The ability of the kinesin-2 heterodimer KIF3AC to navigate microtubule networks is provided by the KIF3A motor domain

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Running title: *KIF3AC navigation of microtubule intersections*

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**ABSTRACT**

Heterodimeric kinesin family member KIF3AC is a mammalian kinesin-2 that is highly expressed in the central nervous system and has been implicated in intracellular transport. KIF3AC is unusual in that the motility characteristics of KIF3C when expressed as a homodimer are exceedingly slow, whereas homodimeric KIF3AA, as well as KIF3AC, have much faster ATPase kinetics and single molecule velocities. Heterodimeric KIF3AC and homodimeric KIF3AA and KIF3CC are processive, although the run length of KIF3AC exceeds that of KIF3AA and KIF3CC. KIF3C is of particular interest because it exhibits a signature 25-residue insert of glycine and serine residues in loop L11 of its motor domain, and this insert is not present in any other kinesin, suggesting that it confers specific properties to mammalian heterodimeric KIF3AC. To gain a better understanding of the mechanochemical potential of KIF3AC, we pursued a single molecule study to characterize the navigation ability of KIF3AC, KIF3AA, and KIF3CC when encountering microtubule intersections. The results show that all three motors exhibited a preference to remain on the same microtubule when approaching an intersection from the top microtubule, and the majority of track switches occurred from the bottom microtubule onto the top microtubule. Heterodimeric KIF3AC and homodimeric KIF3AA displayed a similar likelihood of switching tracks (36.1% and 32.3%, respectively). In contrast, KIF3CC detached at intersections (67.7%) rather than switch tracks. These results indicate that it is the properties of KIF3A that contribute largely to the ability of KIF3AC to switch microtubule tracks to navigate intersections.

Heterodimeric KIF3AC is a mammalian kinesin-2 that is highly expressed in brain and spinal cord, yet we know very little about the cargo that it transports, its adaptors for cargo linkage, or whether there is specificity of KIF3AC polarization either to axons or dendrites (reviewed in (1-7)). The mammalian kinesin-2 motors result from four genes, *kif3a*, *kif3b*, *kif3c*, and *kif17*, and form heterodimeric KIF3AB and KIF3AC and homodimeric KIF17 (8-14). Moreover, KIF3A and KIF3B do not form homodimers, and KIF3B does not heterodimerize with KIF3C suggesting distinct transport functions of KIF3AB, KIF3AC, and KIF17 (10,12,13,15).

KIF3AB and its orthologs across species form a heterotrimeric complex by association with KAP, a distinctive adaptor protein for cargo linkage because of its armadillo repeats (16,17). The essential role of KIF3AB-KAP for intraflagellar transport for ciliogenesis has been well established (18-23). In *Caenorhabditis elegans* the heterotrimeric kinesin-2 transports the intraflagellar transport particles into the cilium and toward the proximal region, but then there is a handoff to OSM-3, the KIF17 homolog, and OSM-3 transports these intraflagellar transport particles to the distal tip (24,25). These results suggest that
both kinesin-2 motors are required for cilia assembly and maintenance. However, Engelke et al. recently discovered that in mammalian primary cilia neither KIF3AC nor KIF17 can rescue intraflagellar transport or ciliogenesis in kif3a/kif3b double-knockout cells (26). This study concluded that KIF3AB-KAP is the only kinesin-2 required for ciliogenesis in mammals, thereby revealing that the utilization of kinesin-2 motors for intraflagellar transport and ciliogenesis may differ across species. Moreover, the inability of KIF3AC to drive intraflagellar transport and ciliogenesis suggests that there are other role(s) for KIF3AC in mammals rather than being redundant for KIF3AB-KAP promoted intraflagellar transport and ciliogenesis.

Early studies indicated that KIF3C expression was enriched in the central nervous system and also suggested that not all KIF3C was associated with KIF3A (11-13,27-29). Moreover, there is evidence for an injury-specific homodimer of KIF3CC in neurons (30), and KIF3CC is a potent catastrophe factor in vitro (31). Both studies suggest a role of KIF3CC in promoting microtubule dynamics. KIF3C has also been of particular interest because it exhibits a signature 25-residue insert of glycines and serines in Loop L11 of the motor domain (3,11,32,33), and the presence of this insert has been shown to decrease KIF3AC processivity (33) and necessary for KIF3CC to target microtubule plus ends and promote microtubule catastrophe in vitro (31). Because this 25-residue insert is not present in any other kinesin, we hypothesized that it confers specific properties to mammalian heterodimeric KIF3AC.

Our single molecule quantum dot (Qdot) motility assays revealed that in the absence of load, KIF3AC is highly processive (run length = 1.23 ± 0.09 µm) with its velocity at 169 ± 5.6 nm/s (33). In contrast, the engineered homodimers were quite different from each other. While the velocity of KIF3AA was quite fast at 239 ± 4.2 nm/s and run length at 0.98 ± 0.09 µm, the velocity of KIF3CC was exceedingly slow at 8.2 ± 0.3 nm/s and the run length decreased to 0.57 ± 0.03 µm. Similarly, the ATPase kinetics of KIF3AC and KIF3AA were significantly faster than the ATPase kinetics of KIF3CC (33-35).

We pursued single molecule experiments to quantify the behavior of KIF3AC at microtubule intersections in comparison to the performance of homodimeric KIF3AA and KIF3CC. Although this microtubule intersection geometry is quite simple, it asks whether the behavior of KIF3AC is driven by KIF3A, KIF3C, or is it an emergent mechanochemical property of KIF3AC not predicted by the motile properties of either KIF3A or KIF3C. Moreover, the ability to navigate microtubule intersections and roadblocks on microtubules is a critical requirement for intracellular transport and has been tested for other processive kinesins (36-52).

The results presented reveal that KIF3AC and KIF3AA were both capable of switching from one microtubule to another at a microtubule intersection. In contrast, KIF3CC detached at microtubule intersections rather than switch microtubules. The results support the interpretation that it is the mechanochemical properties of KIF3A that contribute largely to the ability of KIF3AC to switch microtubule tracks to navigate intersections and that the behavior of KIF3CC at microtubule intersections is more consistent with a role in microtubule plus end targeting and microtubule dynamics than a role as a cargo transporter.

