

Golgi Outposts Shape Dendrite Morphology by Functioning as Sites of Acentrosomal Microtubule Nucleation in Neurons

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SUMMARY

Microtubule nucleation is essential for proper establishment and maintenance of axons and dendrites. Centrosomes, the primary site of nucleation in most cells, lose their function as microtubule organizing centers during neuronal development. How neurons generateacentrosomal microtubules remains unclear. *Drosophila* dendritic arborization (da) neurons lack centrosomes and therefore provide a model system to studyacentrosomal microtubule nucleation. Here, we investigate the origin of microtubules within the elaborate dendritic arbor of class IV da neurons. Using a combination of in vivo and in vitro techniques, we find that Golgi outposts can directly nucleate microtubules throughout the arbor. Thisacentrosomal nucleation requires gamma-tubulin and CP309, the *Drosophila* homolog of AKAP450, and contributes to the complex microtubule organization within the arbor and dendrite branch growth and stability. Together, these results identify a direct mechanism foracentrosomal microtubule nucleation within neurons and reveal a function for Golgi outposts in this process.

INTRODUCTION

Microtubules are organized into dynamic arrays that serve as tracks for directed vesicular transport and are essential for the proper establishment and maintenance of neuronal architecture (Bartolini and Gundersen, 2006; Hoogenraad and Bradke, 2009; Keating and Borisy, 1999; Stiess and Bradke, 2011; Witte and Bradke, 2008). The organization and nucleation of microtubules must be highly regulated in order to generate and maintain such complex arrays (Desai and Mitchison, 1997). Nucleating complexes, in particular, are necessary because spontaneous nucleation of new tubulin polymers is kinetically limiting both in vivo and in vitro (Oegema et al., 1999). Gamma(γ)-tubulin is a core component of microtubule organization centers and has a well-established role in nucleating spindle and cytoplasmic microtubules (Oakley, 2000). Previous studies have proposed that in mammalian neurons, microtubules are nucleated by

γ -tubulin at the centrosome, released by microtubule severing proteins, and then transported into developing neurites by motor proteins (Ahmad et al., 1998; Baas et al., 2005; Wang and Brown, 2002; Yu et al., 1993). Indeed, injection of antibodies against γ -tubulin or severing proteins inhibited axon outgrowth in neurons cultured for one day in vitro (DIV1) (Ahmad et al., 1994, 1999).

However, proper neuron development and maintenance may not rely entirely on centrosomal sites of microtubule nucleation. Although the centrosome is the primary site of microtubule nucleation at DIV2, it loses its function as a microtubule-organizing center during neuronal development (Stiess et al., 2010). In mature cultured mammalian neurons (DIV 11–12), γ -tubulin is depleted from the centrosome, and the majority of microtubules emanate fromacentrosomal sites (Stiess et al., 2010). In *Drosophila dsas-4* mutants that lack centrioles, organization of eye-disc neurons and axon outgrowth are normal in third-instar larvae (Basto et al., 2006). Within the *Drosophila* peripheral nervous system (PNS), although dendritic arborization neurons contain centrioles, they do not form functional centrosomes, and laser ablation of the centrioles does not perturb microtubule growth or orientation (Nguyen et al., 2011). These results indicate thatacentrosomal generation of microtubules contributes to axon development and neuronal polarity. How and whereacentrosomal microtubule nucleation may contribute to the formation and maintenance of the more complex dendrites, and what factors are involved in this nucleation is unknown. Dendritic arborization (da) neurons provide an excellent system for investigating these questions. They are a subtype of multipolar neurons in the PNS of *Drosophila melanogaster* which produce complex dendritic arrays and do not contain centrosomes (Grueber et al., 2002, 2003; Nguyen et al., 2011). Based on their patterns of dendrite projections, the da neurons have been grouped into four classes (I–IV) with branch complexity and arbor size increasing with class number. Class IV da neurons are ideal for studyingacentrosomal microtubule nucleation because they have the most elaborate and dynamic dendritic arbor, raising intriguing questions about the modes of nucleation for its growth and maintenance.

One potential site ofacentrosomal microtubule nucleation within these neurons is the Golgi complex. A number of studies have shown that the Golgi complex can nucleate microtubules in fibroblasts (Chabin-Brion et al., 2001; Efimov et al., 2007; Miller et al., 2009; Rivero et al., 2009). Although, in these cell types, the Golgi is tightly coupled to the centrosome, it does

not require the centrosome for nucleation. It does, however, require γ -tubulin, the centrosomal protein AKAP450, and the microtubule binding proteins CLASPs (Chabin-Brion et al., 2001; Efimov et al., 2007; Hurtado et al., 2011; Miller et al., 2009; Rivero et al., 2009). When the Golgi is fragmented upon treatment with nocodazole, the dispersed Golgi ministacks can still promote microtubule nucleation, indicating that these individual ministacks contain the necessary machinery for nucleation (Efimov et al., 2007; Rivero et al., 2009).

In both mammalian and *Drosophila* neurons, the Golgi complex exists as Golgi stacks located within the soma and Golgi outposts located within the dendrites (Gardioli et al., 1999; Horton and Ehlers, 2003; Pierce et al., 2001). In cultured mammalian hippocampal neurons, these Golgi outposts are predominantly localized in a subset of the primary branches (Horton et al., 2005); however, in *Drosophila* class IV da neurons, the Golgi outposts appear throughout the dendritic arbor, including within the terminal branches (Ye et al., 2007). The Golgi outposts may provide membrane for a growing dendrite branch, as the dynamics of smaller Golgi outposts are highly correlated with dendrite branching and extension (Horton et al., 2005; Ye et al., 2007). However, the majority of larger Golgi outposts remains stationary at dendrite branchpoints and could have additional roles beyond membrane supply (Horton et al., 2005; Ye et al., 2007). It is unknown whether *Drosophila* Golgi outposts contain nucleation machinery similar to mammalian Golgi stacks. Such machinery could conceivably support microtubule nucleation within the complex and dynamic dendritic arbor.

In this study, we identify a direct mechanism for acentrosomal microtubule nucleation within the dendritic arbor and reveal a role for Golgi outposts in this process. Golgi outposts contain both γ -tubulin and CP309, the *Drosophila* homolog of AKAP450, both of which are necessary for Golgi outpost-mediated microtubule nucleation. This type of acentrosomal nucleation contributes not only to the generation of microtubules at remote terminal branches, but also to the complex organization of microtubules within all branches of the dendritic arbor. Golgi outposts are therefore important centers of acentrosomal microtubule nucleation, which is necessary to establish and maintain the complexity of the class IV da neuronal arbor.

