In vivo genetic analysis of Golgi outposts' role in microtubule organization in Drosophila sensory neurons

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Abstract

Microtubules in neurons form a highly organized array. They provide mechanical support for the neurons and serve as the highways for molecular motors and cargos to achieve highly efficient and directional delivery. The neuronal cargos being trafficked using this microtubule-motor system are involved in every part of neuronal function and development. The central feature of neuronal microtubules, also the key to provide the delivery guidance for the cargos, is microtubules' distinct polarity in the axons and dendrites. Although important, there are still many unanswered questions regarding to how such polarized structures are established and maintained in the different neuronal compartments.

In Chapter 1, I aimed to provide a broad overview of neuronal microtubule polarity: first, a discussion on the basis of microtubule polarity; second, our current understanding of microtubule-making machineries in neurons; and finally, an integrated view of how different mechanisms that shape the cytoskeleton collectively make polarized microtubule cytoskeleton over neuronal development. In chapter 2, I focused on the discussion on Golgi's role in microtubule organization. My research has uncovered that, Golgi outposts, a putative microtubule organizing center (MTOC) in neurons, may not be essential to dendrite microtubule cytoskeleton; however, depending on its compartmentalization, Golgi may have the capacities to influence both dendritic and axonal microtubule polarity. In chapter 3, I highlighted the importance of my findings and point out old and new challenges in the study of neuronal microtubule organizations.

List of Abbreviations

γ-tubulin ring complex (γ-TuRC)

microtubule organizing center (MTOC)

end binding 1 (EB1)

+ TIP (plus-end tracking protein)

Chapter I: Building the polarized microtubule architecture in neurons

Introduction

Sorting proteins, mRNAs, organelles, and vesicles to the right cellular destinations is essential to every cell's survival and function. In neurons, the signal-sending axon and the signal-receiving dendrites can extend processes over long distances. They connect to different targets and develop utterly different morphologies. As a result, neuronal cargos that aim for different intracellular destinations need to navigate within these diverse and complex neuronal compartments to reach their targets. Thus, the molecular machinery that is responsible for such tasks needs to be both efficient and specific. Microtubules form highly organized arrays that serve as the highways for the directional trafficking of molecular cargos (Bentley & Banker, 2016; Kelliher et al., 2019). These molecular cargos participate in every facet of cellular activities, regulating ion flow, replenishing or retrieving cellular membranes, and balancing global and local energy supplies. These cellular activities consequently allow neurons to respond to internal and external signals, extend and regenerate neuronal processes, and make dynamic connections with their neighbors (Blanquie & Bradke, 2018; Penazzi et al., 2016). For this reason, building neuronal microtubule architecture is the equivalent of constructing the neurons' lifelines.

The basis of neuronal microtubule polarity

Microtubules are tubular protein structures made of protofilaments assembled from α- and β- tubulin heterodimers. The head-to-tail association of the dimers results in two distinct ends that have disparate biophysical and biochemical properties (Brouhard & Rice, 2018). As a consequence, in cells, these two ends interact with different pools of proteins and exhibit

different dynamics. The α-tubulin-exposed end, also known as the minus-end, is normally capped or stabilized in cells. The β-tubulin-exposed plus-end, in contrast, interacts with plus-end-tracking proteins (+TIPs) that modulate microtubule growth, and is the more dynamic end (Figure 1) (Akhmanova & Steinmetz, 2015).

The importance of microtubule polarity to intracellular transport is exemplified by the direction-specific microtubule and motor interactions. Kinesin and dynein are the two major molecular motor families that walk along microtubules. Cytoplasmic dynein generally walks to the microtubule minus-end. Conversely, the majority of the kinesin family members move towards the microtubule plus-end (Kelliher et al., 2019; Tas et al., 2017). In this way, microtubule polarity provides an integral guidance indicator for motors that determines the direction they will traffic their cargo (Figure 1). Therefore, understanding how neuronal microtubule polarity is essential to the understanding of polarized trafficking in neurons.

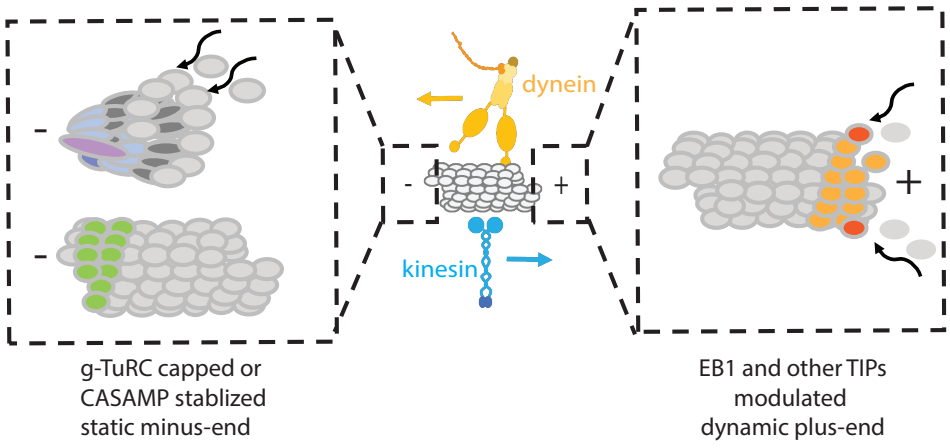

Figure 1. Direction-specific interaction between molecular motors and microtubules; distinct microtubule dynamics at the two microtubule ends.

To study neuronal microtubule organization, visualizing microtubule polarity is key. Microtubules are 25nm in diameter and are densely bundled in neuronal processes (Kapitein & Hoogenraad, 2015). Direct visualization of their orientation is a very challenging task. Early approaches utilized a "hook" assay to detect microtubule orientation by electron microscopy. In this approach, samples were treated with special tubulin buffers that allow new tubulin sheets to form on the side of the existing microtubules. The cross sections of these specially treated microtubules were then examined by electron microscopy to uncover the orientation of the curved newly added tubulin sheet, whose hook-like appearance was used as to read-out orientation (Heidemann & McIntosh, 1980). The sample preparation for this protocol is very lengthy, and the output is relatively low. As a result, while this assay provided the first knowledge of microtubule orientations in developing neurons, it is no longer a widely used protocol for detecting microtubule polarity.

Instead, live imaging of fluorescently tagged proteins that bind to the microtubule plus-ends, called +TIP proteins, has become the most popular tools to assay microtubule orientation. This approach takes advantage of the different dynamics at the two microtubule ends. Microtubule growth primarily occurs at the plus-end, which displays a significantly higher growth rate than the minus-end (Dammermann et al., 2003; Mitchison & Kirschner, 1984). +TIPs such as members of the End-binding protein family (e.g. EB3 in mammals and EB1 in flies) interact specifically with growing microtubule ends that contain unhydrolyzed GTP tubulin (Maurer et al., 2012). As the microtubule grows, the fluorescently tagged EB forms distinguishable comet trajectories that indicate microtubule orientation (Kleele et al., 2014; Rolls et al., 2007; Stepanova et al., 2010). In addition to the +TIPs, proteins that interact with

microtubule minus-ends, such members of the conserved CAMSAP family (del Castillo et al., 2015) and, in flies, a chimeric kinesin-1- Nod fusion protein (Clark et al., 1997; Wang et al., 2019; Zheng et al., 2008), have also been used to assay microtubule orientation. Consequently, microtubule orientation can be easily examined in neurons in primary cultures and in tissues *in vivo* that are accessible to live-imaging.

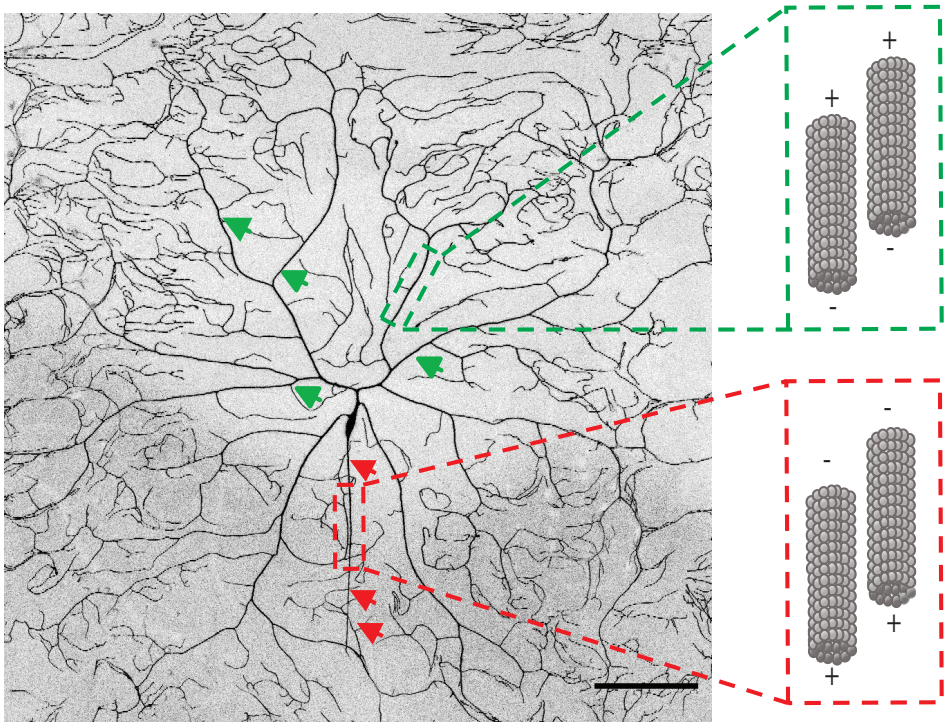

Figure 2. Drosophila Class IV da neuron has primarily minus-end-distal microtubules in proximal dendrites (green) and uniformly aligned plus-end-distal microtubules in the axon (red).

The advance of these microtubule polarity read-out assays has led to a better and more detailed understanding of neuronal microtubule architecture. Mature dendrites and axons have distinct microtubule organization (Baas et al., 1988; Stone et al., 2010). Axons usually contain uniform plus-end-distal microtubules (Baas & Ahmad, 1992). Dendrites, in contrast, have mixed

microtubule polarity; the proportions of minus-end-distal microtubules is dependent on the location within the dendritic arbor, developmental stage, and neuronal type (Baas et al., 1989; Yau et al., 2016). A group of Drosophila sensory neurons, called the ddaC neurons, for instance, contains almost exclusively minus-end-distal microtubules in the proximal dendrite arbor (Figure 2) (Ori-McKenney et al., 2012). Mammalian dendrites, in contrast, contain almost equal plus-end- and minus-end-distal microtubule populations (Baas et al., 1988).

Microtubule-making machineries in neurons

Making new microtubules *de novo* in cells requires nucleators, which lower the kinetic barrier for tubulin dimer addition as the cellular concentration of tubulin dimers is relatively low and insufficient for self-assembly (Wiese & Zheng, 2006). Additional help may be provided by factors that stabilize nascent microtubules (Goodwin & Vale, 2010), enrich local tubulin concentration (Woodruff et al., 2017), or promote microtubule growth (Akhmanova & Steinmetz, 2015). These proteins, which may be present in different combinations in cells, are enriched on cellular structures known as the microtubule organizing centers (MTOCs) (Sanchez & Feldman, 2017). The identity, localization, activity, and geometric orientation of these MTOCs enable the generation of diverse microtubule organizations and polarities that are found in different cell types or in cellular compartments within a cell. For this reason, understanding what cellular structures may serve MTOCs in neurons, and how their activities are regulated, is likely to provide the keys to understanding how the neuronal cytoskeleton is constructed. Therefore, an important first step is to identify neuronal MTOCs.

The centrosome is the first and best characterized MTOC. In interphase and mitotic cells, radially organized microtubules emanate from the centrosome(s), making it one of the most obvious MTOCs. This membraneless organelle has structured proteinaceous layers on which MAPs and microtubule nucleators, such as the γ-tubulin ring complex (γ-TuRC), are concentrated (Magescas et al., 2019; Paz & Lüders, 2018; Wu & Akhmanova, 2017). However, emerging evidence suggests that in highly differentiated cells like neurons, the centrosome's function as an MTOC is compromised (Muroyama & Lechler, 2017; Sanchez & Feldman, 2017). Microtubule nucleation factors, like γ-tubulin, are depleted from the centrosome over development (Leask et al. 1997; Stiess 2019). Moreover, genetic and physical ablation of the centrosome does not significantly perturb microtubule organization in developing neurons (Nguyen et al., 2011; Stiess et al. 2010). For these reasons, microtubules in neurons and other highly differentiated cells are believed to originate from centrosome-independent sources.

While the centrosome has proved non-essential for neuronal microtubule organization, studying it has set up the basic guidelines for finding other organelle-based MTOCs. Putative organelle-based MTOCs are likely to: (1) be enriched with factors that promote microtubule nucleation, e.g. γ-tubulin; and (2) anchor and/or stabilize microtubule minus-ends. Based on these criteria, many cellular structures in a variety of cell types or at specific developmental stages were found to potentially have MTOC activity. The growing list of these cellular structures includes Golgi (mammalian RPE cells, mouse astrocytes, mouse and fly muscles, and fly neurons) (Efimov et al., 2007; Ori-McKenney et al., 2012; Rivero et al., 2009; Zhu & Kaverina, 2013), mitochondria (Drosophila spermatids) (Chen, Buchwalter, Kao, & Megraw, 2017), and endosomes (Drosophila and C. elegans neurons) (Liang et al., 2020; Weiner et al., 2020). This

list of acentrosomal MTOCs provides the next candidate reservoir for identifying neuronal MTOCs.

