule transport is perturbed and the function of PTPN21 as neuronal transport regulator might contribute to the complications in schizophrenia patients with PTPN21 mutations. Further, cAMP/PKA pathway stimulation of kinesin-1 has been shown to reverse aging defects in Drosophila neurons, and a similar age-driven decrease in KIF1C transport of dense-core vesicles and other organelles may have similar effects in neurodegenerative diseases41.

The findings that Hook3 can activate both dynemin/dynactin27,29,30 and KIF1C (this study), and that the binding sites for these opposite directionality motors are non-overlapping29,31, suggests that Hook3 could simultaneously bind to KIF1C and dynemin/dynactin and provide a scaffold for bidirectional cargo transport. Evidence for the existence of a complex of dynemin/dynactin, KIF1C and Hook3 has recently been provided in a preprinted manuscript42. We note that this study did not report an activation of KIF1C upon binding of Hook3; however, this is based solely on the analysis of speed and run lengths, while we find that activation primarily increases KIF1C landing rates. How the directional switching would be orchestrated in such a KIF1C-DDH complex is an exciting question for the future. It is important to note that Hook3 is not the only dynein cargo adapter which binds KIF1C. BICDR1 has been shown to bind to the proline-rich C-terminal region of KIF1C39, and BICD2...
appears to interact with KIF1C biochemically. Whether BICDR1 or BICD2 are able to activate the motor is unclear, but it is possible that different adapters not only mediate linkage to a different set of cargoes, but also recruit opposite polarity motors in different conformations and thus relative activity. For dynein/dynactin, such a difference is seen in BICD2 recruiting only one pair of dynein heavy chains while BICDR1 and Hook3 recruit two pairs and thus are able to exert higher forces.

BICDR1 also binds Rab6 and recruits both dynein/dynactin and KIF1C to participate in the transport of secretory vesicles. Rab6 in turn has been shown to bind and inhibit the KIF1C motor domain. This could provide a potential mechanism for a second layer of regulatory control of KIF1C activity to facilitate its minus end-directed transport with dynein-dynactin-Hook3.

Taken together, we provide mechanistic insight into the regulation of KIF1C, a fast long-distance neuronal transporter. We show that KIF1C is activated by a scaffold function of PTPN21 and the dynein cargo adapter Hook3, but the mechanism of autoinhibition release described here is likely to be universal and we expect cargoes and further scaffold proteins binding to the stalk region around the third coiled-coil in KIF1C to also activate the motor and initiate transport along microtubules. This opens

![Fig. 5 PTPN21 FERM domain activates KIF1C in vitro.](image-url)
up new research avenues into how KIF1C activity is controlled in space and time in cells.

**Methods**

**Plasmids and siRNAs.** The following plasmids used in this study were described previously: pKIF1C-mCherry, pKIF1C-2xFlag, pFlag8, pα5-integrin-GFP44, pNPY-RFP45, and pHA-PTPN21-WT and pHA-PTPN21-C1108S (ref. 25).

pFastBac-M13-6His-KIF1C-GFP was generated by digesting pKIF1C-mCherry with EcoRI–MfeI and replacing it in the backbone of pFastBac-M13 (Invitrogen). A resulting frameshift between the 6His tag and the N-terminus of KIF1C was corrected by cutting with EcoRI, mung bean nuclease treatment and religation of the vector.

KIF1CΔCC3 (i.e. KIF1CΔ623–679) and KIF1CΔS (i.e. KIF1CΔ623–825) deletions were generated by PCR, introducing SalI restriction sites after amino acid position D679 using oligo AS370 and after amino acid position D825 using AS371 with AS83 as the reverse primer (see Supplementary Table 1 for sequences of DNA oligonucleotides used in this study).

