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Kinesin-2 and Apc Function at Dendrite Branch Points to Resolve Microtubule Collisions

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In Drosophila neurons, kinesin-2, EB1 and Apc are required to maintain minus-end-out dendrite microtubule polarity, and we previously proposed they steer microtubules at branch points. Motor-mediated steering of microtubule plus ends could be accomplished in two ways: 1) by linking a growing microtubule tip to the side of an adjacent microtubule as it navigates the branch point (bundling), or 2) by directing a growing microtubule after a collision with a stable microtubule (collision resolution). Using live imaging to distinguish between these two mechanisms, we found that reduction of kinesin-2 did not alter the number of microtubules that grew along the edge of the branch points where stable microtubules are found. However, reduction of kinesin-2 or Apc did affect the number of microtubules that slowed down or depolymerized as they encountered the side of the branch opposite to the entry point. These results are consistent with kinesin-2 functioning with Apc to resolve collisions. However, they do not pinpoint stable microtubules as the collision partner as stable microtubules are typically very close to the membrane. To determine whether growing microtubules were steered along stable ones after a collision, we analyzed the behavior of growing microtubules at dendrite crossroads where stable microtubules run through the middle of the branch point. In control neurons, microtubules turned in the middle of the crossroads. However, when kinesin-2 was reduced some microtubules grew straight through the branch point and failed to turn. We propose that kinesin-2 functions to steer growing microtubules along stable ones following collisions. © 2016 Wiley Periodicals, Inc.

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Introduction

All neuronal mRNAs and most proteins are made in the cell body. These new materials must supply parts of the cell that may be hundreds of microns to more than a meter away. Neuronal microtubules serve as both the tracks and signposts for this long-distance transport. Cargo that is hitched to a motor protein in the cell body can be destined for either axons or dendrites. Motor proteins walk along microtubules towards either the plus or minus end; most kinesins walk towards the plus, or growing end, and dynein walks towards the minus end. Thus microtubule polarity can be read like a signpost by motor proteins.

Axonal microtubules in animals including C. elegans, Drosophila, and mice have plus ends directed away from the cell body [Baas and Lin, 2011; Rolls and Jegla, 2015]. This means that kinesins carry cargo from the site of synthesis in the cell body into the axon, and dynein is the major retrograde motor [Hirokawa et al., 2010]. Dendrites are a little more complicated. In mammalian neurons, dendritic microtubules have mixed polarity with about half minus-end-out and half plus-end-out orientation [Baas et al., 1988; Stepanova et al., 2003]. This means that either kinesins [Hirokawa et al., 2010; Jenkins et al., 2012] or dynein [Kapitein et al., 2010] can act as anterograde motors to take cargo into dendrites. In Drosophila, neurons start off with mixed microtubules in dendrites [Hill et al., 2012], but mature neurons have 90% or more minus-end-out microtubules [Stone et al., 2008]. *C. elegans* neurons have similar internal organization with largely minus-end-out dendritic microtubules [Goodwin et al., 2012; Yan et al., 2013]. This more extreme difference in axonal and dendritic polarity in invertebrate neurons emphasizes the

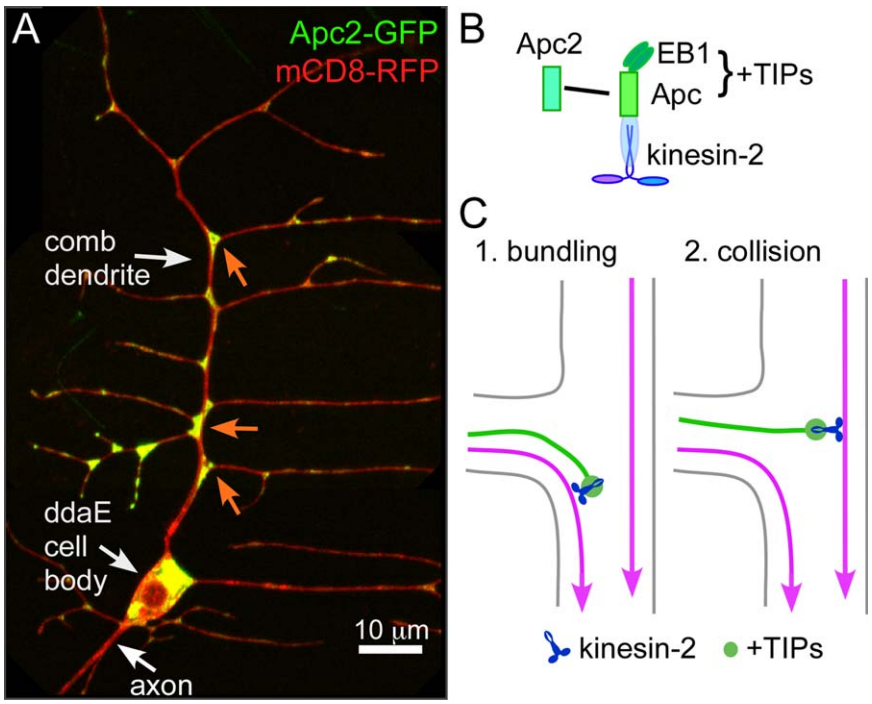

Fig. 1. Dendrite branch points and microtubule steering. The shape of the ddaE dendrite arbor and localization of Apc2-GFP is shown in **A**. The dorsal comb-shaped dendrite was used for imaging experiments. Apc2-GFP is highly concentrated at dendrite branch points (orange arrows). **B**: A summary of interactions between +TIPs and kinesin-2 is shown. C. Kinesin-2 and +TIPs could direct microtubule growth in two different ways: the complex could link the growing microtubule end to the sides of neighboring microtubules (bundling) or it could function when the growing ends of free microtubules collide with the side of a microtubule (collision). In either scenario the outcome would be that stable microtubules guide growing ones towards the cell body at dendrite branch points.