The Golgi apparatus is well characterized as a non-centrosomal MTOC in mammalian fibroblast and muscle cells (Chabin-Brion et al., 2001; Rivero et al., 2009). The Golgi in these cells recruits γ-tubulin on the cis-Golgi membrane through AKAP450, a γ-TuRC adaptor that normally found on the centrosome (Rivero et al., 2009). Super-resolution imaging reveals that microtubule minus-ends are anchored on Golgi membranes (Wu et al., 2016). These findings prompted the examination of Golgi's MTOC potential in neurons. In the soma of a neuron, Golgi forms unlinked mini-stacks; in dendrites, decentralized Golgi satellite structures, termed 'Golgi outposts', can be found in dendritic shafts and branch points (Liu et al., 2017; Ye et al., 2007). In Drosophila ddaC neurons, the Golgi outposts, particularly ones comprised of multiple compartments, coincide with sites of microtubule growth initiation (i.e. the start sites of EB1-GFP comets) (Ori-McKenney et al., 2012; Zhou et al., 2014). Elimination of γ-tubulin and peri-centrin-protein (plp), the fly ortholog of AKAP450, is found to disrupt such coincidence (Ori-McKenney et al., 2012). These findings provide initial support for Golgi as MTOC in neurons. Consistent with this model, centrosomin (cnn; which is the fly ortholog of CDK5RAP2), an activator of γ-tubulin-mediated microtubule nucleation (Choi et al., 2010), is also found to localize to Golgi outpost membranes (Yalgin et al., 2015). However, this model is challenged by a few observations. First, in fly sensory neurons, fluorescently labeled γ-tubulin (both endogenous and transgenic) is not consistently found on Golgi outposts in dendrites (Mukherjee et al., 2019; Nguyen et al., 2014). Second, delocalizing Golgi by fusing an integral Golgi protein with kinesin motor and dragging Golgi to unconventional sites does not affect γ-

tubulin localization (Nguyen et al., 2014). As such, whether Golgi and Golgi outposts function as MTOCs in neurons is still debatable. My work in Chapter 2 will provide a detailed examination of Golgi outposts' role in neuronal microtubule organization.

Recently, endosomes have risen to be another MTOC candidate in neurons. In C. elegans PVD neurons, endogenous γ-tubulin is found concentrated at the dendrite tip, localizing to early endosomes marked by Rab6 and Rab11. Elimination of γ-tubulin in these neurons reduces the proportion of minus-end-distal microtubules in dendrites (Liang et al., 2020). This finding echoes a recent observation in Drosophila ddaE neurons. Axin, which is a Wnt signaling-related protein that is present on endosomes, may serve as an adapter protein for γ-tubulin (Weiner et al., 2020). These studies support a link between γ-tubulin and endosomes.

While organelle-based MTOCs were the focus of the initial searches, decentralized MTOCs, such as augmin-mediated formation of microtubules from the sides of pre-existing microtubules (sometimes referred to as "microtubule branching"), gradually emerged for their importance in making microtubules in a variety of cell types, including neurons. The interaction between the augmin complex and γ-TuRC has been well characterized biochemically through in vitro microtubule branching assays with purified γ-TuRC and augmin components (Goshima et al., 2008; Kamasaki et al., 2013; Petry et al., 2013b). In cultured mammalian neurons, elimination of the augmin complex reduces microtubule density (Sánchez-Huertas et al., 2016). These and other data have led to the model that augmin recruits γ-tubulin ring complex to existing microtubules to allow the formation of new microtubules that align with pre-existing ones, thus reinforcing the polarity of the existing microtubule cytoskeleton (Cunha-Ferreira et al., 2018; Sánchez-Huertas et al., 2016).

Identifying neuronal MTOCs is only the very first step of understanding the ways in which the microtubule cytoskeleton is constructed. Such processes involve a complex and dynamic coordination between different MTOCs. How different MTOCs may collectively construct the microtubule cytoskeleton, and how different MTOCs at a particular stage or location may be activated or deactivated are important future questions.

Establishing and maintaining polarized microtubules in neurons

The central feature of the neuronal microtubule cytoskeleton is the distinct microtubule polarities in dendrites and the axon. In vitro and in vivo studies suggest that young neurites contains microtubules of mixed microtubule polarity, which implies that the observed microtubule polarity in different neuronal compartments of a mature neuron is established during development (del Castillo et al., 2015; Lu, Fox, Lakonishok, Davidson, & Gelfand, 2013; Yau et al., 2016). Once the initial microtubule polarity is established, maintaining the formed microtubule polarity would require molecular machineries, e.g. MTOCs, in the dendrites and the axon, which favor the accumulation of microtubules in a particular direction.

In current models, microtubules in undifferentiated neurites are thought to be generated from centrosomes or other unidentified sources, and transported to the neurites through a molecular motor-dependent sliding mechanism (Winding, Kelliher, Lu, Wildonger, & Gelfand, 2016; W. Yu, Centonze, Ahmad, & Baas, 1993). In S2 cells and isolated Drosophila brain primary cultures, photo-switchable EOS labeled microtubules are photoactivated in the cell body and the labeled microtubules can be subsequently tracked into neurites in a motor depended manner (Lu et al., 2013; Winding et al., 2016). Such activity is thought to be active in

the early stages of neurite development. In neuronal cultures, this type of microtubule sliding activity is ceased within 1h post plating (Del Castillo, Lu, Winding, Lakonishok, & Gelfand, 2015; Lu et al., 2013).

Microtubule polarity is established as the neurites acquire molecular feature of dendrites or axons. In this phase, molecular motor continues playing crucial roles. In Drosophila and mammalian neuronal models, compromised dynein activity alters axonal microtubule polarity (del Castillo et al., 2015; Rao et al., 2017). Reduced kinesin-1 or other plus-end-directed motor activity in C. elegans and cultured neurons, on the other hand, fail to establish dendritic microtubule polarity (Yan et al. 2013; Yu et al. 2000). These observations together with molecular motor's sliding capability lead to the following model: plus-end-directed motors, like kinesin-1, are responsible for sliding/transporting minus-end-distal microtubules to the developing dendrite and sorting out plus-end-distal microtubules from it. The minus-end-directed motor, dynein, does the opposite, accumulating plus-end-distal microtubules in the axon. The important distinction between this polarity establishment period and the initial neurite extension phase may lie in the differential localization or activation of different motor proteins in dendrites and axons. For instance, local activation of dynein at the axon initial segment (AIS) is thought to regulate its compartment-specific activity (Klinman, Tokito, & Holzbaur, 2017). However, it is unknown whether such regulation occurs at initial axon developing stages and whether dendrite possess similar regulation mechanisms for the activation of kinesin motors.

While motor-dependent lateral transport of microtubules plays an important role during the initial neurite extension and polarization, de novo generation of microtubules from other

pathways, like γ-TuRC-mediated microtubule nucleation, are also crucial for this process. In mouse, γ-tubulin is continuingly expressed in the nervous system over the course of development (Sánchez-Huertas et al., 2016), and γ-tub depletion reduces neuronal microtubule density (Sánchez-Huertas et al., 2016).

However, one important question is: how de novo generation of microtubules contributes to microtubule polarity in dendrites and axons? Such a model relies on the microtubule generating mechanisms that favor the accumulation of microtubules in a particular direction within the respective neuronal compartments. In the case of nucleation, γ-tub or γ-TuRC complex, which have limited nucleation activity by themselves, are free floating in the neuronal cytosol (Choi et al., 2010; Sánchez-Huertas et al., 2016); additional nucleation promoting factors could concentrate on a particular MTOC, which is arranged in certain location and geometry, to allow restricted generation of microtubules in certain direction. In axons, augmin complex is thought to interact with microtubules, recruiting γ-TuRC and stimulating microtubule nucleation in a paralleled direction to the existing plus-end-distal microtubule (Cunha-Ferreira et al., 2018; Sánchez-Huertas et al., 2016). Consistent with this model, elevated γ-TuRC activity by increasing CDK5Rap2, a γ-TuRC activator, alters axonal microtubule polarity (Sánchez-Huertas et al., 2016). Similarly, expression of γ-tubulin hypermorph alleles also cause altered microtubule polarity in Drosophila axons (Nguyen et al., 2014). In dendrites, such a mechanism is yet to be characterized. In chapter 2, I have examined whether Golgi outposts, a potential MTOC candidate, may be responsible for minus-end-distal microtubules in dendrites.

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Chapter 2: Golgi outposts locally regulate microtubule orientation in neurons but are not required for the overall polarity of the dendritic cytoskeleton

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Abstract

Microtubule-organizing centers (MTOCs) often play a central role in organizing the cellular microtubule networks that underlie cell function. In neurons, microtubules in axons and dendrites have distinct polarities. Dendrite-specific Golgi outposts, in particular multi-compartment outposts, have emerged as regulators of acentrosomal microtubule growth, raising the question of whether outposts contribute to establishing or maintaining the overall polarity of the dendritic microtubule cytoskeleton. Using a combination of genetic approaches and live imaging in a Drosophila model, we found that dendritic microtubule polarity is unaffected by eliminating known regulators of Golgi-dependent microtubule organization including the *cis*-Golgi matrix protein GM130, the fly AKAP450 ortholog pericentrin-like protein (plp), and centrosomin (cnn). This indicates that Golgi outposts are not essential for the formation or maintenance of a dendrite-specific cytoskeleton. However, the over-expression of GM130, which promotes the formation of ectopic multi-compartment units, is sufficient to alter dendritic microtubule polarity. Axonal microtubule polarity is similarly disrupted by the presence of ectopic multi-compartment Golgi outposts. Notably, multi-compartment outposts alter microtubule polarity independently of microtubule nucleation mediated by the γ-tubulin ring complex (γ-TuRC). Thus, although Golgi outposts are not essential to dendritic microtubule polarity, altering their organization correlates with changes to microtubule polarity. Based on these data, we propose that the organization of Golgi outposts is carefully regulated to ensure proper dendritic microtubule polarity.

Introduction

Proper neuronal structure and function depends on the underlying microtubule cytoskeleton, which is uniquely organized in axons and dendrites. The compartment-specific orientation of microtubules is thought to contribute to the specific morphologies and functions of these compartments. In axons, microtubules are uniformly oriented with their plus-ends positioned distal to the cell body. In contrast, microtubule polarity in dendrites is mixed to varying degrees, and the percentage of microtubules of a particular orientation differs locally within the dendritic arbor. How the distinctly organized cytoskeletons in axons and dendrites are created and maintained remains an open question. Microtubules are often generated at and organized by cellular structures called microtubule-organizing centers (MTOCs) that anchor and stabilize microtubules and support microtubule nucleation (SANCHEZ AND FELDMAN 2017; WU AND AKHMANOVA 2017). The centrosome is one example of a well-studied MTOC. Although neurons have a centrosome, recent work indicates that the neuronal centrosome does not have a major role in either generating or anchoring dendritic or axonal microtubules (STIESS *et al.* 2010; NGUYEN *et al.* 2011; SANCHEZ-HUERTAS *et al.* 2016). The centrosome, however, is not the only organelle that functions as an MTOC (SANCHEZ AND FELDMAN 2017; WU AND AKHMANOVA 2017). The Golgi apparatus and non-conventional Golgi structures such as Golgi elements and Golgi outposts have emerged as potential MTOCs in several cell types, including epithelia, muscles, and neurons (RIOS 2014; SANDERS AND KAVERINA 2015; MARTIN AND AKHMANOVA 2018). Unlike centrosomes, which support the nucleation of microtubules radially, Golgi-based microtubule nucleation can create asymmetric microtubule arrays (EFIMOV *et al.* 2007; ZHU AND KAVERINA 2013). Such asymmetric Golgi-derived microtubule arrays have also been shown to contribute

to cell polarity (ZHU AND KAVERINA 2013; RIOS 2014). Thus, Golgi in neurons have the potential to shape the polarity of the microtubule cytoskeleton, and influence neuronal polarity, by selectively seeding or stabilizing microtubules in a particular orientation.

In developing flies and mammals, many neurons have Golgi in the form of mini stacks called "outposts" that localize specifically to dendrites and have a different structure than the somatic Golgi (GARDIOL *et al.* 1999; PIERCE *et al.* 2001; HORTON AND EHLERS 2003; YE *et al.* 2007; LIU *et al.* 2017; RAO *et al.* 2018; TANN AND MOORE 2019). The term Golgi outposts is used to refer generally to a heterogenous population of dendritic Golgi composed of one or more compartments. In studies using fluorescently tagged End-binding 1 (EB1::GFP), which marks growing microtubule ends, Golgi outposts have been shown to correlate with microtubule growth initiation sites in developing dendrites (ORI-MCKENNEY *et al.* 2012; ZHOU *et al.* 2014; YALGIN *et al.* 2015). Decreasing the levels of proteins involved in microtubule nucleation, such as γ-tubulin and its interactors centrosomin (cnn, the fly ortholog of CDK5RAP2) and pericentrin-like protein (plp, the Drosophila ortholog of AKAP450), disrupts the correlation between microtubule growth initiation sites and outposts and perturbs dendrite branch growth (ORI-MCKENNEY *et al.* 2012; YALGIN *et al.* 2015). This correlation has led to the model that Golgi outposts function as MTOCs that support acentrosomal nucleation and the directional growth of microtubules during dendrite branch formation (DELANDRE *et al.* 2016). These studies have focused on the connection between Golgi outposts, microtubule growth, and dendrite growth, leaving unanswered the broader question of whether outposts have a role in creating and/or maintaining the distinct polarity of the dendritic microtubule cytoskeleton. Notably, the link between microtubule growth and Golgi outposts relies on the compartmental organization of

outposts: multi-compartment dendritic Golgi outposts are more likely than single-compartment outposts to correlate with microtubule growth start sites (ZHOU *et al.* 2014) and dragging individual Golgi compartments into the axon is not sufficient to alter axonal microtubule organization (NGUYEN *et al.* 2014). The dendrite-specific localization of Golgi outposts and their MTOC potential raises the possibility that Golgi outposts, and in particular multi-compartment outposts, may play a role in establishing and/or maintaining the unique polarity of the dendritic microtubule cytoskeleton; this idea, however, has not been tested.

