

Requirement of Cul3 for Axonal Arborization and Dendritic Elaboration in *Drosophila* Mushroom Body Neurons

Sijun Zhu,² Rosanne Perez,¹ Marc Pan,¹ and Tzumin Lee¹

Departments of ¹Cell and Structural Biology and ²Molecular and Integrative Physiology, University of Illinois, Urbana, Illinois 61801

Cul3 belongs to the family of cullin proteins, which function as scaffold proteins of E3 ubiquitin ligase complexes. Here we show cell-autonomous involvement of Cul3 in axonal arborization and dendritic elaboration of *Drosophila* mushroom body neurons. Cul3 mutant neurons are defective in terminal morphogenesis of neurites. Interestingly, mutant axons often terminate around branching points. In addition, dendritic elaboration is severely affected in Cul3 mutant neurons. However, loss of Cul3 function does not affect extension of the axons that rarely arborize. Function of cullin-type proteins has been shown to require covalent attachment of Nedd8 (neural precursor cell-expressed developmentally downregulated), a ubiquitin-like protein. Consistent with this notion, Cul3 is inactivated by a mutation in its conserved neddylation site, and Nedd8 mutant neurons exhibit similar neuronal morphogenetic defects. Together, Cul3 plays an essential role in both axonal arborization and proper elaboration of dendrites and may require neddylation for its proper function.

Key words: *Drosophila*; Cul3; Nedd8; neurite; arborization; mushroom body

Introduction

Most neurons have multiple synaptic partners. Branching/arborization of neurites facilitates such multiparty communications. In addition, arborization/elaboration of neurites determines the ultimate shapes of the neurons, changes of which can drastically alter the computational characteristics of the neurons (Koch and Segev, 2000). Branching and terminal arborization of neurites can be regulated by both extrinsic factors and intrinsic determinants (for review, see Caroni, 1997; Acebes and Ferrus, 2000; Keith and Wilson, 2001). Diverse target-derived diffusible factors have been shown to regulate terminal arborization and branch formation (Cohen-Cory and Fraser, 1995; Wang et al., 1999; Krylova et al., 2002; Dent et al., 2004). Elaboration of neurites is also an intrinsic property of developing neurons, as suggested by the phenomenon that cultured crayfish motor neurons exhibit their characteristic neuronal arborization patterns in the absence of target muscles (Arcaro and Lnenicka, 1995). However, the molecular mechanisms that govern terminal morphogenesis of neurons have just begun to be elucidated.

Mosaic screening is a powerful tool for identifying novel genes required for various aspects of neuronal morphogenesis (Newsome et al., 2000; Reuter et al., 2003; Zugates and Lee, 2004). It basically involves making homozygous mutant neurons within genetically heterozygous brains. MARCM (mosaic analysis with a repressible cell marker) permits visualization of homozygous

mutant cells, making morphological analysis of mutant neurons possible in mosaic fly brains (Lee and Luo, 1999). MARCM labels cells based on lineage and birth timing; thus, it can also be used to characterize normal development of complex tissues (Lee and Luo, 1999; Kiger et al., 2000; Jefferis et al., 2001). MARCM analysis of the *Drosophila* mushroom body (MB), the olfactory learning and memory center (for review, see Davis, 2001; Heisenberg, 2003), has led to identification of various subtypes of MB neurons that are sequentially derived from common precursors (Lee et al., 1999; Zhu et al., 2003). By isolating mutations that disrupt MB development in mosaic organisms, we and others have identified genes involved in various poorly understood neuronal morphogenetic processes, such as remodeling of neuronal projections (Lee et al., 2000; Watts et al., 2003; Zheng et al., 2003) and divergent segregation of axonal branches (Wang et al., 2002).

Here we report recovery of a novel lethal mutation in the *Drosophila Cullin3* [*Cul3*, also named *guftagu* (*gft*)] gene based on morphogenetic defects in MB axonal termini. Mutant MB clones show extension problems selectively in the axonal processes that normally exhibit multiple arbors. Interestingly, mutant processes often stall around the places at which arborization occurs. We also observe defective reelaboration of MB dendrites during metamorphosis. Compared with their wild-type counterparts, most remodeled Cul3 mutant dendrites are much shorter and fail to form typical claw-like structure at their termini. Cul3 belongs to the family of cullin proteins that form E3 ubiquitin ligase complexes together with other components (Ou et al., 2002). We further demonstrate that conserved Nedd8 (neural precursor cell-expressed developmentally downregulated) modification site is essential for Cul3 activity, and Nedd8 mutant MB neurons have similar phenotypes as Cul3 mutant neurons. Together, we suggest that Cul3-based ubiquitin ligase is required for neurite arborization, and its proper function may require neddylation.

Received Jan. 12, 2005; revised Feb. 22, 2005; accepted March 11, 2005.

This work was supported by National Institutes of Health Grant NS42049 (T.L.). T.L. is a Klingenstein fellow and a Sloan fellow. We thank C. T. Chien for *Nedd8^{ANOT5}* flies, the Bloomington Stock Center for other mutant flies, and S. Siechen and members of the Lee laboratory, especially C. T. Zugates, for comments on this manuscript.

Correspondence should be addressed to Tzumin Lee, Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801. E-mail: tzumin@life.uiuc.edu.

DOI:10.1523/JNEUROSCI.0149-05.2005

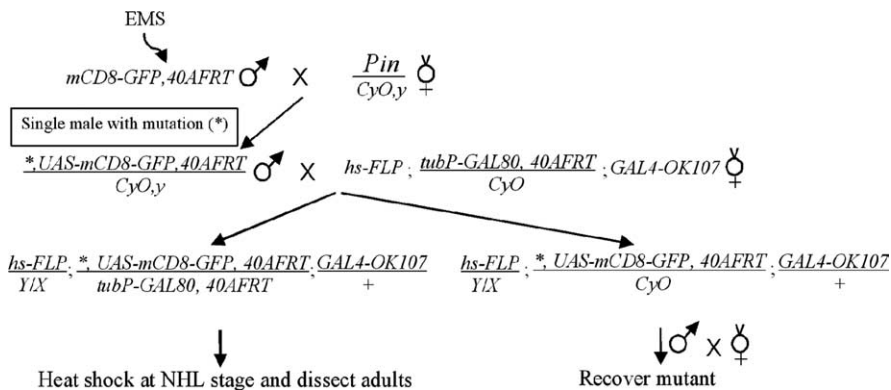
Copyright © 2005 Society for Neuroscience 0270-6474/05/254189-09\$15.00/0

Materials and Methods

Fly strains. GAL80 fly stocks used for generation of MARCM clones include the following: (1) *hs-FLP; tubP-GAL80, FRT40A/CyO, y* and (2) *UAS-mCD8-GFP, hs-FLP; tubP-GAL80, FRT40A/CyO; GAL4-OK107*; (3) *UAS-mCD8-GFP, I(2L)MB397, FRT40A/CyO*, (4) *UAS-mCD8-GFP, I(2L)MB397, FRT40A, GAL4-201Y/CyO, y*, and (5) *Cul3², FRT40A/CyO, y* were used for creation of *Cul3* mutant MARCM clones; (6) *Nedd8^{AN015}, FRT40A/CyO, Kr-GFP* was used for creation of *Nedd8* mutant clones; (7) *UAS-mCD8-GFP, FRT40A* was used for generating wild-type clones; ectopic expression of transgenes in MARCM clones involves (8) *UAS-GFP-Cul3* and (9) *UAS-GFP-Cul3^{K717R}*.

Other fly stocks collected for this study include *Df(2L)TE35BC-24* (BL-3588), *Df(2L)b87e25* (BL-3138), *Df(2L)r10* (BL-1491), *Df(2L)rd9* (BL-3592), *stc⁰⁵⁴⁴¹/CyO* (BL-11408), and *Cul3⁰⁶⁴³⁰/CyO* (BL-12175).

MARCM-based genetic screen. *UAS-mCD8-GFP, FRT40A* male flies were subjected to chemical mutagenesis according standard procedures (Lewis and Bacher, 1968) with ethyl methanesulfonate (EMS) at 40 mM. Individual male progenies derived from mutagenized flies were then crossed with *hs-FLP; tubP-GAL80, FRT40A/CyO; GAL4-OK107* for MARCM analysis of MB neurons homozygous for the mutations induced on the *UAS-mCD8-GFP, FRT40A* chromosome arms in the newly hatched larvae (NHL), as shown in the following genetic flow.