**Fig. 6** Deletion of KIF1C stalk results in a hyperactive motor. a Primary structure of hyperactive KIF1C deletion mutants used here. b Representative RPE1 cells co-transfected with full-length KIF1C-mCherry and either KIF1C-GFP, KIF1CΔCC3-GFP or KIF1CΔS-GFP and imaged 36 h post transfection. Scale bar is 20 µm. c Box plot shows ratio of KIF1C enrichment at the tail relative to cytoplasmic levels for GFP vs. mCherry channel. ***p < 0.0005 (Kolmogorov–Smirnov). n = 26, 32, 39 cells. Data are provided in Source Data file. d Coomassie-stained SDS-PAGE of purified KIF1C-GFP and deletion mutants. e–i Representative kymographs from single-molecule experiments. Grey lines indicate immobile motors, green lines running motors and orange dots landing events. j, k Frequency of KIF1C landing events and average velocity of running motors (>25 nm/s). n = 17, 82, 58, 22, 24 microtubules, respectively, pooled from three experiments. ***p < 0.0005 (t-test with Bonferroni correction). Data are provided in Source Data file.
pKIF1CΔ3-GFP was replaced with the truncated fragments to create plasmids pKIF1CΔ3-ACC3-GFP and pKIF1CΔ3-AS-GFP for mammalian expression. pFastBac-M13-6His-KIF1CΔ3-GFP and pFastBac-M13-6His-KIF1CΔ3-GFP were generated by digesting pKIF1CΔ3-ACC3-GFP and pKIF1CΔ3-AS-GFP with BsiWI-BamHI and replacing KIF1C in pFastBac-M13-6His-KIF1CΔ3-GFP. A human codon-optimised BioID2-HA construct was synthesised to include 5’ BamHI and 3’ NotI restriction sites allowing direct substitution of the C-terminal GFP of KIF1C with BioID2-HA tag to generate pKIF1CΔ3- BioID2-HA and pKIF1CΔ3- BioID2-HA.

The motor-GFP construct (pET22b-KIF1C (1-349)-NTTVSN-GFP-6His) was generated by amplifying the first 349 amino acids of KIF1C using primers UT157 and AS813, digesting with Ndel and NotI and replacing EB1 in pET22b-mEB1-GFP-6His47. The KIF1C stalk construct was cloned into a modified pGEX-6P2 vector (pGEX-6P2-LTLT) which contains an insertion of a tandem TEV cleavage site between the BamHI and EcoRI sites. Amino acids 612 to 922 from human KIF1C were amplified from pKIF1C-2xFlag using AS696 and AS778, digested with EcoRI and NotI, and ligated into pGEX-6P2-LTLT to create pGEX-6P2-LTLT-KIF1C612-922.

RNAi-protected PTP21 was made using pHAA-PTP21-WT as a template in a three-step mutagenesis PCR using AS937, AS380 and AS264. The fragment containing the mutation was replaced in pHAA-PTP21-WT using Ndel to generate pHAA-PTP21ΔRIP. RNAi-protected inactive PTP21, pHAA-PTP21ΔRIP-C1108S was generated by replacing the fragment containing mutation C1108S in pHAA-PTP21ΔRIP using Bgl.

pFERM expressing HA-tagged PTPN21ΔC77 was generated by digesting pHAA-PTP21ΔRIP and pEGFP-N1 (Clontech) with HindIII and inserting the fragment in pEGFP-N1. As the GFP is not in frame, the FERM domain is expressed from this plasmid with an N-terminal HA-tag only.

To express and purify FERM domains from E. coli, pET22b-HA-PTP21ΔC77-6His was generated by amplifying pHAA-PTP21ΔRIP by PCR with primers AS558 and AS559, digesting with Ndel and NotI and replacing EB1 in pET22b-mEB1-6His47. For labelling FERM domain, SNAPf-26 was codon optimised for E. coli, synthesised, and ligated into a PCR cloning vector generating pCAPS-SNAPf-26 with the addition of 5’ Ndel and KpnI sites. The HA tag in pET22b-HA-PTP21ΔC77-6His was exchanged for SNAPf-26 in a three-fragment ligation where the backbone was digested with PvuII and Ndel and PvuI and KpnI, and the SNAPf-26 insert was digested with Ndel and KpnI.