importance of dynein as a dendritic motor; an interpretation that is supported by severe dendrite growth and trafficking defects in dynein mutants [Liu et al., 2000; Satoh et al., 2008; Zheng et al., 2008]. It also led us to think about the relationship between dendrite branching and microtubule organization.

Dendrites, including Drosophila dendrites with minus-end-out microtubules, are often highly branched. We realized that for uniform microtubule polarity to be maintained in branched dendrites, the direction of microtubule growth must be controlled at branch points; if microtubules were allowed to grow in any direction when they encountered a branch junction, some would grow away from the cell body and result in a plus-end-out microtubule. When we examined growing microtubules navigating branch points, we observed that almost all turned towards the cell body [Mattie et al., 2010]. Based on phenotypic analysis, protein-protein interactions and protein localization we proposed a model for directed growth of microtubules through branch points. Reduction of any of the three subunits of kinesin-2, Klp64D, Klp68D or Kap3, resulted in mixed polarity in dendrites. Based on an interaction between Kap3 and Apc in mammalian cells [Jimbo et al., 2002], we tested whether the Drosophila homologs interact, and whether Apc was required for microtubule polarity. A summary of protein-protein interactions identified in our study is shown in Fig. 1B. All of these proteins, Apc, Apc2 and EB1 are required to maintain polarity like kinesin-2 [Mattie et al., 2010]. In addition Apc2-GFP localizes strongly to dendrite branch points (Fig. 1A) and can recruit Apc there [Mattie et al., 2010]. We therefore hypothesized that the microtubule plus end-binding protein (+TIP), EB1, links the end of growing microtubules to kinesin-2 through Apc. This would allow growing microtubules to be directed along stable ones, and since kinesins walk towards microtubule plus ends, the growing microtubules would be forced into the same orientation as existing microtubules.

Although this model is satisfying and fits with the phenotypic analysis, we were not able at that time to directly demonstrate that this complex functioned at branch points, and several important questions remained. First, the association of +TIPs with microtubule plus ends is very transient [Chen et al., 2014; Dixit et al., 2009; Maurer et al., 2014], and so it was not clear that sufficient force could be transferred through this temporary cloud of +TIPs to steer one microtubule along another. In vitro reconstitution of microtubule steering using kinesins linked to +TIPs demonstrated that the proposed mechanism is sufficient to steer growing microtubules along stable ones [Chen et al., 2014; Doodhi et al., 2014]. Second, the initial in vivo study left open two alternate mechanisms by which kinesin-2 might steer microtubules through branch points. The motor could

pull the growing microtubule along the side of a stable one, bundling the two together in parallel orientation (Fig. 1C). Alternately, the motor might only engage with a stable microtubule to determine the direction of growth after a collision (Fig. 1C). In this study our goal was to determine whether we could pinpoint the activity of the kinesin-2-+TIP complex to branch points, and to determine whether this complex acts to bundle microtubules, to resolve collisions, or both.

Results

Microtubule Turning and Apc-RFP Comets Are Consistent with kinesin-2 and +TIP Function at Branch Points

Our first goal was to determine whether we could find direct evidence for kinesin-2 functioning with +TIPs at dendrite branch points. We previously showed that in the comb dendrite of ddaE neurons (Fig. 1A), RNA hairpins targeting any of the three kinesin-2 subunits resulted in mixed polarity, and that mutant and RNAi phenotypes are similar [Mattie et al., 2010]. These neurons are sensory neurons in the body wall of Drosophila that have a fairly simple and stereotyped branching pattern [Grueber et al., 2002]. In addition, the close to right angle branches mean that they are more reliant on kinesin-2 to maintain polarity than dendrites with more acute branch angles [Mattie et al., 2010]. To determine whether mixed polarity in these dendrites is associated with misdirection of growing microtubules as they traverse branch points, we monitored the pattern of microtubule growth at branch points in control and Klp64D RNAi ddaE neurons. Growing microtubules were labeled with low levels of EB1-GFP; high levels of this transgene can have dominant-negative effects [Mattie et al., 2010]. Timelapse movies of EB1-GFP comets moving through the comb dendrite were analyzed and comets that passed through a branch point were scored as either turning towards the cell body or away from the cell body. In control neurons, almost all microtubules grew towards the cell body at the branch point, while in Klp64D RNAi neurons more than half turned away from the cell body (Figs. 2A and 2B). This result is consistent with kinesin-2 functioning at dendrite branch points.