Mapping by complementation tests. After learning that the *I(2L)MB397* line was homozygous lethal, we tried to map the lethal mutation(s) using the 2L deficiency kit from the Bloomington Stock Center (Indiana University, Bloomington, IN). *I(2L)MB397* only failed to complement with *Df(2L)TE35BC-24*, localizing one lethal mutation to the region 35B04-35F07. Additional complementation tests using *Df(2L)b87e25*, *Df(2L)r10*, and *Df(2L)rd9* indicated that the lethal mutation is located in either the 35B10-35C2 region or the 35C5-35D1 region. By crossing with existing lethal mutation within these regions, we finally found that *I(2L)MB397* failed to complement with two independent *Cul3* mutations, indicating that *I(2L)MB397* carries a new recessive lethal allele of *Cul3* (*Cul3⁸*).

Molecular characterization of *I(2L)MB397*. To determine the nature of mutation of *Cul3⁸*, genomic DNAs were isolated from both wild-type and *I(2L)MB397* homozygous mutant second-instar larvae using standard procedures. Genomic DNAs for individual exons and their flanking intron splicing sites of *Cul3* were then amplified by PCR and sequenced by the ABI sequencing system (Applied Biosystems, Foster City, CA).

Construction of *UAS-GFP-Cul3* and *UAS-GFP-Cul3^{K717R}*. For *pUAST-GFP-Cul3*, *Cul3* cDNAs amplified by reverse transcription-PCR using a pair of primers flanking the entire open reading frame were first cloned into the *EcRV* site of pBSK vector. After confirming the sequence, *Cul3* cDNAs were connected to the 3'-end of GFP cDNA through a *Bgl*III site and were subsequently cloned into *EcoRI-XhoI* of pUAST. For *pUAST-GFP-Cul3^{K717R}*, the above pBSK-*Cul3* construct was used for PCR-based site-directed mutagenesis to generate a Lys-to-Arg substitution at Lys-717. Then the *Cul3^{K717R}* cDNA was ligated to the GFP and cloned into pUAST using the same restriction sites as the wild-type *Cul3* cDNAs.

Phenotypic analysis and rescue of MARCM clones. MARCM clones of

MB neurons were induced and examined at specific developmental stages as described previously (Lee and Luo, 1999; Lee et al., 1999). Rescuing the phenotypes of the MARCM clones involves coexpression of *UAS-GFP-Cul3* or *UAS-GFP-Cul3^{K717R}* with *UAS-mCD8-GFP* in GAL80-minus neurons. MARCM clones were detected by rat anti-mCD8 monoclonal antibody (1:100; Caltag, Burlingame, CA). Fluorescent images were collected using a Zeiss (Oberkochen, Germany) LSM510 confocal microscope and processed with Adobe Photoshop (Adobe Systems, San Jose, CA). Midlines were labeled according to the positions of esophagi that were visualized by enhancing the intensity of images.

Results

I(2L)MB397 homozygous mutant MB neurons show axon terminal morphogenetic defects

Using MARCM technology (Fig. 1A), we have been screening for novel mutations that cause specific developmental defects in the MB neurons by generating homozygous mutant clones in otherwise phenotypically wild-type brains (Lee et al., 2000; Wang et al., 2002; Zheng et al., 2003). The MBs are paired neuropil structures in the *Drosophila* protocerebrum. One adult MB is derived from four indistinguishable neuroblasts (Nbs), each of which sequentially generates three major types of MB neurons that, respectively, project their axonal processes into three distinct sets of MB lobes (Fig. 1B) (Ito et al., 1997; Lee et al., 1999). γ neurons, born before the mid-third instar stage, extend axons medially toward the midline of protocerebrum into the γ lobes, whereas all later-derived MB neurons, including α' / β' and α / β neurons, have bifurcated primary axons that project into specific pairs of perpendicularly oriented lobes (Lee et al., 1999). Using GAL4-OK107 (Connolly et al., 1996) in MARCM, one can selectively label all GAL80-minus MB neurons in mosaic brains. Our primary screening involved the induction of mitotic recombination in NHL,

followed by analysis of MB clones at the adult stage. We observe three distinct sizes of clones: large multi-cellular Nb clones innervating all five axonal lobes (Fig. 1B,D) and single-cell/two-cell clones consisting purely of γ neurons (Fig. 1C,E). We screened 1200 mutagenized 2L chromosome arms and recovered one line, *I(2L)MB397*, that shows truncated γ and β' axonal processes in homozygous mutant MB clones (Fig. 1D,E).

The axon terminal defects of *I(2L)MB397* are observed in mutant Nb clones, as well as in single-cell/two-cell clones of mutant γ neurons. First, mutant Nb clones acquired shorter γ and β' lobes than wild-type clones (Fig. 1D). It appears that most γ processes in the mutant Nb clones are only approximately two-thirds the length of wild-type γ processes (Figs. 1D, arrow, 2I). Second, in contrast to the truncated γ and β' lobes, only subtle morphological defects are observed in the α , α' , and β lobes. These three lobes extend as far as the wild-type counterparts, but their tips are rough and less dense (Fig. 1D, arrowheads). Third, \sim 76% of single-cell/two-cell clones of mutant γ neurons, unlike their wild-type controls, fail to project axons to the tips of the γ lobes (Fig. 1, compare E, arrows, with C). Together, *I(2L)MB397* selectively affects terminal morphogenesis of MB axons, especially the γ and β' processes.

Short axons of *I(2L)MB397* mutant γ neurons result from extension defect

Formation of the γ lobe involves pruning of larval-specific axonal branches, followed by reextension of the pruned γ axons (Fig. 2A) (Lee et al., 1999). Thus, it is possible that mutant γ axons stop