FERM domain from Ezrin, pET22b-HA-EzrinΔC77-6His was generated by PCR from random-prepared cDNA reverse transcribed from RPE1 RNA using primers AS556 and AS557, digesting with NotI and KpnI and replacing in pET22b-mEB1-6His.

Hook3 was cloned by PCR from random-prepared cDNA reverse transcribed from RPE1 RNA using primers AS690 and AS691, which was incorporated between the EcoRI and BamHI site of pKIF1CΔ3-GFP, replacing the KIF1C and generating pKIF1CΔ3-AS-GFP. A multi-tag version of pFastBacM13 (Invitrogen) was created by ligating an insect-cell codon optimised synthesised cDNA for 8His-2LTL-BICDR1-SNAPf-26 between the NotI and KpnI sites creating pFastBacM13-8His-2LTL-BICDR1-SNAPf. Hook3 was cloned into this by PCR from random-prepared cDNA reverse transcribed from RPE1 RNA using...
primers AS689 and AS691 which allowed direct ligation into the multi-tag pFastBacM13 vector creating pFastBacM13-8His-ZZ-LTLT-Hook3-SNAPf, which was subsequently used for production of a recombinant baculovirus.

Duplex siRNA oligos targeting KIF1C, KIF16B and PTPN21 were custom synthesised by Sigma and were: siControl 5′-GGACCUGGAGGUCUGCUGU-[dT]-[dT]-3′ (ref. 3), siKIF1C-2 (targeting both rat and human KIF1C) 5′-GUGACUGAUAUGGAGAUCU-[dA]-[dT]-3′ (refs. 3,5), siPTPN21 (targeting both rat and human PTPN21) 5′-UUCAGCCUCUGGUACUACA-[dT]-[dT]-3′, siKIF16B (targeting rat KIF16B) 5′-GAACUACAGCGACGUGGAG-[dT]-[dT]-3′.

Recombinant protein purification. For baculovirus expression, pFastBac-M13-6His-KIF1C-GFP, pFastBac-M13-6His-KIF1CΔS-GFP, pFastBac-M13-6His-KIF1CΔS-GFP and pFastBacM13-8His-ZZ-LTLT-Hook3-SNAPf plasmids were transformed into DH10BacYFP competent cells48 and plated on LB-Agar supplemented with 30 µg/ml kanamycin (#K4000, Sigma), 7 µg/ml gentamycin (#G1372, Sigma), 10 µg/ml tetracycline (#T3258, Sigma) and 100 µg/ml X-Gal (#MB1001, Melford). Positive transformants (white colonies) were screened by PCR using M13 forward and reverse primers for the integration into the viral genome. The bacmid DNA was isolated from the positive transformants by the alkaline lysis method and transfected into SF9 cells (Invitrogen) with Escort IV (#L-3287, Sigma) according to the manufacturer’s protocols. After 5–7 days, the virus (passage 1, P1) is harvested by centrifugation at 300 × g for 5 min in a swing out 5804 S-4-72 rotor (Eppendorf). Baculovirus infected insect cell (BIIC) stocks were made by infecting SF9 cells with P1 virus and freezing cells before lysis (typically around 36 h) in a 1°C cooling rate rack (#NU200 Nalgene) at −80°C. P1 virus was propagated to passage 2 (P2) by infecting 50 ml of SF9 (VWR, #EM71104-3) culture and harvesting after 5–7 days as described above49. For large-scale expression, 500 ml of SF9 cells at a density of 1–1.5 × 10^6 cells/ml were infected with one vial of BIIC or P2 virus. Cells