RESULTS

Microtubule Orientation Differs in Primary versus Terminal Branches of *Drosophila* Class IV Dendritic Arborization Neurons

In order to understand how the microtubule cytoskeleton is organized in the branches of class IV dendritic arborization (da) neurons, we analyzed the dynamics of EB1-GFP comets throughout the entire dendritic arbor in vivo. We expressed *UAS-EB1-GFP* using the class IV specific promoter, *ppk-Gal4* and focused on third-instar larvae 96 hr after egg laying, because although the arbor is well established and the primary branches are stable, the terminal branches are still dynamic (Lee et al., 2011; Parrish et al., 2009; Ye et al., 2007). Using EB1-GFP to mark the growing plus ends of microtubules, we found that microtubules grew predominantly in the retrograde direction toward the cell body in long (>50 μ m) primary branches, consis-

tent with previous reports in other classes of neurons (Figure 1A; Mattie et al., 2010; Rolls et al., 2007; Satoh et al., 2008; Song et al., 2012; Stone et al., 2008; Zheng et al., 2008). However, in shorter branches (20–30 μ m), we detected mixed microtubule polarity (Figure 1B), similar to that defined in mammalian neurons (Baas et al., 1988; Kapitein et al., 2010). Branches of this length corresponded to higher order branches, such as the secondary and tertiary branches, from which terminal branches originate (see Figure S1 available online). In even shorter terminal branches (<20 μ m), EB1 comets grew predominantly in the anterograde direction toward the distal tip of the branch (Figures 1C and 1D). Therefore, microtubule orientation within the dendritic arbor correlates with the length of the dendrite branch. Longer, more established branches contain predominately retrograde EB1 comets and shorter branches are composed of mainly anterograde EB1 comets (Figure 1E). This held true for branches in both the proximal and distal regions of the arbor. The speeds of the anterograde and retrograde comets were comparable in all branches, and closely matched the growth rates of microtubules in other systems (Figure 1F; Akhmanova et al., 2001; Stepanova et al., 2003).

Microtubules Originate from Distinct Sites within the Terminal Branches

In order to generate different patterns of microtubule polarity throughout the dendritic arbor, there likely exist a variety of mechanisms for microtubule nucleation. We therefore wanted to understand the origins of anterograde and retrograde EB1 comets growing specifically within the terminal branches. Anterograde comets originated from three main sources: the parent branch (Figure 2A), the branchpoint (Figure 2B), and within the terminal branch (Figure 2C). EB1 comets growing retrogradely along the parent branch could be directed into a smaller daughter branch and grow anterogradely toward the distal tip; however, this was the least common source of EB1 comets for the terminal branches (20% and 5% of anterograde comets in <10 μ m and >10 μ m branches, respectively) (Figures 2A, 2D, and 2E). Anterograde comets that originated from the branchpoint were most common for branches under 10 μ m (58% of comets), while comets that originated within the branch were most common for branches over 10 μ m (79% of comets) (Figures 2B–2E; Movie S1). Intriguingly, in both instances, the EB1 comets grew from very specific sites, indicating these microtubules may be nucleated from a common structure located at the branchpoint or within the branch (Figures 2B and 2C). Though retrograde comets were quite rare in these terminal branches, we also wanted to understand their origins. Retrograde comets either grew from the distal tip of the branch, which was more common in shorter branches, or within the branch, which was more common in longer branches (Figure 2F–2H and S2A–S2C). Again, multiple EB1 comets emanated from the same site, suggesting that these microtubules may be nucleated from specific structures within the branch.

Golgi Outposts Nucleate Microtubules Both In Vivo and In Vitro

Previous studies have reported that Golgi outposts are often enriched at dendritic branchpoints (Horton et al., 2005;

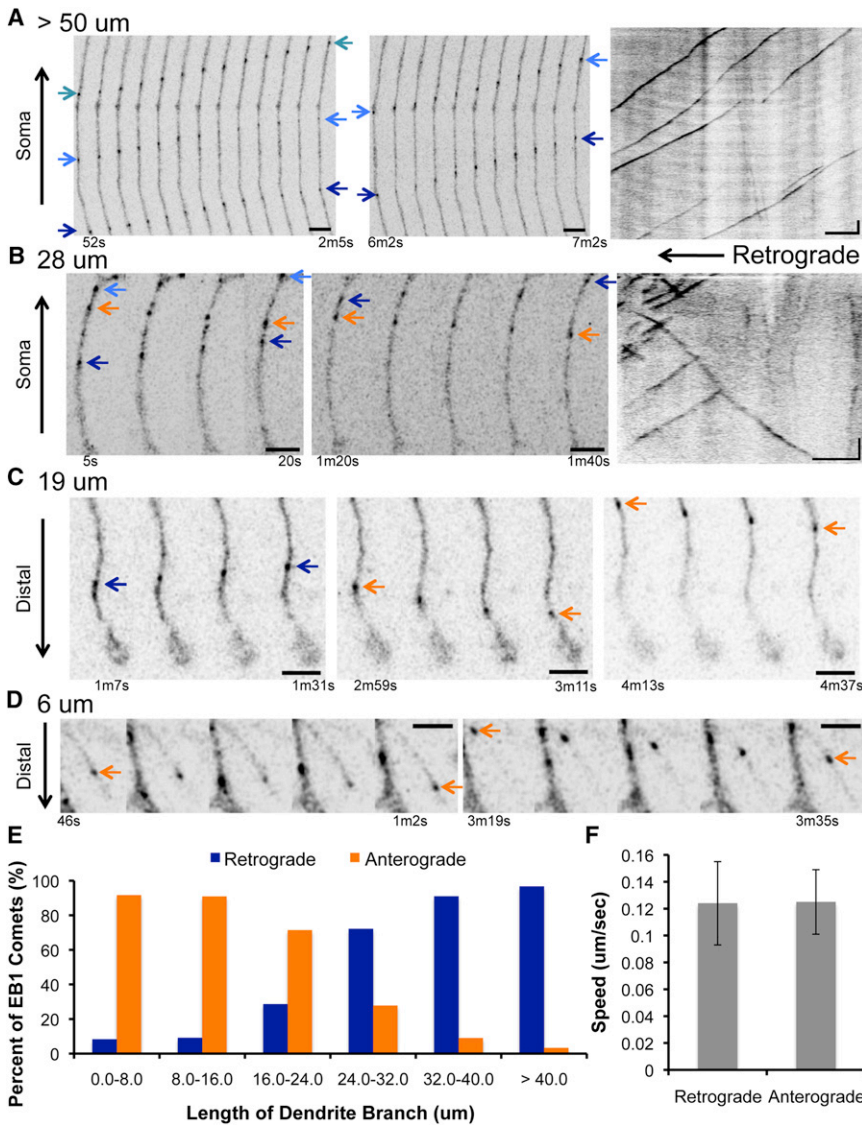


Figure 1. Microtubule Orientation Correlates with Dendrite Branch Length in Class IV Dendritic Arborization Neurons

Movie montages and/or kymographs depict the trajectory of EB1-GFP comets in dendrite processes that are >50 μm (A), 28 μm (B), 19 μm (C), or 6 μm (D) (see Figure S1 for branch classification). Black arrows indicate the direction of the soma or distal tip for each montage. *UAS-EB1-GFP* was expressed in class IV neurons by *ppk-Gal4*. In long primary branches, the majority of the comets move retrogradely toward the cell body, but in shorter terminal branches, most of the comets move anterogradely toward the distal tip of the process. Blue and orange arrows indicate the starting and ending positions of comets growing retrogradely or anterogradely, respectively. Montage scale bars are 3 μm for (A) and (B) and 2.5 μm for (C) and (D). Kymograph scale bars are 5 μm (x axis) and 30 s (y axis).