Drosophila Apc can bind to both kinesin-2 and EB1 [Mattie et al., 2010], potentially positioning it as the linker between the growing plus end and the motor. We therefore imaged Apc-RFP to see if it might track microtubule plus ends. Without extra expression of Apc2, very little Apc-RFP is present in dendrites [Mattie et al., 2010]. We therefore expressed Apc-RFP with Apc2-GFP. In some cases we could see spots of Apc-RFP moving through branch points at the speed expected for growing microtubules (Fig. 2C). Although consistent with our model, these events were rare,

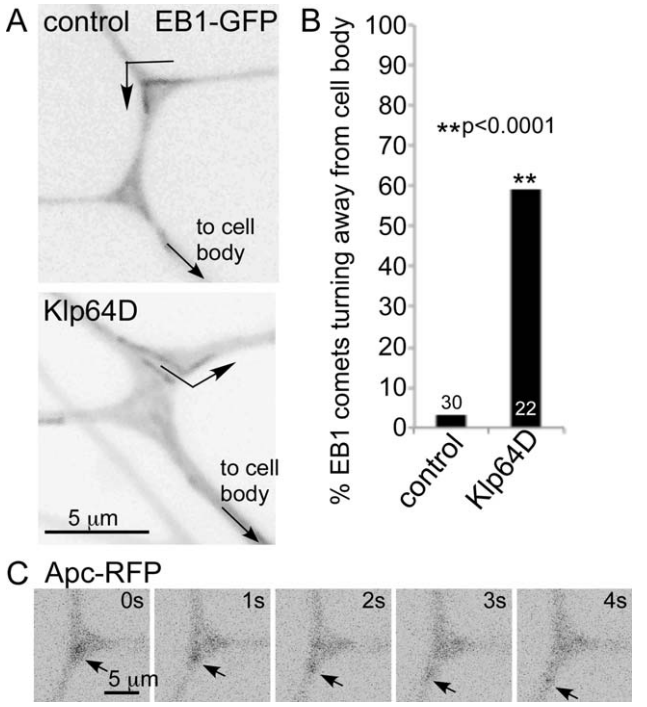

Fig. 2. Behavior of tagged +TIPs at branch points. AL EB1-GFP was used to track growing microtubule in the comb dendrite of ddaE in control (Rtnl2) RNAi and Klp64D RNAi neurons. Maximum projections of timeseries movies are shown and the tracks of individual microtubules through the branch points are marked with arrows. In all images the cell body is at the bottom. **B**: Comets were scored based on whether they turned towards the cell body or away from it at branch points. The numbers of the bars in the graph are the number of comets scored. A Fisher's exact test was performed to determine statistical significance. **C**: Apc-RFP was expressed with Apc2-GFP and live imaging of the RFP channel was performed. An example of an Apc-RFP comet moving through a branch point is shown.

so we wished to use other approaches to determine whether and how this complex functions at branch points.

Kinesin-2 Does Not Influence the Number of Growing Microtubules That Cross Branch Points in Smooth Arcs

One way that kinesin-2 and +TIPs could guide growing microtubules towards the cell body is by bundling the growing tip along existing microtubules (Fig. 1C). To test this idea we first needed to map the pattern of stable regions of microtubules in branch points. We expressed Jupiter-RFP [Cabernard and Doe 2009], a microtubule-associated protein (MAP) and imaged its distribution in living ddaE neurons. Jupiter localizes similarly to other MAPs, including tau, in Drosophila cells, and like these MAPs that bind to stable regions of microtubules is highly expressed in neurons [Karpova et al., 2006]. Almost all branch points had microtubules running in smooth arcs along the outside edges

