Microtubule-severing protein Katanin regulates neuromuscular junction development and dendritic elaboration in Drosophila
Chuan-Xi Mao1,2, Ying Xiong2, Zhaohuan Xiong1, Qifu Wang2, Yong Q. Zhang2,* and Shan Jin1,*

ABSTRACT
Microtubules (MTs) are crucial for diverse biological processes including cell division, cell growth and motility, intracellular transport and the maintenance of cell shape. MT abnormalities are associated with neurodevelopmental and neurodegenerative diseases such as hereditary spastic paraplegia. Among many MT regulators, katanin was the first identified MT-severing protein, but its neuronal functions have not yet been examined in a multicellular organism. Katanin consists of two subunits; the catalytic subunit katanin 60 contains an AAA (ATPases associated with a variety of cellular activities) domain and breaks MT fibers while hydrolyzing ATP, whereas katanin 80 is a targeting and regulatory subunit. To dissect the in vivo functions of Katanin, we generated mutations in Drosophila Katanin 60 and manipulated its expression in a tissue-specific manner. Null mutants of Katanin 60 are pupal lethal, demonstrating that it is essential for viability. Loss-of-function mutants of Katanin 60 showed excess satellite boutons, reduced neurotransmission efficacy, and more enlarged cisternae at neuromuscular junctions. In peripheral sensory neurons, loss of Katanin 60 led to increased elaboration of dendrites, whereas overexpression of Katanin 60 resulted in the opposite. Genetic interaction analyses indicated that increased levels of MT acetylation increase its susceptibility to Katanin-mediated severing in neuronal and non-neuronal systems. Taken together, our results demonstrate for the first time that Katanin 60 is required for the normal development of neuromuscular synapses and dendrites.

KEY WORDS: Drosophila melanogaster, Katanin, Spastin, HDAC6, Neuromuscular junction

INTRODUCTION
Microtubules (MTs) play a crucial role in a diverse array of fundamental cellular processes including cell division, cell growth and the maintenance of cell shape. MT dynamics are tightly regulated by a host of proteins that stabilize or destabilize MTs. Three MT-severing proteins, katanin, spastin and fidgetin, are members of the diverse ATPases associated with a variety of cellular activities (AAA) protein superfamily (Roll-Mecak and McNally, 2010; Sharp and Ross, 2012). Katanin, the founding member of the MT-severing protein family, was initially identified from Xenopus egg extracts exhibiting MT-severing activity (Vale, 1991). It consists of two subunits, katanin 60 and katanin 80 (McNally and Vale, 1993). Katanin 60 is the catalytic subunit that breaks MT fibers while hydrolyzing ATP, whereas katanin 80 is a targeting and regulatory subunit (Hartman and Vale, 1999).

The in vivo functions of katanin 60 have been characterized in a range of organisms. Genetic analyses of katanin 60 in Tetrahymena showed that it plays a crucial role in the formation of cilia and is essential for locomotion (Sharma et al., 2007). Caenorhabditis elegans mutant for the katanin 60 homolog mei-1 show meiotic spindle abnormalities (Srayko et al., 2006). In Drosophila S2 cells, overexpression of Katanin 60 results in MT severing and depolymerization (Díaz-Valencia et al., 2011; Zhang et al., 2007; Zhang et al., 2011). The susceptibility of MTs to katanin-mediated severing is controlled by many factors; for example, acetylation of MTs sensitizes their severing by katanin (Sudo and Baas, 2010) whereas tau binding to MTs protects them from katanin severing (Qiang et al., 2006; Qiang et al., 2010; Sudo and Baas, 2010; Yu et al., 2008).