Fig. 8 Activation and cotransport with KIF1C. a–d Both PTPN21-FERM domain and Hook3 cargo adapter are co-transported with KIF1C if the stalk domain is present. e, f Model for KIF1C activation. KIF1C is an autoinhibited dimer in solution. Intramolecular interactions between stalk and motor domain prevent the inhibited motor from interacting with microtubules. Upon binding of PTPN21, the intramolecular interactions are released, KIF1C can engage with microtubules and will step processively towards the plus end. The cargo adapter Hook3 also binds to the stalk of KIF1C and activates it, thereby mediating cargo-activated transport along microtubules.
were harvested when 90% infection rate was achieved as observed by YFP fluorescence, typically between 48 and 72 h. Cells were pelleted at 252 × g in a S3-3000 rotor (for 20 min. For infection with 20 mM IPTG, cells were inoculated with 0.5% (v/v) TEV cleavage buffer and resuspended in 150 mM NaCl, 50 mM Imidazole and 10% glycerol. Purified proteins were flash frozen and stored in liquid nitrogen.

Hydrodynamic analysis. Size exclusion chromatography was carried out using a Superdex 200 column (GE Healthcare) at 15 °C with a flow rate of 0.5 ml/min. The elution volume (Ve) was determined from the following formula, Ve = [S + Mr(1 – ν)]/S, where S is the sedimentation coefficient, M is the molecular weight of the standard and ν is the frictional ratio or the Ion trap at 120 K. MS/MS analysis was performed using a Nano LC-20AC HPLC system (Shimadzu) coupled to a Q-Exactive high-resolution mass spectrometer (Thermo Scientific).

Crosslinking mass spectrometry. Two crosslinkers, BS3 (bis (sulpho-succinimidyl) suberate) (#21580, Thermo Scientific) and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (#22980, Thermo Scientific) were used to analyse protein-protein interactions using crosslinking mass spectrometry. Crosslinker was freshly prepared in MilliQ water and was mixed in the ratio of 1:2 with the protein(s) of interest by pipetting. KIFIC-GFP concentration was 1 mM when crosslinked alone. For crosslinking in the presence of PTPN21-FERM, both proteins were at 0.5 mM. Final concentration NaCl in the crosslinking reaction was 150 mM NaCl, diluted to 500 mM for high salt samples and reduced to 500 mM for high salt crosslinking, 3 mM N-hydroxy sulfosuccinimidyl (#24510, Thermo Scientific) was added to the reaction with EDC to improve efficiency of the crosslinking. The reaction was incubated shaking at 400 rpm for an hour at room temperature and then quenched with 50 mM Tris-HCl pH 7.5. Next, the protein was diluted in equal volume of 50 mM ammonium bicarbonate (#A9048, Sigma) and reduced to 500 mM for high salt crosslinking.

were induced with 0.5 mM or 1 mM IPTG and incubated at 16 °C or 18 °C overnight. For expression in SF9 cells, the pellet was resuspended in 4 ml of SF9 tail lysis buffer (50 mM sodium phosphate pH 7.5, 500 mM NaCl, 20 mM Imidazole, 150 mM glucose, 10% glycerol) supplemented with 20 mM reduced glutathione and 10% glycerol. Puriﬁed proteins were flash frozen and stored in liquid nitrogen.