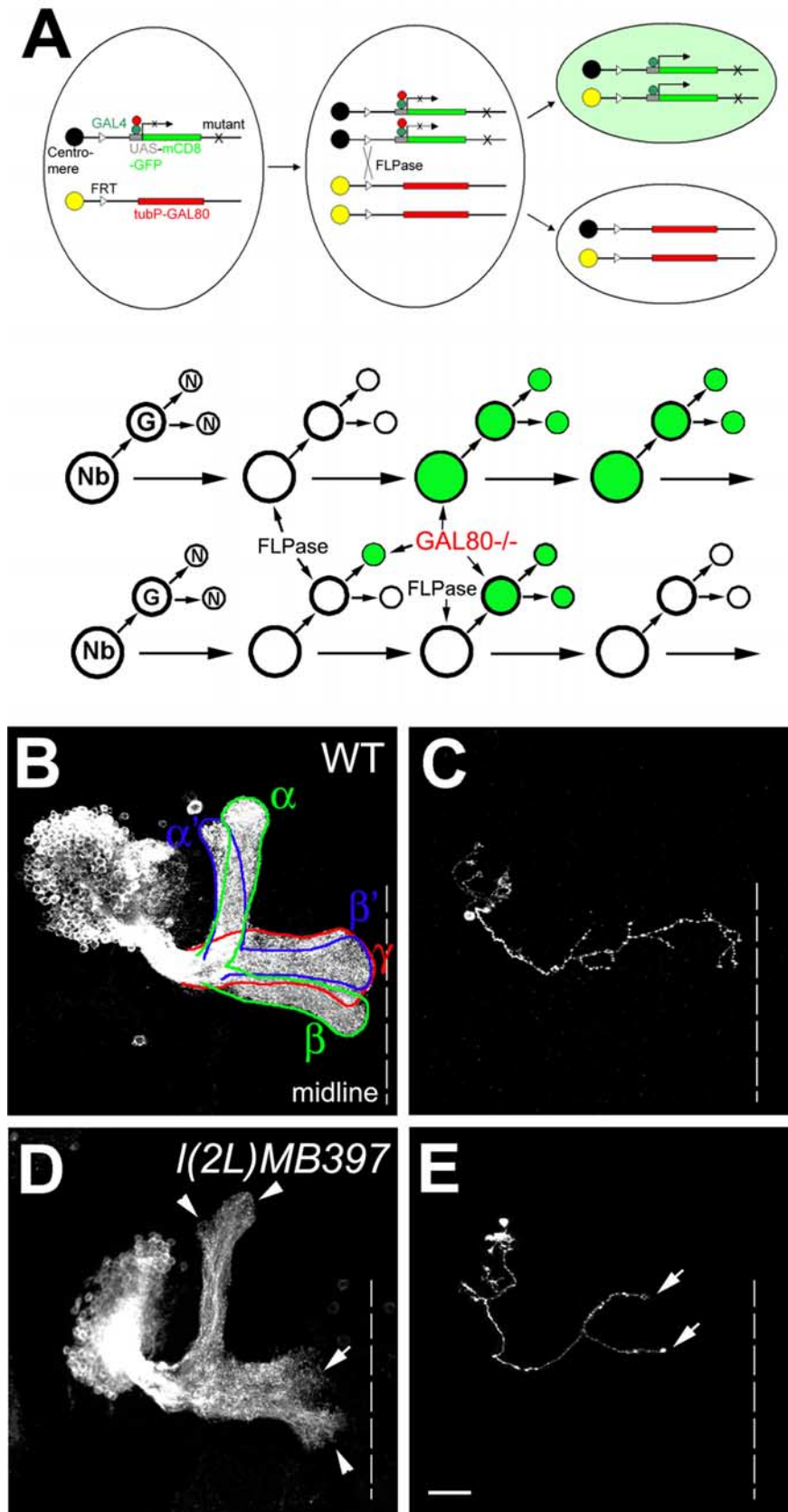


Figure 1. *I(2L)MB397* exhibits axon terminal morphogenetic defects in MB neurons. **A**, Schematic diagram of MARCM. Top, Heat shock-induced FLP recombinase (FLPase) mediates the FRT site-specific mitotic recombination, which results in the expression of mCD8-GFP driven by GAL4 in one of the daughter cells attributable to loss of the repressor transgene GAL80. If the mother cell is heterozygous for a mutant gene (X), the daughter cell homozygous for the mutant gene will be positively labeled. Bottom, An Nb or single-cell/two-cell MARCM clone can be generated in *Drosophila* CNS, depending on whether GAL80 is lost in the newly

short because of reextension problems. Alternatively, short γ processes might be derived from partially pruned larval-specific axonal branches or result from degeneration of fully reextended axons. To distinguish these possibilities, we examined the morphologies of mutant γ neurons at different developmental stages.

GAL4-201Y-marked Nb clones were generated in NHL and analyzed at different developmental stages. GAL4-201Y selectively labels γ neurons and late-pupal-born α/β neurons (Yang et al., 1995), permitting detailed examination of γ neurons through metamorphosis. In wandering larvae, *I(2L)MB397* mutant clones acquire larval MB-specific morphological features, including two perpendicularly oriented lobes (Fig. 2*B, C*, large arrowheads), as well as two short bundles of terminal branches (Fig. 2*B, C*, small arrowheads). No dramatic morphogenetic defects are observed in *I(2L)MB397* mutant clones at this stage, except subtle deformation of the termini of the medial axon bundles (Fig. 2, compare arrows in *B, C*; similar to the terminal morphogenetic defects of adult MBs). By 18 h after puparium formation (APF), most larval-specific axonal branches are pruned in mutant clones and look morphologically indistinguishable from their wild-type controls (Fig. 2*D, E*, arrows). In contrast to normal pruning, we observe severe regeneration problems in *I(2L)MB397* mutant clones. For instance, by 48 h APF, wild-type clones have reextended axons to the midline (Fig. 2*F*, arrow), but regrowth of the majority of mutant axons is incomplete and appears unsynchronized (Fig. 2*G*, arrow). In addition, no age-dependent morphological changes could

←
generated Nb, in the postmitotic neurons (N), or in the ganglion mother cell (GMC). **B, C**, Wild-type Nb (WT; **B**) and single-cell (**C**) clones of MB neurons, generated in NHL, were examined in adults. Note that one Nb clone contains all five MB lobes (outlined by solid lines in **B**) and that all of the medial lobes (γ , β , and β') can reach the midline (dashed lines in this and all of the following figures). **D, E**, *I(2L)MB397* mutant Nb (**D**) and single-cell (**E**) clones, generated in NHL, were examined in adults. Note that the γ and β' lobes are significantly shortened (arrow in **D**), but other lobes have only subtle morphological defects at the termini (arrowheads in **D**). Truncated γ axons are also observed in single-cell clones (arrows in **E**). Scale bar (in this and all of the following figures), 20 μ m. All unilateral MB clones are oriented such that their medial processes project from left to right toward the midline. All images are processed from composite confocal images. Genotype: **B, C**, *hs-FLP/X;UAS-mCD8-GFP, FRT40A/tubP-GAL80, FRT40A;GAL4-OK107/+*; **D, E**, *hs-FLP/X;I(2L)MB397,UAS-mCD8-GFP,FRT40A/tubP-GAL80,FRT40A;GAL4-OK107/+*.

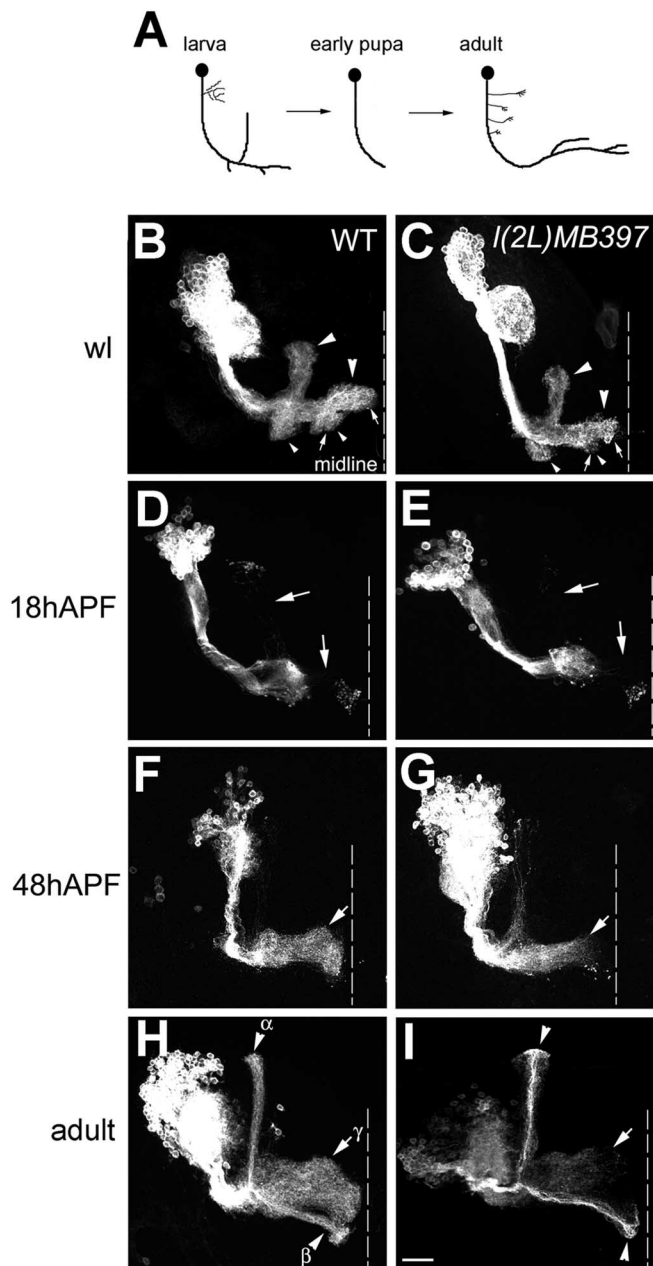


Figure 2. *I(2L)MB397* impedes reextension of γ axons during metamorphosis of the MBs. **A**, Schematic drawing of γ neuron remodeling. **B–I**, GAL4-201Y-labeled wild-type (WT; **B, D, F, H**) and *I(2L)MB397* mutant (**C, E, G, I**) Nb clones were generated in NHL and examined at the wandering larval stage (**B, C**), 18 h APF (**D, E**), 48 h APF (**F, G**), and in adults (**H, I**). Larval MB neurons have two perpendicular oriented lobes (large arrowheads in **B** and **C**) and two short bundles of terminal branches (small arrowheads in **B** and **C**). *I(2L)MB397* mutant clones exhibit subtle deformation of lobe termini at the wandering larval (wl) stage (arrows in **C** compared with arrows in **B**), normal pruning of larval lobes by 18 h APF (arrows in **E** compared with arrows in **D**) but incomplete axon regeneration, even after eclosion (arrows in **G** and **I** compared with arrows in **F** and **H**). Note that late-pupal-born α/β axons have no obvious terminal morphogenetic defects (arrowheads in **I** compared with arrowheads in **H**). Genotype: **B, D, F, H**, *FRTG13,UAS-mCD8-GFP,GAL4-201Y/FRTG13,hs-FLP,tubP-GAL80*; **C, E, G, I**, *hs-FLP/X;I(2L)MB397,UAS-mCD8-GFP,FRT40A,GAL4-201Y/tubP-GAL80,FRT40A*.

be detected in *I(2L)MB397* mutant clones after eclosion (Fig. 2, compare arrows in **H, I**; and data not shown). These results suggest that *I(2L)MB397* mutant γ appear truncated because of reextension problems rather than partial pruning or progressive degeneration.