Katanin is distributed in all neuronal compartments including axons, dendrites and cell bodies, and is particularly abundant in axons, whereas spastin is mostly concentrated at axonal branching points (Ahmad et al., 1999; Karabay et al., 2004; Sharp and Ross, 2012; Yu et al., 2008). In the rodent brain, katanin levels are high during rapid phases of axonal growth but diminish as axons reach their targets (Karabay et al., 2004). In cultured rat sympathetic neurons, expression of a dominant-negative form of katanin 60 inhibits MT severing and axonal growth, whereas overexpression of wild-type katanin 60 results in excess MT severing, but can also be deleterious to axonal growth in a subset of neurons (Karabay et al., 2004). Thus, katanin is a crucial regulator of axonal growth.

Dendrites play an essential role in information processing in the nervous system as they are involved in synapse formation and signal integration (Jan and Jan, 2010). MTs are crucial for dendrite elaboration. However, how MT regulators including MT-severing proteins affect dendritic development is poorly understood.

Previous studies have uncovered an important role for spastin in neuromuscular junction (NMJ) growth and dendritic elaboration (Jinushi-Nakao et al., 2007; Ozdowski et al., 2011; Trotta et al., 2004; Sherwood et al., 2004; Yao et al., 2011; Ye et al., 2011). However, how katanin affects neuronal development has not been characterized at an organism level. To dissect the neuronal function of katanin we generated mutants of Drosophila melanogaster Katanin 60 and transgenic lines that could be used to overexpress Katanin 60 in a tissue-specific manner. We then examined the effects of altered Katanin 60 expressions on NMJ synapses, neuronal morphogenesis and the MT cytoskeleton. We report for the first time that Katanin 60 is required for the normal development of NMJs and dendrites in Drosophila.

RESULTS
The Drosophila genome encodes an ortholog of human katanin 60
Sequence comparisons revealed that the Drosophila genome contains a gene that encodes an ortholog of human katanin 60. Drosophila Katanin 60 protein is overall 50% identical and 63%...
similar to human katanin 60 (Fig. 1A). The identity and similarity of the AAA domains of the two homologs are 80% and 93%, respectively. Indeed, katanin 60 is well conserved from C. elegans to humans, and the high degree of homology suggests that katanin 60 function has been conserved through evolution.

As a first step to understanding the in vivo function of Katanin 60 we generated two deletions, 77 and 721, which remove the N-terminus of Katanin 60 (Fig. 1B), by imprecise excision of the P-element insertion EY09078. We also used ends-out targeting to produce deletion 17A that deletes 252 amino acids containing the entire AAA domain in the Katanin 60 C-terminus (Fig. 1A, B). These deletions are likely to be nulls because there was no detectable protein as evaluated by western blotting with an antibody against Katanin 60 (Fig. 1C). Immunostaining with the antibody detected no specific signals of endogenous Katanin 60 in the ventral nerve cord, NMJs and sensory neurons of wild-type larvae, but cytoplasmic Katanin 60 was observed when it was neuronally overexpressed driven by elav-Gal4 or ppk-Gal4 (Fig. 1D; supplementary material Fig. S1). Heteroallelic 77, 721 and 17A combinations or hemizygous mutants that carry each of the deletions on one chromosome and the deficiency Df(3R)ED5138 that removes the entire Katanin 60 gene on the other, were all early pupal lethal. Specifically, 80% of homozygous Katanin 6017A mutants died at larval stages, whereas the remaining 20% lived to early pupal stage. The surviving mutant larvae appeared sluggish. As a control, the precise excision line 66 was fully viable as either homozygous or hemizygous over Df(3R)ED5138.

To determine whether the mutant phenotype could be rescued by re-expression of Katanin 60, we generated multiple transgenic lines carrying UAS-Katanin 60 insertions at 51D through site-directed integration (Bischof et al., 2007) as well as at non-targeted sites. Ubiquitous and neuronal overexpression of Katanin 60 in Drosophila background by da-Gal4 and elav-Gal4, respectively, delayed the lethality of mutants to adult stage. More importantly, one or two copies of the targeted UAS-Katanin 60 insertion without Gal4 drivers but not the original ZH-attP-51D line effectively rescued the lethality of homozygous Katanin 6017A mutants to adulthood, suggesting that a low-level expression of Katanin 60 from the targeted UAS insertion as detected by western analysis (Fig. 2H) is sufficient to rescue the lethality.