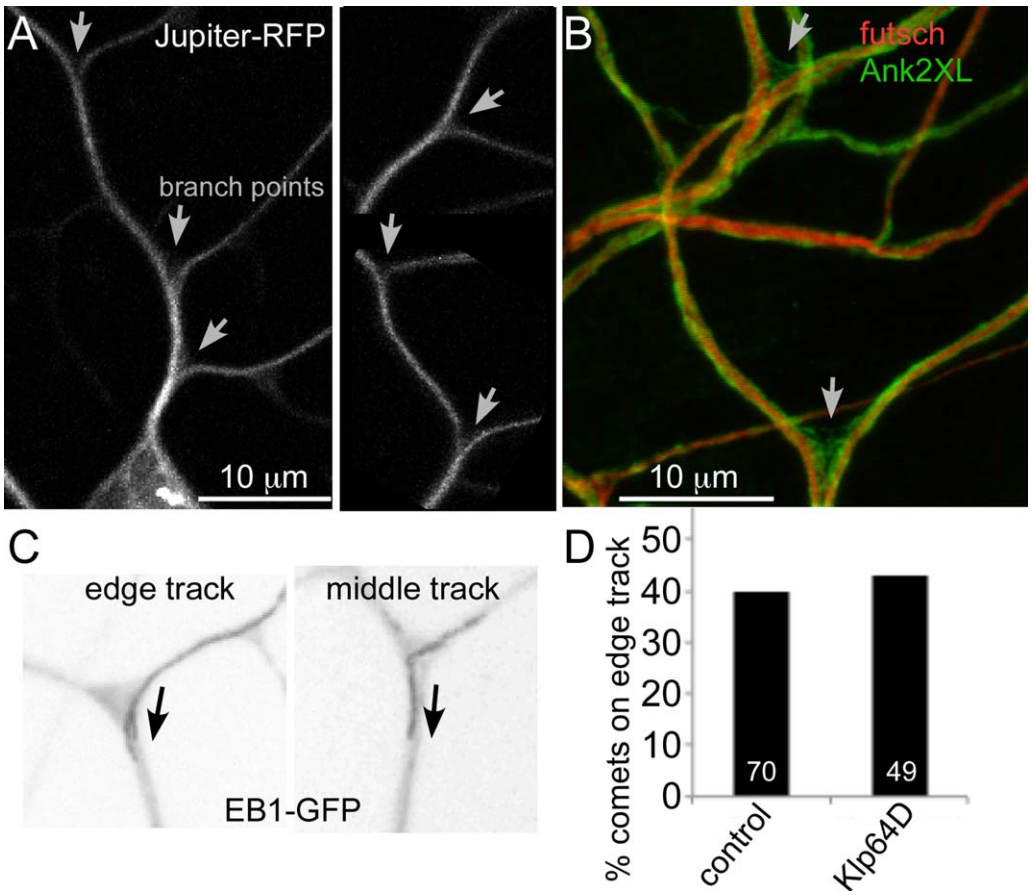

Fig. 3. Kinesin-2 does not influence the number of microtubules that track the position of stable microtubules in branch points. A, B: Stable microtubules in da neurons were visualized in live animals expressing Jupiter-RFP (A) or fixed animals stained with anti-futsch (B). Fixed animals were also stained with anti-Ank2XL antibodies to outline the cell by labeling the submembrane skeleton. **C, D**: Maximum projection images of EB1-GFP comets traveling through branch points were generated to determine whether they followed the edge of the branch where stable microtubules lie or go through the middle of the branch. Examples of both types of paths are shown in C. Based on these projections, comets in control (Rtnl2) RNAi and Klp64D RNAi neurons were classified as either tracking along the edge of the branch or not; the numbers in the graph are the ones that moved along the edge of the branch point and the remainder moved through the middle of the branch (D). Numbers of comets analyzed are indicated on the bars.

and meeting in a sharp V at the cell body side of the branch (Fig. 3A). This is similar to the pattern of stable microtubules we previously observed with endogenous GFP-labeled tau [Stone et al., 2008]. To ensure that this V pattern represented the overall distribution of stable microtubules and was not specific to the tagged Jupiter, we also performed immunostaining of fixed and fileted larvae. Because we were detecting endogenous futsch, a microtubule-associated protein that binds stable microtubules and is similar to MAP1B [Hummel et al., 2000], all neurons in the body wall are observed. Again, stable regions of microtubules can be seen to arc smoothly along the edges of branch points (Fig. 3B). Co-staining for Ankyrin2 allowed us to visualize neuron shape. In many of the branch points, microtubules occupy only the edge leaving open space in the middle that does not contain stable microtubules (see the schematic in Fig. 1C).

Using this information about the layout of stable microtubules in branch points, we classified EB1-GFP comets into i) those that took paths along the edge of the branch point where stable microtubules are found ii) and those that entered the middle of the branch away from stable microtubules. For this analysis we only considered comets that turned from a side branch into the main dendrite trunk of the ddaE comb dendrite. We distinguished these two classes based on time projections of comet movement through the branch point (Fig. 3C). In control animals, about 40% of comets that passed through a branch point did so in a smooth arc along the outer edge, and this number was unchanged in Klp64D RNAi neurons (Fig. 3D), although this RNAi had a strong effect on turning direction (Fig. 2B). Thus kinesin-2 is not required for growing microtubules to travel along the outside of branch points where stable microtubules are found.

Kinesin-2 and Apc Prevent Microtubule Growth from Slowing When the Microtubule Encounters the Edge of a Branch Point

As we found no evidence that kinesin-2 guides growing microtubules along stable ones, we tested whether there were other parameters of microtubule growth through branch points that depended on the motor. One possibility we examined was that interaction with the motor might change the speed at which the microtubule grew through the branch point. We therefore generated kymographs to track individual comets through time and marked the point at which the comet contacted the opposite branch wall with a white line (Supporting Information Figs. S1A and 4B). Comets moving at constant speed appear as a straight diagonal line on a kymograph, and changes in speed are seen as a change the angle of the line. The speed change for comets passing through a branch point was calculated from the difference in angles (Supporting Information Fig. S1B). We separated the data into two pools based on the origin point of the comet: from a side branch versus within the main trunk of the comb dendrite. The set of comets originating from a side branch was also analyzed in Fig. 3D; here we break down the behavior based on speed rather than path through the branch. The trajectory of comets within the main trunk tended to be quite straight, while the ones arising in the periphery typically had to turn more as they navigated the branch point. The majority of microtubules growing within the dendrite trunk did not change speed in branch points, and this did not change in Klp64D RNAi neurons (Supporting Information Figs. S1B and 4A). However, for the set of microtubules that had to turn more as they entered the trunk from the periphery, the average speed decreased in Klp64D RNAi neurons (Fig. S1B). When we binned microtubules into categories (no change, decrease in speed or increase in speed), we could see that the overall decrease in velocity was due to more comets slowing down in the branch points in Klp64D RNAi neurons than in control neurons (Fig. 4A).