Here we investigated whether Golgi outposts are necessary for the compartment-specific orientation of dendritic microtubules. In particular, we asked whether outposts are necessary for the formation of the minus-end-distal microtubules that are specific to the dendritic cytoskeleton. To test this notion, we leveraged a combination of genetics and live imaging and used the class IV dendritic arborization (da) neurons in Drosophila as a model. In the class IV da neurons, dendritic microtubules are predominately oriented with their minus-ends distal to the cell body, providing an advantageous paradigm to detect changes in microtubule orientation. If Golgi outposts regulate the overall polarity of the dendritic microtubule cytoskeleton, then blocking the MTOC activity of Golgi should disrupt the stereotyped minus-ends-distal orientation of dendritic microtubules. We targeted the *cis*-Golgi matrix protein GM130, which has two key roles: first, work done in mammalian cells has shown that GM130 recruits AKAP450, which in turn recruits protein complexes that nucleate, tether, and stabilize microtubules (RIVERO *et al.* 2009; HURTADO *et al.* 2011; ROUBIN *et al.* 2013; WU AND AKHMANOVA 2017). Second, GM130 is needed for proper Golgi structure and connects Golgi compartments to form multi-compartment units, including outposts (NAKAMURA *et al.* 1995;

BARR *et al.* 1997; KONDYLIS *et al.* 2005; ZHOU *et al.* 2014; LIU *et al.* 2017; LOWE 2019). We found that the global orientation of dendritic microtubules is unaffected by the loss of GM130 or the fly AKAP450 ortholog plp. Cnn, which is proposed to orient Golgi outpost-associated microtubule growth (YALGIN *et al.* 2015), is also dispensable. This suggests that Golgi outposts do not have an essential role in establishing the overall polarity of the dendritic microtubule cytoskeleton. Our studies of GM130 over-expression, however, reveal that inducing the formation of ectopic multi-compartment units correlates with a disruption in microtubule polarity. This suggests that compartment connectedness is critical to the microtubule organization capacity of outposts. Interestingly, the ability of multi-compartment outposts to organize microtubules is independent of γ-TuRC-mediated microtubule nucleation, which suggests that outposts can regulate microtubule polarity via microtubule anchoring or stabilizing.

Results

Golgi outposts are not essential to the overall polarity of the dendritic microtubule cytoskeleton

To determine whether Golgi outposts play a critical role in determining the unique polarity of the dendritic microtubule cytoskeleton, we turned to the class IV da neurons in Drosophila as a model. The da neurons are an ideal model as they are easily accessible for live imaging, the orientation of microtubules in the dendritic microtubule cytoskeleton is well-defined, and there is a wealth of tools for labeling and manipulating Golgi, microtubules, and microtubule regulators. In the class IV da neurons, Golgi compartments are present in the cell body and throughout the dendritic arbor, but most are found close to the cell body (Figure 1, A-C). Indeed, GalNacT2-positive *trans* Golgi compartments are seldom present beyond 95 µm of the cell body. Next, we quantified the number of multi-compartment outposts in the arbor. Consistent with previous work, we defined multi-compartment Golgi outposts as units with at least two compartments (ZHOU *et al.* 2014). Here, we used markers of the *medial* and *trans* Golgi, namely ManII::GFP and GalNacT2::TagRFP, respectively. Fewer than half of the ManII-positive Golgi outposts colocalized with GalNacT2, indicating ManII::GFP predominantly labels single-compartment Golgi outposts (Figure 1D). In contrast, the majority of GalNacT2-positive outposts colocalized with ManII::GFP (Figure 1D), which suggests that most multi-compartment Golgi outposts cluster relatively close to the cell body. Given that Golgi-associated microtubule growth correlates predominantly with multi-compartment outposts (ZHOU *et al.* 2014), this suggests that outposts in the proximal arbor may be most likely to regulate microtubule organization.

To determine whether Golgi outposts function to create or maintain the dendrite-specific orientation of microtubules, we analyzed microtubule polarity in neurons in which we eliminated the *cis*-Golgi matrix protein GM130. GM130 both recruits the protein machinery for MTOC activities (microtubule nucleation, anchoring, and stabilization) and contributes to forming multi-compartment Golgi units (NAKAMURA *et al.* 1995; BARR *et al.* 1997; KONDYLIS *et al.* 2005; ZHOU *et al.* 2014; SANDERS AND KAVERINA 2015; LIU *et al.* 2017; MARTIN AND AKHMANOVA 2018; LOWE 2019). We found that the percentage of multi-compartment outposts decreases when GM130 is absent, which supports the model that GM130 participates in connecting Golgi compartments in neurons (Figure 1D)(ZHOU *et al.* 2014). The loss of GM130 also reduces the number of ManII- and GalNacT2-positive Golgi outposts, consistent with previous reports (LIU *et al.* 2017). To read-out microtubule orientation, we used EB1::GFP, whose binding to growing microtubule ends produces a comet-like trajectory. The majority of microtubule growth occurs at plus-ends, which also grow faster than minus-ends, enabling a clear distinction of plus- and minus-end growth and thus microtubule polarity (FENG *et al.* 2019). As previously reported, in control neurons dendritic microtubules are oriented predominantly minus-ends-distal (Figure 1E). Strikingly, eliminating GM130 had no effect on the overall polarity of microtubules within the proximal dendritic arbor where multi-compartment Golgi outposts clustered (Figure 1E). The overall frequency of microtubule growth was also unaffected by the loss of GM130 (Figure 1F). In mammalian cells, GM130 affects microtubule organization through the recruitment of AKAP450, whose fly ortholog is plp (MARTINEZ-CAMPOS *et al.* 2004; RIOS 2014; SANDERS AND KAVERINA 2015; MARTIN AND AKHMANOVA 2018). Plp in fly neurons has likewise been implicated in the MTOC activity of Golgi outposts (ORI-MCKENNEY *et al.* 2012). Similar to the loss of GM130,

eliminating plp had no effect on dendritic microtubule polarity (Figure 1E). Plp is proposed to regulate microtubule growth at Golgi outposts in conjunction with cnn and γ-tubulin, the latter of whose activity is controlled by the γ-tubulin ring complex (γ-TuRC) (ORI-MCKENNEY *et al.* 2012; YALGIN *et al.* 2015). Eliminating either cnn or the γ-TuRC component dGrip75 does not alter dendritic microtubule polarity (Figure 1E). Thus, the results of our experiments provide compelling evidence that Golgi outposts do not play an essential role in creating or maintaining the overall polarity of the dendritic microtubule cytoskeleton.

Loss of GM130 significantly reduces misoriented microtubules in *nudE*$^{-}$ axons

The results of our GM130 loss-of-function experiments in da neurons suggest that the overall polarity of the dendritic microtubule cytoskeleton does not depend on Golgi outposts. Microtubule polarity, however, varies within the dendritic arbors of da neurons (STONE *et al.* 2008), and it is possible that Golgi outposts are nevertheless sufficient to locally influence microtubule polarity as previously reported (ORI-MCKENNEY *et al.* 2012; YALGIN *et al.* 2015; DELANDRE *et al.* 2016). To test the idea that outposts have the capacity to affect microtubule polarity, we used a paradigm in which Golgi outposts are ectopically localized to axons, a compartment from which outposts are normally excluded and in which microtubules are uniformly oriented with their plus-ends distal. If Golgi outposts are capable of regulating microtubule organization, we reasoned that their ectopic presence in axons should disrupt the uniform plus-ends-distal array of axonal microtubules. To ectopically localize Golgi outposts to axons, we relied on mutations that disrupt the activity of the molecular motors dynein and kinesin-1, which transport outposts (YE *et al.* 2007; ZHENG *et al.* 2008; ARTHUR *et al.* 2015; LIN *et*

al. 2015; KELLIHER *et al.* 2019). While disrupting the activity of either motor results in Golgi outposts invading axons, we and others have previously shown that these ectopic outposts do not always correlate with a change in axonal microtubule polarity (YE *et al.* 2007; NGUYEN *et al.* 2014; KELLIHER *et al.* 2019). Analyzing these different motor mutants and the outposts in their axons enables us to determine whether Golgi outposts are sufficient to affect microtubule polarity and to then identify the factors that are essential for this activity.

The loss of dynein activity alters both Golgi outpost localization and axonal microtubule polarity (ZHENG *et al.* 2008; ARTHUR *et al.* 2015; DEL CASTILLO *et al.* 2015; KLINMAN *et al.* 2017; RAO *et al.* 2017). We first asked whether the misoriented microtubules in the axons of dynein loss-of-function neurons depend on the ectopic Golgi outposts. The multi-subunit dynein motor complex has several cofactors that are important for its activity (RECK-PETERSON *et al.* 2018); in Drosophila neurons, this includes the conserved cofactor nudE (ARTHUR *et al.* 2015). In the absence of nudE, axons are infiltrated by multi-compartment Golgi outposts (Figure 2, A and B)(ARTHUR *et al.* 2015). In the axons of these $nudE^{39A/Df}$ mutant neurons, EB1::GFP comets travel both anterograde and retrograde, which indicates a disruption in axonal microtubule polarity (Figure 2, C and D). Although EB1::GFP can also bind to slowly growing microtubule minus-ends (FENG *et al.* 2019), the speed of the retrograde comets in the $nudE^{39A/Df}$ mutant axons is indicative of microtubule plus-end growth (Figure 2E). Thus, the ectopic axonal Golgi outposts in the $nudE^{39A/Df}$ mutant neurons correlate with a change in axonal microtubule polarity.

To determine whether ectopic Golgi outposts might contribute to the alteration in axonal microtubule polarity in $nudE^{39A/Df}$ mutant axons, we eliminated GM130. By itself, the loss of GM130 did not affect either the orientation of axonal microtubules or the localization of

Golgi outposts (Figure 2, A-D). However, eliminating GM130 significantly reduced the number of misoriented minus-end-distal microtubules in *nudE*$^{39A/Df}$ mutant axons (Figure 2, C and D). ManII-positive Golgi were still present in the *nudE*$^{39A/Df}$ *GM130*$^{23/Df}$ double-mutant axons, but, strikingly, the loss of GM130 suppressed the axonal mislocalization of GalNacT2-positive outposts in *nudE*$^{39A/Df}$ mutant neurons (Figure 2, A and B). A little over half of the ManII-positive outposts in the *nudE*$^{39A/Df}$ mutant axons were multi-compartment, but there were virtually no multi-compartment outposts in the axons of *nudE*$^{39A/Df}$ *GM130*$^{23/Df}$ double-mutant neurons, likely because the loss of GM130 suppressed the axonal mislocalization of the GalNacT2-positive compartments. These data suggest that microtubule polarity may be affected by multi-compartment, but not single-compartment, Golgi outposts.

Notably, the loss of GM130 did not completely suppress the appearance of misoriented microtubules. This may be due to the "microtubule gatekeeper" role that dynein is proposed to play in maintaining axonal microtubule polarity. In addition to carrying cargo, dynein also transports, or slides, microtubules (RAO AND BAAS 2018). Dynein anchored in the proximal axon translocates microtubules into or out of the axon and prevents the entry of minus-end-distal microtubules (DEL CASTILLO *et al.* 2015; RAO *et al.* 2017; RAO AND BAAS 2018). Our data suggest that dynein also maintains axonal microtubule polarity by excluding Golgi outposts that have the capacity to induce changes in microtubule organization.

The presence of ectopic Golgi outposts in the *nudE*$^{39A/Df}$ mutant axons also correlates with the formation of ectopic axonal branches (ARTHUR *et al.* 2015). The axons of neurons lacking nudE develop multiple fine branches that run parallel to the main axon but terminate before reaching the ventral nerve cord; occasionally ectopic branches even extend back toward

the cell body and dendrites (Figure 2, F and G). Since dendritic Golgi outposts have been correlated with dendrite branch formation and stability and loss of GM130 decreases branch number (YE *et al.* 2007; ORI-MCKENNEY *et al.* 2012; ZHOU *et al.* 2014; YALGIN *et al.* 2015; LIU *et al.* 2017), we tested whether Golgi outposts might be implicated in the formation of ectopic branches that sprout from the *nudE*$^{39A/Df}$ mutant axons. We found that loss of GM130 suppressed the axonal morphology defects of the *nudE*$^{39A/Df}$ mutant axons, implicating the mislocalized Golgi outposts in the growth of ectopic axonal branches (Figure 2, F and G). Together, these data suggest that the ectopic Golgi outposts may be a key contributing factor to both the cytoskeletal and morphological defects of the *nudE*$^{39A/Df}$ mutant axons. Thus, Golgi outposts are likely capable of inducing changes both in the microtubule cytoskeleton and neurite branching.

Golgi outposts affect microtubule polarity independently of γ-TuRC-mediated microtubule nucleation

Dendritic Golgi outposts have been reported to serve as platforms for oriented microtubule growth during dendrite branch extension (ORI-MCKENNEY *et al.* 2012; YALGIN *et al.* 2015). This suggests that Golgi outposts might influence microtubule polarity by controlling microtubule nucleation. Therefore, we tested whether the misoriented microtubules in the *nudE*$^{39A/Df}$ mutant axons resulted from ectopic nucleation at Golgi outposts. Microtubule nucleation at Golgi membranes is templated by γ-tubulin whose nucleation activity is regulated by additional proteins, including cnn and γ-TuRC components (SANDERS AND KAVERINA 2015; MARTIN AND AKHMANOVA 2018; TANN AND MOORE 2019). In dendrites, cnn has been implicated in

regulating the directional growth of Golgi-derived microtubules during branching (YALGIN *et al.* 2015). Thus, we focused on whether γ-TuRC-mediated microtubule nucleation might mediate the Golgi-induced change in microtubule polarity in $nudE^{39A/Df}$ mutant axons.

We have previously shown that reducing γ-tubulin does not suppress the appearance of minus-end-distal microtubules in $nudE^{39A/Df}$ mutant axons (ARTHUR *et al.* 2015). Nonetheless, we followed-up our earlier findings by testing the γ-tubulin regulator cnn and the γ-TuRC component GCP4, known as dGrip75 in Drosophila (VEROLLET *et al.* 2006). Consistent with our prior report, we found that eliminating either cnn ($cnn^{HK21/Df}$) or dGrip75 ($dGrip75^{175/Df}$) does not suppress the formation of misoriented microtubules in $nudE^{39A/Df}$ mutant axons (Figure 3, A and B). Altogether, our results suggest that the ectopic Golgi outposts have the capacity to affect microtubule polarity but do so through a pathway that is independent of γ-TuRC-mediated microtubule nucleation.