**Results**

**Kinesin motors exhibit three possible behaviors when encountering microtubule intersections**

We pursued single-molecule quantum dot (Qdot) experiments using Total Internal Reflection Fluorescence (TIRF) microscopy to quantify the behavior of KIF3AC, KIF3AA, and KIF3CC at microtubule intersections composed of two microtubules (Fig. 1A, B). The design of the truncated kinesins is shown in Supporting Fig. S1, and these motor constructs have been characterized previously (31,33-35,53,54). Fig. 1B illustrates the three potential fates of the motor upon encountering a microtubule intersection. A motor can continue on the same microtubule track through the intersection (Fig. 1B, 1), detach from the microtubule at the intersection (Fig. 1B, 2), or turn onto the second intersecting microtubule (Fig. 1B, 3). Pausing at the intersection point was also observed and scored. As shown in Fig. 1C, dual color composite kymographs provided a visual representation of motor runs on each microtubule as separate colors. Individual kymographs for each constituent microtubule were constructed: for one microtubule, motor runs were represented in red on the kymograph, and for the second microtubule of the
intersection motor runs were represented in green. The red and green kymographs for the constituent microtubules were overlaid at the point along their x-axes corresponding to the point at which the two microtubules intersected. This process generated a composite kymograph in which a red run color represents Qdot motion along one of the microtubules, while a green run color represents Qdot motion on the second microtubule. These composite kymographs were constructed for each intersection, as described in Experimental Procedures, to confirm Qdot behaviors observed in the experimental videos (Supporting Information, Movies S1-S5). Qdot behavior at a given intersection was observed in the video and compared to the corresponding composite kymograph to confirm which of the three possible outcomes occurred in the motor’s run.

Fig. 1C provides examples of the three potential fates of a motor at the intersection. Qdots that continued on the same microtubule through the intersection showed the same color run on the kymograph before and after the intersection (Fig. 1C, panel 1). Qdots which detached at the intersection ended their runs at the intersection point on the kymograph x-axis (Fig. 1C, panel 2). Qdots which switched microtubule tracks exhibited a change in run color after encountering the intersection (Fig. 1C, panel 3). The change in run color from red before the intersection point to green thereafter represents a track switch or a turn onto the intersecting microtubule. For all three outcomes, a motor that pauses at the microtubule intersection exhibits a yellow vertical line at the point of intersection along the x-axis of the composite kymograph (Fig. 1C, panel 1). Because the Qdot is paused at the point of microtubule overlap, its run appears in both the red and green kymographs. Therefore, the overlaid, dual color kymograph shows the yellow pause. Qdots were also observed to be paused on microtubules but not at the intersection. These appear as either a red or green vertical line on the kymographs.

**Differential fluorescence labeling allows a distinction between two intersecting microtubules based on their relative position**

Two populations of fluorescent X-rhodamine labeled microtubules were incorporated into the perfusion chamber for imaging. One microtubule population was diluted with unlabeled tubulin, as described in Experimental Procedures, yielding a microtubule population with a relatively low fluorescence intensity (“dim” microtubules). Upon TIRF imaging, these microtubules were distinct from the second population of microtubules, which was polymerized from undiluted X-rhodamine tubulin and appeared brighter upon imaging (“bright” microtubules). In these experiments, the dim microtubules were always introduced into the perfusion chamber first, followed by the addition of the bright microtubule population. This procedure yielded microtubule intersections with a dim microtubule on the bottom and a bright microtubule on top. The TIRF images shown in Fig. 2-4 and corresponding videos (Movies S1-S4) demonstrate the contrasting fluorescence intensities of the bottom microtubule versus the top microtubule that form an intersection. This distinction allowed an analysis of whether the relative position of the microtubule from which a motor approaches an intersection has any impact on the outcome executed by the motor at the intersection.

For the kinesin-2 experiments, red was assigned to the kymograph representing the dim (bottom) microtubule, while green was assigned to the kymograph representing the bright (top) microtubule. The composite kymograph for these intersections therefore represented Qdot runs along the bottom microtubule in red, and Qdot runs along the top microtubule in green.

**KIF3AC behavior at an intersection is dependent on the microtubule from which the run was initiated**

Fig. 2 presents the single molecule Qdot results for KIF3AC at microtubule intersections. Fig. 2A shows the motion of a Qdot starting from the top microtubule and continuing straight through the intersection, whereas Fig. 2B is an example in which KIF3AC began on the bottom microtubule and at the intersection switched to the top microtubule. These KIF3AC examples are also shown in Supporting Information, Movie S1. The results for the KIF3AC population are shown as pie charts in Fig. 2C and a table in Fig. 2D. The overall KIF3AC population (N = 310) exhibited a 36.1% frequency of switching microtubule tracks at an intersection. This proportion was statistically different (p < 0.001) from the 52.6% of the population which continued straight at the
intersection. As shown in pie chart 1 of Fig. 2C and Fig. 2D, the 11.3% probability of detaching at the intersection was significantly different from the population which switched tracks as well as the population which continued straight (p << 0.001).

However, when the results were subdivided into KIF3AC motors that began on the top microtubule versus the bottom microtubule, distinct behavioral differences were observed. KIF3AC motors which started on the top microtubule continued straight through the intersection in 71.8% of observations, compared to 34.8% of motors which continued straight when starting on the bottom microtubule (p << 0.001, Fig. 2C, 2-3; Fig. 2D). Motors which started on the bottom microtubule demonstrated a 57.1% tendency to switch tracks, as opposed to 13.4% of motors which started on the top microtubule (p << 0.001, Fig. 2C, 2-3; Fig. 2D).

KIF3AC exhibits a statistically significant likelihood to continue straight through the intersection when approaching an intersection from the top microtubule (Fig. 2A), as opposed to “stepping down” from the top microtubule to switch onto the bottom microtubule track. KIF3AC also exhibits a significant tendency to turn from the bottom microtubule onto the top microtubule (Fig. 2B), as opposed to negotiating such an intersection by maneuvering around the top microtubule to remain on its incident bottom microtubule. KIF3AC behavior at intersections is therefore influenced by the position of the microtubule upon which the motor initiates its run to approach the intersection. However, the likelihood of detaching at the intersection point appeared to be independent of the microtubule position, remaining relatively consistent whether the Qdot run began on the top (14.8%) or bottom (8.1%) microtubule (p = 0.0929, Fig. 2C, 2-3; Fig. 2D).

Furthermore, KIF3AC exhibited an overall average pause duration of 7.8 ± 0.7 s at microtubule intersections (N = 100, Fig. 2D). A significant difference was observed (p = 0.00436) between the mean pause durations for runs beginning on the top (9.6 ± 1.3 s) versus those initiated on the bottom microtubule (5.7 ± 0.5 s, Fig. 2D). Mean pause durations prior to detaching at the intersection were longer than the overall average, and this trend was observed regardless of whether the run initiated on the top or bottom microtubule (Fig. 2D). However, the longer mean pause durations and standard errors reported may be due to the extremely low number of observations (N = 12) for pause durations prior to detaching.