To understand where microtubules were slowing down in the absence of kinesin-2 we analyzed the position of stalling, which includes microtubules that slowed down and microtubules that depolymerized. We used two different control RNAi hairpins, as well as RNAi hairpins that targeted kinesin-2 subunits and Apc. In all genotypes roughly 20% of comets that entered the branch point stalled in the middle (Fig. 4C). In control neurons 15 − 20% of the comets that entered the branch stalled close near the wall of the branch point opposite to the side of entry (Fig. 4C). However, when any of the kinesin-2 subunits or Apc was targeted by RNAi, this number increased to 35-45% of the total. This result suggests that kinesin-2 and Apc function when a growing microtubule collides with a structure near the wall of the branch point; in the absence of kinesin-2 more of these collisions result in slowed or terminated microtubule growth.

Kinesin-2 Steers Microtubules at Dendrite Crossroads

In V-shaped branch points, stable microtubules run along the edge close to the plasma membrane. It was therefore not possible to distinguish between growing plus ends colliding with the side of stable microtubules versus with the membrane or submembrane skeleton. While most branches along the main trunk of the ddaE comb dendrite were V-shaped, some of these neurons also contained 4-way or crossroad branch points (Fig. 5A). Jupiter-RFP labeled stable microtubules ran through the middle of these crossroads (Fig. 5A). In branch points with this geometry the stable tracks are therefore spatially separated from the plasma membrane.

To further characterize the layout of microtubules in crossroad branch points, we took advantage of EB1 labeled with GFP at an internal site rather than at the C-terminus (EB1int-GFP). EB1 has a low affinity for the microtubule lattice [Berrueta et al., 1998; Morrison et al., 1998], and the C-terminally tagged EB1-GFP used to label growing plus ends also has low background fluorescence along stable microtubules. However, when we generated transgenic flies that expressed a different tagged version of EB1 with the GFP inserted into a loop before the C-terminus, microtubules were often labeled quite clearly. This labeling provided further confirmation that in crossroad branch points, microtubules often ran through the middle (Fig. 5B). There were also often smaller bundles of microtubules that ran along the outside edge of the branch point as in V-shaped branch points (Fig. 5B).

We hypothesized that if kinesin-2 acted to steer growing microtubules along stable ones after a collision, then EB1-GFP comets would turn in the middle of crossroads at the stable track. We selected dendrites that contained crossroads and performed live imaging of EB1-GFP. Of the microtubules that entered from a side process, and that did not hug the outside edge of the branch point, many turned towards the cell body, often turning before reaching the far side of the branch point (Figs. 5C and 5D). When we compared the behavior of EB1-GFP comets in crossroads of control neurons and Klp64D RNAi neurons, the most striking difference was a population of microtubules that grew straight through the crossroads only in the absence of Klp64D (Fig. 5D). There was a corresponding reduction of microtubules that turned into the main branch. This result suggests that in the presence of kinesin-2 growing microtubules that encountered stable tracks in the middle of the crossroads can steer into the main dendrite trunk (Fig. 5E). In the absence of kinesin-2, this encounter would be more likely