I(2L)MB397 mutant axons often stall around the places at which arborization normally occurs

Because *I(2L)MB397* mutation selectively affects γ and β' lobes, it is unlikely that *I(2L)MB397* generally blocks axon extension. Furthermore, it is also unlikely that *I(2L)MB397* specifically impedes reextension of axons in that β' lobes do not undergo pruning and reextension as γ lobes. Given that most γ and β' processes undergo repeated arborization and few arbors exist in the other three lobes, one possibility is that failure in axon arborization underlies *I(2L)MB397* terminal morphogenetic defects of mutant axons. To examine this possibility, we collected isolated single-cell clones of γ neurons, generated in NHL, for detailed phenotypic analysis. We observe that mutant γ axons have few arbors. For quantitative analysis, we arbitrarily defined major arbors as branches longer than 15 μm (Fig. 3*F–H*, arrows). Because fewer axon arbors in the mutant γ neurons could result from extension defects instead of arborization problems, our initial analysis was focused on the mutant γ neurons that had extended axons to the lobe termini. We found that 57% (12 of 21) of the fully extended mutant γ neurons have no major axon arbor (Fig. 3*A*). In contrast, only 15% (15 of 100) of wild-type γ neurons have axons without major arbors (Fig. 3*E*). These phenomena suggest that arborization is suppressed in mutant γ axons. Interestingly, when we analyzed all single-cell clones (including those whose axons did not reach lobe termini), similar percentages of γ neurons (14% of mutant clones versus 15% of wild-type clones) acquired fully extended “arbor-free” axons, regardless of their genotypes. Thus, *I(2L)MB397* mutation might cause axons to stall during the process of branching but have no effect on the axons that do not form arbors. In support of this notion, mutant GAL4-201Y-positive late-pupal-born α/β axons, which do not arborize (S. Zhu and T. Lee, unpublished data), have no obvious terminal morphogenetic defects (Fig. 2, compare arrowheads in **H, I**).

To examine further whether mutant axons stall during the process of branching, we tried to determine where axon arborization normally occurs and where mutant axons have stalled in single-cell clones of early-larval-born γ neurons, as well as α'/β' neurons. We found that mutant γ processes could terminate within any part of the γ lobes (Fig. 3*B–D*) (see Fig. 5). Analogously, wild-type γ axons form arbors at stochastic positions (Fig. 3*F–H*) (see Fig. 5). However, such complex and highly variable axon arborization patterns make it difficult to ascribe *I(2L)MB397* the terminal morphogenetic defects of axons to extension or arborization problems. Unlike γ neurons, most α'/β' neurons exhibit invariant patterns of axon arborization. Typical β' processes acquire two widely separate clusters of arbors, one within the proximal one-third segment of the β' lobe and another within the distal one-third segment (Figs. 4*A, 5*), whereas their sister neurites, the α' processes, rarely branch (Fig. 4*A*). To determine whether axons tend to stall at the positions at which arborization normally occurs in mutant α'/β' neurons, we collected 100 isolated single-cell clones of α'/β' neurons after brief induction of mitotic recombination in *I(2L)MB397* heterozygous mid-third-instar larvae and analyzed their phenotypes. First, only two α' processes stop short, consistent with the subtle phenotypes in the α' lobes of mutant Nb clones (data not shown). Second, truncation occurs in one-quarter of mutant β' processes. Low penetrance of phenotypes in single-cell clones of mutant α'/β' neurons, which undergo morphogenesis shortly after mitotic recombination, is probably attributable to perdurance of preexisting wild-type molecules. However, in the mutant γ neurons, wild-type molecules may not exist any more when reextension of their pruned axons occurs several days after loss of het-

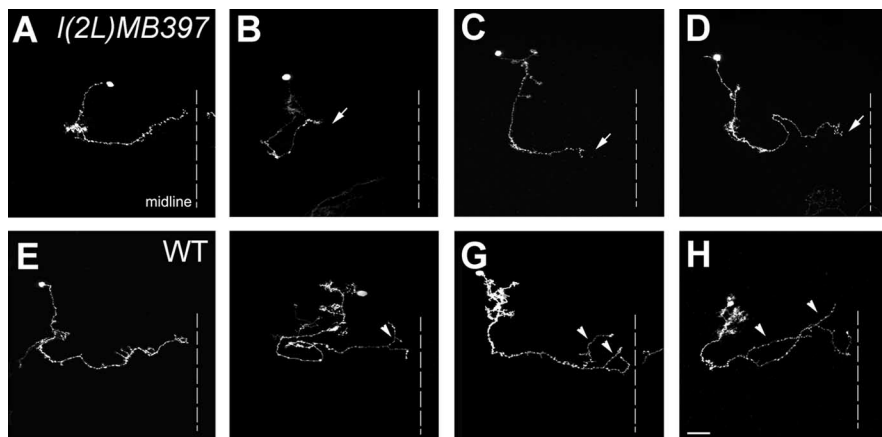


Figure 3. *I(2L)MB397* mutant γ axons have few arbors and stall randomly within γ lobes. Single-cell clones of *I(2L)MB397* mutant (A–D) and wild-type (WT; E–H) γ neurons, generated in NHL, were examined in adults. Mutant γ axons can extend to the tip of γ lobe (A) or stall at any position within the γ lobes (arrows in B–D). Few arbors can be found even in fully extended mutant γ processes (A). In contrast, only a small part of wild-type γ axons have no major arbors (E). The majority of wild-type γ axons often have multiple major arbors that are randomly distributed along the γ lobes (arrowheads in F–H). Genotype: A–D, *hs-FLP/X;I(2L)MB397,UAS-mCD8-GFP,FRT40A/tubP-GAL80,FRT40A;GAL4-OK107/+*; E–H, *hs-FLP/X;UAS-mCD8-GFP,FRT40A/tubP-GAL80,FRT40A;GAL4-OK107/+*.

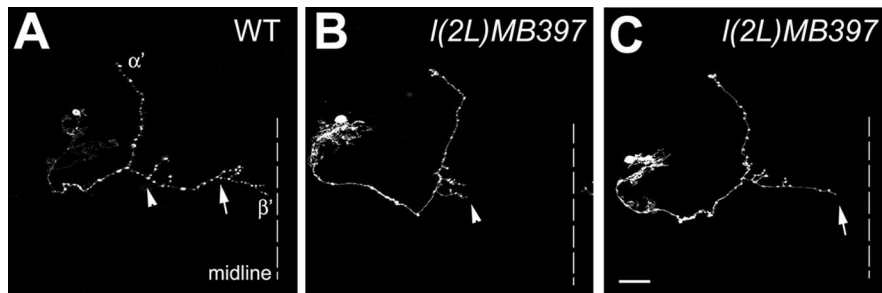


Figure 4. *I(2L)MB397* mutant β' axons often terminate around the places at which arborization normally occurs. Single-cell clones of wild-type (WT; A) and *I(2L)MB397* mutant (B, C) α' / β' neurons, generated at the mid-third-instar larval stage, were examined in adults. Wild-type β' axons form arbors within the proximal one-third segment (arrowhead in A) and the distal one-third segment (arrow in A). Mutant β' axons stall either near the proximal position of the arborization (arrowhead in B) or close to the distal locus of the arborization (arrow in C). Genotype: A, *hs-FLP/X;UAS-mCD8-GFP,FRT40A/tubP-GAL80,FRT40A;GAL4-OK107/+*; B, C, *hs-FLP/X;I(2L)MB397,UAS-mCD8-GFP,FRT40A/tubP-GAL80,FRT40A;GAL4-OK107/+*.

erozygosity. Third, in great contrast with mutant γ axons that often stall randomly within γ lobes, all truncated β' axons stall within either the proximal one-third segments (Figs. 4B, 5) or the distal one-third segments (Figs. 4C, 5) of the β' lobes, close to the positions at which two clusters of arbors form in wild-type β' processes. Together, we suggest that *I(2L)MB397* mutant axons often stall around branching points, and it is possible that mutant neurites become truncated because of defects in arborization rather than extension.