KIF3AA exhibits a similar behavioral pattern as KIF3AC at microtubule intersections

Fig. 3 presents the single molecule Qdot results for KIF3AA at microtubule intersections. Note that there was no statistically significant difference between the overall tendencies of the KIF3AA population (N = 310, Fig. 3D) and the KIF3AC population (N = 310, Fig. 2D): KIF3AA exhibited a 32.3% probability of track switching (p = 0.352), a 59.0% probability of continuing straight (p = 0.124), and an 8.7% probability of detaching at a microtubule intersection (p = 0.349). These KIF3AA behaviors are shown in Fig. 3C (pie chart 1) and Fig. 3D.

Furthermore, KIF3AA demonstrated no statistically significant difference in behavior from KIF3AC when divided into motor populations which initiated runs on the top microtubule versus those which began runs on the bottom microtubule. Similar to KIF3AC, KIF3AA exhibited a 76.7% probability of continuing straight through the intersection when starting on the top microtubule (p = 0.408), and a 42.5% probability of continuing straight when starting on the bottom microtubule (p = 0.192, Fig. 3C, pie charts 2-3). Additionally, like KIF3AC, KIF3AA demonstrated a 49.4% tendency to switch tracks when starting on the bottom microtubule (p = 0.199), and a 14.0% likelihood of turning when starting on the top microtubule (p = 1.0, Fig. 3C, pie charts 2-3; Fig. 3D). Furthermore, KIF3AA demonstrated no statistically significant difference (p = 0.861) in the probability of detaching at the intersection, regardless of whether the run began on the top or bottom microtubule, and the motor detached with a likelihood of 9.3% and 8.1%, respectively (Fig. 3C, pie charts 2-3; Fig. 3D). This consistent frequency of detachment despite the incident microtubule position is an additional common feature observed in both KIF3AC and KIF3AA. (See summary Fig. 6 also.)

Fig. 3A-B show dual color kymographs and corresponding TIRF images which illustrate KIF3AA behavior. See also Movie S2. KIF3AA exhibits a preference for continuing straight when approaching an intersection from the top microtubule (Fig. 3A), as well as a tendency to turn from the bottom microtubule onto the top
microtubule (Fig. 3B). The behavior of KIF3AA at intersections is consistent with the observed behavior of KIF3AC, with both motors impacted by the orientation position of the microtubule from which the motor approaches the intersection.

Pause durations for KIF3AA, with an overall average of 2.5 ± 0.1 s (N = 54), were significantly shorter than those exhibited by KIF3AC at 7.8 ± 0.1 s (p << 0.001, Fig. 3D). There was no significant difference between the overall mean pause duration and the mean pause duration prior to detaching, nor was there a significant difference between mean pause durations at intersections when starting on the top versus the bottom microtubule (Fig. 3D). However, the extremely low numbers of observed pauses prior to detaching (N = 3), and when starting on the top microtubule (N = 14) render statistical tests between these subpopulations unreliable.

**KIF3CC exhibits unique tendency to detach at microtubule intersections**

Fig. 4 presents the single molecule Qdot results for KIF3CC at microtubule intersections with representative examples of a KIF3CC start from the top microtubule in Fig. 4A and Movie S3, and from the bottom microtubule in Fig. 4B and Movie S4. As shown in pie chart 1 of Fig. 4C and Fig. 4D, the KIF3CC population (N = 310) exhibited a track switch frequency of 7.1%, which was significantly lower than the proportions of track switching for both KIF3AC and KIF3AA (p << 0.001, for both comparisons). Rather, the KIF3CC population exhibited a 67.7% likelihood of detaching from its associated microtubule at the intersection point, which was significantly greater than the likelihood of KIF3AC and KIF3AA detaching at intersections (p << 0.001, for both comparisons).

KIF3CC exhibited a high probability of detachments at the intersection point, and this was observed regardless of whether the run was initiated on the top or on the bottom microtubule. Fig. 4C pie charts 2 and 3 and Fig. 4D show this trend, with KIF3CC exhibiting a 62.5% probability of detaching when starting on the top microtubule and a similar 73.3% probability of detaching when starting on the bottom microtubule (p = 0.0552). Furthermore, the likelihood of KIF3CC to detach is relatively consistent regardless of whether the run was initiated on the top or bottom microtubule.

Additionally, there was a statistically significant difference between the proportion of the KIF3CC population (33.8%) which continued straight when starting on the top microtubule, and the proportion (16.0%) which continued straight when starting on the bottom microtubule (p < 0.001, Fig. 4C, pie charts 2-3; Fig. 4D). KIF3CC, therefore, displayed the same tendency exhibited by KIF3AC and KIF3AA to continue straight when approaching an intersection on the top microtubule.

KIF3CC also exhibited the longest pause durations of all three kinesin-2 motors tested, with an overall average pause duration of 126.4 ± 11.3 s (N = 67, Fig. 4D). Unlike the outcomes observed in KIF3AC, statistical tests for KIF3CC showed no significant difference between the mean pause duration for runs initiated on the top microtubule and the mean pause duration for KIF3CC runs initiated on the bottom microtubule (p = 0.264, Fig. 4D). For KIF3CC, the likelihood of pausing appeared to be highest prior to detaching at the intersection (Fig. 4D). However, the small number of observed pauses before switching tracks or continuing straight through an intersection on the same microtubule renders statistical comparisons between pausing subpopulations unreliable.

**Motor behaviors are independent of the angle of intersection**

Intersections comprised of microtubules at an array of acute and obtuse angles were selected for analysis. The angle formed by the intersecting microtubules was tabulated in order to examine the impact of the angle of incidence upon motor behavior at the intersection. “Acute” intersections were defined as those in which the intersecting microtubules formed an angle less than 90 degrees. “Obtuse” intersections were defined as those in which the intersecting microtubules formed an angle greater than 90 degrees. For these experiments, we included a similarly designed kinesin-1, KIF5B K439 as a control (54).

For each of the four motors, the subpopulations which executed a microtubule track switch were further divided based on the angle created by the intersecting microtubules (Fig. 5A-D). For each motor population, the number of total events at acute and obtuse intersections were tabulated, as well as the number of switching events at each intersection type. These data were used to calculate the percentage of track switching events.
at acute intersections versus at obtuse intersections, which enabled direct comparison of KIF3AC, KIF3AA, and KIF3CC with kinesin-1 KIF5B (Fig. 5E). See also Fig. S2 and Movie S5.

Each of the four motor populations failed to exhibit a statistically significant difference in the probability of switching tracks, whether the intersecting microtubules lay at acute or obtuse angles. KIF3AC demonstrated a 38.9% likelihood of switching at acute intersections and a 34.2% likelihood of switching at obtuse intersections \((p = 0.473, \text{Fig. 5B})\). KIF3AA demonstrated a 29.8% likelihood of switching at acute intersections and a 35.2% likelihood of switching at obtuse intersections \((p = 0.368, \text{Fig. 5C})\). KIF3CC demonstrated a 7.0% likelihood of switching at acute intersections and a 7.2% likelihood of switching at obtuse intersections \((p = 1.0, \text{Fig. 5D})\).