Elevating GM130 levels increases the number of multi-compartment Golgi and alters microtubule polarity

Our manipulations of GM130 in $nudE^{39A/Df}$ mutant neurons indicate that ectopic multi-compartment, but not single-compartment, Golgi outposts disrupt axonal microtubule polarity (Figure 2, A and B). As previously mentioned, GM130 is implicated in both the MTOC activity of Golgi and in promoting the formation of multi-compartment Golgi units (ZHOU *et al.* 2014; MARTIN AND AKHMANOVA 2018). Connectedness between Golgi compartments may be important to the coordinated regulation of microtubules by protein complexes that are present on the *cis* and *trans* Golgi compartments (RIOS 2014; SANDERS AND KAVERINA 2015). Thus, our results and the

work of others suggests that the ability of Golgi to regulate microtubule polarity may depend on the formation of multi-compartment units. We asked whether increasing GM130 levels would be sufficient to increase the number of multi-compartment Golgi outposts and to alter microtubule polarity in dendrites. We found that GM130 over-expression increased the number of multi-compartment outposts in dendrites as previously reported (ZHOU *et al.* 2014) and that these additional outposts were more prevalent in dendrite branches than branch points (Figure 4A). Thus, our results provide additional support to the idea that GM130 is integral to the connectedness of Golgi compartments in neurons (ZHOU *et al.* 2014; LIU *et al.* 2017); this is significant given that the role of GM130 in Golgi stack formation in other cell types has been debated (KONDYLIS AND RABOUILLE 2003; PUTHENVEEDU *et al.* 2006; MARRA *et al.* 2007; BASCHIERI *et al.* 2014; TORMANEN *et al.* 2019). Notably, this increase in multi-compartment units within branches correlated with an increase in anterograde EB1::GFP comets that originated within branches (there was also a mild but significant increase in the anterograde comets that originated from the cell body; Figure 4B). There was no significant increase in anterograde comets that originated at branch points and the frequency of comets that changed direction at branch points was also unaffected (Figure 4B). Others have suggested that multi-compartment Golgi outposts are prime sites of EB1::GFP comet initiation (ZHOU *et al.* 2014), leading us to propose that the additional anterograde EB1::GFP comets we observe in the neurons over-expressing GM130 originate from ectopic multi-compartment outposts. Altogether, these data are consistent with the model that the formation of ectopic multi-compartment outposts is sufficient to alter microtubule polarity in dendrites.

To further test the idea that Golgi compartmentalization correlates with an effect on microtubule polarity, we turned to a mutation in *Kinesin heavy chain* (*Khc*^{E177K}) that enhances kinesin-1 activity by disrupting motor autoinhibition (KELLIHER *et al.* 2018). Golgi outposts mislocalize to *Khc*^{E177K} mutant axons; however, unlike in *nudE*$^{39A/Df}$ mutant axons, the polarity of axonal microtubules is not significantly affected (KELLIHER *et al.* 2018). Our results indicate that the compartmental organization of Golgi outposts is key to their ability to affect microtubule polarity in dendrites. This led us to characterize the compartmental organization of the ectopic Golgi outposts in the *Khc*^{E177K} and *nudE*$^{39A/Df}$ mutant axons to determine whether the differences in axonal microtubule polarity in the two mutants might correlate with differences in Golgi compartmentalization. More specifically, we reasoned that there may be a higher number of multi-compartment Golgi outposts in *nudE*$^{39A/Df}$ mutant axons, which have altered axonal microtubule polarity, than in the *Khc*^{E177K} mutant axons, which do not.

We analyzed the distribution of ManII::GFP and GalNacT2::TagRFP in *Khc*^{E177K} and *nudE*$^{39A/Df}$ mutant axons. Consistent with the idea that compartment connectedness enables Golgi to influence microtubule polarity, we found that there was a higher percentage of multi-compartment Golgi outposts in the *nudE*$^{39A/Df}$ mutant axons than the *Khc*$^{E177K/27}$ mutant axons (Figure 5, A and B). Notably, *Khc*$^{E177K/27}$ mutant axons contained equal numbers of GalNacT2-positive outposts and more ManII-positive outposts than *nudE*$^{39A/Df}$ mutant axons (Figure 5B). This indicates that the *Khc*$^{E177K/27}$ mutant axons have just as many Golgi compartments as the *nudE*$^{39A/Df}$ mutant axons, but that the compartments are not as connected. Correspondingly, microtubule polarity is largely normal in *Khc*$^{E177K/27}$ mutant axons, which is in contrast to the *nudE*$^{39A/Df}$ mutant axons (Figure 5C). Combined, these results suggest that nudE (and dynein

activity) are needed for the proper localization, but not the formation, of multi-compartment Golgi outposts. In contrast, enhancing kinesin-1 activity both perturbs Golgi localization and antagonizes the connectedness of Golgi compartments.

We then asked whether increasing GM130 might alter the polarity of axonal microtubules in the *Khc*$^{E177K/27}$ mutant axons, which contain predominantly single-compartment Golgi outposts. In control neurons, the over-expression of GM130 alone did not affect axonal microtubule polarity (Figure 5D). The over-expression of GM130 in *Khc*$^{E177K/27}$ mutant neurons both increased the percentage of multi-compartment outposts and resulted in the appearance of ectopic minus-end-distal microtubules (Figure 5, A and D). Altogether, our data support the idea that multi-compartment Golgi outposts have the capacity to remodel microtubule polarity locally even if they are not essential to the overall polarity of the dendritic cytoskeleton.

Discussion

Microtubules in axons and dendrites have distinct polarities. In a variety of cell types microtubule orientation is regulated by MTOCs, raising the question of whether neurons have MTOCs that carry out a similar function. In dendrites, Golgi outposts are likely candidates (ORI-MCKENNEY *et al.* 2012; ZHOU *et al.* 2014; YALGIN *et al.* 2015). The compartmental organization of Golgi outposts and their correlation with microtubule growth initiation sites were recently shown to depend on the *cis*-Golgi matrix protein GM130, the fly AKAP450 ortholog plp, and cnn (ORI-MCKENNEY *et al.* 2012; ZHOU *et al.* 2014; YALGIN *et al.* 2015). We found that the elimination of these factors does not affect the predominantly minus-end-distal orientation of microtubules in class IV da neuron dendrites, suggesting that Golgi outposts are not necessary for the unique polarity of the dendritic microtubule network. This raises the question of whether Golgi outposts might have any capacity to affect microtubule orientation. Our analysis of outposts in dendrites and outposts mislocalized to axons suggests that ectopic multi-compartment Golgi outposts are sufficient to alter microtubule polarity and likely do so independently of microtubule nucleation. We propose that the unique polarity of the dendritic microtubule cytoskeleton is established and maintained independently of Golgi outposts, but that multi-compartment Golgi outposts may have the capacity to locally influence microtubule polarity during events such as dendrite branch extension.

Studies carried out in mammalian cells have shown that Golgi serve as platforms for microtubule nucleation, anchoring, and stabilization (SANDERS AND KAVERINA 2015; MARTIN AND AKHMANOVA 2018; FU *et al.* 2019). These activities arise from distinct protein complexes, whose recruitment to the *cis* Golgi depends on GM130. Golgi are generally thought to anchor and

stabilize microtubules that have been generated at Golgi membranes, although the molecular mechanism by which Golgi would selectively capture these microtubules is unclear (ZHU AND KAVERINA 2013; MARTIN AND AKHMANOVA 2018). Our studies indicate that γ-TuRC-mediated microtubule nucleation is dispensable for Golgi outposts to affect microtubule polarity. Consistently, a new study indicates that γ-tubulin only rarely associates with outposts (MUKHERJEE *et al.* 2019). One possibility is that microtubules are nucleated at Golgi independently of γ-tubulin. For example, it was recently reported that the mammalian tubulin polymerization promoting protein (TPPP) nucleates microtubules at Golgi outposts independently of γ-tubulin; however, TPPP is enriched in glia cells, not neurons, in the mammalian nervous system (FU *et al.* 2019). Another possibility is that Golgi outposts may be able to capture and stabilize microtubules that are growing in the vicinity of outposts in the relatively confined spaces of dendrites (and axons). For example, in the *nudE* mutant axons, ectopic multi-compartment Golgi outposts may alter microtubule polarity by stabilizing nearby misoriented microtubules that would otherwise be eliminated. Thus, outposts may influence microtubule polarity by tethering and stabilizing microtubules that are not generated at Golgi.

Our results raise the question of what molecular players may organize microtubules at Golgi outposts in neurons. A key component of the complexes that tether and stabilize microtubules on Golgi in vertebrate cells is myomegalin (ROUBIN *et al.* 2013; WANG *et al.* 2014; WU *et al.* 2016). However, there is no clear Drosophila ortholog of myomegalin, making it difficult to directly test this model. The most closely related family member in flies is cnn, which is implicated in activating γ-tubulin-templated microtubule assembly (CHOI *et al.* 2010; ROUBIN *et al.* 2013) and which we have shown is not necessary for Golgi outposts to alter axonal

microtubule polarity. Moreover, new findings call into question whether cnn strongly associates with Golgi in fly neurons (MUKHERJEE *et al.* 2019). Another component of the Golgi-associated complex that anchors and stabilizes microtubules is the microtubule minus-end-binding protein CAMSAP2, whose fly ortholog is Patronin. Recent studies using da neurons have shown that Patronin is needed for minus-end-distal microtubules in dendrites, and likely acts by antagonizing the kinesin-13 microtubule depolymerase KLP10A (FENG *et al.* 2019; WANG *et al.* 2019). Our preliminary analysis of Patronin localization, however, makes it unclear whether Patronin localizes to or functions at Golgi outposts. In work using mitotic mammalian cells, GM130 has also been implicated in the stabilization of microtubules on Golgi through a mechanism that depends on the microtubule-associated protein TPX2; however, TPX2 structure and function are likely not conserved between mammals and flies (GOSHIMA 2011; HAYWARD *et al.* 2014; WEI *et al.* 2015). Thus, additional studies are needed to identify the molecular players that tether and stabilize microtubules on Golgi in Drosophila neurons.

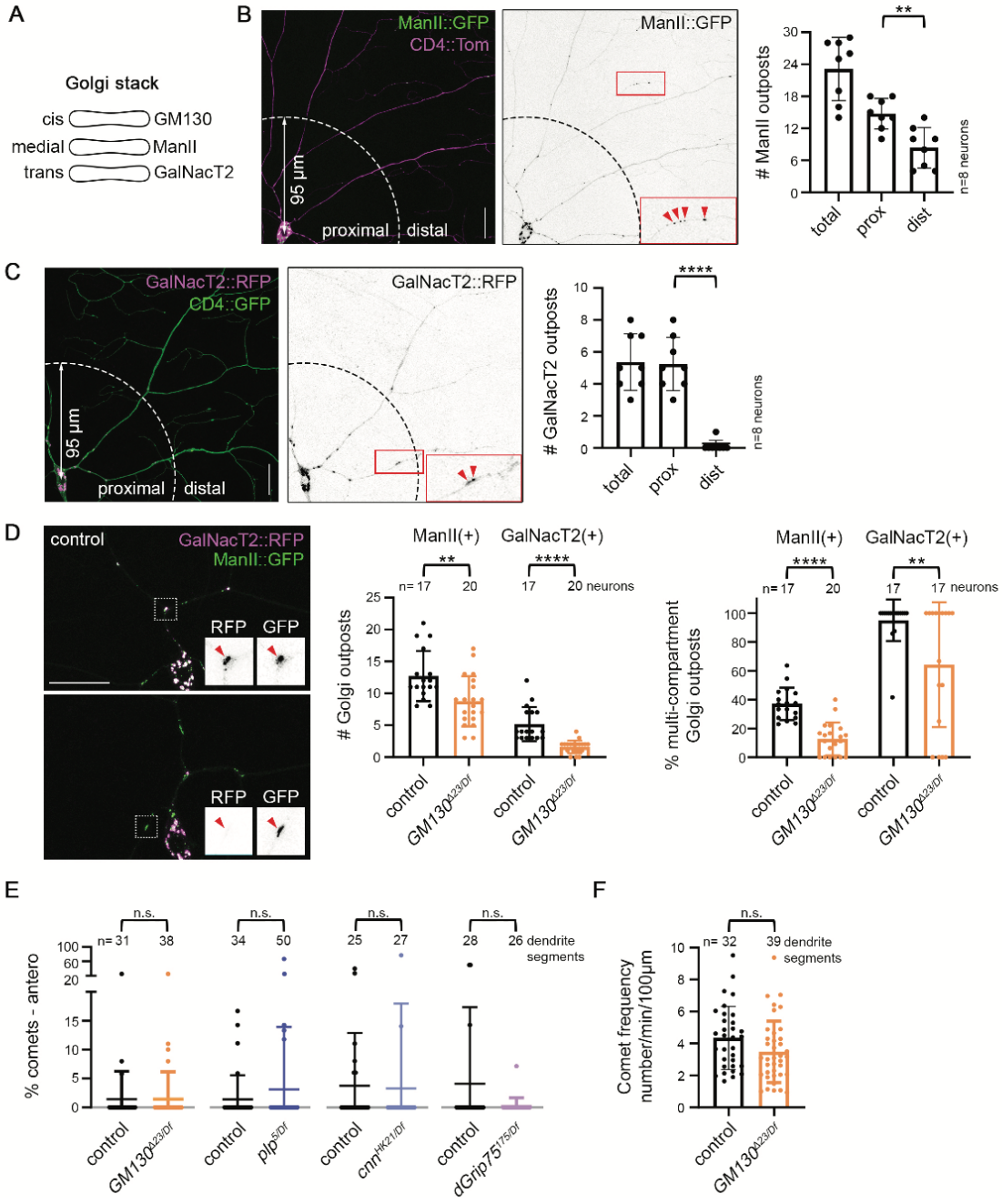
The organization of the Golgi apparatus into a multi-compartment stack gives it a morphological and functional polarity. Correspondingly, microtubules associated with Golgi are proposed to be oriented in a particular direction relative to the Golgi compartments (EFIMOV *et al.* 2007; ZHU AND KAVERINA 2013; MARTIN AND AKHMANOVA 2018). In da neurons, the correlation between microtubule polarity and Golgi compartment organization is supported by findings that microtubule growth typically initiates in a single direction from an outpost (ORI-MCKENNEY *et al.* 2012; YALGIN *et al.* 2015). This suggests a relationship between the polarity of the Golgi stack and the associated microtubules. Thus, the relative orientation of the Golgi outpost stack likely influences the polarity of Golgi-associated microtubules (DELANDRE *et al.* 2016). Our finding

that elevated GM130 levels altered microtubule polarity in dendrites may suggest that GM130 instigated the formation of misoriented Golgi stacks that in turn stabilized misoriented microtubules. Given the potential of multi-compartment outposts to influence microtubule polarity, it will be interesting to determine how Golgi outpost compartmentalization in dendrites is controlled to ensure proper dendritic microtubule polarity.