(E) Histogram revealing the correlation between EB1-GFP comet directionality and length of the dendrite branch. Longer branches contain predominantly retrogradely moving comets, while shorter branches frequently possess anterogradely moving comets. $n = 14$ neurons with 71 anterograde and 62 retrograde comets.

(F) Bar graph showing comparable speeds (means \pm SD) for retrograde and anterograde EB1-GFP comets ($p = 0.799$; $n = 44$ neurons and 204 comets).

Ye et al., 2007), the most common site for anterograde EB1 comets growing into short terminal branches (Figure 2E). In class IV da neurons, Golgi outposts were located at 47% of branchpoints, but they also appeared throughout the entire dendritic arbor, including at 25% of distal tips (Figures S3A–S3D). The Golgi complex has been shown to support microtubule nucleation in fibroblasts, and Golgi outposts contain both *cis* and *trans* elements (Horton and Ehlers, 2003). Therefore, we asked whether the Golgi outposts in these da neurons could also be sites of microtubule nucleation within the dendritic arbor. We expressed *UAS-ManII-mCherry* to label Golgi outposts (Ye et al., 2007) and *UAS-EB1-GFP* using class IV neuron-specific drivers and noticed a striking correlation between the site of Golgi outposts and the site of EB1 comet formation. We observed that Golgi outposts correlated with EB1 comet formation at dendrite branchpoints (Figure 3A; Movie S2), distal tips (Figure 3B), and within the terminal branch (Figure 3C). Other organ-

elles, such as Rab11-positive endosomes and mitochondria, did not correlate with EB1 comet formation in vivo (data not shown). Multiple EB1 comets could originate from a single Golgi outpost, with an average time of 66 s between formation of the first and second comets. Strikingly, multiple EB1 comets always emanated from the Golgi outposts in a particular direction, suggesting specific polarity in the nucleation machinery, and a role for the outposts in establishing or maintaining microtubule orientation within the terminal branch.

We also wanted to determine if there was a similar correlation within the primary branch, where EB1 comets predominantly move in the retrograde direction and Golgi outposts are rapidly transported (Golgi outposts moved at an average speed of 0.8 $\mu\text{m}/\text{s}$ compared with the EB1 comet growth speed of 0.1 $\mu\text{m}/\text{s}$; Figure S3E). Even in primary branches, stationary Golgi outposts colocalized with sites of EB1 comet formation, and again, all of the EB1 comets grew specifically in the retrograde direction (Figure 3D). The population of rapidly transported outposts did not colocalize with EB1 comet origins; however, we did observe that some moving outposts became stationary followed by nucleation of EB1 comets (Figures 3A and 3D). Overall, we observed 45% of total EB1 comets originating from Golgi outposts and 44% of total Golgi outposts correlating with EB1 comet formation (Figures 3E and 3F). These results strongly

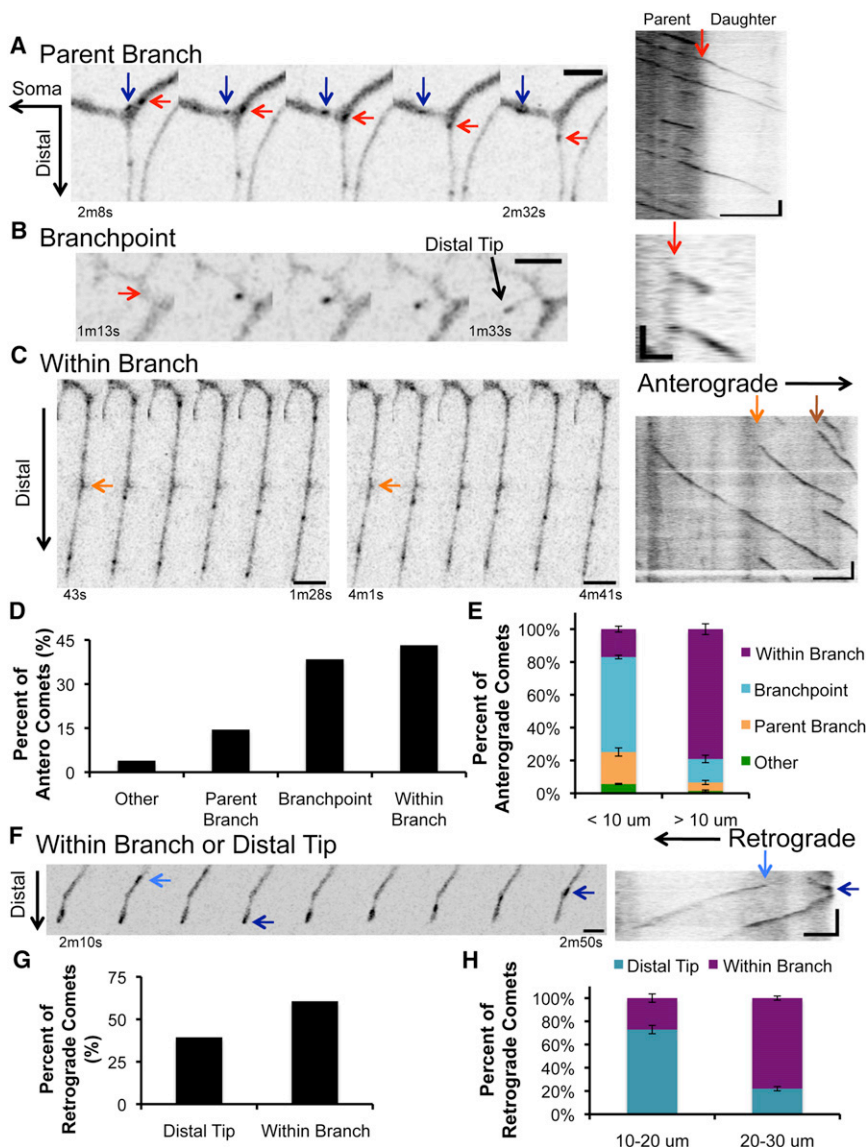


Figure 2. Anterograde and Retrograde Comets Originate from Specific Sites within the Dendritic Arbor

EB1-GFP comets growing anterogradely into a branch primarily originate from one of three sources: a parent branch, within the branch, or a branchpoint. Black arrows indicate the direction of the soma or distal tip for each montage.