Similar to the kinesin-2 motors, kinesin-1 KIF5B failed to demonstrate a statistically significant difference in the probability of track switching, with a 14.5% likelihood of switching at acute intersections and a 22.5% likelihood of switching at obtuse intersections \((p = 0.141, \text{Fig. 5A})\).

This consistency across all four motor types indicates that the probability of track switching at intersections is independent of the angle formed by the two intersecting microtubules with a nearly equal likelihood of turning at acute and obtuse intersections. Therefore, angle of incidence appears to be a characteristic of the intersection yet not a significant influence on the motor’s behavior at the intersection. Note though that these results are in contrast to those reported in other studies in which multiple motors or different motors were associated with the cargo or the motors were performing under load (42,45,48).

**Discussion**

**Navigation abilities appear to be influenced by the specific kinesin motor domain**

The results presented show that although KIF3AC and KIF5B are both processive kinesins, they differ in their ability to navigate microtubule intersections even though both transport cargoes in neurons. KIF5B tends to do so by continuing on the same microtubule track (Fig. S2), whereas KIF3AC has a greater capacity to switch tracks in order to navigate obstacles posed by intersecting microtubules (Fig. 2). These distinct behaviors represent two mechanisms to successfully transport cargo to their cellular destination.

Among the kinesin-2 motors examined, KIF3AC and KIF3AA exhibited the greatest tendency to execute a track switch at a microtubule intersection, and the lowest likelihood of detaching at the intersection point. The strong similarities between the single molecule results for KIF3AC and KIF3AA and not KIF3CC suggests that the track-switching capability is encoded by the motor domain and that it is the KIF3A motor domain that enables KIF3AC to switch microtubules at intersections (Fig. 2-4, 6). The results for KIF5B also reinforce the hypothesis that it is the specific motor domain that encodes the track switching behavior with KIF5B exhibiting a very low percentage of track switch events at intersections (Fig. 5 and Fig. S2).

In contrast, dimerization of the KIF3C polypeptide results in a homodimer that lacks the same ability as KIF3AC and KIF3AA to switch microtubule tracks at intersections. KIF3CC predominantly detached at microtubule intersections and exhibited substantially longer mean pause durations at the intersection point than either KIF3AA or KIF3AC. These single-molecule results indicate a tendency of KIF3CC to stall its motion and end its run at the more restrictive geometry of a microtubule intersection. These motility properties would render KIF3CC an inefficient motor for long-range cargo transport in a cellular environment, yet these properties are consistent with a role in promoting microtubule dynamics at the microtubule plus end as indicated by previous studies (13,30,31). Guzik-Lendrum et al. reported that KIF3CC, but not KIF3AC, was targeted to the microtubule plus ends, acted as a potent catastrophe factor, and modulated the tapered structure typically seen in growing dynamic microtubules (31). Moreover, this activity required ATP turnover, and KIF3CC tended to pause at the microtubule tip prior to catastrophe. In these microtubule dynamics assays, KIF3CC maintained the ability to collide with the microtubule and step processively, but the velocity was extremely slow and the microtubule plus end events resulted from KIF3CC association at the microtubule plus end rather than KIF3CC stepping to the microtubule plus end. These combined results support the interpretation that the mechanochemical capability of KIF3C is very different from that of KIF3A.
Navigation abilities, across all motors, appear to be similarly influenced by the structure of a microtubule intersection

The single molecule results also indicate that kinesin-2 KIF3AC, KIFAA, and KIFCC share common behaviors at intersections (Fig. 6), suggesting that the geometry or structure of the intersection plays a role in the navigation ability of the motor. In each case, if the approach to the microtubule intersection is from the top microtubule, all three kinesins tend to continue through the intersection on the top microtubule. However, if the motor is approaching the intersection from the bottom microtubule, it is less likely to continue on the bottom microtubule through the intersection. Rather, each motor that approached the microtubule intersection from the bottom microtubule exhibited a significantly higher probability to switch or turn onto the upper microtubule. These behaviors were also consistent for KIF3CC although it predominantly detached at microtubule intersections.

The influence of the microtubule intersection on a motor’s behavior can be explained at the level of microtubule protofilaments. The majority of protofilaments of the top microtubule do not contact the bottom microtubule. Therefore, it is much more likely that a motor will continue stepping on the top microtubule because of the low probability of contacting the bottom microtubule for a track change at the microtubule intersection. Thus, a motor’s approach to the intersection has a large influence on how the motor will navigate the intersection.

This study has also revealed a profound difference in the behavior of KIF3AC and KIF3AA in comparison to homodimeric KIF3CC at microtubule intersections. A recent report by Bensel et al. provides insight (55). The authors showed that in the absence of load, KIF3CC homodimers moved much slower than KIF3AA and KIF3AC, and both KIF3CC and KIF3AC backstep as single back steps more frequently than observed for KIF3AA. Moreover, both KIF3A and an assisting load accelerate the stepping kinetics of KIF3C in a strain-dependent manner. The results from Bensel et al. suggest that it is the decreased interhead tension within KIF3CC that decreases its run length and its ability to switch tracks at microtubule intersections. Rather, KIF3CC detaches at microtubule intersections, which is consistent with its role as a catastrophe factor that is targeted to the microtubule plus end to promote microtubule dynamics after injury. Moreover, the results of Bensel et al. support the argument presented here that the ability to navigate microtubule intersections is encoded in the catalytic motor domain of KIF3A rather than KIF3C. In conclusion, the results presented emphasize unique adaptations that have occurred to optimize kinesin motors for specific cellular functions.

**Experimental procedures**

**Kinesin-1 K439 plasmid design and expression**

The expression construct for human kinesin-1 K439 was a custom gene synthesis from GenScript and inserted into the pET24d expression vector between NcoI and BamHI restriction sites as reported previously (54). This construct encodes the first 439 residues of *Homo sapiens* KIF5B, followed by the C-terminally fused end binding protein 1 (EB1) dimerization motif (bold) whose sequence matched the coiled-coil registry of the helix to generate stable dimers. A TEV protease site (italiced) followed with linker residues (plain font) and His8 tag (underlined).

\[ Hs \text{ KIF5B K439 (Met}^1\text{-Ile}^{439}\text{-DFYFGKLRNIELICQENEGENDPVLQRIVDILYATDETTSENLYFQGASHHHHHHHHH} \]

(predicted MW = 56,538).