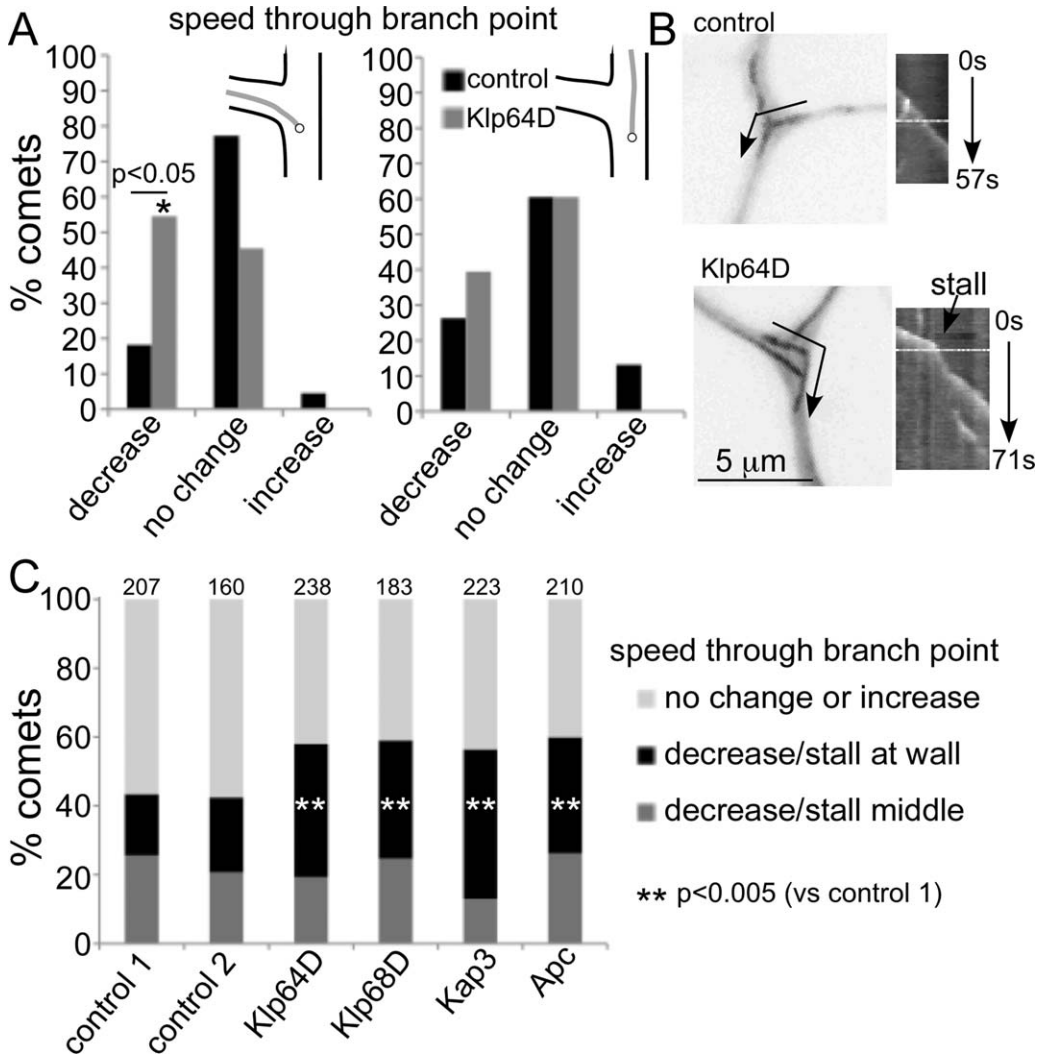

Fig. 4. Kinesin-2 and Apc are required for a subset of microtubules to grow through branch points without stalling. A: Speed changes of microtubules growing from peripheral processes were measured through branch points (left graph), and the same was done for microtubules in the main trunk (right graph). Numbers of microtubules analyzed for control RNAi (Rtnl1) and Klp64D RNAi were the same, 22 comets of each genotype were analyzed for the left graph, and 38 for the right graph. **B**: Examples of microtubule behavior seen with EB1-GFP comets are shown in time projections and kymographs. The white line indicates the time when the comet encountered the edge of the branch point opposite to the point of entry. **C**: EB1-GFP comet behavior was analyzed in six different RNAi conditions. Control 1 is Rtnl2 and control 2 is γ-tubulin37C RNAi. Klp64D, Klp68D and Kap3 are the three subunits of kinesin-2. Comets that entered the branch point from a peripheral dendrite were categorized based on whether they changed speed, and for the ones that slowed this was further broken down into the position where the change occurred. The number of microtubules analyzed for each genotype is shown above the bars. A Fisher's exact test was used to determine statistical significance for each type of behavior. Only decrease at the edge was altered by reducing kinesin-2 and Apc. The significance shown on the graph is a comparison to control 1, but the differences were significant when compared to either control.

to be non-productive and the microtubule would proceed straight through the crossroad (Fig. 5E).

Discussion

In this study we analyzed stable and growing microtubules at dendrite branch points to determine how kinesin-2 and +TIPs contribute to dendritic microtubule organization. Our previous phenotypic analysis suggested that these proteins were likely to control microtubule polarity by controlling the direction of growth at branch points, but left open two modes of action: aligning parallel microtubules or resolving microtubule collisions. We found no evidence that kinesin-2 helps growing microtubules to bundle in parallel. Instead, we found that when kinesin-2 or Apc levels were reduced, more microtubules slowed or stopped growing when they encountered the back wall of the branch point. By taking advantage of crossroad branch points, we pinpointed the key difference in microtubule behavior in the absence of kinesin-2: fewer microtubules were directed along the bundles in the motor's absence, and instead microtubules grew straight through the intersections.

In many differentiated cells microtubules take on specific arrangements that are essential to cellular organization and function [Bartolini and Gundersen, 2006]. Lateral interactions between microtubules are used in a number of different scenarios to generate aligned bundles. In mammalian neurons, tau and MAP2 generate microtubule bundles with characteristic spacing in axons and dendrites [Chen et al., 1992]. As muscle cells differentiate, microtubules align into an array parallel with the long axis of the cell. An isoform of MAP4, oMAP4, is required for this alignment [Mogessie et al., 2015]. Using in vitro experiments with purified oMAP4 and dynamic microtubules, it was shown that oMAP4 acts to zipper together microtubules that encounter one another at shallow angles, but not wider angles [Mogessie et al., 2015]. Similar angle-dependent zippering has been observed in the plant cortical microtubule array [Dixit and Cyr, 2004]. This zippering behavior is quite distinct from that facilitated by kinesin and +TIP complexes, which allow microtubules that grow into one another at wide angles to become aligned [Chen et al., 2014; Doodhi et al., 2014]. Our data support the idea that kinesin-2 works with +TIPs in vivo to resolve microtubule collisions rather than to mediate lateral interactions.