(A) EB1-GFP comets can be directed from the parent branch into a smaller branch. The red arrow identifies a comet that is guided into the smaller daughter branch from the parent branch, while the blue arrow follows a comet that bypasses the daughter branch. The red arrow in the corresponding kymograph indicates the point at which three comets are directed into the daughter branch.

(B) Anterograde comets can originate from a specific branchpoint structure of a nascent branch. Red arrows indicate the branchpoint site of nucleation in both the montage and kymograph. (C) Anterograde comets originating within a branch also grow from specific sites (see also [Movie S1](#)). The orange arrows point to the identical origin of multiple EB1 comets.

(D and E) Bar graphs depicting the percent of total anterograde comets that originate from each source, and that the source of EB1 comets for a branch depends on its length.

(F) Retrograde EB1-GFP comets growing from a terminal branch originate from one of two sources: within the branch or from the distal tip (light and dark blue arrows, respectively, in the montage and kymograph) (see also [Figure S2](#)).

(G and H) Bar graphs depicting the percent of total retrograde comets that originate from each source and that the source of EB1 comets for a branch depends on its length.

Montage scale bars are 3 μ m. Kymograph scale bars are 5 μ m (x axis) and 30 s (y axis) for (A) and (C), 1.5 μ m and 20 s for (B), and 2 μ m and 30 s for (F). Data are means \pm SEM; n = 26 neurons and 133 comets for anterograde quantification and 21 neurons and 50 comets for retrograde quantification.

support a role for Golgi outposts as microtubule nucleation sites within da neurons.

We next asked whether these Golgi outposts could support microtubule nucleation in vitro. We partially purified Golgi vesicles from *ppk-Gal4 > UAS-ManII-eGFP* fly embryos. We separated the vesicles from other membranous structures via vesicle flotation through a sucrose step gradient, and then tested the ability of these vesicles to nucleate microtubules when incubated with purified tubulin and GTP (Hendricks et al., 2010; Kollman et al., 2010; Macurek et al., 2008; Mitchison and Kirschner, 1984; Ori-McKenney et al., 2010). We observed numerous microtubules extending from GFP-labeled Golgi vesicles, indicating that these vesicles are competent to promote microtubule nucleation in vitro (Figures 4A–4D). Quantification revealed that 54% of the GFP-labeled Golgi vesicles were associated with one or more microtubules, and 60% of the micro-

tubules extended from Golgi vesicles, consistent with our in vivo results (Figures 4A–4C). To investigate the differences between the Golgi vesicles that could support nucleation and those that could not, we immunostained the vesicles for γ -tubulin and CP309, the *Drosophila* homolog of AKAP450 (Figures 4B and 4C; Kawaguchi and Zheng, 2004). Both proteins were present on the Golgi outpost vesicles associated with microtubules. Strikingly, 85% of the γ -tubulin positive vesicles and 68% of the CP309 positive vesicles nucleated microtubules (Figure 4D). In contrast, we did not observe microtubule nucleation from any of the γ -tubulin negative vesicles. We were also able to inhibit microtubule nucleation from Golgi vesicles with a γ -tubulin function blocking antibody (Figure S4; Joshi et al., 1992). Together these results reveal that Golgi outposts are novel sites of microtubule nucleation both in vivo and in vitro, and that this activity requires γ -tubulin and CP309.

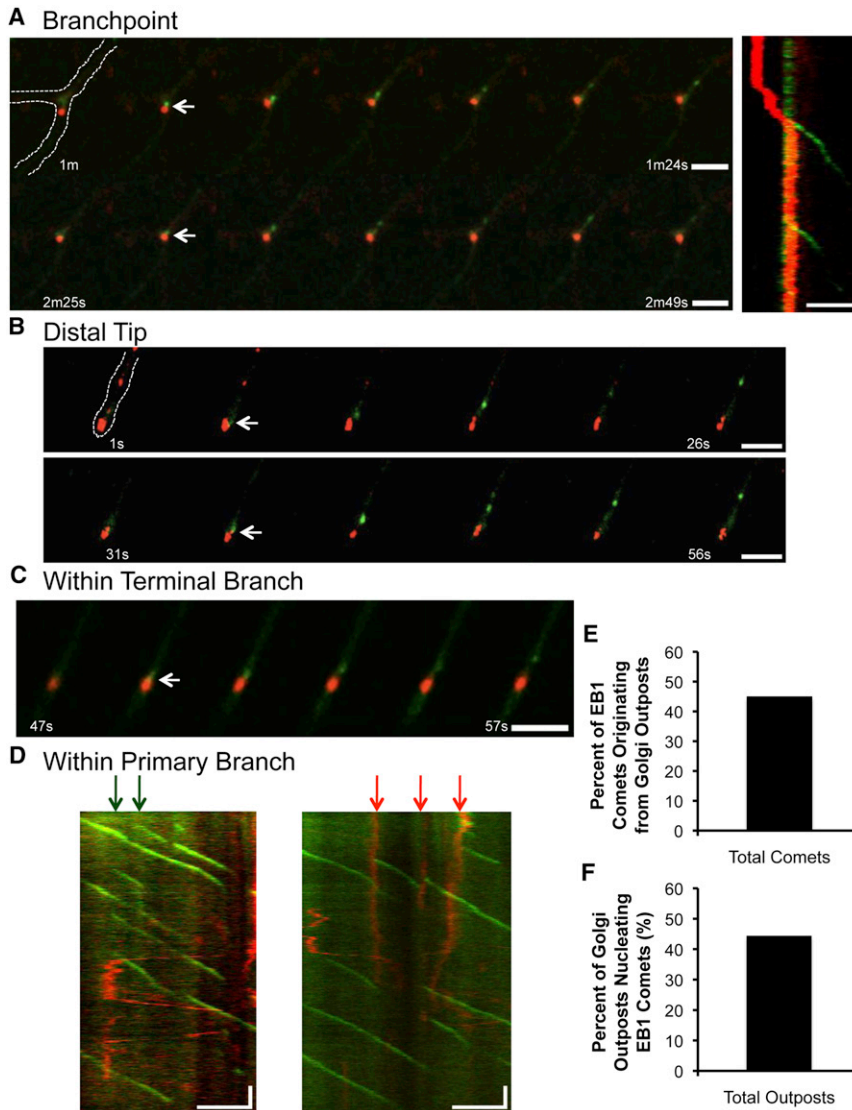


Figure 3. Golgi Outposts Colocalize with Sites of EB1-GFP Comet Formation In Vivo

EB1-GFP comets emanate from Golgi outposts at branchpoints (A), distal tips (B), and within the terminal branches (C) of class IV da neurons (see also [Movie S2](#)). *UAS-EB1-GFP* and *UAS-Man1-mCherry* were expressed in class IV neurons by *Gal4⁴⁻⁷⁷*. White arrows indicate the generation of EB1 comets (green) at the location of Golgi outposts (red).