*Escherichia coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene) were transformed with the *K439* plasmid followed by selection on lysogeny broth (LB) plates containing 50 µg/mL kanamycin (selection for *K439* plasmid) plus 10 µg/mL chloramphenicol. Colonies were selected and grown in LB liquid culture containing the same selective antibiotics at 37 °C until they reached an A600 of 0.4-0.5. The cultures were chilled in an ice bath to lower the temperature to 16 °C (~30 min). Subsequently, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added dropwise to induce protein expression with continued shaking at 16 °C at 185 rpm for ~15 h. Cells were collected by centrifugation and resuspended in lysis buffer at 10 mL/g of cells. Lysis Buffer: 10 mM...
sodium phosphate buffer, pH 7.2, 300 mM NaCl, 2 mM MgCl₂, 0.1 mM EGTA, 0.02 mM ATP, 1 mM DTT, 10 mM phenylmethylsulfonyl fluoride (PMSF), and 30 mM imidazole.

**Kinesin-2 KIF3 plasmid design and expression**

The *Mus musculus* KIF3A and KIF3C plasmids for KIF3AC, KIF3AA, and KIF3CC protein expression were described in detail previously (33). Briefly, each expression plasmid encoded the native N-terminal motor domain, neck linker, helix α7 to initiate coiled-coil formation, followed by the EB1 dimerization motif (bold) that matched the coiled-coil registry of the native α7 helix, followed by the TEV protease site (italicized) with linker residues (plain font) and affinity purification tags (underlined). For KIF3AC, the use of the StrepII tag on KIF3A combined with a His₈ tag on KIF3C allowed for sequential purification steps to generate pure, stable heterodimeric KIF3AC.

KIF3A (Met¹-Leu³⁷⁴)-

DFYFGKLRNIELCQENEGENPDVQLRIVD
ILYATDETTSENLYFQGASNWSPQPFEK
(predicted MW = 48,559).

KIF3C when expressed for heterodimeric KIF3AC or homodimeric KIF3CC includes the native N-terminal motor domain, neck linker, and helix α7, followed by the EB1 dimerization motif, and the C-terminal TEV protease cleavable His₈ tag.

KIF3C (Met¹-Leu⁰⁹⁶)-

DFYFGKLRNIELCQENEGENPDVQLRIVD
ILYATDETTSENLYFQGASHHHHHHHHH
(predicted MW = 49,759).

Expression of homodimeric KIF3AA is similarly designed with the KIF3A motor domain (Met¹-Leu³⁷⁴)-

DFYFGKLRNIELCQENEGENPDVQLRIVD
ILYATDE TTSENLYFQGASHHHHHHHHH
(predicted MW = 48,502).

KIF3AC, KIF3AA, and KIF3CC were expressed in *E. coli* BL21-CodonPlus (DE3)-RIL cells (33-35,53). KIF3AC heterodimers resulted from co-transformation of the KIF3A and KIF3C plasmids each with different antibiotic resistance (*KIF3A*: ampicillin and *KIF3C*, kanamycin). Transformed cells were selected on lysogeny broth (LB) plates containing 100 µg/mL ampicillin, 50 µg/mL kanamycin plus 10 µg/mL chloramphenicol. For homodimeric KIF3AA and KIF3CC, each plasmid was selected by 50 µg/mL kanamycin plus 10 µg/mL chloramphenicol. Colonies were selected and grown in LB liquid culture in the presence of the antibiotics at 37 °C until they reached A₆₀₀ of 0.4-0.5. Cultures were chilled in an ice bath to 16 °C, and then expression was induced by dropwise addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside. Incubation continued at 16 °C with shaking at 185 rpm for ~15 h. Cells were harvested by centrifugation and resuspended in lysis buffer at 10 mL/g cells. The homodimers of KIF3AA and KIF3CC were expressed following the same protocol with the appropriate antibiotic selection. The construct design for each of the expressed motor proteins is illustrated in Fig. S1.

**Purification of kinesin dimers**

The purification strategy for these motors has been described previously (33,34,53,54) with modifications included here. An aliquot of the cell suspension (~10 g/100 mL lysis buffer plus 0.1 mg/mL lysozyme) was incubated with gentle stirring in an ice bath for 45 min. Cell lysis was achieved by three replicates of freezing in liquid N₂ and thawing in a 37 °C water bath. Subsequently, the lysate was clarified through ultracentrifugation and applied to a HisTrap™ FF Ni²⁺-NTA column (GE Healthcare) that was pre-equilibrated with Ni²⁺-NTA binding buffer (20 mM sodium phosphate buffer, pH 7.2, 300 mM NaCl, 2 mM MgCl₂, 0.1 mM EGTA, 1 mM DTT, 0.02 mM ATP, and 30 mM imidazole). After loading, the column was washed with Ni²⁺-NTA binding buffer until the absorbance returned to baseline. The his8-tagged kinesins were eluted with a linear gradient (30 mM imidazole to 300 mM imidazole in Ni²⁺-NTA binding buffer, pH 7.2). The fractions enriched in the specific his8-tagged kinesin were identified through SDS-PAGE. For KIF5B, KIF3AA, and KIF3CC, these fractions were pooled, concentrated, and dialyzed in 20 mM HEPES, pH 7.2 with KOH, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT plus NaCl in sequential buffers containing 300 mM, 200 mM, and 100 mM NaCl.
The final dialysis buffer included 100 mM NaCl plus 5% sucrose (w/v).

For KIF3AC, the fractions from the HisTrap™ FF Ni²⁺-NTA column that were enriched in both KIF3A with the StrepII-tag and KIF3C with the his8- tag, were identified through SDS-PAGE, pooled, and transferred directly to the StrepTrap™ HP column (GE Healthcare) which was pre-equilibrated with StrepII column buffer: 20 mM sodium phosphate buffer, pH 7.2, 300 mM NaCl, 2 mM MgCl₂, 0.1 mM EGTA, 1 mM DTT, and 0.02 mM ATP. The loaded StrepTrap™ HP column was washed with excess buffer to return to baseline absorbance, followed by elution with StrepII column buffer plus 2.5 mM desthiobiotin. Fractions were analyzed by SDS-PAGE to identify fractions that contained a 1:1 ratio of KIF3A and KIF3C polypeptides. These fractions were then pooled, concentrated, and dialyzed at 4 °C in 20 mM sodium phosphate buffer pH 7.2, 2 mM MgCl₂, 0.1 mM EGTA, and 1 mM DTT plus NaCl in sequential dialysis steps: 200 mM NaCl, 100 mM NaCl, and 100 mM NaCl plus 5% sucrose (w/v).

The proteins were concentrated, clarified by ultracentrifugation, and aliquoted prior to freezing in liquid N₂, and storage at -80 °C. The purity of all preparations was confirmed by both analytical gel filtration on an HPLC gel filtration column (Superose™ 10/300, GE Healthcare Life Sciences) and SDS-PAGE. Note that the purification tags were not cleaved for the experiments reported herein. Typical yields for these purified motors were 1.7-3 mg/g of E. coli. Predicted molecular weights of dimeric motors based on amino acid residue sequence are as follows: KIF5B at 113,076; KIF3AC at 98,318; KIF3AA at 97,004; and KIF3CC at 99,518.