Acknowledgments

We thank the following laboratories and stock centers for generously sharing Drosophila strains: Drs. Paul Conduit (University of Cambridge) and Jordan Raff (University of Oxford) for *dGrip75* flies, Bing Ye (University of Michigan) for *UAS-GalNacT2::TagRFP* flies, and the Bloomington Drosophila Stock Center (supported by NIH P40OD018537). We thank Erik Dent and Mary Halloran (University of Wisconsin-Madison), Adrian Moore (Riken CBS), Paul Conduit (University of Cambridge), and members of the Wildonger lab for insightful discussions and feedback; and Lindsay Mosher and Dena Johnson-Schlitz for technical assistance. This work is supported by the National Institutes of Health grant R01NS102385.

Figures


A
Golgi stack
cis
medial
trans
GM130
ManII
GalNacT2
B
ManII::GFP
CD4::Tom
95 µm
proximal
distal
ManII::GFP
# ManII outposts
n=8 neurons
total
prox
dist
C
GalNacT2::RFP
CD4::GFP
95 µm
proximal
distal
GalNacT2::RFP
# GalNacT2 outposts
n=8 neurons
total
prox
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D
control
GalNacT2::RFP
ManII::GFP
RFP
GFP
RFP
GFP
ManII(+)
GalNacT2(+)
# Golgi outposts
n= 17
20
17
20 neurons
control
GM130 23/Df
% multi-compartment Golgi outposts
E
% comets - antero
n= 31
38
34
50
25
27
28
26 dendrite segments
control
GM130 23/Df
plp 5/Df
cnn HK21/Df
dGrip75 175/Df
F
Comet frequency number/min/100µm
n= 32
39 dendrite segments
control
GM130 23/Df

Fig. 1. Global dendritic microtubule polarity does not depend on Golgi outposts.

(A) Cartoon showing the compartmental distribution of GM130, ManII and GalNacT2 in a Golgi stack. (B-D) ManII::GFP-positive outposts are present throughout the class IV da neuron dendritic arbor, but fewer than half of these outposts are multi-compartment units (B, D). Multi-compartment outposts are defined as those that have overlapping ManII::GFP and GalNacT2::TagRFP signal. In contrast to ManII::GFP-positive outposts, GalNacT2::TagRFP-positive outposts cluster in the proximal arbor and nearly all GalNacT2::TagRFP-positive outposts are multi-compartment (C, D). Eliminating GM130 reduces the overall number of Golgi outposts and the percentage of outposts that are multi-compartment in the proximal arbor. The proximal arbor (prox) encompasses a radius of 95 µm from the cell body; distal (dist) is beyond this radius. Red arrowheads indicate Golgi outposts. The left side of each graph represents the fraction of ManII::GFP-positive outposts that are multi-compartment and the right side of each graph represents GalNacT2::TagRFP-positive outposts that are multi-compartment (D). **P*=0.05–0.01, ***P*=0.01–0.001, and ****P<0.0001; Student's unpaired t tests. (E) Dendritic microtubule polarity is not affected by the loss of GM130, plp, cnn, or dGrip75. Antero = anterograde. n.s.=not significant, Mann-Whitney test. (F) EB1::GFP comet frequency is normal in the dendrites of neurons lacking GM130. n.s.=not significant, Student's unpaired t-test. Microtubule polarity and EB1::GFP comet frequency were quantified in the proximal dendritic arbor, which contained the majority of multi-compartment outposts. Scale bars: 25 µm. All data are mean ± standard deviation.

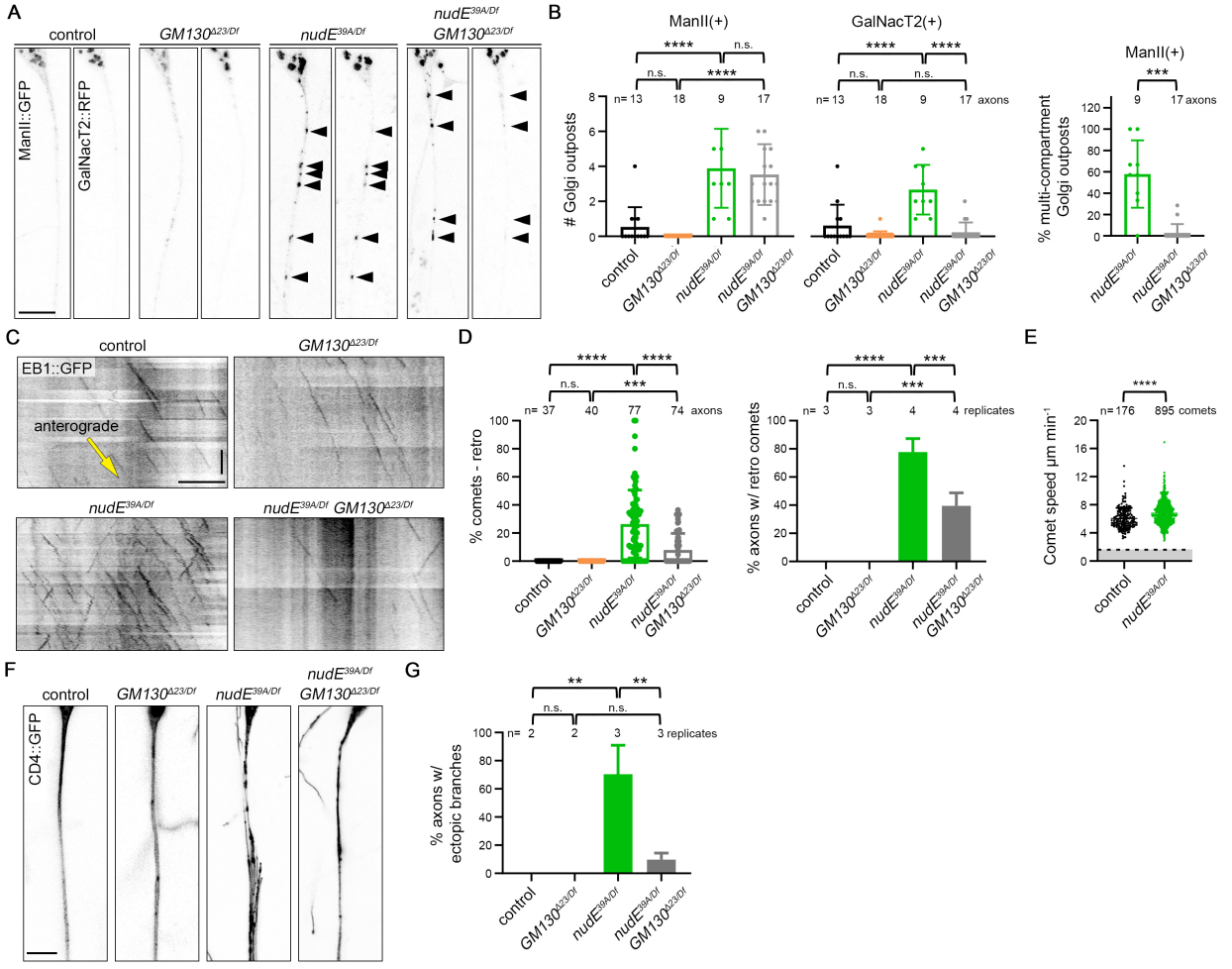

Fig. 2. Misoriented microtubules in *nudE* mutant axons are significantly reduced when GM130 is eliminated.

(A,B) Golgi outposts (marked by ManII::GFP and GalNacT2::RFP; arrowheads) mislocalize to axons in *nudE*$^{39A/Df}$ mutant neurons. In *nudE*$^{39A/Df}$ mutant neurons, the loss of GM130 does not affect the mislocalization of ManII-positive Golgi outposts, but suppresses the mislocalization of GalNacT2-positive outposts; as a result, the percentage of multi-compartment ManII-positive outposts is dramatically reduced. n.s.=not significant, ***P=0.001–0.0001, and ****P<0.0001, one-way ANOVA with Tukey's post-hoc analysis. Scale bar: 10 µm. (C,D) In the absence of nudE, axonal microtubule polarity is perturbed. Eliminating GM130 reduces the number of misoriented microtubules in *nudE*$^{39A/Df}$ mutant axons. Cell body is to the left; yellow arrow indicates the direction of anterograde comet movement. n.s.=not significant, ***P=0.001–0.0001, and

****P<0.0001, Kruskal-Wallis with post-hoc Dunn's multiple comparison analysis (% retro comets) and one-way ANOVA with Tukey's post-hoc analysis (% axons). Scale bars: 10 µm (x-axis) and 30 sec (y-axis). Antero = anterograde, retro = retrograde. (E) The speed of EB1::GFP comets in control and $nudE^{39A/Df}$ mutant axons is consistent with microtubule plus-end growth, indicating $nudE^{39A/Df}$ mutant axons indeed contain microtubules with mixed polarity (comets moving at speeds below the dotted line would be consistent with microtubule minus-end growth). ****P<0.0001, Mann-Whitney test. (F,G) The ectopic branches that sprout from $nudE^{39A/Df}$ mutant axons are suppressed by removing GM130. n=16 (control), 27 ($GM130^{\Delta 23/Df}$), 59 ($nudE^{39A/Df}$), and 41 ($GM130^{\Delta 23/Df}$; $nudE^{39A/Df}$) axons in replicates as indicated (G). n.s.=not significant and **P=0.01–0.001, one-way ANOVA with Tukey's post-hoc analysis. Scale bar: 10 µm. All data are mean ± standard deviation.

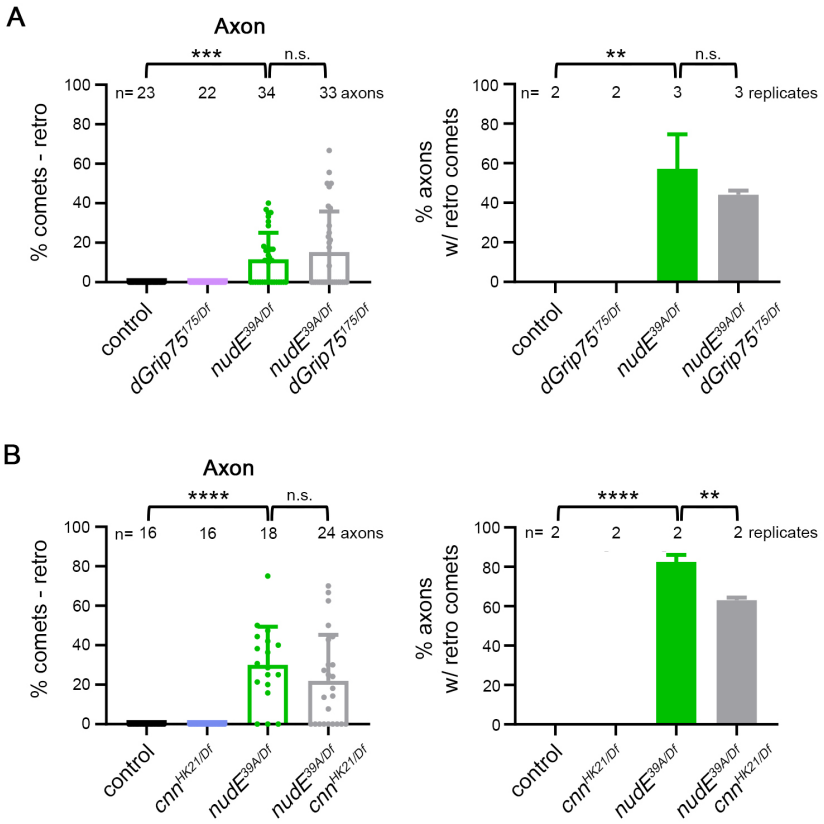

Fig. 3. Appearance of misoriented microtubules in *nudE* mutant axons does not depend on microtubule nucleation machinery.

(A,B) Eliminating either dGrip75 (A) or cnn (B) does not affect the microtubule polarity phenotype of $nudE^{39A/Df}$ mutant axons. n=23 (control), 22 ($dGrip75^{175/Df}$), 34 ($nudE^{39A/Df}$), and 33 ($dGrip75^{175/Df}$; $nudE^{39A/Df}$) axons (A) and n=16 (control), 16 ($cnn^{HK21/Df}$), 18 ($nudE^{39A/Df}$), and 24 ($cnn^{HK21/Df}$; $nudE^{39A/Df}$) axons (B) in replicates as indicated. n.s.= not significant, **P=0.01–0.001, and ***P=0.001–0.0001, Kruskal-Wallis with post-hoc Dunn's multiple comparison analysis. n.s.=not significant, Mann-Whitney test. All data are mean ± standard deviation.