Motor proteins do, however, also mediate lateral interactions between microtubules in vivo. In neurons, kinesin-6 is important for arranging antiparallel microtubules characteristic of mammalian dendrites [Lin et al., 2012; Sharp et al., 1997; Yu et al., 2000]. Kinesins-5 and −12 also regulate microtubules in mammalian neurons [Liu et al., 2010], probably by limiting sliding forces of microtubules against one another generated by other motors [Myers and Baas 2007]. One of the motors that generates sliding forces during early neurite extension in Drosophila neurons is kinesin-1 [Lu et al., 2013], which is also important for microtubule organization in C. elegans neurons [Yan et al., 2013]. In all of these examples of kinesin actions on neuronal microtubules, microtubules are lined up against one another either in parallel or anti-parallel, and the motors mediate lateral interactions. This type of motor activity is quite different from the resolution of collisions between non-aligned microtubules by kinesin-2.

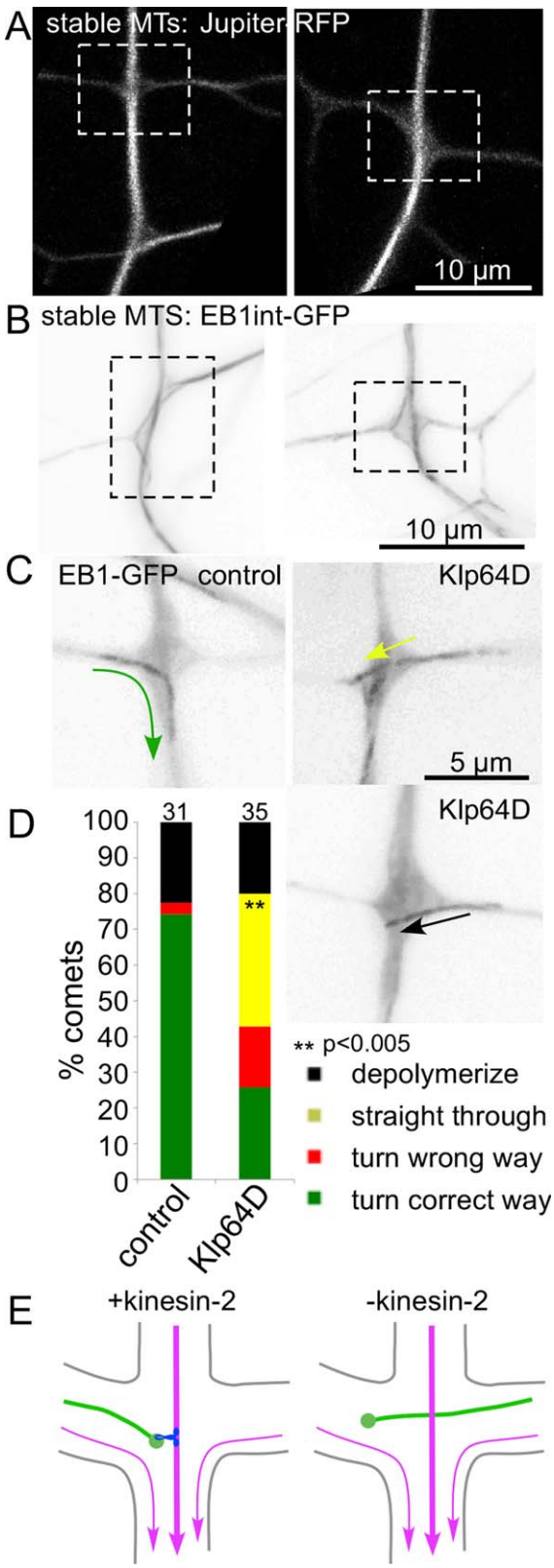

Fig. 5. Microtubule behavior at dendrite crossroads. The layout of stable microtubules at 4-way or crossroad branch points in ddaE comb dendrites was visualized in living animals either with Jupiter-RFP (**A**) or EB1int-GFP (**B**). **C**: Examples of EB1-GFP comet behavior at dendrite branch points are shown. The green arrow tracks a microtubule that turns towards the cell body; a yellow arrow indicates a microtubule that grows across the crossroad from one peripheral branch to the other, and the black arrow tracks a microtubule that depolymerizes in the branch point. The percentages of these types of behavior in control (Rtnl2) and Klp64D RNAi are shown in **D**. Only comets that entered the middle area of the branch were considered for this analysis. Numbers of comets analyzed for each genotype are shown above the bars. A chi-squared test was used to compare the distribution of all 4 outcomes between the two genotypes; the p value was less than 0.0001. In addition, a Fisher's exact test was used to specifically compare the "straight through" outcome between the two genotypes and the *p* value was less than 0.005. **E**: Diagrams of the behavior of microtubules growing through crossroads are shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The mode of kinesin-2 action at dendrite branch points is perhaps most similar to entry of growing microtubules into dendritic spines mediated by interactions between microtubules and actin [Merriam et al. 2013]. However, because motor proteins are quite ubiquitous regulators of microtubule organization, and because specific arrangements of microtubules are required in many cell types, we anticipate that guidance of growing microtubules by motors at the plus end will be discovered in other contexts as well.