Before each experiment, the purified motors were clarified through high-speed centrifugation for 5 min at 4 °C (Beckman Coulter Optima TLX Ultracentrifuge, TLA-100 rotor, 313,000 x g). Motor concentration was determined using the Bio-Rad Protein Assay with IgG as the protein standard (Bio-Rad Laboratories, Inc.).

**Quantum-dot motor attachment**

Streptavidin-coated quantum dots (Qdot 525-Streptavidin conjugate, Life Technologies) at 200 nM were incubated with 200 nM biotinylated-Penta-His antibody (Qiagen, Valencia, CA) for 60 min in PME80 buffer (80 mM PIPES, pH 6.9 with KOH, 5 mM MgCl₂, and 1 mM EGTA) at room temperature (33,35). Subsequently, His₈-tagged kinesin motors were added to the Qdot-antibody complex to a final concentration of 20 nM kinesin dimer. This mixture results in a 1:10 ratio of kinesin dimer to Qdot complex (20 nM kinesin dimer, 200 nM Qdot-antibody complex). Based on a Poisson distribution, this procedure yields a working stock in which 9% of the Qdots were estimated to have one motor bound and 0.5% of the Qdots with ≥ 2 motors bound. Additional single molecule experiments at 1:20 ratio of kinesin dimer to Qdot complex showed that there was no difference in the run length or velocity at 1:10 or at a 1:20 ratio of kinesin dimer to Qdots, thereby providing additional evidence that the experiments at a 1:10 ratio of kinesin dimer to Qdots were at single molecule conditions (33).

**Microtubule preparation**

Microtubules of different fluorescence intensities were prepared using X-rhodamine tubulin (Cytoskeleton, Inc.). The microtubules of higher fluorescence intensity were prepared with X-rhodamine tubulin and polymerized to yield microtubule tracks of approximately 13-30 µm for the experiments. The X-rhodamine tubulin was resuspended in PME80 buffer containing 10% glycerol, incubated on ice with 1 mM MgGTP for 5 min, and then centrifuged at 16,000 x g for 10 min at 4 °C (Galaxy 16D Micro 1816 centrifuge, VWR). The supernatant containing the soluble tubulin was transferred to a new tube, and microtubule polymerization was initiated at 37 °C for 25 min. The microtubules were stabilized with paclitaxel (33 µM final concentration) and were incubated for an additional 10 min at 37 °C, yielding a final stock of highly fluorescent microtubules at 30 µM tubulin polymer.

To generate microtubules of lower fluorescence intensity, both X-rhodamine tubulin and bovine brain tubulin were combined to yield a 1:10 ratio of X-rhodamine tubulin to unlabeled tubulin. The bovine brain tubulin was mixed with PME80 buffer containing 10% glycerol and incubated on ice with 1 mM MgGTP for 5 min, followed by centrifugation at 16,000 x g for 10 min.
at 4 °C. The supernatant, containing soluble unlabeled tubulin was transferred to a second tube. In a separate tube, 9 µL unlabeled bovine brain tubulin was mixed with 1 µL X-rhodamine tubulin, and polymerization was initiated at 37 °C for 25 min. These microtubules were stabilized with paclitaxel (33 µM final concentration) and were incubated for an additional 10 min, resulting in a final microtubule stock of low-fluorescence intensity microtubules at 30 µM tubulin polymer. Note that control experiments showed that the relative velocity and run length using either population of fluorescent microtubules were not statistically different.

**Microtubule intersection assay**

Perfusion chambers were formed by mounting a silanized coverslip on a glass slide separated by strips of double-sided tape to generate 10-µL flow cells (33,53). The chamber was incubated with 0.4% rat anti-α-tubulin antibody (ABD Serotec) for 5 min, followed by a 5-min incubation with 5% Pluronic F-127 (Sigma-Aldrich) to block the surface. A dilution of the lower-fluorescence intensity microtubule stock (1:200 in PME80 supplemented with paclitaxel to 22 µM) was introduced into the chamber and incubated for 6 min. PME80 with 10 mM DTT and 20 µM paclitaxel was then flowed into the chamber to remove unbound microtubules. A dilution of the brighter-fluorescence intensity microtubule stock (1:800 in PME80 supplemented with paclitaxel to 22 µM) was introduced into the chamber and incubated for 6 min, followed by perfusion of PME80 with 10 mM DTT and 20 µM paclitaxel into the chamber to remove unbound microtubules. The working stock of Qdot-motor complexes (20 nM kinesin dimer-200 nM Qdot-antibody complex) was diluted in Activity Buffer (PME80, 0.5% Pluronic F-127, 30 µM paclitaxel, 125 µg/mL bovine serum albumin, 50 µM DTT, 25 mM glucose, 0.2 mg/mL glucose oxidase, 175 µg/mL catalase, 0.3 mg/mL creatine phosphokinase, 2 mM phosphocreatine, 1 mM ATP) to achieve the final concentration in the perfusion chamber of 2 nM kinesin-Qdot complex. Note that only the high fluorescence intensity microtubules were used for the kinesin-1 KIF5B control experiments (Fig. S2).

**Total internal reflection fluorescence microscopy and image acquisition**

Immediately upon addition of the kinesin-Qdot complexes, the chambers were imaged by total internal reflection fluorescence (TIRF) microscopy using a Zeiss Inverted Axio Observer Z1 MOT fluorescence microscope with the 100X oil 1.46 N.A. Plan-Apochromat objective (Carl Zeiss Microscopy) at 25 °C. A Hamamatsu electron multiplier EM-CCD digital camera was used to collect digital images using the ZEN 2.3 software package. This setup yielded 512 x 512-pixel images with 0.16 µm per pixel in both x and y planes. The kinesin-Qdot complexes were tracked by imaging at 488 nm (5% laser power) every ~0.6 s for 5 mins using 150 ms exposure. Video frames were collected for kinesin-2 KIF3AC and KIF3AA as well as kinesin-1 KIF5B at an acquisition rate of 1.7 Hz. Because of the extremely slow velocity of KIF3CC (33), videos were acquired over 30 min with 488-nm exposure (150 ms) at 5 s intervals, at an acquisition rate of 0.2 Hz.

Reference images of the X-rhodamine microtubule tracks were acquired at 561 nm (2% laser power) with a 180 ms-exposure. These microtubule images were overlaid with the corresponding kinesin-Qdot videos using NIH ImageJ software.

**Data analysis**

Analysis of the kinesin-Qdot motility was conducted using the MultipleKymograph plugin for ImageJ (J. Rietdorf and A Seitz, European Molecular Biology Laboratory, Heidelberg, Germany). For a given intersection of two microtubules, a dual color composite kymograph was generated, enabling the visualization and tabulation of all kinesin-Qdots which encountered the intersection of interest.