were equilibrated with the lysis buffer and the cleared lysate obtained is mixed with the 1.5 ml Eppendorf tube and incubated with 3.5 µM SNAP-Surface Alexa Fluor 647 substrate (#S9136S, New England Biolabs) and sonicated at 50% amplitude for 30 s in a 10 s on off cycle repeated thrice. The peak fraction was run on a SDS-PAGE gel for visualisation and protein was aliquoted, flash frozen and stored in liquid nitrogen. Hooke3-647 was puriﬁed and labelled in a two-step process utilising both the His and ZZ afﬁnity tags at 4 °C. A pellet corresponding to 500 ml insect culture was resuspended in 40 ml Adaptor Lysis Buffer (50 mM HEPEs pH 7.2, 150 mM NaCl, 20 mM Imidazole, 10% glycerol) and stored in liquid nitrogen. For puriﬁcation, 20 strokes in a douncer. Lysates were cleared of insoluble material by centrifugation at 38,000 × g 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 2 ml Ni-NTA beads for 2 h, and subsequently washed with 200 CV of TEV cleavage buffer and resuspended in a 1.5 ml Eppendorf tube and incubated with 3.5 µM SNAP-Surface Alexa Fluor 647 substrate (#S9136S, New England Biolabs) and sonicated at 50% amplitude for 30 s in a 10 s on off cycle repeated thrice. The peak fraction was run on a SDS-PAGE gel for visualisation and protein was aliquoted, flash frozen and stored in liquid nitrogen.

Cell lysis was bound to 2 ml Ni-NTA beads for 2 h, and subsequently washed with 200 CV of lysis buffer followed by 200 CV lysis buffer supplemented with 60 mM imidazole. Protein was eluted in 5 CV of lysis buffer containing 350 mM Imidazole, and the eluate was batched and 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 0.1 mM ATP) and 10% glycerol. The peak fraction was run on a SDS-PAGE gel for visualisation and protein was aliquoted, flash frozen and stored in liquid nitrogen. The peak fraction was run on a SDS-PAGE gel for visualisation and protein was aliquoted, flash frozen and stored in liquid nitrogen.
selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 2 x 2 cycles.

Raw data files were converted to mgf format using the ProteoWizard mconvert toolkit. Sequences are visualized using Scaffold (Proteome software) for percentage coverage and purity followed by analysis using StavroX. Crosslinked peptides were identified using the StavroX software with appropriately defined parameters for the crosslinker used. A crosslinked peptide was considered as valid if it passed the 5% false discovery rate (FDR) cut-off along with stavroX score of at least 100, which is based on: (i) the presence of ion series fragmentation for both peptides, specifically those fragment ions that include the crosslinker and the attached second peptide, (ii) the proximity of observed fragmentation ion mass to expected fragment ion mass (within 10 ppm), (iii) number of crosslinked fragment ions for the a peptide, (iv) number of unidentified high intensity signals in the spectra. The MS/MS spectra were also manually inspected and only those crosslinks were accepted for which fragmentation ions were observed for both peptides and three or more fragments for b or y ions in the alpha peptide were required to match. Any crosslinks of continuous peptides, which could indicate intradimer interactions, were rejected as these could not be distinguished from partially cleaved peptides that have been modified by the crosslinker. Once a significant crosslink was identified in at least one sample, we used the information on retention time and mass of the precursor ion to verify the presence of the crosslinked peptide in other samples.

Label-free quantification of crosslinked precursor ions and unmodified peptides was done using MaxQuant (V1.5.5.1). The LFQ module calculates the integrated peak area of the precursor ion based on a retention time window of 4 min and ppm error window of 10 ppm to quantify the presence and absence of a precursor ion of interest in different mass spectrometry output files. LFQ intensity data for each sample were normalised to the sum of unmodified and modified peptides containing LKEGANINKS. RFS experiments were repeated 3–5 times and EDC experiments 2–3 times for each sample.

Microscale thermophoresis. GST-stalk was rebuffered into MST buffer (50 mM Tris pH 7.4, 150 mM NaCl 10 mM MgCl2, 0.05 % Tween- 20) using Zeba Spin Desalting Columns (Thermo Scientific). The resulting 37 µM stock solution was used to prepare a twofold dilution series. Motor-GFP was diluted in MST buffer to a final concentration of 250 nM, 2 µl of which was mixed with 8 µl of each GST-stalk diluted twofold series. Capillaries were filled with 10 µl protein mix and thermophoresis was performed in a Monolith NT.115 (NanoTemper) using 30% Nano Blue detection power at 25 °C. The experiment was repeated three times. Traces were analysed in 0.5 and 1.5 s heating window and the KD model was fitted using MO.Affinity Analysis software (NanoTemper).