I(2L)MB397 MB phenotypes are caused by a splicing mutation in *Drosophila* Cul3

To determine whether a lethal mutation in 2L is responsible for *I(2L)MB397* MB phenotypes, we first identified and mapped an *I(2L)MB397* lethal mutation to the 35B10–35D01 cytogenomic region based on complementation experiments with the 2L deficiency kit (see Materials and Methods). Additional complementation tests using additional deficiency lines mapped the lethal mutation to either 35B10–35C02 or 35C05–35D01. By crossing with existing lethal mutations within these two regions, we found that *I(2L)MB397* fails to complement with two previously identified *Drosophila* Cul3 [*Cul3*, also named *guftagu* (*gft*)] loss-of-function alleles (*Cul3*² and *Cul3*⁰⁶⁴³⁰) (Fig. 6A). Cul3 belongs to

the family of cullin proteins, which function as E3 ubiquitin ligases by forming complexes with other proteins (Ou et al., 2002; Chiba and Tanaka, 2004). In the fly genome, there are six different cullin genes: *Cul1* (also called *lin19*), *Cul2*, *Cul3*, *Cul4*, *Cul5*, and *CG11261*, which is close to *Cul1* and *Cul2* (Ou et al., 2002). The *Cul3* gene consists of 11 exons and encodes a protein of 773 amino acids. Sequence analysis demonstrated that the *I(2L)MB397* chromosome has a point mutation at the splice acceptor site of the intron between exon 6 and exon 7 of *Cul3* (Fig. 6B). This splicing defect may lead to truncation and/or open reading frame shift of Cul3 after amino acid 516, which may eliminate several C-terminal conserved domains, including Roc1 binding site, Nedd8 modification site (also see below), and Cullin motif (Wada et al., 1999; Mistry et al., 2004). To confirm that all *I(2L)MB397* MB phenotypes resulted from loss of Cul3 function, we created clones of MB neurons that are homozygous for *Cul3*² and observed similar axon terminal morphogenetic defects as in *I(2L)MB397* mutant clones (Fig. 6C). Moreover, *I(2L)MB397* MB phenotypes were fully rescued in the presence of an autonomously expressed wild-type *Cul3* cDNA (Fig. 6D). Thus, *I(2L)MB397* is a novel *Cul3* mutation (*Cul3*⁸).

Neddylation may be required for Cul3 function

It has been shown that Cul1-based ubiquitin ligase activity requires the covalent attachment of Nedd8, a small ubiquitin-like protein, to a lysine residue in the C-terminal region of Cul1 (Amir et al., 2002) (for review, see Chiba and Tanaka, 2004). Biochemical studies have also demonstrated that all known members of human cullin family proteins can be conjugated to Nedd8 *in vitro* (Hori et al., 1999). Sequence alignment reveals that Cul3, like other cullin proteins, has a putative Nedd8 conjugation site (Lys-717) (Fig. 6E) (Wada et al., 1999). To examine possible involvement of Nedd8 covalent modification in regulating Cul3 function, we investigated whether mutation of Lys-717 to arginine in Cul3 eliminates its biological activity. Analogous mutations have inactivated other cullin proteins (Wada et al., 1999; Amir et al., 2002). We repeated earlier rescue experiments using a mutated *Cul3* cDNA, encoding *Cul3*^{K717R}. Multiple independently inserted transgenic lines were checked, and no mutant *Cul3*^{K717R} transgenic line could rescue *Cul3*⁸ MB phenotypes (Fig. 6F). These results indicate that normal Cul3 function requires an intact Nedd8 conjugation site. Elimination of neddylation site could at least partially explain the loss of function of *Cul3*⁸ allele.

To directly examine the requirement of Nedd8 for MB morphogenesis, we further investigated whether and how loss of Nedd8 affects MB development. MARCM clones of MB neurons that are homozygous for a Nedd8 loss-of-function allele (*Nedd8*^{AN015}) (Ou et al., 2002) were generated in NHL and exam-

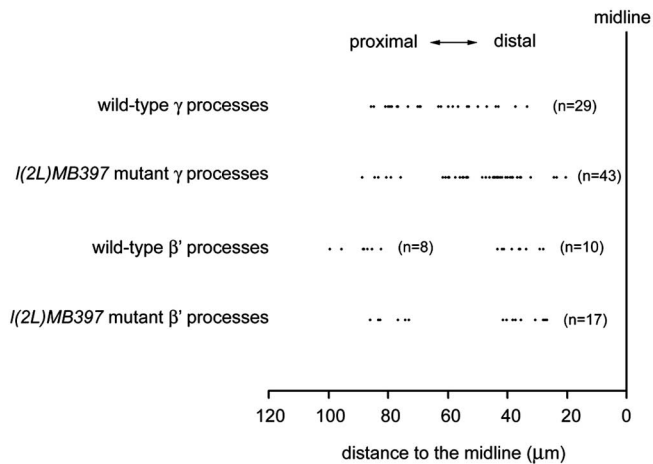


Figure 5. Comparison of ending points of *I(2L)MB397* mutant axons and arborization points of wild-type axons. The distance from the ending points of *I(2L)MB397* mutant axons or the branch points of wild-type axons to the midline were measured and compared. In the wild-type γ axons, each dot represents the distance from the first branch point of a single neuron to the midline of the brain. In the *I(2L)MB397* mutant γ axons, each dot represents the distance from the ending points of axons to the midline. Only truncated *I(2L)MB397* mutant γ axons without major axon arbors were examined. In the case of wild-type β' axons, the distance from the proximal arborization point (the right cluster) or the first arborization point of the distal cluster of arbors (the left cluster) to the midline were measured separately. Note that ending points of *I(2L)MB397* mutant β' axons fall into two clusters, close to the arborization point of wild-type β' axons. Numbers in parentheses represent the total number of neurons analyzed.

ined at the adult stage. Knocking out *Nedd8* may disable various cullin proteins with distinct functions (Amir et al., 2002). Consistent with this notion, *Nedd8* mutant Nb clones exhibit more complex phenotypes than *Cul3* mutant clones. For instance, *Nedd8* mutant MB Nb clones consist of only small numbers of γ -type neurons (Fig. 7C), indicating severe defects in MB neurogenesis. Such failure in MB neurogenesis might result from loss of Cul1 ubiquitin ligase activity that leads to cell cycle arrest at the G_1/S transition attributable to abnormal accumulation of Cul1 ligase substrates, such as p27^{Kip1} (Tsvetkov et al., 1999). Nevertheless, axonal extension defects are observed in *Nedd8* mutant clones as in *Cul3* mutant clones (Fig. 7C). γ neurons homozygous for *Nedd8*^{ANO15} fail to extend their axons to the midline in both Nb clones and single-cell/two-cell clones (Fig. 7, compare A, B with C, D). Therefore, with respect to γ neurons, *Nedd8* mutant clones qualitatively phenocopy *Cul3* mutant clones, further suggesting that proper function of Cul3 requires Nedd8 modification.