For each of the two distinct microtubules that formed an intersection, a unique kymograph was generated. To create these individual kymographs, microtubule polarity was determined through observation of the kinesin-Qdot motility in the microscopy videos. Using the reference images of the microtubule tracks, the full length from the minus end to the plus end of a single microtubule was measured. The length from the minus end to the intersection of interest was also measured. This provided a measurement of the distance along the kymograph x-axis at which the microtubule...
interacted with the second microtubule. This process generated a kymograph depicting all kinesin-Qdot motion along a single microtubule, with the point of microtubule intersection marked on the x-axis. This process was repeated for each of the two intersecting microtubules, resulting in two distinct kymographs.

The two single kymographs were differentiated by assigning the color red to one kymograph, and the color green to the other. In these experiments, the kymograph corresponding to the dim microtubule was always assigned the color red while the kymograph corresponding to the bright microtubule was always assigned the color green. The distances along the x-axis from the microtubule minus end to the point of intersection were compared among the two kymographs. The kymograph x-axis lengths were then adjusted by adding the appropriate number of pixels to the ends of the shorter kymograph. This rendered the two kymographs equal in overall length, and allowed them to be overlaid by aligning the kymographs at the x-axis coordinates corresponding to the point of intersection. This process of merging the red and green kymographs generated a single dual color composite kymograph.

This composite kymograph provided a visual representation of all kinesin-Qdot runs which occurred at a particular microtubule intersection. The point of intersection between two microtubules could be clearly represented as a single coordinate along the x-axis of the composite kymograph. Furthermore, on such a composite kymograph, kinesin-Qdot runs on the dim microtubule were represented in red and were distinguishable from runs on the bright microtubule, represented in green. This allowed a distinction between kinesin-Qdot runs initiated on the dim microtubule (the bottom microtubule in the intersection) versus runs initiated on the bright microtubule (the microtubule lying on top).

For the kinesin-1 KIF5B experiments, the same process was used to generate a dual color composite kymograph to represent a microtubule intersection. However, a single population of microtubules with the same fluorescence intensity was used, rendering the orientation of the intersecting microtubules unknown. Therefore, the single kymographs were assigned the colors red and green arbitrarily without regard to microtubule orientation.

For each intersection examined, a dual color kymograph was constructed and was used to score the behavior of all kinesin-Qdots as they encountered the intersection. The N value for each motor includes only kinesin-Qdot runs that were visible within the time of imaging and reached the point of intersection. Intersections of two distinct microtubules oriented at a variety of angles were examined. However, intersections with microtubule bundles were excluded as well as intersections with both microtubules of either high or low fluorescence intensity for the kinesin-2 experiments. Moreover, the minimum pause duration scored for KIF3AC, KIF3AA, and KIF5B was defined as a vertical on the kymograph for at least 2 frames or ~1.1 s. An event at the intersection was scored as a detachment provided the frame was not significantly different from the background for at least one frame or ~0.6 s. Because of the very slow velocity of KIF3CC, the shortest pause scored was 3 frames or 15 sec and a detachment was defined as not being significantly different from background for one frame or greater, which corresponds to ≥ 5 s.

For each of the three kinesin-2 motors, the overall N value of 310 was composed of 150-160 kinesin-Qdots which approached the intersection from the dim (bottom) microtubule, and 150-160 Qdots which approached the intersection from the bright (top) microtubule. For KIF5B, the overall N value of 251 was not further subdivided based on the orientation of the incident microtubule. These data resulted from 4-6 days of independent experiments for KIF3AC, KIF3AA, and KIF5B and 9 days of independent experiments for KIF3CC.

**Statistical analysis**

The R language and environment for statistical computing was used (R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria URL https://www.R-project.org/) to test for the statistical significance of differences in the behaviors of the KIF3AC, KIF3AA, and KIF3CC populations. The R software was used to compare the kinesin-2 populations by conducting a two-sample test for equality of proportions with a continuity correction. This test was also applied for each motor population to compare the proportions of track switching at acute versus at obtuse angles of intersecting
microtubules. The IBM SPSS Statistics software (Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) was used to remove outliers from the sets of pause duration data for each motor population, compute mean pause durations and standard errors, and conduct independent samples t-tests to compare and determine statistically significant differences in mean pause durations. For each comparison, a two-tailed $p$ value was computed and evaluated at a significance level of $\alpha = 0.01$

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FOOTNOTES
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Figure 1. Kinesin behavior at microtubule intersections as observed in single-molecule experiments. (A) Illustration of the experimental design with a kinesin-2 motor poised to step towards the microtubule plus end. Rhodamine-labeled microtubules were anchored to the coverslip via anti-tubulin antibodies. Motors were visualized by a streptavidin-coated Qdot attached to the motor C-terminal His-tag through biotinylated-Penta-His antibodies. (B) The possible fates of a Qdot at a microtubule intersection are shown: (1) a motor can continue through the intersection on the same microtubule track, (2) a motor can detach at the intersection, or (3) a motor can switch onto a new microtubule track. For each of these behaviors, the possibility exists for pausing at the intersection point. (C) Examples of kinesin behaviors at microtubule intersections. The left panels show dual color composite kymographs where for one microtubule the motor runs are presented in red on the kymograph and for the second microtubule of the intersection motor runs are represented in green. The yellow, vertical dotted line represents the point of intersection of the two microtubules. The right panel for each example includes sequential frames captured through TIRF microscopy that show examples of the motor behaviors illustrated in Fig. 1B. Illustrations of these frames, shown underneath, depict the motor position at a given time. The three examples correspond to each of potential outcomes in panel B. (1) A motor that continues on the same microtubule has the same color run before and after the intersection. (2) A motor that detaches will end its run at the intersection. (3) A motor that switches microtubule tracks will exhibit a change in run color after encountering the intersection. A motor that pauses at the intersection under any of these circumstances exhibits a yellow vertical line at the intersection as illustrated in example 1 of panel C.
Figure 2. KIF3AC navigation of microtubule intersections. To determine the behavior of KIF3AC at intersections, microtubules of lower fluorescence were added first to the coverslip followed by perfusion of microtubules of higher fluorescence. Therefore, the behavior of KIF3AC at intersections could be assessed based on whether the run started on the top microtubule or the bottom microtubule track. For the dual color composite kymographs (left panels), the top microtubule was assigned the color green, the bottom microtubule red, with the yellow, vertical dotted line the point of intersection of the two microtubules. (A) A KIF3AC run initiated from the top microtubule. The left panel shows an example in which KIF3AC began its run on the top microtubule and at the intersection, and it remained on the top microtubule. The right panel shows sequential frames captured through TIRF microscopy with the illustrations below each of the frames. “T” corresponds to the top microtubule and “B” corresponds to the bottom microtubule. The dual color kymograph (left) shows a lack of color change before and after the intersection, indicating that the motor remained on the same microtubule track. (B) A KIF3AC run initiated from the bottom microtubule. The left panel shows an example in which KIF3AC began its run on the bottom microtubule and at the intersection, and it remained on the bottom microtubule. The right panel shows sequential frames captured through TIRF microscopy with the illustrations below each of the frames. “T” corresponds to the top microtubule and “B” corresponds to the bottom microtubule. The dual color kymograph (left) shows a lack of color change before and after the intersection, indicating that the motor remained on the same microtubule track.