BioID protein interaction analysis. For each BioID experiment, three 14.5 cm dishes of RPE1 cells were grown, and each was transfected at 90% confluence with 20 µg DNA diluted in 1 ml Optimem (#18958062, Fisher) with 60 µg poly-ethyleneimine (PEI, #408727, Sigma). After 24 h, media was replaced and supple- mented with 500 µg/ml Geneticin (#G8168 Sigma). After a further 12 h, cells were harvested by trypsinisation, washed with PBS and lysed in RIPA buffer (#98065, New England Biolabs) supplemented with 250 U of benzonase (#E1014, Sigma). After a further 12 h, cells were incubated for 2 min with 0.1% Triton X-100 (#T8787, Fisher Scientific) diluted in PBS. Coverslips were then washed with PBS and incubated for 30 min with 1:50 BSA, washed again and incubated for 30 min at room temperature or overnight at 4 °C with 1:100 anti-cortactin 4F11, (#05-180, Lot 2290226, Millipore) primary antibodies diluted in 0.5% BSA-PBST solution. Coverslips were washed with PBST and incubated for 45 min at room temperature with 1:300 anti-mouse IgG 647 conjugate (#A31571, Lot 47755A, Molecular Probes) secondary antibodies and 1:1000 Acti-stain 555 phalloidin (#PHDH1-A, CytoDiol) diluted in 0.5% BSA in PBST. Nuclei were stained with 5 µg/mL DAPI (#D9542, Sigma) for 1 min and coverslips washed with PBST prior to mounting on glass slides with Vectashield (#H1000, Vector Laboratories).

Cells were imaged using a DeltaVision Elite Wide-field microscope and Z-stacks of individual cells were acquired using a ×40 NA 1.49 objective and a Z-spacing of 0.2 µm.

To determine the number of podosomes formed in each cell, images of the cortactin channel were first transformed in a Z-projection. Z-projection images were then segmented using the ImagePro Analyzer 7 software by applying a threshold and a minimal size filter of 16 pixels (2.58 µm). Individual objects identified in the cortactin Z-projection were then visualized compared to the actin channel to confirm that they are podosomes, removed if the cortactin staining did not coincide with an actin spot and podosome clusters were split into individual podosomes. All experiments were repeated three times with 30 cells being analysed for each condition in each experiment.

Live cell imaging. Live cells were imaged using a ×60 oil NA 1.4 objective 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). The environment was maintained at 37 °C and 5% CO2 using a stage-top incubator (Tokai Hit) and a weather station (Precision control). RPE1 a5-integrin-GFP cells were seeded 24 h before imaging into quadrant glass-bottom dishes (#627975, Greiner) coated with 10 µg/ml Fibronectin. Cells with tails were selected and the mid region of the tail was bleached using the 488 nm laser. Vesicle movement was imaged for 200 time points at 0.7–1.0 s per frame. a5-integrin-GFP trafficking was analysed using ImageJ. Kymographs were generated by drawing lines from parallel 21 pixel wide lines passing through a location in the neurite in anterograde and retrograde direction. Vesicle movement was extracted manually from all kymographs using the selected line tool. Vesicles moving less than a total of 1.5 µm over the period of imaging were classified as stationary.

Primary mouse hippocampal neurons transfected with pNPy-RFP and either pERK control or pERK-RFP and imaged at DIV5 or DIV6. Images were acquired with 500 ms exposure every 1.5 s for 160 s. The frequency of NPY-positive vesicles passing through a location in the neurite in anterograde and retrograde direction was determined manually from kymographs.