(D) EB1 comets also originate from Golgi outposts within primary branches. In the kymographs, red arrows indicate the generation of EB1 comets (green) at stationary Golgi outposts (red), while green arrows indicate the EB1 comets that are not generated at Golgi outposts. Montage scale bars are 3 μ m. Kymograph scale bars are 5 μ m (x axis) and 30 s (y axis).

(E and F) Bar graphs showing the total percent of Golgi outposts that colocalize with sites of EB1 comet formation and the total percent of EB1 comets that originate from Golgi structures in vivo, $n = 26$ neurons.

See also [Figure S3](#).

γ -tubulin and CP309 Are Necessary for Golgi Outpost-Mediated Microtubule Nucleation In Vivo

Next, we asked whether γ -tubulin and CP309 were essential for microtubule nucleation by Golgi outposts in vivo. We combined the *UAS- γ -tubulin-23C* RNAi with the loss of function allele, *γ -tubulin-23C^(A15-2)*, to generate viable mutant larvae with a robust phenotype. We found a dramatic reduction in the percent of Golgi outposts that correlate with EB1 comet formation, and in the percent of EB1 comets emanating from Golgi outposts in neurons of these γ -tubulin mutants as well as *CP309⁽⁵⁾/CP309⁽⁵⁾* loss of function mutant larvae ([Figures 5A](#) and [5B](#)). Interestingly, the Golgi outposts were still localized to branchpoints and scattered throughout the arbor in both mutants ([Figure 5C](#)). The number of EB1 comets was also unchanged in the primary branches; however, there were fewer comets entering the terminal branches of these mutant neurons ([Figures 5D](#) and [5E](#)). We next examined the neuronal morphology in the absence of Golgi outpost mediated microtubule nucleation.

Sholl analysis revealed an overall decrease in the complexity of the arbor of both γ -tubulin and CP309 mutant neurons, with a reduction in total dendrite length and in the number of branchpoints ([Figures 6A–6E](#)). Remarkably, the terminal branches were most affected, while the primary and secondary branches seemed to develop relatively normally ([Figure 6A](#)). Maternally contributed γ -tubulin (*γ -tubulin-37C*) could be necessary for the initial development of the primary arbor, but it is reportedly degraded by the 3rd larval instar ([Basto et al., 2006](#); [Wiese and Zheng, 2006](#)).

Our data indicate that Golgi outpost associated γ -tubulin-23C could be necessary for the maturation of the rest of the arbor, especially for terminal branch growth.

Microtubules Are Necessary for the Growth and Stability of Terminal Branches within the da Neuron Arbor

In order to understand how microtubule nucleation could affect terminal branch dynamics, we compared the dynamics of terminal branches that contained EB1 comets with those that did not over the course of 30 min in wild-type larval neurons. We found that when EB1 comets entered a terminal branch, the branch either extended or remained stable and rarely retracted ([Figures 7A](#) and [7B](#); 40.7% extended and 7.4% retracted). On the other hand, the majority of terminal branches that lacked EB1 comets retracted ([Figures 7B](#), [S5A](#), and [S5B](#); 13.6% extended and 50.8% retracted). We noticed far fewer EB1 comets entering terminal branches in the γ -tubulin and CP309 mutant neurons ([Figure 5E](#)), indicating the ability of a terminal branch to extend or remain stable could be

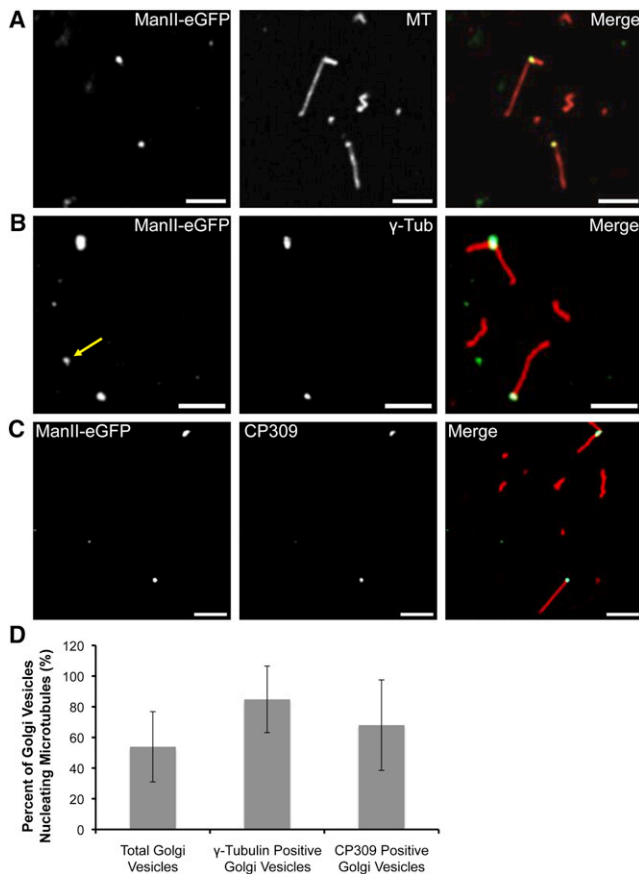


Figure 4. Golgi Vesicles Promote Microtubule Nucleation In Vitro

Golgi vesicles were partially purified from *ppk-Gal4 > UAS-ManII-eGFP* embryo lysate by membrane flotation, then incubated with 10 μ M tubulin and 1 mM GTP at 25°C for 15 min., then 29°C for 15 min. After fixation, samples were centrifuged onto coverslips, which were used for immunostaining.

(A) Multiple microtubules (MT) extend from ManII-eGFP Golgi structures.

(B and C) Golgi vesicles that nucleate microtubules are positive for γ -tubulin and CP309. Yellow arrow points to a vesicle negative for γ -tubulin that is not associated with microtubules.

(D) Bar graph showing the percent of total Golgi vesicles that nucleate microtubules in vitro and the percent of γ -tubulin and CP309 positive vesicles that nucleate microtubules in vitro.