Mutation of *Cul3* blocks reelaboration of γ dendrites

Analogous mechanisms may underlie arborization of axons and elaboration of dendrites (for review, see Keith and Wilson, 2001; Jan and Jan, 2003). To determine the role(s) of Cul3 in MB dendritic morphogenesis, we examined and tried to quantify patterns of dendritic elaboration in isolated single-cell clones of *Cul3* mutant γ neurons. Like γ axons, dendrites of γ neurons also undergo remodeling during metamorphosis (Lee et al., 1999, 2000; Zheng et al., 2003), and we recognized stage-specific dendritic elaboration patterns in larval versus adult γ neurons (Zhu et al., 2003). First, consistent with normal pruning of larval neurites in *Cul3* mutant Nb clones, most adult mutant neurons possess three to five primary dendritic processes, comparable with what γ neurons normally have after remodeling (Fig. 8, compare A, B with C, D) (Zhu et al., 2003). Second, in contrast to no change in the numbers of primary dendrites, the averaged total dendritic length of mutant γ neurons is only $32.9 \pm 15.9 \mu\text{m}$ ($n = 25$) (Fig. 8C,D),

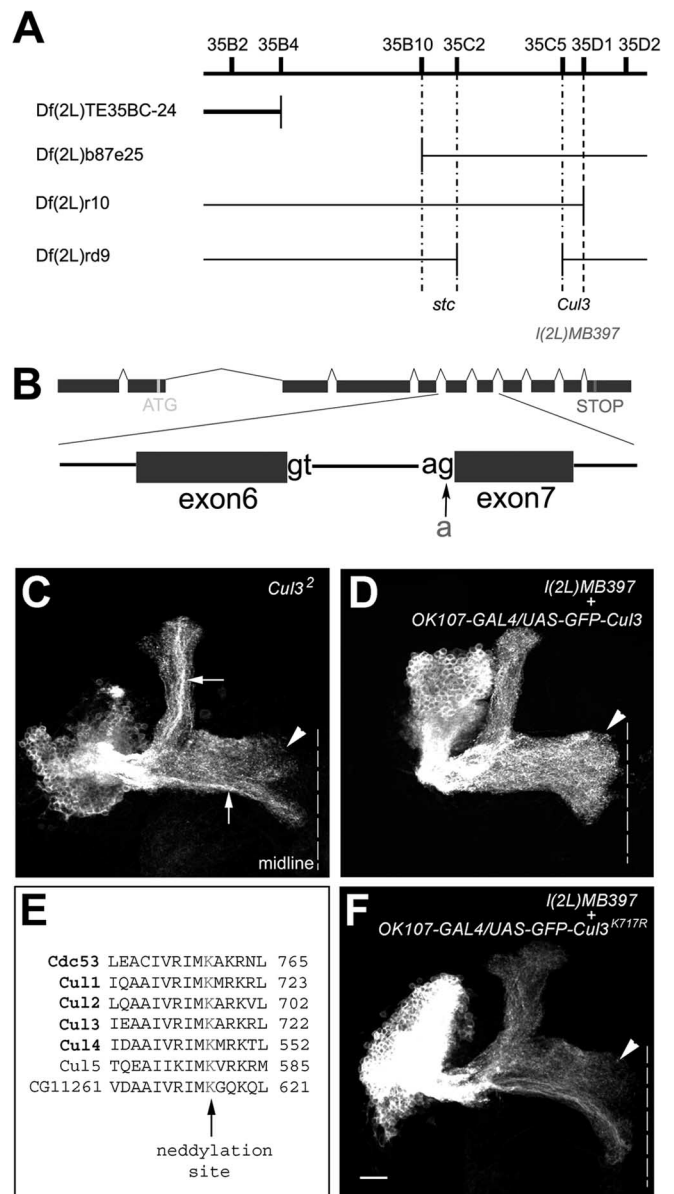


Figure 6. *I(2L)MB397* carries a splicing mutation in *Cul3*, and the conserved neddylation site is essential for Cul3 activity. **A**, *I(2L)MB397* fails to complement with the deficiency line whose deficiency region is indicated by the gap in the thick line but complemented with the deficiency lines whose deficiency regions are indicated by gaps in thin lines. Additional complementation tests assign *I(2L)MB397* to the *Cul3* complementation group. **B**, *I(2L)MB397* carries a g-to-a mutation at the acceptor site of the intron between exon 6 and exon 7 of *Cul3*. **C**, The *Cul3*² mutant Nb clone shows terminal morphogenetic defects in the γ and β' lobes (arrowhead). Also note the dense axon fascicles within the α and β lobes (arrows). **D**, All of the medial lobes are fully extended (arrowhead) in the *I(2L)MB397* mutant Nb clone in which GFP-tagged wild-type Cul3 is autonomously expressed. **E**, Partial sequence alignment among yeast Cul1 (Cdc53) and six *Drosophila* cullin proteins. Consensus neddylation sites are highlighted in red. **F**, The terminal defects of the γ and β' lobes (arrowhead) remain in the *I(2L)MB397* mutant Nb clones that autonomously express GFP-tagged *Cul3*^{K717R}. Genotype: **C**, *hs-FLP,UAS-mCD8-GFP/X; Cul3²,FRT40A/tubP-GAL80,FRT40A;GAL4-OK107/+*; **D**, *hs-FLP/UAS-GFP-Cul3;(2L)MB397,UAS-mCD8-GFP,FRT40A/tubP-GAL80,FRT40A;GAL4-OK107/+*; **F**, *hs-FLP/X;(2L)MB397,UAS-mCD8-GFP,FRT40A/tubP-GAL80,FRT40A;UAS-GFP-Cul3^{K717R}/+;GAL4-OK107/+*.

much shorter than $61.3 \pm 24.9 \mu\text{m}$ ($n = 25$), the normal dendritic length per γ neuron (Fig. 8A,B). We noticed that quite a few (42.5%; $n = 33$) mutant dendrites are extremely short and hardly separable from their primary neurites (Fig. 8D). In addition, terminal elaboration looks abnormal in mutant dendrites.

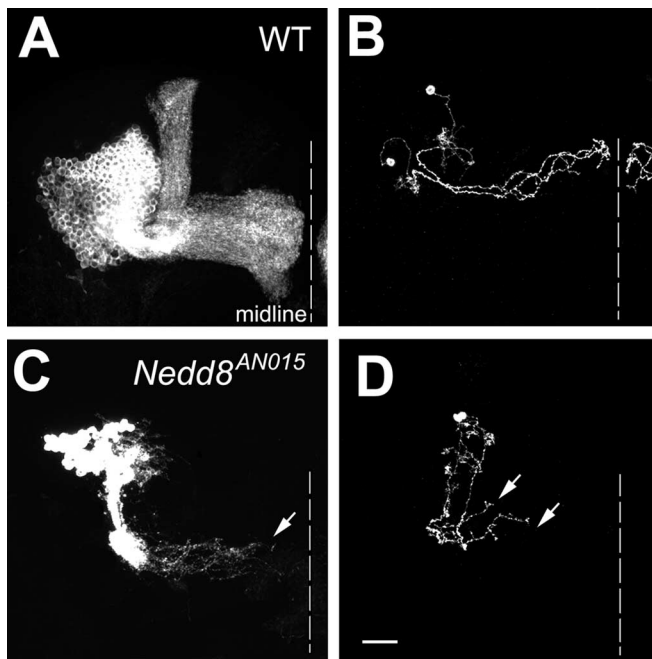


Figure 7. *Nedd8* mutant MB clones show axon terminal morphogenetic defects. Wild-type (WT; **A, B**) and *Nedd8*^{AN015} mutant (**C, D**) clones of MB neurons, generated in NHL, were examined in adults. Only a small number of γ neurons exist in the mutant Nb clone (**C**), and most mutant γ axons stall before reaching the midline (e.g., arrows in **C** and **D**). Genotype: **A, B**, *hs-FLP/X;UAS-mCD8-GFP,FRT40A/tubP-GAL80,FRT40A;GAL4-OK107/+*; **C, D**, *hs-FLP/X;UAS-mCD8-GFP/X;Nedd8*^{AN015},*FRT40A/tubP-GAL80, FRT40A;GAL4-OK107/+*.

Unlike wild-type dendrites that mostly terminate with typical claw-like structures (Fig. 8*A, B*, arrows), *Cul3* mutant γ dendrites often stall with dense irregular endings (Fig. 8*D*, arrows). These results demonstrate that elaboration of adult-specific dendritic branches is also impaired in *Cul3*⁸ mutant γ neurons.