RPE1 cells co-transfected with pKIF1C-mCherry and either pKIF1C-GFP, pKIF1C-GFP or pKIF1C-GFP were imaged 24 h post-transfection. Images were acquired with 500 ms exposure in the eGFP channel and 1 s exposure in the mCherry channel. To determine the ratio of enrichment at the tail, a region of interest was drawn manually surrounding the accumulation observed and the mean intensity was measured at the tail, in the cytoplasm near the nucleus and the background. The ratio of the mCherry to the background intensity at the tail was calculated as IntmCherry / Intbackground / Intcytoplasm / Intbackground for mCherry vs. mCherry channel.
**Co-immunoprecipitation.** HEK293 cells (Agilent, #240073) were seeded at a density of 1 x 10^4 cells onto one 10 cm dish 24 h before transfection with pKIFC-GFP, pGFP-Flag and either pHook3-GFP or pHAT-PTPN21GST. Two micrograms of DNA was mixed with 6 µl 1 mg/ml PEI (Sigma) in 200 µl PBS, incubated for 15 min and then added onto cells for transfection. Transiently transfected cells were collected 20–24 h after transfection by decanting the media and washing the cells off the dish with 5 ml ice cold 1× PBS. Cells were collected by centrifugation at 300 × g for 5 min and washed twice with 1× PBS. The cell pellets were lysed in 130 µl of dynecine lysis buffer (DLB: 30 mM HEPES, pH 7.4; 50 mM KOAc; 2 mM MgOAc; 1 mM EGTA, pH 7.5; 10% glycerol) supplemented with 1 mM DTT, 0.2% Triton X-100, 1× protease inhibitor cocktail (Expedion) by sonication in the bath of the Bioruptor (Diagenode) at medium setting for 5 min (30 s on, 30 s off cycle). Lysates were cleared at maximum speed in Eppendorf microcentrifuge (5417 R) at 4 °C for 15 min. For each reaction, 10 µl of Dynabeads Protein G (Invitrogen 100.03D) were incubated with 1 µg anti-Flag antibodies (#F13615, Lot 058K6113, Sigma, Cytoskeleton Inc.), 1:5000 anti-HA (C29F4) (#3724 S, Lot, 9 Cell Signalling Technology) and 1:3000 anti-Hook3 (#15457-1-AP, Proteintech) as primary antibodies and anti-rabbit-HRP (#40118, Lot 237671, Promega). Detection with SuperSignal West Pico Plus substrate (Pierce) was followed by imaging chemiluminescence in G-Box (Syngene) or using blue-sensitive film.

**Single-molecule motility and binding assays.** Microtubules were assembled from 8 µl of 3.4 mg/ml unlabelled pig tubulin, 0.2 µl of 1 mg/ml HiLyte Fluor 670 tubulin (#TL670M, Cytoskeleton) and 0.5 µl of 0.5 mg/ml biotin tubulin (#TT335P, Cytoskeleton) in MRB80 (200 mM PIPES pH 6.8, 4 mM MgCl2, 1 mM EGTA, 1 mM DTT). The mixture was incubated on ice for 5 min before adding 8.5 µl of polymerisation buffer (2× RR880 buffer plus 20% (v/v) DMSO and 2 mM MgGTP). Microtubules were polymerised at 37 °C for 30–60 min. The sample was diluted with 100 µl MT-buffer (MRB80 plus 30 µM paclitaxel). Unincorporated tubulin was removed by pelleting microtubules at 20,238 × g for 8.5 min at room temperature with the pelleted 100 µl MT-buffer and re-pelleting as before. The microtubule pellet was resuspended in 50 µl of MT-buffer and stored at RT covered from light for at least half a day (maximum 3 days) before use.