Data are means \pm SD; n = 5, 3, and 3 experiments for (A), (B), and (C), respectively. Scale bars are 5 μ m. See also Figure S4.

compromised in these mutant neurons. We therefore analyzed the branch dynamics of γ -tubulin and CP309 mutant neurons and indeed found that the terminal branches were less stable than those of wild-type neurons, with the majority of the branches retracting (Figures 7C and 7D; 69% for γ -tubulin mutant and 53% for CP309 mutant versus 34% for wild-type). Together these results reveal that γ -tubulin positive Golgi outposts may be especially important at branchpoints for nucleating microtubules into the terminal branches to promote their growth and stability. Without this mechanism of generating microtubules, the terminal branches are deficient in their ability to extend and fill in the arbor (Figure 6A).

DISCUSSION

We have addressed how microtubules are organized and nucleated within the complex arbor of class IV da neurons and how essential these processes are for dendrite growth and stability. Microtubule organization within different subsets of branches in da neurons must require many levels of regulation. In this current study, we have identified the first direct mechanism for acentsosomal microtubule nucleation within these complex neurons and uncovered a role for Golgi outposts in this process. Our data are consistent with the observation that pericentriolar material is redistributed to the dendrites in mammalian neurons (Ferreira et al., 1993) and that γ -tubulin is depleted from the centrosome in mature mammalian neurons (Stiess et al., 2010). This suggests that the Golgi outposts may be one structure involved in the transport of centriole proteins such as γ -tubulin and CP309. We find that microtubule nucleation from these Golgi outposts correlates with the extension and stability of terminal branches, which is consistent with the observation that EB3 comet entry into dendritic spines accompanies spine enlargement in mammalian neurons (Jaworski et al., 2009). It is striking that microtubule organization in shorter branches, but not primary branches, mimics the organization in mammalian dendrites, with a mixed microtubule polarity in the secondary branches and a uniform plus end distal polarity in the terminal branches (Baas et al., 1988). Kinesin-2 and certain +TIPS are necessary for uniform minus end distal microtubule polarity in the primary dendrites of da neurons (Mattie et al., 2010). Golgi outpost mediated microtubule nucleation could also contribute to establishing or maintaining this polarity both in the terminal branches and in the primary branches. It will be of interest to identify other factors that may be involved in organizing microtubules in different subsets of branches in the future.

Our in vivo and in vitro data support a role for Golgi outposts in nucleating microtubules at specific sites within terminal and primary branches. However, we note that not all EB1 comets originate from Golgi outposts, indicating other possible mechanisms of generating microtubules (Figure 3; Rogers et al., 2008). One potentially important source of microtubules is the severing of existing microtubules by such enzymes as katanin and spastin, both of which are necessary for proper neuronal development (Ahmad et al., 1999; Jinushi-Nakao et al., 2007; Stewart et al., 2012; Yu et al., 2008). It is likely that both microtubule nucleation and microtubule severing contribute to the formation of new microtubules within the dendritic arbor; however, our studies suggest that Golgi-mediated nucleation is especially important for the growth and maintenance of the terminal arbor. In γ -tubulin and CP309 mutant neurons, the primary branches contain a similar number of EB1 comets, but only a small fraction of the terminal branches still contain EB1 comets. This result indicates that severing activity or other sources of nucleation may suffice for microtubule generation within the primary branches, but γ -tubulin mediated nucleation is crucial in the terminal branches. As a result, the terminal branch arbor is dramatically reduced by mutations compromising the γ -tubulin nucleation activity at Golgi outposts (Figure 6).

It is important to note that Golgi outposts are present in the dendrites, but not in the axons of da neurons; thus, this mode

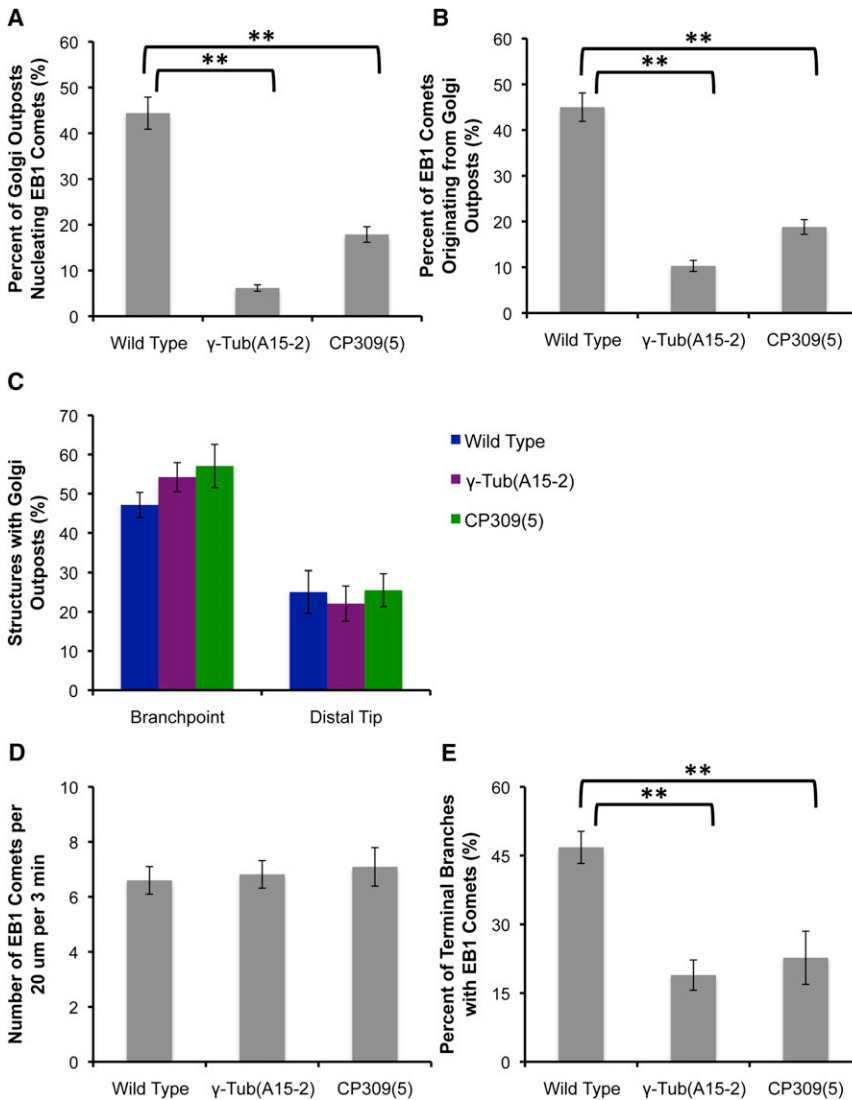


Figure 5. Golgi-Dependent Microtubule Nucleation Requires γ -Tubulin and CP309

The percent of Golgi outposts that colocalize with EB1 comets and the percent of EB1 comets that emanate from Golgi outposts *in vivo* is dramatically decreased in γ -tubulin23C RNAi/ γ -tubulin23C^(A15-2) and CP309⁽⁵⁾/CP309⁽⁵⁾ mutant larval neurons as shown in (A) and (B). Data are means \pm SEM; n = 93, 96, and 93 Golgi outposts and n = 127, 99, and 115 EB1 comets for wild-type, γ -tubulin mutant, and CP309 mutant, respectively. Stars indicate p < 0.001.