Katanin 60 regulates NMJ synapse development

Misregulated MTs lead to abnormal NMJ synapses in Drosophila (Jin et al., 2009; Nahm et al., 2013; Roos et al., 2000; Ruiz-Canada et al., 2004; Sherwood et al., 2004; Trotta et al., 2004; Wang et al., 2007; Yao et al., 2011; Zhang et al., 2001). To determine whether Katanin 60 plays a role in synapse development, we co-stained larval NMJs with anti-horseradish peroxidase (HRP) to reveal the neuronal branches (Dickman et al., 2006). The satellite boutons started to form in the middle third instar larval stages of Katanin 6017A mutants (supplementary material Fig. S2). The excess number of total boutons, largely due to the formation of satellite boutons, was also present in Katanin 6017A hemizygous larvae which carry the 17A deletion on one chromosome and Df(3R)ED5138 on the other (P<0.001; Fig. 2C, F, G). The excess of satellite boutons was highly penetrant and was also observed in other NMJs such as NMJ6/7 of homozygous and hemizygous Katanin 6017A mutants.

To confirm that loss of Katanin 60 produced the observed NMJ alterations, we carried out rescue experiments by expressing Katanin...
60 in the Katanin 60\(^{17A}\) background. Overexpression of Katanin 60 driven by the pan-neuronal elav-Gal4, the muscle-specific C57-Gal4, or the ubiquitous da-Gal4 in the mutant background showed no rescue of the Katanin 60\(^{17A}\) NMJ phenotypes. This is not surprising as overexpression of Katanin 60 from multiple independent UAS lines by elav-Gal4 in the wild-type background produced NMJ phenotypes with more satellite boutons, whereas muscular overexpression of Katanin 60 by C57-Gal4 had a minor effect on NMJ growth (see below in Fig. 6). However, as with the lethality rescue shown above, heterozygous or homozygous targeted UAS-Katanin 60 insertion without Gal4 drivers rescued the excess satellite boutons of homozygous (Fig. 2E-G) and hemizygous (data not shown) Katanin 60\(^{17A}\) mutants, whereas the original ZH-attP-51D line did not. As a control, the number of satellite boutons in NMJ terminals of the homozygous UAS-Katanin 60 line was as in the wild type (Fig. 2D). The excess satellite boutons in homozygous, hemizygous and trans-allelic Katanin 60 mutants, together with the effective rescue of the phenotypes by a low-level expression of satellite boutons, respectively. (F,G) Quantification of the total number of boutons (F) and satellite boutons (G) from different genotypes (n=20 NMJs).

Increased Futsch-positive loops in the NMJ terminals of Katanin 60 mutants

To evaluate the effects of Katanin 60 on MTs at NMJ synapses, we stained NMJ terminals with antibodies against Futsch, the Drosophila ortholog of microtubule-associated protein MAP1B (Hummel et al., 2000; Zhang et al., 2001), and \(\alpha\)-tubulin. At the wild-type muscle 4 NMJ, continuous MT bundles run along the NMJ axes. MTs within boutons are occasionally arranged in loops. MT loops are associated with an arrest in synaptic bouton division, whereas irregular and branched MT arrays indicate an actively growing or budding bouton (Conde and Caceres, 2009). There were significantly more Futsch-positive MT loops in Katanin 60\(^{17A}\) NMJ terminals than in controls (P<0.001; Fig. 3A,B,E). A homozygous UAS-Katanin 60 insertion fully reversed the effect on the MTs in Katanin 60\(^{17A}\) mutants to the wild-type level (Fig. 3C,E). Overexpression of Katanin 60 in wild-type background by elav-Gal4 did not alter the number of Futsch-positive loops at NMJs (Fig. 3D,E). Using anti-\(\alpha\)-tubulin staining, we were able to clearly see more MT loops within synaptic boutons of Katanin 60\(^{17A}\) mutants than the wild type (Fig. 3F-G*). When Katanin 60 was overexpressed in presynaptic neurons by elav-Gal4, we observed short MT fragments within the terminal boutons (Fig. 3I-II). MTs within synaptic boutons of different genotypes are schematically presented in Fig. 3"".