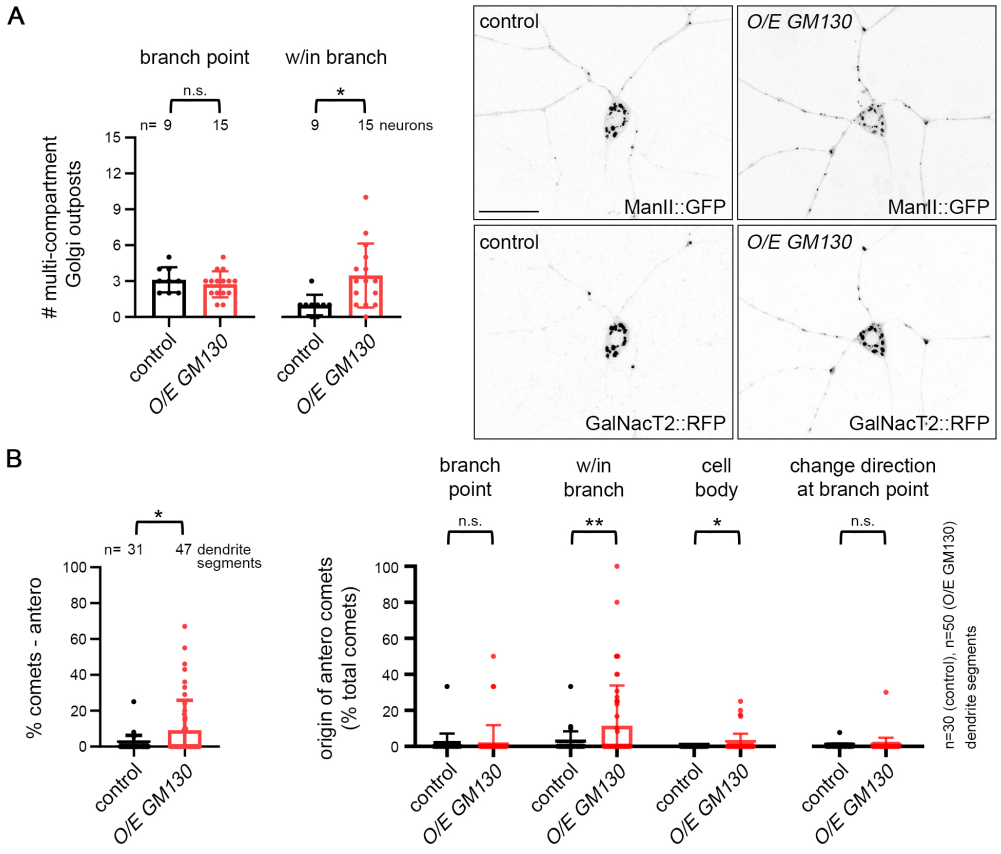

Fig. 4. Multi-compartment Golgi are sufficient to alter microtubule polarity.

(A) The over-expression of GM130 increases the number of multi-compartment Golgi outposts within dendrite branches but not branch points. Scale bar: 25 µm. n.s.=not significant and **P*=0.05–0.01, Student's unpaired t tests. (B) The percentage of anterograde comets increases in the dendrites of neurons over-expressing GM130. The percentage of anterograde comets that originate from within branches increases, paralleling the increase in multi-compartment outposts within branches. The graph on the right represents the number of anterograde comets that fall into a designated category divided by the total number of comets in the dendrite segment (% total comets). n.s.=not significant, **P*=0.05–0.01, and ***P*=0.01–0.001, Mann-Whitney test. All data are mean ± standard deviation.

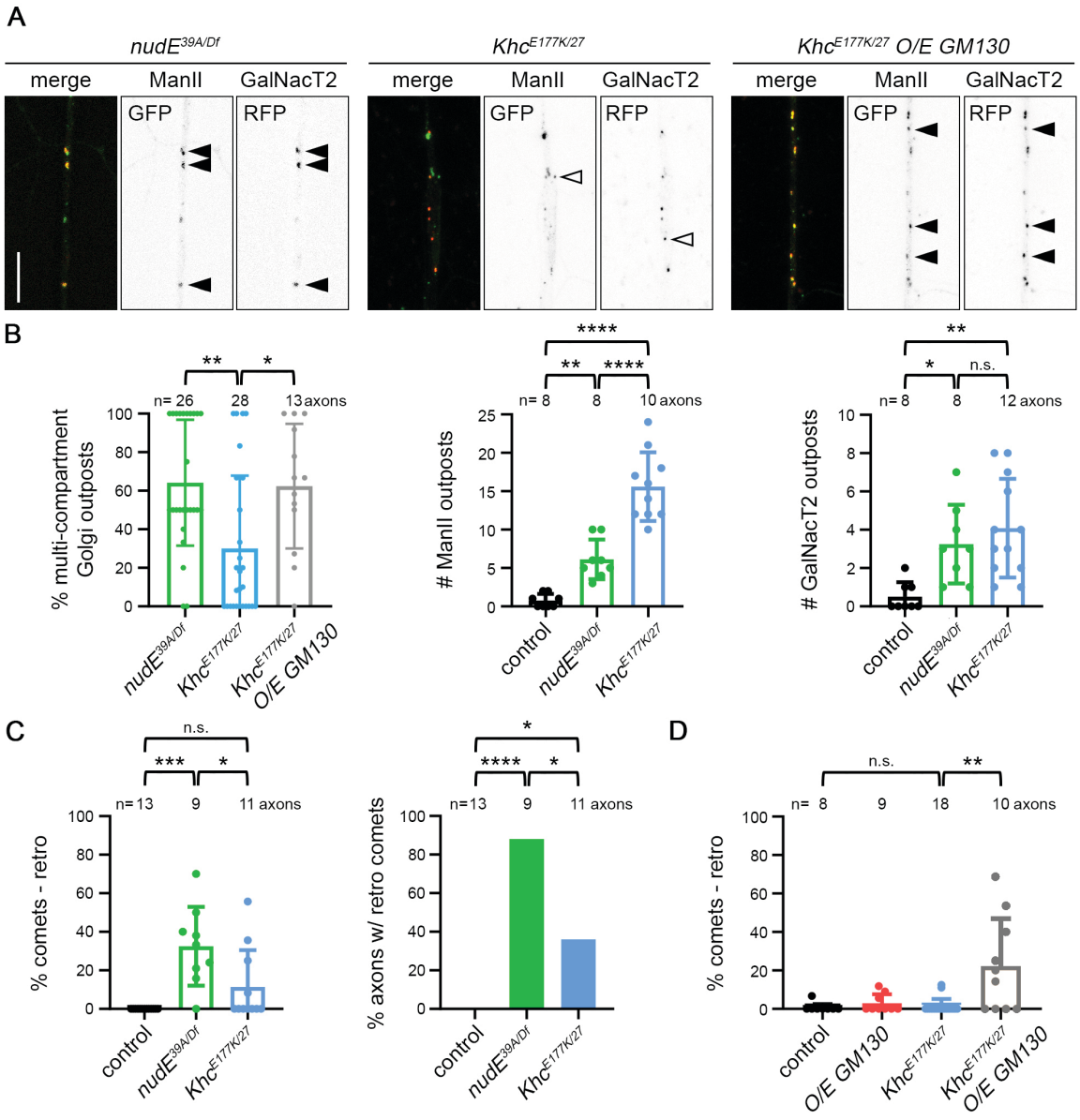

Fig. 5. Inducing the formation of multi-compartment Golgi alters microtubule polarity in the axons of *Khc* mutant neurons. (A,B) Axons of *Khc*$^{E177K/27}$ mutant neurons contain fewer multi-compartment Golgi outposts than *nudE*$^{39A/Df}$ mutants, despite have similar or more numbers of ManII- and GalNacT2-positive compartments. Over-expressing GM130 increases the percentage of multi-compartment outposts in *Khc*$^{E177K/27}$ mutant axons. n.s.=not significant, **P*=0.05–0.01, ***P*=0.01–0.001, ****P*=0.001–0.0001 and *****P*<0.0001; Kruskal-Wallis with post-hoc Dunn's multiple comparison analysis (% multi-compartment Golgi outposts) and one-way ANOVA with Tukey's post-hoc analysis (# outposts). Scale bar: 10 µm. Closed arrowheads indicate multi-

compartment outposts and open arrowheads indicate single compartments. (C) In contrast to *nudE*$^{39A/Df}$ mutants, *Khc*$^{E177K/27}$ mutant axons have normal microtubule polarity. n.s.=not significant, **P*=0.05–0.01, ****P*=0.001–0.0001 and *****P*<0.0001; Kruskal-Wallis with post-hoc Dunn's multiple comparison analysis. (D) The increase in multi-compartment Golgi outposts in *Khc*$^{E177K/27}$ mutant axons that results from the over-expression of GM130 is accompanied by an increase in misoriented axonal microtubules. n.s.=not significant and ***P*=0.01–0.001, Kruskal-Wallis with post-hoc Dunn's multiple comparison analysis. All data are mean ± standard deviation.

Materials and methods

Fly stocks

The following alleles and transgenic fly strains from the Bloomington Drosophila Stock Center (BDSC) and individual laboratories were used as follows: *cnn*^{HK21} (MEGRAW *et al.* 1999; VAIZEL-OHAYON AND SCHEJTER 1999) (BDSC 5039), which produces a drastically truncated ~106 amino acid-long protein that is not detectable by western blot, and *Df(2R)BSC306* (BDSC 23689) were used to eliminate cnn; *GM130*$^{\Delta 23}$, a protein null allele (ZHOU *et al.* 2014) (BDSC 65255), and *Df(2R)Exel7170* (BDSC 7901) were used to eliminate GM130; *UAS-GM130::eBFP* (ZHOU *et al.* 2014) (BDSC 65254) and *ppk-Gal4* (BDSC 32079) were used to over-express GM130; *dGrip75*^{175} (SCHNORRER *et al.* 2002) (Conduit lab, University of Cambridge; Raff lab, University of Oxford) and *Df(2L)Exel7048 (BDSC 7999)* were used to eliminate dGrip75; *Khc*^{E177K} (KELLIHER *et al.* 2018) was used in trans to the null allele *Khc*^{27} (BRENDZA *et al.* 1999) (Saxton lab, UC-Santa Cruz); *nudE*^{39A} (WAINMAN *et al.* 2009) (Goldberg lab, Cornell University), a protein null allele, and *Df(3L)BSC673* (BDSC 26525) were used eliminate nudE; *plp*^{5} (BDSC 9567)(MARTINEZ-CAMPOS *et al.* 2004), an EMS-induced loss-of-function allele that strongly reduces plp levels, and *Df(3L)Brd15, pp* (BDSC 5354) were used to eliminate plp activity; *ppk-ManII::GFP* (JENKINS *et al.* 2017) and *UAS-GalNacT2::TagRFP* (ZHOU *et al.* 2014) (Ye lab, University of Michigan) were used to label *medial* and *trans* Golgi compartments, respectively; *ppk-CD4::GFP* (BDSC 35842, BDSC 35843) and *ppk-CD4::tdTom* (HAN *et al.* 2011) (BDSC 35844, BDSC 35845) were used to visualize neuron morphology; *ppk-EB1::GFP* (ARTHUR *et al.* 2015) was used to analyze microtubule polarity and dynamics.

Live imaging

Fly crosses for live imaging were set up using 5-8 virgin females and 4-6 young males; larvae were collected in 12-h intervals and aged to the desired developmental stage (larvae produced in the first 24-48 h after mating were not used). Larvae of the desired genotype were washed with 1X phosphate-buffered saline (PBS), mounted in a 50:50 1X PBS:glycerol solution on a slide between two strips of vacuum grease, and immobilized by pressing on a coverslip mounted on top of the larva and vacuum grease spacers. The dorsal class IV ddaC neurons within abdominal segments 2-4 were imaged with a 40x1.3 NA oil immersion objective. All imaging was performed on a Leica SP5 (Leica Microsystems) using HyD photodetectors.

Microtubule polarity and growth analysis

Microtubule polarity and growth were analyzed by scoring EB1::GFP comets within 150 μm of the cell body in dendrites or axons. EB1::GFP comet trajectories were captured at a resolution of 1024 x 512 pixels and a rate of 0.86s per frame for 5-7min. One or two ddaC neurons per larva were imaged at 96 -120 h after egg laying (AEL). Videos were stabilized with the stabilizer plugin in FIJI ImageJ (ImageJ; National Institutes of Health); kymographs were generated in Metamorph (Molecular Devices). Movies that did not contain at least two comets were excluded. Comet trajectories were manually traced, and the position and time coordinates were recorded to calculate comet direction (microtubule orientation) and frequency. A comet trajectory was included only if it could be clearly traced in at least 12 continuous frames (~11s). Anterograde comets traveled away from the cell body whereas retrograde comets traveled toward the cell body. The frequency of EB1::GFP comets was calculated as the number of comets present in a 100 μm segment (axons) per minute. To

calculate microtubule polarity in dendrites, EB1::GFP comets were scored in a segment ≥ 30 µm between two branchpoints. To identify the origin of anterograde EB1::GFP comets in dendrites, dendrite segments within 100 µm of the cell body that contained at least one comet were selected for analysis. Comet trajectories were identified in kymographs, and any anterograde comets were then traced back to their origin in the corresponding movie.

Axon branching analysis

Axon morphology was visualized at late 3rd instar (120-144h AEL) using CD4::GFP and CD4::Tomato. Images were captured at 1024x1024 resolution and 1 µm z-steps (5-15 steps total). Analysis was performed on axons within 150 µm of the cell body. Z-stacks 5-15 µm thick were max projected for analysis. Axon branching was assessed manually by determining whether an axon split into one or more branches.

Golgi compartment analysis

Images of ManII::GFP and GalNacT2::TagRFP puncta in the dendrites and axons of neurons in 96-120h AEL larvae were captured at a resolution of 1024 x 1024 pixels and 0.75 µm z-steps over 5-15 µm. Analysis of Golgi outposts in axons included outposts within 100 µm of the cell body. For dendrite analysis, Golgi outposts throughout the entire arbors were included and split into two groups: (1) those within a radius of 95 µm of the cell body and (2) those outside this radius. One to two ddaC neurons were imaged per larva. Analysis was performed on the max-projected images. Signal outside the regions of interest (ROI) in axons and dendrites were masked. Masked images were subjected to a threshold gray value of 100 for

segmentation. The segmented signals were quantified with the ImageJ particle analysis function with the size cutoff of 0.10-15 μm^2. Puncta outlines were saved as ROIs. The resulting particle numbers and sizes were exported to Excel for analysis. For multi-compartment analysis, overlapping compartments were manually scored by overlaying the puncta outlines from each channel, namely ManII::GFP and GalNacT2::TagRFP. Golgi units were scored as multi-compartment when the ManII::GFP and GalNacT2::TagRFP signal overlapped.