### Table: KIF3AC Navigation of Microtubule Intersections

|                  | Overall | Top Start | Bottom Start |
|------------------|---------|-----------|--------------|
| N                | 310     | 149       | 161          |
| Straight (%)     | 52.6    | 71.8      | 34.8         |
| Straight without pause (%) | 38.7    | 52.3      | 26.1         |
| Straight with pause (%) | 13.9    | 19.5      | 8.7          |
| Switch (%)       | 36.1    | 13.4      | 57.1         |
| Switch without pause (%) | 19.3    | 6.7       | 31.0         |
| Switch with pause (%) | 16.8    | 6.7       | 26.1         |
| Detach (%)       | 11.3    | 14.8      | 8.1          |
| Detach without pause (%) | 7.1     | 9.4       | 5.0          |
| Detach with pause (%) | 4.2     | 5.4       | 3.1          |
| Pause duration (s) | 14.7 ± 4.5 | 16.3 ± 6.6 | 11.7 ± 3.8 |
| Overall average pause duration (s) | 7.8 ± 0.7 | 9.6 ± 1.3 | 5.7 ± 0.5 |
panel shows the dual color kymograph which indicates that this KIF3AC run began on the bottom microtubule (red) and at the intersection switched to the top microtubule (green). The right panel presents the TIRF microscopy frames with the corresponding illustrations below. These motor runs can be viewed in the corresponding video (Movie S1).

(C, D) Individual KIF3AC motors were scored based on the three behaviors at microtubule intersections, and the results shown as either a pie chart or table. Pause durations at intersections are reported as mean ± SEM.
Figure 3. KIF3AA navigation of microtubule intersections. Microtubule intersections were generated in which the microtubules of higher fluorescence formed the top microtubule of the intersection and assigned the kymograph color green and the bottom microtubule of lower fluorescence was assigned the kymograph color red. Examples are shown of a KIF3AA run beginning from the top microtubule (green) in panel A and from the bottom microtubule (red) in panel B. (A) The right panel shows sequential frames captured through TIRF microscopy with a motor initiating a run (arrowhead) on the top (bright) microtubule with illustrations shown beneath. The corresponding dual color kymograph (left) shows a lack of color change before and after the intersection indicating that the motor did not switch to the intersecting microtubule. A second motor on the top microtubule, indicated by an asterisk, leaves the field of view of the TIRF frames and therefore was not scored. This motor corresponds to the green run on the kymograph marked with an asterisk. A third motor, marked with an asterisk and not scored, runs along the bottom microtubule, and the corresponding red run in the kymograph is marked with an asterisk. These motor runs can be viewed in the corresponding video (Movie S2). (B) The dual color kymograph (left) indicates that this motor began on the bottom
microtubule (red) and switched to the top microtubule at the intersection (green). Right panel: TIRF microscopy frames and representative illustrations show a motor initiating a run on the bottom microtubule (right). See Movie S2 also. (C, D) The behavior of the KIF3AA motors at microtubule intersections were scored and presented as pie charts or the table. Pause durations at microtubule intersections are reported as mean ± SEM.
Figure 4. KIF3CC navigation of intersections composed of microtubules with different fluorescence intensities. (A) On the right panel, sequential frames captured through TIRF microscopy show a KIF3CC motor (arrowhead) initiating a run on the top (bright) microtubule with illustrations shown beneath. On the left, the corresponding dual color kymograph shows that the run (green) ended at the intersection point. The short red run on the kymograph, marked with an asterisk, indicates a second motor moving along the bottom microtubule. This run is not seen in the TIRF frames on the right, but can be observed in the corresponding video (Movie S3). (B) On the right panel, TIRF microscopy frames and representative illustrations show a KIF3CC motor (arrowhead) initiating a run on the bottom microtubule. On the left, the corresponding dual color kymograph shows the run (red) ends at the intersection point. A second motor on the top microtubule is indicated with an asterisk, and the corresponding green run on the kymograph is marked with an asterisk. The red run in the kymograph marked with an asterisk indicates an additional motor running along the bottom microtubule. This run is not seen in the TIRF frames on the right but can
be observed in the corresponding video (Movie S4). (C, D) The KIF3CC behaviors at microtubule intersections were scored and shown in pie charts and the table. Pause durations at intersections are reported as mean ± SEM.
Figure 5. Microtubule track switching behavior based on the angle generated by the intersecting microtubules. 

(A-D) Pie charts are shown for each kinesin motor and the processive run behavior at microtubule intersections. The pink fractions, corresponding to the subset of motors which switched tracks, are subdivided based on the angle of incidence. The subset of motors which navigated a turn at an acute angle (< 90°) is shown as the striped pink fraction. The subset of motors which navigated a turn at an obtuse angle (> 90°) is shown as the solid pink fraction. (E) The table reports the track switching events based on the angle upon entry into the intersection.

|                | KIF5B | KIF3AC | KIF3AA | KIF3CC |
|----------------|-------|--------|--------|--------|
| **N**          | 251   | 310    | 310    | 310    |
| **Acute intersections** |       |        |        |        |
| Number of events         | 131   | 126    | 168    | 157    |
| Number of switch events  | 19    | 49     | 50     | 11     |
| % switching              | 14.5  | 38.9   | 29.8   | 7.0    |
| **Obtuse intersections** |       |        |        |        |
| Number of events         | 120   | 184    | 142    | 153    |
| Number of switch events  | 27    | 63     | 50     | 11     |
| % switching              | 22.5  | 34.2   | 35.2   | 7.2    |

*Switching populations are calculated based on the number of events observed at acute versus obtuse intersections.
Figure 6. Summary of KIF3 motor behavior at microtubule intersections. Side-by-side comparisons are shown of the three behaviors (straight, switch, or detach) observed for KIF3AC, KIF3AA, and KIF3CC as each approached the microtubule intersection from the top (A) or bottom (B) microtubule. See Fig. 2-4 for complete data sets of each KIF3 motor.
The ability of the kinesin-2 heterodimer KIF3AC to navigate microtubule networks is provided by the KIF3A motor domain

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