PERSPECTIVE

The roles of tubulin-folding cofactors in neuronal morphogenesis and disease

Microtubules play important roles in neuronal morphogenesis, including cellular polarization, neurite growth, and branching. A microtubule is a polymer of α- and β-tubulin heterodimers that are formed by a multistep process assisted by at least five tubulin-folding cofactors (TBCA–E) (Lopez-Fanarraga et al., 2001). Newly synthesized α- and β-tubulins associate with the cytosolic chaperonin complex (CCT), and then the quasi-native tubulins interact with five tubulin-folding cofactors. The α- and β-tubulins first interact with TBCB and TBCA, which are further transferred to TBCE and TBCD, respectively. The α- and β-tubulins, TBCC, TBCD, and TBCE form a supercomplex, and guanosine triphosphate (GTP) hydrolysis occurs within the supercomplex, releasing a tubulin heterodimer. In addition to their functions in tubulin folding and tubulin heterodimer formation, excess amounts of TBCD or TBCE may interact with tubulin heterodimers, leading to their degradation. Thus, tubulin-folding cofactors may play a role in both the synthesis and degradation of tubulin heterodimers.

Recently, the functions of tubulin-folding cofactors in the nervous system have been revealed. The human *TBCE* mutation is a known cause of the hypoparathyroidism-retardation-dysmorphism and Kenny-Caffey syndromes (Parvari et al., 2002). The progressive motor neuronopathy (*pmn*) mouse, a model for a fast developing human motor neuron disorder, is caused by a missense mutation in *TBCE* (Martin et al., 2002). The axons of *pmn* mice possess a reduced number of microtubules and progressively degenerate; eventually, the *pmn* mice die approximately 4–6 weeks after birth (Martin et al., 2002). *Drosophila* TBCE has also been shown to regulate the development and function of neuromuscular synapses and promote microtubule formation (Jin et al., 2009). Giant axonal neuropathy (GAN) is caused by mutations in *GAN*, which encodes for the protein gigaxonin that controls the protein degradation of TBCB (Wang et al., 2005). TBCB is localized at the transition zones of the growth cones of growing neurites. Knockdown of *TBCB* promotes axon growth and TBCB overexpression leads to microtubule depolymerization, growth cone retraction, and axon degeneration (Lopez-Fanarraga et al., 2007). The accumulation of TBCB may be a causative factor of cytoskeletal pathology in GAN. However, the functions and regulations of the other tubulin-folding cofactors in neurons remain largely unknown.

We recently reported that one of the tubulin-folding cofactors, TBCD, regulates neuronal morphogenesis by mediating Down syndrome cell adhesion molecule (Dscam) functions (Okumura et al., 2015). We utilized *Drosophila* olfactory projection neurons as a model for understanding the molecular mechanisms of neuronal morphogenesis. Mosaic analysis with a repressible cell marker (MARCM) system enabled us to generate labeled, homozygous cells with single-cell resolution *in vivo*. Using the MARCM system, we found that TBCD is required for the dendrite and axon morphogenesis in projection neurons. Wild-type projection neurons sent their dendrites to a single glomerulus, out of approximately 50 glomeruli, in the antennal lobe (**Figure 1A**). In contrast, *TBCD* mutant projection neurons exhibited ectopic dendrite branching, that is, the dendrite targeted the correct glomerulus and additional glomeruli (**Figure 1B**). Axons of the wild-type projection neurons innervated the mushroom body and lateral horn with stereotypical terminal arborization (**Figure 1A**). Axons of the *TBCD* mutant projection neurons elongated to the mushroom body and lateral horn during development, but degenerated soon after eclosion (**Figure 1B**). The ectopic dendrite branching defect was also observed in the loss of other tubulin-folding cofactors, that is, TBCB and TBCE, suggesting that the dendrite defect in the *TBCD* mutant is likely caused by the loss of TBCD function in tubulin heterodimer formation. Consistent with these results, the tubulin levels were found to be significantly reduced in the *TBCD* mutant projection neurons. Interestingly, the overexpression of TBCD in projection neurons also showed ectopic dendrite branching. Both the loss- and gain-of-function studies suggested that the amount and dynamics of tubulin heterodimers must be tightly regulated for correct dendrite morphogenesis.