Discussion

Like phosphorylation, ubiquitination of target proteins is a potent posttranslational modification pathway that actively controls stability, trafficking, and activity of proteins involved in various cellular processes, such as cell cycle progression, apoptosis, and signal transduction (for review, see Pickart, 2001; Glickman and Ciechanover, 2002). More recently, the ubiquitin-proteasome system has been implicated in governing various aspects of neural development, including synaptogenesis, axon guidance, and axon pruning (Wan et al., 2000; DiAntonio et al., 2001; Keleman et al., 2002; Myat et al., 2002; Watts et al., 2003; Konishi et al., 2004; Liao et al., 2004). Here we demonstrate that Cul3 is a positive regulator for axon arborization and dendritic development in MB neurons. Distinct MB axons arborize differentially. Interestingly, mutation of *Cul3* selectively blocked terminal morphogenesis of the axons that arborize extensively. Axons that rarely arborize were minimally affected, and mutant axons often stalled around the positions at which arborization normally occurs. Furthermore, *Cul3* mutant β lobes, which only show subtle phenotypes at the tips, basically project along the right path and do not curl around and project away from the midline. These observations suggest involvement of Cul3 in proper arborization rather than general extension or midline attraction/repulsion of axons. Requirement of Cul3 for reelaboration of γ dendrites implies that common mechanisms may be used to govern branching of both axons and dendrites.

Cullin family proteins function as E3 ubiquitin ligases, which

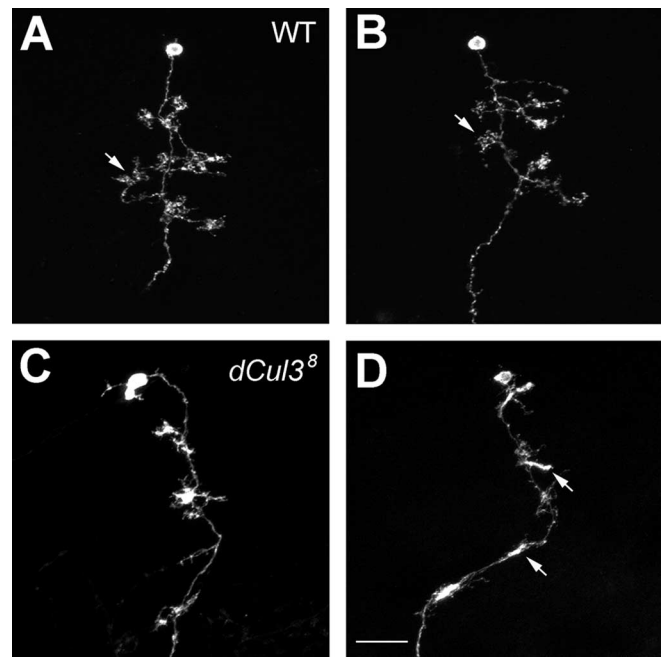


Figure 8. Poor elaboration of dendrites in *Cul3* mutant γ neurons. Close-up views of the dendritic regions of MARCM-labeled adult γ neurons. Wild-type γ neurons (WT; **A, B**) acquired longer dendritic branches than *Cul3* mutant γ neurons (**C, D**). Arrows in **A** and **B** point to the typical claw-like structures at the ends of wild-type dendritic processes. Note that mutant dendrites often stall with dense irregular endings (arrows in **D**). Genotype: **A, B**, *hs-FLP/X;UAS-mCD8-GFP,FRT40A/tubP-GAL80,FRT40A;GAL4-OK107/+*; **C, D**, *hs-FLP/X;Cul3*⁸,*UAS-mCD8-GFP,FRT40A/tubP-GAL80, FRT40A;GAL4-OK107/+*.

target specific substrates for degradation (Chiba and Tanaka, 2004). Cul3 promotes axon arborization likely via increasing turnover of its target protein(s). So far, there is one documented *Drosophila* Cul3 substrate, the full-length Cubitus interruptus (Ci155) (Ou et al., 2002; Mistry et al., 2004). Cul3-dependent degradation of Ci155 has been shown to modulate Hedgehog signaling in the posterior cells of *Drosophila* eye discs (Ou et al., 2002; Mistry et al., 2004). However, overexpression of Ci155 through development did not affect either wild-type or *Cul3* mutant MB neurons (Zhu and Lee, unpublished results), suggesting that Ci155 is not involved in Cul3-governed neurite terminal morphogenesis. In *Caenorhabditis elegans*, Cul3 mediates meiosis-to-mitosis transition by targeting meiotic-1 (MEI-1)/Katanin for degradation (Pintard et al., 2003). Interestingly, Katanin60 (Kat60), the smaller subunit of the Katanin heterodimeric ATPase, possesses microtubule severing activities and exists in neuronal processes, as well as the centrosomes (Ahmad et al., 1999). Further inspired by the phenomena that levels of Kat60 are critical for axonal outgrowth (Karabay et al., 2004), we examined the candidacy of Kat60 for the physiological substrate of Cul3 in MB neurons. Again, we observe that GAL4-OK107-driven overexpression of Kat60 exert no detectable effect on either wild-type or *Cul3* mutant MB morphogenesis (our unpublished results). In addition, Nicola et al. (2003) have reported that crossing a Kat60 *UY* element line with *7B P{GAL4}* causes the dorsal lobes of *Drosophila* MBs to stop short (Nicola et al., 2003), not mimicking *Cul3* mutant phenotypes at all. Therefore, in the MBs, Kat60 is unlikely to be the substrate of Cul3, either.

Levels of cell adhesion molecules (CAMs) may help regulate defasciculation as well as arborization of axons. As reported by Rutishauser's laboratory, blocking functions of L1 and neural CAM (N-CAM) leads to abnormal defasciculation and excessive

arborization of neurites in developing chicken embryos (Landmesser et al., 1988). Conversely, aberrant accumulation of cell adhesion molecules, such as Fasciclin II, the *Drosophila* homolog of N-CAM, promotes fasciculation of axons and impedes neurite arborization (Lin and Goodman, 1994). Interestingly, *Cul3* mutant Nb clones occasionally contain dense axon fascicles within normally homogeneous MB lobes (Fig. 6C, arrows), suggesting involvement of Cul3 in fasciculation/defasciculation of axons as well. It is possible that Cul3 mediates neurite arborization and prevents abnormal fasciculation of axons via a common mechanism, perhaps involving regulation of dynamic changes in the levels of cell adhesion molecules.

Given the involvement of cullins in more and more biological processes, it is no surprise that activities of cullin proteins need to be dynamically regulated. One common mechanism for regulation of cullin family proteins involves Nedd8 modification, a cascade of enzymatic reactions similar to ubiquitination (Chiba and Tanaka, 2004). Previous studies have suggested that Nedd8 regulates Cul1 ligase activity by facilitating the dissociation of p120 (CAND1) from Cul1 and enhancing recruitment of E2 ubiquitin to the Cul1-based ubiquitin ligase complex termed SCF (Skp1-Cul1/Cdc53-F-box) (Kawakami et al., 2001; Liu et al., 2002). In *C. elegans*, neddylation is required in Cul3 to target MEI-1 for degradation at the meiosis-to-mitosis transition (Pintard et al., 2003). However, in the developing *Drosophila* eye discs, Cul3-mediated degradation of Ci155 only partially requires Nedd8 modification (Ou et al., 2002), contrasting with the essential role of Nedd8 in Cul3-governed MB neurite terminal morphogenesis. Nedd8 was originally isolated from neural precursor cells, and its expression is developmentally downregulated (Kumar et al., 1993). Together with the requirement of Nedd8 for multiple aspects of MB development, neddylation of cullin family proteins is likely used repeatedly to govern diverse neuronal developmental processes. Studying how neddylation of cullins is regulated as well as identifying the physiological substrates of cullins promises to help further elucidate the molecular mechanisms underlying development of the brain.