Coverslips (22 × 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 15 min in 0.1% detergent (TensoJet). The wash cycle was repeated five times. The coverslips were dried using a Spin Clean (Technical video) and plasma cleaned using Susos AG). Biotin-647-microtubules were attached to this surface with streptavidin (C8040). Microtubules were immobilised on the coverslip with (0.2 mg/ml) 25 mM KCl, 100 µM taxol, 0.2 mg/ml BSA, 7 U/ml creatine phosphokinase (#C3755 Sigma), 0.2 mg/ml κ-casein) and 100 µM GDP-taxol microtubules, and microtubule intensities in ten random fields-of-view were captured with an exposure time of 400 ms for 21.4% 488 nm laser power. Control and experimental chambers were prepared in parallel, and we alternated which chamber was imaged first to account for time effects. For analysis, microtubules were traced by hand with a line ROI in ImageJ, and the mean intensity was taken. A region close to the microtubule was selected as the local background, and subtracted from the mean to generate a local-background corrected intensity.

**Bleach step analysis.** A flow chamber was prepared as described above with biotin-647-microtubules immobilised on the coverslip. Six hundred picomolar KIFC1-GFP was flowed into a chamber with MRB80, 1 mM AMP-PNP, 100 µM taxol and 0.2 mg/ml κ-casein. Images were acquired every 500 ms for 600 s at an exposure of 848 nm laser line at 50% laser power. Images were analysed using the Plot Profile function in ImageJ for each individual spot and manually counting bleach steps. A mixed binomial distribution for dimers and tetramers of KIFC1 was fitted to the data using Eq. (1), where \( p(k) \) is the probability to find k bleach steps, \( p \) is the fraction of active GFP and \( x \) is the fraction of tetramers.

\[
P(k) = \frac{2^k}{k!} \left( \frac{x}{1-x} \right)^k \left( 1 - \frac{x}{1-x} \right)^{k-x} \cdot \left( \frac{1}{k!} \right) \cdot \left( \frac{x}{1-x} \right)^{k-x} \cdot \left( \frac{1}{k!} \right)
\]

**Statistical analysis and figure preparation.** Statistical data analyses and graphs were generated using Origin Pro 8.5 (OriginLab), python’s Matplotlib and Scipy packages or R. Box plots show quartiles with whiskers indicating 10% and 90% of data as well as the mean indicated with a circle. All statistical significance analyses were carried out using two-tailed two-sample t-tests assuming equal variance (related to a t-test is legends), Mann–Whitney U-test, ANOVA with post hoc Tukey, or Kolmogorov–Smirnov test. Where necessary, p-values were adjusted for multiple comparisons using Bonferroni or Holm–Sidak corrections. Figures were prepared by adjusting min/max and analysis and carrying and inverting look up tables using ImageJ and assembled using Adobe Illustrator.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request. A reporting summary for this Article is available as a Supplementary Information file. The source data underlying Figs. 1a, 1e, 3c, 3d, 3g, 3h, 4c, 4d, 4f, 5d, 5e, 6a, 6b and 7c and Supplementary Figs 2e and 6 are provided as a Source Data file. Our BioID mass spectrometry data are provided in Supplementary Data 1. The crosslinking mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE\(^\text{a}\) repository partner registry with the dataset identifier PXD013939.

**Code availability**

Custom ImageJ macros will be available at the CMCB Github (https://github.com/cmcb-warwick) and on our website (http://mechanomicschemistry.org/Schubert/tab so#) upon publication.

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Author contributions

A.S. and I.K. perceived the project, N.S. purified KIF1C, KIF1CΔCC3 and FERM domains, performed biochemical characterisation, single molecule experiments, crosslink mass spectrometry, KIF1C localisation in cells and data analysis. A.J.Z. performed BioID, purified Hook3, KIF1CΔS, KIF1C motor and stalk constructs, performed single-molecule experiments and data analysis, A.B. performed all cell biology experiments except the KIF1C/KIF16B double depletion performed by J.B., D.R. generated resources, performed coIPs and image analysis, H.H. analysed crosslink mass spectrometry data, A.S. managed the project, analysed data and wrote the manuscript with contributions from all authors.

Additional information

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