We also set out to understand how the function of TBCD is regulated in neuronal morphogenesis. We identified Dscam, an immunoglobulin family protein, as a binding partner of TBCD (**Figure 1C**). *Drosophila* Dscam possesses extraordinary diversity because of the alternative splicing of extracellular and transmembrane domains, which can generate 38,016 isoforms, and has been reported to function in several processes of neural development, such as axon guidance and dendrite self-avoidance (Hattori et al., 2008). These isoforms show isoform-specific binding, which mediates homophilic repulsion. Although the function of Dscam in the nervous system has been studied extensively, the link between Dscam and cytoskeletal proteins (especially microtubules) remains unclear.

We hypothesized that TBCD functions downstream of Dscam in neuronal morphogenesis, which was supported by the following results: First, Dscam overexpression caused ectopic dendrite branching, similar to that seen for *TBCD* mutant and TBCD overexpression. Second, the overexpression of Dscam in cultured *Drosophila* S2 cells led to a decrease in microtubules projecting radially from the cell center. Third, the overexpression of Dscam in cultured *Drosophila* primary neurons reduced α-tubulin staining locally at the site where Dscam accumulated. Fourth, the Dscam gain-of-function phenotype in mushroom body neurons was suppressed by the reduction of TBCD levels. Additionally, the reduction in TBCD levels did not affect the expression and localization of Dscam. These results suggest that TBCD is essential for Dscam function during neuronal morphogenesis.

We also examined how TBCD regulates dendrite morphogenesis. Phenotypic analysis of *TBCD* mutant projection neurons during their development showed that their dendrites first targeted only the correct glomerulus; however, ectopic dendrite branches were observed at a later stage, suggesting that TBCD is required for the maintenance of correct dendrite morphology. Additional dendrite arborization in *TBCD* mutant projection neurons was seen between the cell body and the correct glomerulus (**Figure 1B**), implying that, in wild-type projection neurons, dendrite arborization within the dendrite stalk region (between the cell body and the correct glomerulus) is suppressed by the regulation of microtubule stabilization. In *TBCD* mutant, microtubule destabilization disrupts cellular transport; therefore, factors that inhibit the ectopic branching may not be transported to their proper sites. It is also possible that the disruption of cellular transport may cause proximal localization of factors regulating dendrite branching in the distal site (Sekine et al., 2009). A possible cargo to be transported is the Golgi outpost. In *Drosophila* dendrite arborization (da) neurons, Golgi outposts are localized at the dendrite branching point. The disruption of microtubule-dependent transport may result in the proximal accumulation of Golgi outposts, which induces ectopic dendrite branching. Another candidate cargo is the endosome. In da neurons, mutants of motor proteins, such as dynein, decrease the distal dendrite branches and increase the proximal branches. In this situation, Rab5, an early endosome marker, is proximally localized. Consistent with these results, we previously reported that the loss of *strip*, a regulator of early endosome organization, causes ectopic dendrite branching in projection neurons (Sakuma et al., 2014). Thus, tubulin-folding cofactors could affect dendrite branching by regulating microtubule-dependent endosome transport.