(C) Bar graph revealing a similar percent of branchpoints and distal tips that contain Golgi outposts throughout the arbors of wild-type, γ -tubulin23C RNAi/ γ -tubulin23C^(A15-2), and CP309⁽⁵⁾/CP309⁽⁵⁾ mutant neurons. Data are means \pm SEM; n = 22, 10, and 10 neurons for wild-type, γ -tubulin mutant, and CP309 mutant, respectively.

(D and E) Bar graphs showing that although there are a similar number of EB1-GFP comets in the primary branches of γ -tubulin23C RNAi/ γ -tubulin23C^(A15-2) and CP309⁽⁵⁾/CP309⁽⁵⁾ mutant neurons compared with wild-type, there is a dramatic reduction in the percent of terminal branches that have EB1-GFP comets. Data are means \pm SEM; n = 11, 11, and 10 neurons for (D) and n = 20, 15, and 15 neurons for (E) for wild-type, γ -tubulin mutant, and CP309 mutant, respectively. Stars indicate p < 0.001.

of nucleation is dendrite specific and likely contributes to the difference in microtubule arrays in axons and dendrites. While the axon is one long primary branch with uniform microtubule polarity, the dendrite arbor is an intricate array of branches where microtubule polarity depends on branch length (Figure 1). Therefore, this more elaborate branched structure may have evolved a variety of nucleation mechanisms, including Golgi outpost nucleation and microtubule severing. Intriguingly, in *da* neurons lacking cytoplasmic dynein function, the Golgi outposts are mislocalized to the axon, which appears branched and contains microtubules of mixed polarity (Zheng et al., 2008). We speculate that in these mutants, Golgi-mediated microtubule nucleation within the axon is contributing to the mixed microtubule orientation and formation of ectopic dendrite-like branches.

Only a subpopulation of Golgi outposts could support microtubule nucleation both *in vivo* and *in vitro*. Our results show that Golgi outpost mediated microtubule nucleation is restricted

trafficked into terminal branches that are 150–300 nm in diameter (Han et al., 2012; Ye et al., 2007), and therefore may provide an excellent vehicle for transporting nucleation machinery to these remote areas of the arbor. It will be interesting to determine how these nucleation factors are recruited to the Golgi outposts. It has been previously shown that GM130 can recruit AKAP450 to the Golgi complex, but whether the first coiled-coil domain of the *Drosophila* AKAP450 homolog, CP309, can also bind GM130 is unknown (Hurtado et al., 2011; Kawaguchi and Zheng, 2004; Rivero et al., 2009). Interestingly, we observed that predominantly stationary Golgi outposts correlated with EB1 comet formation, indicating that this specific subpopulation may contain γ -tubulin and CP309. What other factors may be necessary to properly position the Golgi outposts at sites such as branchpoints, and how this is achieved will be a fascinating direction for future studies.

Whether the acentrosomal microtubule nucleation uncovered in our study also occurs in the dendrites of mammalian neurons

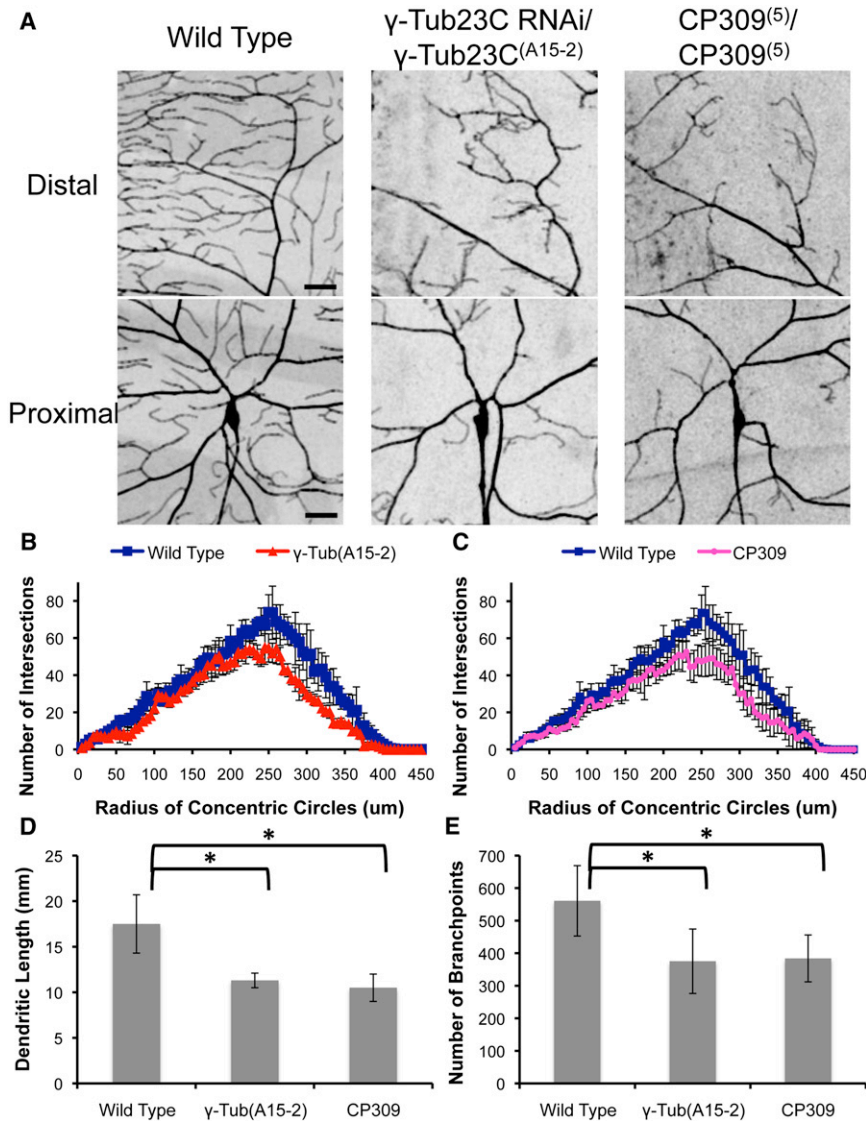


Figure 6. Loss of γ -Tubulin or CP309 Function Alters the Dendritic Morphology of Class IV da Neurons

(A) Dendrite defects in γ -tubulin23C RNAi/ γ -tubulin23C^(A15-2) and CP309⁽⁵⁾/CP309⁽⁵⁾ mutant class IV da neurons, visualized by *ppk-cd4-tdtom*. Scale bars are 25 μ m.