References

- Acebes A, Ferrus A (2000) Cellular and molecular features of axon collaterals and dendrites. *Trends Neurosci* 23:557–565.
- Ahmad FJ, Yu W, McNally FJ, Baas PW (1999) An essential role for katanin in severing microtubules in the neuron. *J Cell Biol* 145:305–315.
- Amir RE, Iwai K, Ciechanover A (2002) The NEDD8 pathway is essential for SCF(beta-TrCP)-mediated ubiquitination and processing of the NF-kappa B precursor p105. *J Biol Chem* 277:23253–23259.
- Arcaro KF, Lnenicka GA (1995) Intrinsic differences in axonal growth from crayfish fast and slow motoneurons. *Dev Biol* 168:272–283.
- Caroni P (1997) Intrinsic neuronal determinants that promote axonal sprouting and elongation. *BioEssays* 19:767–775.
- Chiba T, Tanaka K (2004) Cullin-based ubiquitin ligase and its control by NEDD8-conjugating system. *Curr Protein Pept Sci* 5:177–184.
- Cohen-Cory S, Fraser SE (1995) Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. *Nature* 378:192–196.
- Connolly JB, Roberts IJ, Armstrong JD, Kaiser K, Forte M, Tully T, O'Kane CJ (1996) Associative learning disrupted by impaired Gs signaling in *Drosophila* mushroom bodies. *Science* 274:2104–2107.
- Davis RL (2001) Mushroom bodies, Ca²⁺ oscillations, and the memory gene amnesiac. *Neuron* 30:653–656.
- Dent EW, Barnes AM, Tang F, Kalil K (2004) Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. *J Neurosci* 24:3002–3012.
- DiAntonio A, Haghighi AP, Portman SL, Lee JD, Amaranto AM, Goodman CS (2001) Ubiquitination-dependent mechanisms regulate synaptic growth and function. *Nature* 412:449–452.
- Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* 82:373–428.
- Heisenberg M (2003) Mushroom body memoir: from maps to models. *Nat Rev Neurosci* 4:266–275.
- Hori T, Osaka F, Chiba T, Miyamoto C, Okabayashi K, Shimbara N, Kato S, Tanaka K (1999) Covalent modification of all members of human cullin family proteins by NEDD8. *Oncogene* 18:6829–6834.
- Ito K, Awano W, Suzuki K, Hiroami Y, Yamamoto D (1997) The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* 124:761–771.
- Jan YN, Jan LY (2003) The control of dendrite development. *Neuron* 40:229–242.
- Jefferis GS, Marin EC, Stocker RF, Luo L (2001) Target neuron prespecification in the olfactory map of *Drosophila*. *Nature* 414:204–208.
- Karabay A, Yu W, Solowska JM, Baird DH, Baas PW (2004) Axonal growth is sensitive to the levels of katanin, a protein that severs microtubules. *J Neurosci* 24:5778–5788.
- Kawakami T, Chiba T, Suzuki T, Iwai K, Yamanaka K, Minato N, Suzuki H, Shimbara N, Hidaka Y, Osaka F, Omata M, Tanaka K (2001) NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J* 20:4003–4012.
- Keith CH, Wilson MT (2001) Factors controlling axonal and dendritic arbors. *Int Rev Cytol* 205:77–147.
- Keleman K, Rajagopalan S, Cleppien D, Teis D, Paiha K, Huber LA, Technau GM, Dickson BJ (2002) Comm sorts robo to control axon guidance at the *Drosophila* midline. *Cell* 110:415–427.
- Kiger AA, White-Cooper H, Fuller MT (2000) Somatic support cells restrict germline stem cell self-renewal and promote differentiation. *Nature* 407:750–754.
- Koch C, Segev I (2000) The role of single neurons in information processing. *Nat Neurosci [Suppl]* 3:1171–1177.
- Konishi Y, Stegmuller J, Matsuda T, Bonni S, Bonni A (2004) Cdh1-APC controls axonal growth and patterning in the mammalian brain. *Science* 303:1026–1030.
- Krylova O, Herreros J, Cleverley KE, Ehler E, Henriquez JP, Hughes SM, Salinas PC (2002) WNT-3, expressed by motoneurons, regulates terminal arborization of neurotrophin-3-responsive spinal sensory neurons. *Neuron* 35:1043–1056.
- Kumar S, Yoshida Y, Noda M (1993) Cloning of a cDNA which encodes a novel ubiquitin-like protein. *Biochem Biophys Res Commun* 195:393–399.
- Landmesser L, Dahm L, Schultz K, Rutishauser U (1988) Distinct roles for adhesion molecules during innervation of embryonic chick muscle. *Dev Biol* 130:645–670.
- Lee T, Luo L (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* 22:451–461.
- Lee T, Lee A, Luo L (1999) Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development* 126:4065–4076.
- Lee T, Marticke S, Sung C, Robinow S, Luo L (2000) Cell-autonomous requirement of the USP/Ecr-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. *Neuron* 28:807–818.
- Lewis EB, Bacher F (1968) Method of feeding ethylmethane sulfonate (EMS) to *Drosophila* males. *Dros Info Ser* 43:193.
- Liao EH, Hung W, Abrams B, Zhen M (2004) An SCF-like ubiquitin ligase complex that controls presynaptic differentiation. *Nature* 430:345–350.
- Lin DM, Goodman CS (1994) Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron* 13:507–523.
- Liu J, Furukawa M, Matsumoto T, Xiong Y (2002) NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. *Mol Cell* 10:1511–1518.
- Mistry H, Wilson BA, Roberts IJ, O'Kane CJ, Skeath JB (2004) Cullin-3 regulates pattern formation, external sensory organ development and cell survival during *Drosophila* development. *Mech Dev* 121:1495–1507.
- Myat A, Henry P, McCabe V, Flintoft L, Rotin D, Tear G (2002) *Drosophila* Nedd4, a ubiquitin ligase, is recruited by Commissureless to control cell surface levels of the roundabout receptor. *Neuron* 35:447–459.
- Newsome TP, Asling B, Dickson BJ (2000) Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. *Development* 127:851–860.
- Nicolai M, Lasbleiz C, Dura JM (2003) Gain-of-function screen identifies a role of the Src64 oncogene in *Drosophila* mushroom body development. *J Neurobiol* 57:291–302.

- Ou CY, Lin YF, Chen YJ, Chien CT (2002) Distinct protein degradation mechanisms mediated by Cul1 and Cul3 controlling Ci stability in *Drosophila* eye development. *Genes Dev* 16:2403–2414.
- Pickart CM (2001) Mechanisms underlying ubiquitination. *Annu Rev Biochem* 70:503–533.
- Pintard L, Kurz T, Glaser S, Willis JH, Peter M, Bowerman B (2003) Neddylation and deneddylation of CUL-3 is required to target MEI-1/Katanin for degradation at the meiosis-to-mitosis transition in *C. elegans*. *Curr Biol* 13:911–921.
- Reuter JE, Nardine TM, Penton A, Billuart P, Scott EK, Usui T, Uemura T, Luo L (2003) A mosaic genetic screen for genes necessary for *Drosophila* mushroom body neuronal morphogenesis. *Development* 130:1203–1213.
- Tsvetkov LM, Yeh KH, Lee SJ, Sun H, Zhang H (1999) p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol* 9:661–664.
- Wada H, Yeh ET, Kamitani T (1999) Identification of NEDD8-conjugation site in human cullin-2. *Biochem Biophys Res Commun* 257:100–105.
- Wan HI, DiAntonio A, Fetter RD, Bergstrom K, Strauss R, Goodman CS (2000) Highwire regulates synaptic growth in *Drosophila*. *Neuron* 26:313–329.
- Wang J, Zugates CT, Liang IH, Lee C-HJ, Lee T (2002) *Drosophila* Dscam is required for divergent segregation of sister branches and suppresses ectopic bifurcation of axons. *Neuron* 33:559–571.
- Wang KH, Brose K, Arnott D, Kidd T, Goodman CS, Henzel W, Tessier-Lavigne M (1999) Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. *Cell* 96:771–784.
- Watts RJ, Hoopfer ED, Luo L (2003) Axon pruning during *Drosophila* metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. *Neuron* 38:871–885.
- Yang MY, Armstrong JD, Vilinsky I, Strausfeld NJ, Kaiser K (1995) Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns. *Neuron* 15:45–54.
- Zheng X, Wang J, Haerry TE, Wu AY, Martin J, O'Connor MB, Lee CH, Lee T (2003) TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. *Cell* 112:303–315.
- Zhu S, Chiang AS, Lee T (2003) Development of the *Drosophila* mushroom bodies: elaboration, remodeling and spatial organization of dendrites in the calyx. *Development* 130:2603–2610.
- Zugates CT, Lee T (2004) Genetic mosaic analysis in the nervous system. *Curr Opin Neurobiol* 14:647–653.