Materials and Methods

Drosophila Lines

Standard genetic methods were used to generate lines containing multiple transgenes. Transgenic lines used in this study include: UAS-Apc2-GFP and UAS-Dicer2 from the Bloomington Drosophila Stock Center, 221-Gal4 from Dr. Wes Grueber,

For EB1 comet assays in da neurons, line containing UAS-Dicer2; 221-Gal4, UAS-EB1-GFP/TM6 was crossed with RNAi lines from the Vienna Drosophila RNAi Center (VDRC). The RNAi lines used as controls were Rtnl2 RNAi (#33320) and γ-tubulin37C (a maternal γ-tubulin not expressed in somatic cells [Wiese, 2008]) RNAi (#25271). We have previously validated these as controls [Mattie et al., 2010; Chen et al., 2012]. Other RNAi lines from VDRC were: Klp64D (#45373), Klp68D (#27943), Kap3 (#45400), and Apc (#51469). Larvae were grown on standard fly media consisting of cornmeal, yeast, dextrose, sucrose, and agar. Caps of food with fly embryos were collected every 24h and aged 3 days at 25°C. On the third day imaging experiments were performed.

Generation of EB1int-GFP Transgenic Flies

N-terminal and C-terminal fragments of Drosophila EB1 were amplified from cDNA. The N-terminal EB1 fragment (amino acids 1-257) was cloned into pUAST containing 3 copies of Emerald GFP with EcoRI and KpnI. The N-terminal part of EB1 replaced the first GFP coding sequence. The C-terminal EB1 fragment (amino acids 258-291) was then cloned using AgeI and XbaI to replace the third GFP. The result was the coding sequence of Emerald GFP inserted between amino acids 257 and 258 of EB1. Injections of the plasmid into Drosophila embryos to generate transgenics ere performed by BestGene.

Live Imaging of Drosophila Larvae

Third instar larvae were selected from their food cap and washed in PBS. They were then transferred to a circle of dried agarose on a microscope slide. Once a larva oriented with its dorsal side up and started to move, a 22x40 mm coverslip was anchored on top of it with sticky tape. Larvae were imaged with a Zeiss ImagerM2 with a Colibri LED light source, Zeiss LSM510 confocal microscope, or an Olympus FV1000 confocal microscope. Timeseries of ddaE neurons were acquired for 300 frames at one frame per second. After images were collected, they were analyzed in ImageJ. Each movie was inverted and aligned using the TurboReg plugin (http://bigwww.epfl.ch/thevenaz/turboreg/ [Thevenaz et al., 1998]) to reduce the impact of larva movement. Comets moving through branch points were selected for analysis.

Immunofluorescence of Drosophila Larvae

Third instar larvae were dissected to generate body wall filets, and then fixed in 4% paraformaldehyde. Staining was performed as described [Koch et al., 2008] using rabbit anti-Ank2XL at 1:1000. Microtubules were stained with the 22C10 primary antibody (1:100 mouse anti-futsch, from the Developmental Studies Hybridoma Bank). Goat secondary antibodies were obtained from Jackson ImmunoResearch.

Blind Analysis of EB1 Comet Behavior

Using Microsoft Excel, random IDs were assigned to each EB1 comet analyzed in the experiment in Figs. 3B and 3C. From each comet, a maximum projection plot of the path through the branch point was generated in ImageJ, a line was drawn on this, and a kymograph was generated from the line (shown in Supporting Information Fig. S1). The time when the comet encountered the far side of the branch point was indicated on the kymograph by deleting a frame, which then leaves a line at this point in the kymograph. The shape of the maximum projection path (edge track or middle track) was determined without knowing the genotype of the animal. Velocity measurements (Figs. 4 and Supporting Information S1) were made using the kymographs (see Supporting Information Fig. S1).

Measurement of Comet Speed

The velocity change was calculated from the slope of the line generated by the comet in a kymograph before and after it encountered the back of the branch point (see diagram in Supporting Information Fig. S1). Each slope was calculated as an angle drawn using the line tool in ImageJ. In Excel, the formula, =((TAN(RADIANS(90-ABS(slope before))))/9.767)-((TAN(RADIANS(90-ABS(slope after))))/9.767) was used to convert the slopes into a velocity difference (Supporting Information Fig. S1). Velocities were subsequently binned into categories: increase in velocity, a decrease, or no change (Fig. 4A).

Position of Stalling in a Branch Point

Every comet in the dendrite that entered a branch point along the main backbone of the ddaE comb was analyzed. The behavior of comets was binned into three categories: those that depolymerized or slowed at the back wall, those that depolymerized or slowed anywhere else in the branch point, and those that did not slow down as they traveled through the branch.

Analysis of Comet Behavior at Crossroads

ddaE neurons containing 4-way branch points were selected at low magnification, and timelapse movies of EB1-GFP were captured in these cells. In the movies, comets that entered form the peripheral processes into the 4-way branch point were counted. As a first step comets were categorized into ones that stayed near the edge of the branch (edge track as in Fig. 3) and comets that entered the middle of the branch. Only the ones that entered the middle of the branch were considered further. These were then categorized into one of the following groups: comets that turned toward the cell body, comets that turned away from the cell body, comets that remained free and continued into the opposite peripheral process, and those that depolymerized within the branch point.

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