(B and C) Sholl analysis reveals decreased complexity of the dendritic arbor in the absence of γ -tubulin or CP309. Data are means \pm SD; n = 4 neurons per genotype.

(D and E) Total dendrite length and number of branchpoints were reduced in γ -tubulin23C RNAi/ γ -tubulin23C^(A15-2) and CP309⁽⁵⁾/CP309⁽⁵⁾ mutant neurons. Data are means \pm SD; n = 4 neurons per genotype. Stars indicate $p < 0.05$.

overall arbor maintenance of class IV da neurons, and that Golgi outposts are important nucleation centers within the dendritic arbor.

EXPERIMENTAL PROCEDURES

Additional details about the Experimental Procedures used for this paper can be found in the Supplemental Experimental Procedures.

Fly Stocks

We used Gal4 driver lines *ppk-Gal4* and *Gal4⁴⁷⁷* (Grueber et al., 2007) to drive the expression of *UAS-ManII-mCherry* and *UAS-ManII-eGFP* (Ye et al., 2007) to visualize Golgi outposts and *UAS-EB1-GFP* (Sato et al., 2008; Zheng et al., 2008) to visualize the growing plus ends of microtubules in class IV da neurons. We used the reporter line, *ppk-CD4-tdTomato* (Han et al., 2011) to observe the dendrite morphology of class IV da neurons in wild-type and mutant backgrounds. The mutant lines γ -tubulin23C^(A15-2) (Stock 7042) and CP309⁽⁵⁾ (Stock 9567), as well as the *UAS- γ -tubulin23C* RNAi line (Stock 31204) were obtained from the Bloomington

Drosophila Stock Center (Department of Biology, Indiana University, Bloomington, IN).

Live Imaging and Analysis

Whole, live, third-instar larvae were mounted in 90% glycerol under coverslips sealed with grease and imaged using a Leica SP5 laser scanning confocal microscope. Live imaging and analysis of EB1-GFP were performed as previously described (Zheng et al., 2008).

In Vitro Microtubule Nucleation Assay

The microtubule nucleation solution assays were performed as described with a few modifications (Kollman et al., 2010; Macurek et al., 2008; Mitchison and Kirschner, 1984). GFP-labeled Golgi vesicles were partially purified by membrane flotation through a sucrose step gradient, then incubated with 10 μ M tubulin and 1 mM GTP at 25 $^\circ$ for 15 min, then 29 $^\circ$ for 15 min. The solution was spun onto a coverslip, which was immunostained for α -tubulin (monoclonal T9026, Sigma, St. Louis, MO), γ -tubulin (monoclonal GTU88 or polyclonal T3559, Sigma, St. Louis, MO), or CP309 (polyclonal, gift from Yixian Zheng, Carnegie Institute, HHMI, Baltimore, MD).

is a question of great interest. Golgi outpost distribution in cultured hippocampal neurons is significantly different than that in da neurons (Horton et al., 2005; Ye et al., 2007), and hippocampal neurons do not form as elaborate arbors as da neurons. However, other types of mammalian neurons form much more complex dendritic arbors and may conceivably require acentrosomal nucleation for the growth and perpetuation of the dendrite branches.

Our study provides the first evidence that Golgi outposts can nucleate microtubules at acentrosomal sites in neurons, shedding new light on the longstanding question about the origin of the microtubule polymer in elongated neuronal processes. This source of nucleation contributes to the complex organization of microtubules within all branches of the neuron, but is specifically necessary for terminal branch development. We thus conclude that acentrosomal microtubule nucleation is essential for dendritic branch growth and

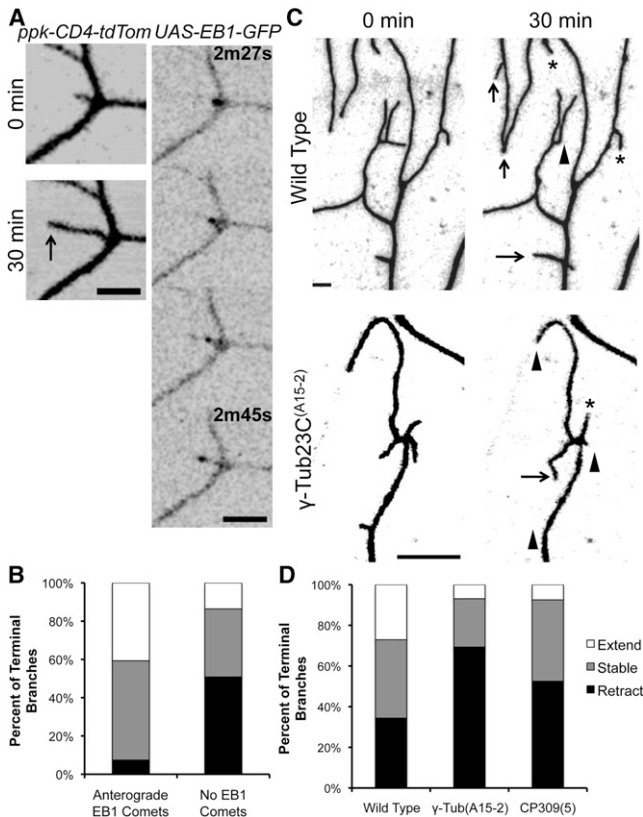


Figure 7. Microtubule Dynamics Are Important for Dendrite Branch Extension and Stability

(A) Movie montage shows EB1 comets grow into branches that extend and remain stable in a wild-type neuron (arrow indicates point of branch extension within 30 min) (see also Figure S5).

(B) Quantification of the correlation between EB1 comet growth within a branch and branch dynamics; $n = 86$ branches.

(C) Representative images of dendrite branch dynamics in wild-type or γ -tubulin23C RNAi/ γ -tubulin23C^(A15-2) neurons within 30 min. Arrows, arrowheads, and stars indicate points of branch extension, retraction, and stability after 30 min for each genotype.

(D) Quantification of total branch dynamics in wild-type, γ -tubulin23C RNAi/ γ -tubulin23C^(A15-2) and CP309⁽⁵⁾/CP309⁽⁵⁾ neurons. $n = 96, 101,$ and 120 branches for wild-type, γ -tubulin mutant, and CP309 mutant, respectively.

Statistical Analysis

All statistical tests were performed with two-tailed Student's *t* test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two movies, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2012.10.008>.

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