We have found that TBCD cooperates with Dscam during neuronal morphogenesis and that TBCD may function downstream of Dscam. The pathways acting downstream of Dscam, especially their effects on microtubules, are not fully understood, and only the Dock/Pak signaling pathway is known to function downstream

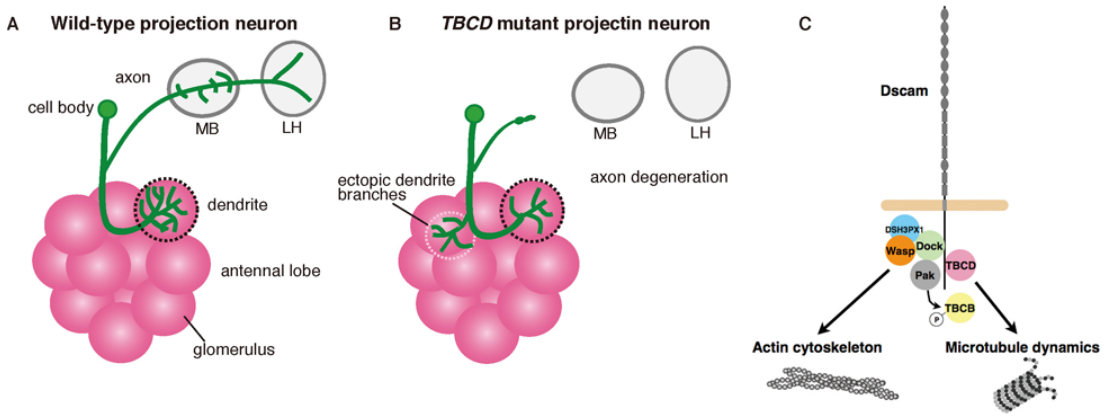

Figure 1 Tubulin folding cofactor D (TBCD) cooperates with Dscam during neuronal morphogenesis.
(A, B) Schemes of wild-type (A) and *TBCD* mutant (B) projection neurons. (A) The dendrite of a wild-type projection neuron targets a single glomerulus (black circle) in the antennal lobe (pink circles), and the axon elongates to the mushroom body (MB; gray circle) and the lateral horn (LH; gray circle). (B) The dendrite of a *TBCD* mutant projection neuron targets the correct glomerulus (black circle) and makes ectopic branches (white circle). The axon of the *TBCD* mutant projection neuron degenerates soon after eclosion. (C) Model for the downstream of Dscam. Dscam makes a complex with Dock, DSH3PX, and Wasp, affecting the actin cytoskeleton. The intracellular domain of Dscam interacts with TBCD, regulating microtubule dynamics. In human, PAK1 binds and phosphorylates TBCB. The Dock/Pak signaling pathway and tubulin-folding cofactors may coordinate with each other during neuronal morphogenesis.

of Dscam. Dscam forms a complex with Dock, DSH3PX (a sorting nexin), and Wasp (a protein component of the actin polymerization machinery) that may affect the actin cytoskeleton (**Figure 1C**, Worby et al., 2001). In *Drosophila* projection neurons, *Dock* and *Pak* mutants do not show obvious dendrite and axon morphological defects; therefore, TBCD may function downstream of Dscam, independently of the Dock/Pak signaling pathway in projection neurons. However, it is still possible that the tubulin-folding pathway coordinates with the Dock/Pak signaling pathway. For example, PAK1 (a human ortholog of Pak) phosphorylates human TBCB, and the knockdown of *TBCB* or P*AK1* reduces microtubule polymerization (**Figure 1C**, Vadlamudi et al., 2005). Further studies are required in order to understand how the tubulin-folding pathway is regulated by Dscam and how the tubulin-folding pathway and other signaling pathways coordinate the dynamics of actin and microtubules under stimulus from the other guidance receptors.

Microtubule dynamics regulated by microtubule-associated proteins are important for neural development. Growing evidence suggests that regulation of the amount of free tubulins helps modulate microtubule dynamics. As we have described here, TBCD is an essential factor for neuronal morphogenesis. Although specific human disorders caused by *TBCD* mutation have not been identified, human DSCAM is located in the Down syndrome critical region and is implicated in the cognitive disabilities seen in Down syndrome. We found that the gain-of-function phenotype of Dscam was suppressed by reduction of TBCD. Therefore, TBCD may contribute to structural alterations, functional alterations, or both of neural circuits in Down syndrome and other neurological disorders.

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