Statistical analysis

Statistical analyses were performed using GraphPad Prism8. The Anderson-Darling and Shapiro-Wilk tests were used to determine whether data were normally distributed. For normally distributed data, Student's unpaired t tests were used to compare two groups; one-way ANOVA with post hoc Tukey was used for multiple comparisons. For non-normally distributed data, a Mann-Whitney test was used to compare two groups and Kruskal-Wallis test followed by post hoc Dunn's was used for multiple comparisons. Fisher's Exact test was used for comparing proportions. P=0.05 was used as a cutoff for significance. Significance levels are represented as: *P=0.05–0.01, **P=0.01–0.001, ***P=0.001–0.0001 and ****P<0.0001. n.s.=not significant.

Data Availability

All strains are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are represented fully within the article and its figures.

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Chapter 3: Concluding Remarks and Future directions

Introduction

Microtubule cytoskeleton in neurons are highly organized and polarized. The cytoskeleton not only provides mechanical support for neurons; it is also fundamentally important for guiding motor-cargo machineries to achieve polarized cargo delivery. With increasing imaging capacity, the intricate details of the neuronal cytoskeleton are being revealed through electron microscopy and super-resolution microscopy (Kapitein & Hoogenraad, 2015; Leterrier et al., 2017; Tas et al., 2017). In vitro biophysical and biochemical assays provide insight into the details of microtubule assembly and reveal their dynamic interaction with MAPs (Brouhard & Rice, 2018). However, we still know very little about how such structures are made in neurons. The challenge is how a particular microtubule-making mechanism is capable of promoting the accumulation of microtubules of a particular orientation to result in the distinct dendritic and axonal microtubule polarities.

Current challenges and limitations in the search of neuronal MTOCs

Organelle-based microtubule organizing centers (MTOCs) have been the main focus of past searches. The centrosome, the earliest known and best characterized MTOC in many cell types, has been the model. It emanates microtubules, whose minus-ends are anchored at the centrosome, and is enriched in factors that promote microtubule nucleation, stabilization, and polymerization. Although the centrosome is not essential for building neuronal microtubules, these characteristics of a centrosome are used as a guideline for finding other MTOCs (Dammermann et al., 2003; Woodruff et al., 2017).

In practice, these criteria can be difficult to use to identify other MTOCs. First of all, while microtubule filaments can be visualized by super-resolution microscopy in cultured neurons, visualizing the ends of individual microtubule is still challenging. Super-resolution imaging still has limited axial resolution; thus, continuous sampling of an entire microtubule filament in a neuronal process to identify a microtubule end can be difficult. On the other hand, looking for enrichment of the microtubule nucleator γ-tubulin is also not an easy task. γ-tubulin is free floating in the cytosol in its inactive form and may not have the same level of enrichment on a non-centrosomal MTOC as on the centrosome (Sánchez-Huertas et al., 2016).

For these reasons, the search for non-centrosomal MTOCs has been approached in less directed ways. For example, in one common approach, microtubules are eliminated with cold or nocodazole treatment that depolymerizes microtubules; during the subsequent recovery period microtubule regrowth is monitored to reveal sites at which microtubule growth initiates. The Golgi's MTOC potential was discovered through such an assay (Rivero et al., 2009). However, it is important to note that this type of approach is usually not able to clear all the microtubules; therefore, the regrowth could potentially occur from the ends of residual microtubules, rather than de novo growth seeded from a microtubule nucleator. Also, under such treatment, the peri-nucleus Golgi stacks are dismantled into Golgi fragments, making it unclear whether Golgi has remained its original identity. In neurons, this assay can have additional difficulty, as some neuronal microtubules are exceptionally stable (Yamada & Hayashi, 2019). Therefore, alternative assays have been developed and used to search for neuronal MTOCs. For example, putative microtubule nucleation sites have been identified using fluorescently labeled +TIP markers such as EB1::GFP and identifying the initiation sites of

EB1::GFP comets. However, EB1 does not only track microtubule growth from sites of nucleation; it also tracks microtubule growth that results from microtubule catastrophe and rescue events. Consequently, there is inherently ambiguity in such assays. For this reason, although Golgi outposts in dendrites are found to coincide with EB1::GFP initiation hotspots in dendrites, its status as a neuronal MTOC was still under debate.

Heterogeneous Golgi population in neurons have different capacities to influence microtubule organization

My thesis work examines the role of Golgi outposts in neuronal microtubule organization and its potential function as a neuronal MTOC. My research has revealed that while Golgi outposts support microtubule growth during dendrite development, they are surprisingly dispensable for the overall polarity of the dendritic cytoskeleton. I was able to show that, using multiple ways to disrupt Golgi outposts' compartmental structure and its potential MTOC activity, dendrite microtubule polarity is unaffected. Despite that Golgi outposts are not essential for dendritic cytoskeleton, my work shows that ectopic Golgi outposts have the capacities to locally influence dendritic and axonal microtubule polarity. In genetic backgrounds that induce formation of ectopic Golgi in the axons and dendrites, we have found multicompartmental Golgi outposts correlate with appearance of mis-oriented microtubules (Figure 1). Importantly, we also found that Golgi's ability to impact local microtubule polarity is independent of γ-TuRC, the microtubule nucleation complex. These findings suggest that neurons have a heterogenous population of Golgi, differentiated by their localization and/or compartmentalization, can have different abilities to impact local microtubule organization. The

heterogeneity of Golgi as well as its γ-TuRC independent MTOC nature provides explanations for the contradictory findings in the previous studies.

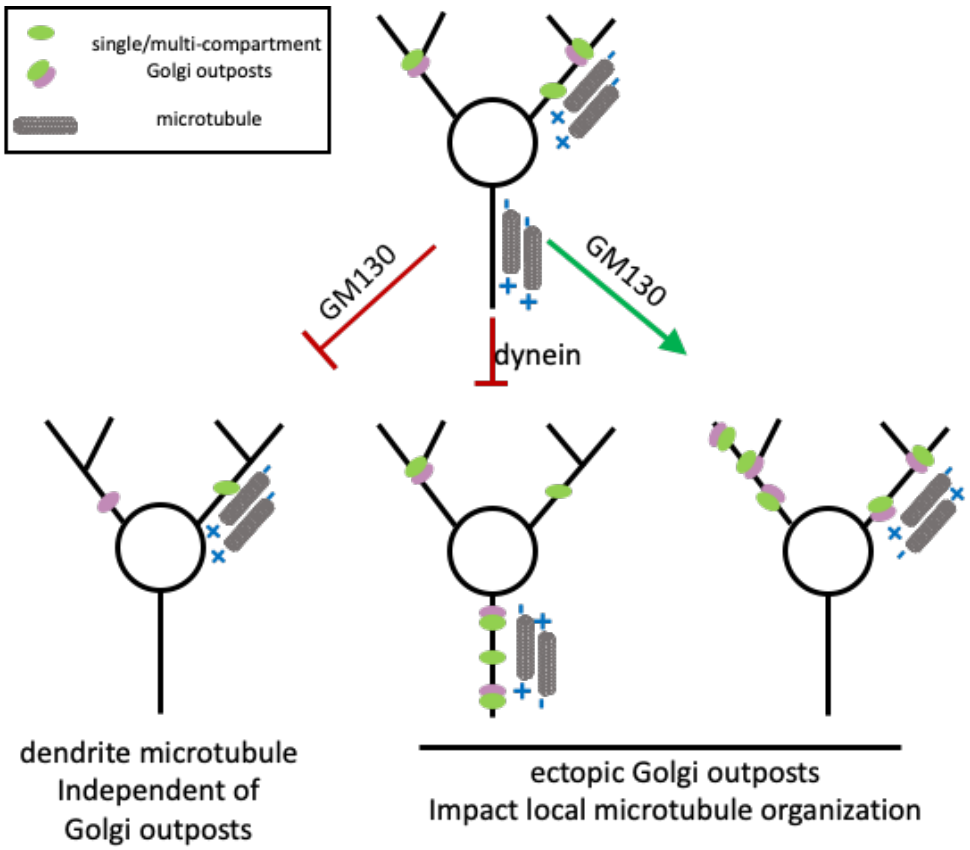

Fig. 1. Golgi is not required for dendrite microtubule polarity but its ectopic localization in dendrites and axons locally impact microtubule organization.

Disruption of Golgi outposts compartmentalization and MTOC pathway by depleting GM130 does not impact microtubule organization in the dendrites. Ectopic Golgi outposts in axons with reduced dynein activity and dendrites with GM130 overexpression induced local microtubule change in axons and dendrites respectively.

A recent research that focused on visualizing fluorescently labeled endogenous γ-tubulin found complementary evidences to support differential MTOC capacities of distinct Golgi

populations in neurons (Mukherjee, 2019). When γ-tubulin is examined in relative to Golgi markers in Drosophila sensory neurons, colocalization is only found on somatic Golgi but not consistently on dendritic Golgi outposts. If γ-tubulin-mediated microtubule nucleation is necessary for Golgi's MTOC activity, this result would again support that lacking γ-tubulin, Golgi outposts in dendrites are not essential for dendritic microtubule organization. One important unresolved question from this study is whether γ-tubulin is necessary for Golgi-based MTOC activity.

An important question that arises from our study is that if Golgi outposts are not essential for dendritic microtubule organization, what else may perform this function. A recent new study in C. elegans PVD neurons may shed light on this question. In developing dendrite, tip localized endosome is found colocalize with γ-tubulin, and elimination of γ-tubulin in this neuron is sufficient to perturb dendritic microtubule polarity (Liang et al., 2020). This observation made endosome a new candidate MTOC for making minus-end-distal microtubules in the dendrites. The endosome markers used in this study partially overlap with trans-Golgi network (TGN). It would be interesting to examine the relative localization of endosome compartments with different Golgi populations, so as to see whether the Golgi that has MTOC activity is also overlap with endosomes.

Diverse pathways of microtubule-making: γ-tubulin is not the only key

Most studies seek to identify a MTOC by looking for its colocalization with the canonical microtubule nucleator γ-tubulin. However, it is important to recognize that such criteria will miss two potential types of MTOCs: first, MTOCs that do not concentrate γ-tubulin at one

particular site (Augmin-mediated microtubule growth is such an example (Cunha-Ferreira et al., 2018; Sánchez-Huertas et al., 2016); Augmin-anchored γ-TuRC are scattered at different positions along microtubules); second, MTOCs that use microtubule-making pathways that are independent of γ-tubulin. Accumulating evidences suggest that elimination of γ-tubulin, using a variety of knockdown methods, does not strongly deplete microtubules in Drosophila and mammalian neurons (Liang et al., 2020; Nguyen et al., 2014; Ori-McKenney et al., 2012; Sánchez-Huertas et al., 2016). Such observation is not unique to neurons. Knocking down γ-tubulin in C. elegans intestinal epithelium similarly does not deplete majority of microtubules, despite causing an elevated microtubule dynamics (Sallee et al., 2018). These findings collectively suggest that other microtubule-making pathways may exist to compensate for loss of γ-tubulin.

TPPP (tubulin polymerization promoting protein) in oligodendrocytes, by recruiting and enriching local tubulin concentration, is recently found as a nucleation-competent protein (Fu et al., 2019). While TPPP may not function as a microtubule nucleator in neuron, it opens up the possibility of finding pathways that nucleate microtubules independent of γ-tubulin. Microtubule stabilizing enzyme CAMSAPs and severing proteins, such as Katanin and Spastin, represent another important category of microtubule interacting proteins that may create and modify the cytoskeleton (Tang et al., 2020; Wang et al., 2019). Altering the cellular level of these proteins individually is sufficient to affect neuronal cytoskeleton polarity. The exact mechanisms by which these proteins may impact on microtubule polarity is still unknown.

Outlook

Building a highly organized cellular microtubule architecture in neurons or in any cell is a complex process. It is a result of actions from likely a combination of different MTOCs that make microtubules, together with microtubule interacting proteins that stabilize, anchor and sever microtubules over the course of development. Finding the components that fall in these categories would be an important first step. To fully understand how microtubules are built in neurons, we would like to know how these pathways that generate and modify microtubules are turned on or off; how these pathways cross-talk with one another; and, finally, how these factors may collectively contribute to the overall cytoskeleton.

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Appendix. A. Visualizing EB1 and Grasp65 at endogenous level with tissue-specific reconstituted splitGFP

Introduction

Microtubule-based cargo trafficking in neurons is essential to neuron's function and survival. Visualization of the key components involved in this process is crucial to our understanding of how polarized and highly efficient cargo delivery is achieved. Microtubule, the highway and asymmetrically distributed organelle cargo, such as Golgi, are such key components. While these structures were previously visualized by antibody staining or transgenically expressed protein fused to fluorescent tags, examining endogenous state of these proteins will provide more precise information about their localization and dynamics.

Using CRISPR, we edited the gene locus that encode for +TIP EB1 and Golgi matrix protein GRASAP65 by adding the splitGFP11 sequence at 3' end of the last exon (Figure A.2). To reconstitute GFP signal in a specific tissue, in Class IV da neurons for instance, we expressed the GFP11 counterpart GFP1-10 under pickpocket (ppk) promoter (Figure A.1). As such, we are able to track dynamic endogenous EB1 comet as well as visualize endogenous Golgi structure. These engineered EB1 and Golgi marker provide great new tools to visualize endogenous microtubule and Golgi dynamics. This also exemplifies application of CRISPR and split fluorophore in visualizing endogenous protein.

Preliminary results

Dynamic EB1 comets are visualized with reconstituted split-GFPs.

We tested the splitGFP tagged endogenous EB1 alleles by reconstituting it with GFP1-10 that expressed in Drosophila Class IV sensory neurons. The reconstituted EB1 has a cytosolic distribution in dendrite, axon and the cell body (Figure A.2B). In dendrites, its signal is brighter

in the major branches, and much weaker in terminal dendrites. To analyze its dynamic property, we also performed time-lapse live imaging in dendrites and axons (Figure A.2B). Like transgenic EB1::GFP, the reconstitute EB1::GFP11 and GFP1-10 forms very dynamic comets; these comets move at comparable rate as the EB1::GFP transgenic construct. Similar to previously found, the reconstituted EB1 comets in dendrites move in the retrograde direction, and oppositely in the axons (Figure A.2B).

GRASP65 tagged with reconstituted split-GFP localize to branch point and soma

We examined edited GRAPSP65 allele by testing reconstituted GFP signal in the Class IV da neurons. The reconstituted GRASP65 GFP signal localize to branch point and soma (Figure A2.D), which is similar to the localization of GM130, a cis-Golgi matrix protein that directly interact with GRASP65 .

Materials and Methods

Generation of GFP11 labeled EB1 and Grasp65 loci

The tagged EB1 and Grasp65 locus were generated in a two-step fashion (Figure A.3): CRISPR-Cas9-mediated genome editing is used to introduce linker-GFP11x7-pBac-3xP3-dsRED-pBac at the end of the respective loci; the screening marker is excised with piggyBac transposase. To generate the initial CRISPR-Cas9 edited loci, guideRNA expression plasmid (pBSK-U63) and respected repair template are injected to nos-Cas9 fly (BDSC: 78782) embryos by BestGene; the progenies that has 3xP3 DsRed markers are considered as hits and were subsequently crossed to transposase fly line to excise the pBac flanked sequences. The targeted loci were fully sequenced.

Sequences

EB1 guide sequence	5'TAATACTCCTCGTCCTCTGG3'
Grasp65 guide sequence	5'GCAGGCAACGACGAACTATC3'
linker sequence	5'GGCGGATCCGGCGGA3'
GFP11 x 7	5'CGTGACCACATGGTCCTTCATGAGTATGTAAATGCTGCTGGGATTACAGGTGGCTCTGGAGGTAGAGATCAT ATGGTTCTCCACGAATACGTTAACGCCGCAGGCATCACTGGCGGTAGTGGAGGACGCGACCATATGGTACTAC ATGAATATGTCAATGCAGCCGGAATAACCGGAGGGTCCGGAGGCCGGGATCACATGGTGCTGCATGAGTATG TGAACGCGGCGGGTATAACTGGTGGGTCGGGCGGACGAGACCATATGGTGCTTCACGAATACGTAAACGCAG CTGGCATTACTGGCGGATCAGGTGGCAGGGATCACATGGTACTCCATGAGTACGTGAACGCTGCTGGAATCAC AGGCGGTAGCGGCGGTCGGGACCATATGGTCCTGCACGAATATGTCAATGCTGCCGGTATCACC 3'

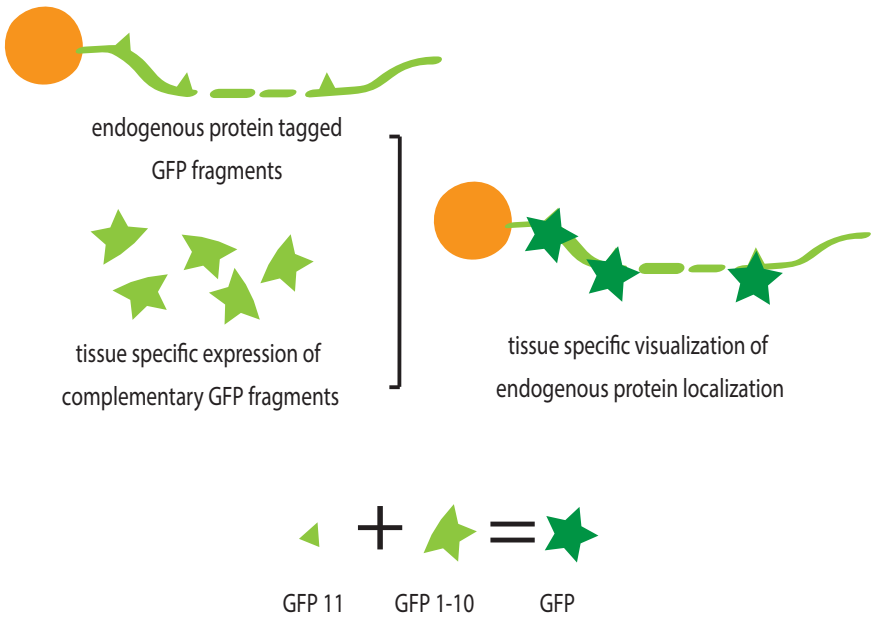

Figure A.1: Basic strategies to visualize proteins at endogenous level with tissue-specific split-fluorescent protein reconstitution. Protein of interest is modified at endogenous locus to add single or multiple copies of fluorescent fragments of GFP11. The complementary GFP1-10 is expressed in a selective tissue under the respective tissue-specific promoter. The fluorophore is only excitable when it has both peptide fluorophore pieces, which enables tissue-specific visualization of targeted protein at its endogenous level with the reconstituted fluorescent proteins.

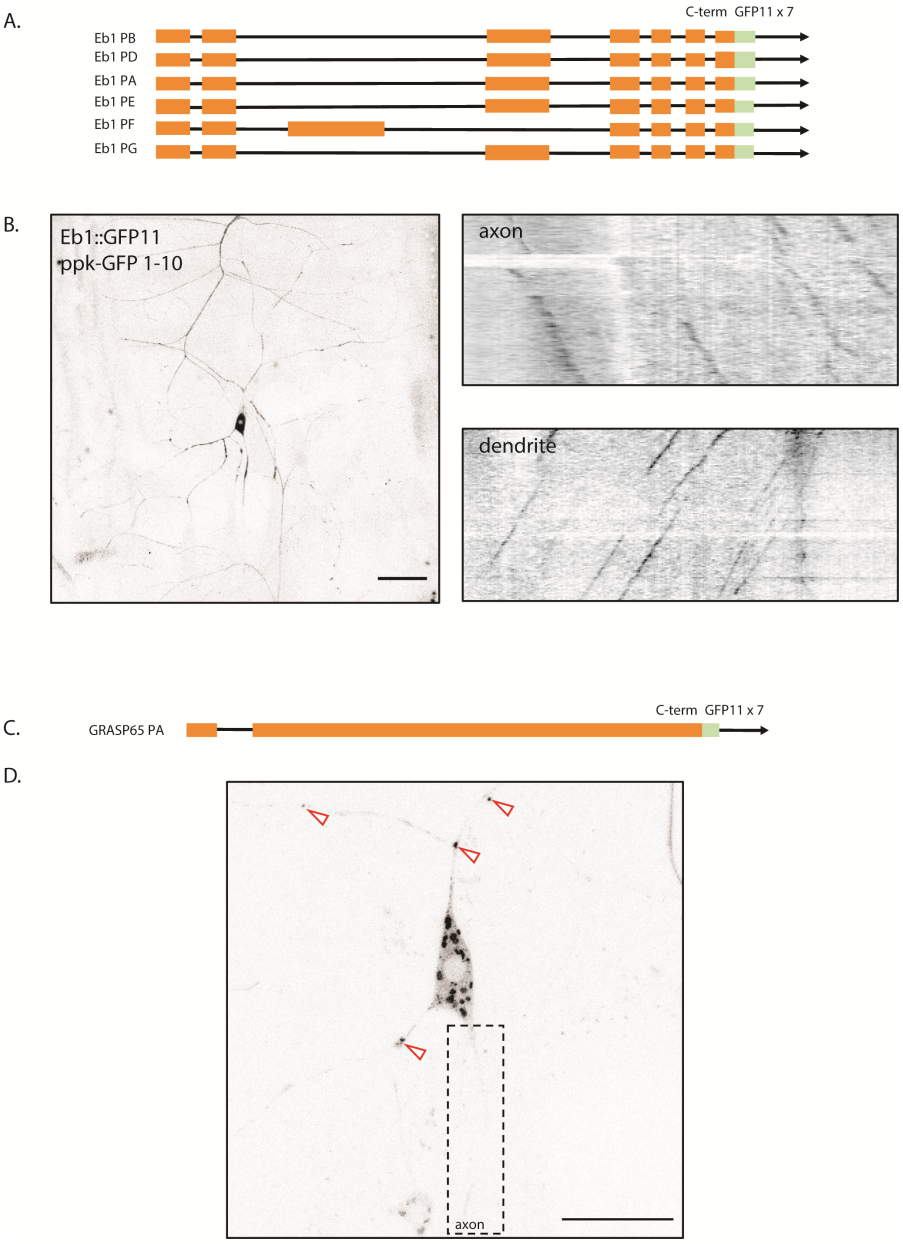

Figure A.2: Reconstituted GFP labels EB1 and Grasp65 at their endogenous level. A) and C) Sequences that encode for seven copies of GFP11(GFP11 x7) are added to EB1 and GRASP65 transcripts at the end of the last axon, respectively. B) Reconstituted EB1 signal in Class IV da neuron. Z-stack taken over the whole arbor. Scale bar = 50μm. Kymographs of reconstituted EB1::GFP signal in an axon and a dendrite segments. D) Reconstituted GRASP65 signal in Class IV da neuron. Scale bar = 50μm.

Appendix. B. Generating *Grip75*$^{KO\text{-}attp}$ allele for expediated genetic manipulation

Introduction

Generating microtubules in cells is crucially dependent on the γ-TuRC complex, which is so far the only well-characterized microtubule nucleator. This multi-component complex assembles into a cone-shaped structure, providing a stable platform for tubulin dimer addition. The γ-TuRC is made of two subcomplex, γ-TuSC, composed of GCP1,GPC2 and GCP3, and γ-TuRC specific components GCP4, 5, and 6. The γ-TuSCs form the direct interface between γ-TuRC and incoming tubulin dimers. The g-TuRC-specific components regulate the overall structural integrity as well as the activity and localization of the γ-TuRC complex. While this complex has been intensively studied in in vitro we know little about this complex's location and regulation in vivo.

Here, we have taken advantage of CRISPR-mediated genome editing to generate an allele of the Drosophila GCP4/Grip75 in which the original Grip75 gene sequence is replaced by an attP site. This manipulation of the site will allow the easy and rapid generation of tagged Grip75 and mutant Grip75 alleles to visualize its localization and manipulate its activity, respectively.

Materials and Methods

Generation of *Grip75$^{KO\text{-}attP}$* locus with CRISPR-Cas9

CRISPR-Cas9 was used to generate the *Grip75$^{KO\text{-}attP}$* locus. The endogenous *Grip75* gene and the flanking intergenic regions are replaced with attP-DsRed-pBac through homologous recombination using a donor template. The donor template contains homology arms, which facilitates knock-in of new Grip75 alleles, and CRISPR selection marker 3xP3-DsRed. This will result in the deletion of the entire Grip75 gene and part of the overlapping Cog4 gene.

Sequences

Grip75 guide1	5'GGTGCAAGGGGATGTCCTCG3'
Grip75 guide2	5'gAAAAATGTGCGAACTTCGTT3' (an additional g is added to enhance guideRNA transcription)
Homology arm 1	5' TTAACGTTAAAAATGTGCGAACCTCATTA - (961bp)- AGTGCGTTCACAAAATCAC 3' (T-C mutation*)
Homology arm 2	5' AAGGACTGTAAGACCACATTT - (951bp) - GGTGCAAGGGGATGTCC 3'
	* mutation in repair template to prevent recursive editing events

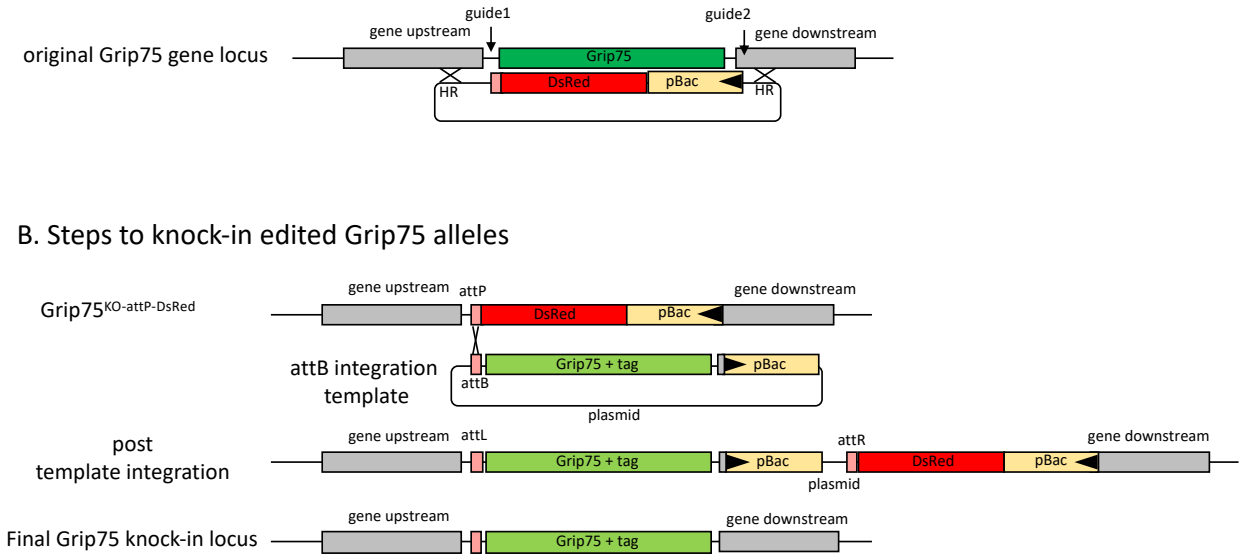

Figure B.1: Engineering the Grip75 gene to facilitate knock-in of Grip75 alleles. A) CRISPR-Cas9 in combination with homologous recombination was used to replace the endogenous Grip75 gene with attP, DsRed and pBac sequences. B) Knock-in template that carries attB-Grip75 alleles and pBac was injected *Grip75*$^{KO\text{-}attP\text{-}DsRed}$ embryos expressing φC31 integrase, which mediates recombination between attP and attB. The pBac sequences from the Girp75 KO allele and the knock-in template would facilitate the removal of attR and DsRed and additional exogenous plasmid sequences post integration.