The ultrastructure of NMJ synapses is altered in Katanin 60 mutants

To investigate further the effects of Katanin 60 on the structure of NMJ synapses, we examined larval NMJ6 and 7 by electron microscopy (Fig. 4A-G). The most common organelles in wild-type NMJ are active zones, mitochondria, and small core-core synaptic vesicles 35-50 nm in diameter. The profile of presynaptic boutons containing active zones of transmitter release in Katanin 60\(^{17A}\) mutants appeared largely normal (Fig. 4A,B). We detected no obvious alterations in presynaptic T-bars, postsynaptic densities, or the subsynaptic reticulum (SSR) (Fig. 4A,B,D,E). However, a large number of enlarged vesicles up to 200 nm in diameter were observed throughout the boutons of Katanin 60\(^{17A}\) animals (Fig. 4B,F-H). Some of the large vesicles were horse-shoe shaped (Fig. 4F,G). There were ten times more enlarged vesicles, with diameter ≥80 nm, in the NMJ boutons of Katanin 60\(^{17A}\) mutants than the wild type (P<0.001; Fig. 4A,B,F-H). Homozygous UAS-Katanin 60 insertion fully rescued the enlarged vesicle phenotype of Katanin 60\(^{17A}\) mutants (Fig. 4C,H), indicating that the increased number of cisternae at NMJs is specifically caused by Katanin 60 mutations. How Katanin 60 affects cisterna formation in the NMJs remains unknown at present. However, we note that the other MT-severing protein spastin affects vesicle formation by interacting with integral membrane proteins or membrane-associated proteins (Blackstone et al., 2011).

Katanin 60 is required for normal neurotransmission at NMJ synapses

Given the morphological and structural abnormalities in NMJs of Katanin 60 mutants (Figs 2, 3), synaptic transmission might be also defective. To test this possibility we examined both evoked and spontaneous glutamate release using intracellular recordings.

Fig. 2. Katanin 60 regulates NMJ synapse development. (A-E) NMJs from wandering third instar larvae double-stained with anti-HRP (green) and anti-CSP (red). Representative muscle 4 NMJs of the abdominal segment A3 are shown for wild type (A), Katanin 60\(^{17A}\) (B), Katanin 60\(^{17A}\)/Df(3R)ED5138 (C), homozygous targeted UAS-Katanin 60 insertion without a Gal4 driver (D), and homozygous UAS-Katanin 60 insertion in a Katanin 60\(^{17A}\) background (E). Scale bar: 5 μm. Arrows and arrowheads in B indicate synaptic and satellite boutons, respectively. (F,G) Quantification of the total number of boutons (F) and satellite boutons (G) from different genotypes (n=20 NMJs). *P<0.05, ***P<0.001. Error bars indicate s.e.m. (H) Western analysis of larval carcasses with anti-Katanin 60. A low level of Katanin 60 was detected from homozygous UAS-Katanin 60 insertion at 51D without Gal4 drivers in the Katanin 60\(^{17A}\) background. Five times more protein was loaded for the two mutant genotypes than the wild type. Actin was used as a loading control.
from muscle 6 in the abdominal segment A3. Compared with the wild type, Katanin 60^{17A} mutants showed a significant decrease in excitatory junctional potential (EJP) amplitudes (P<0.001; Fig. 5A,B). The decreased EJP amplitudes in Katanin 60^{17A} mutants were partially rescued by one copy, and completely rescued to wild-type levels by two copies, of the UAS-Katanin 60 insertion (Fig. 5C,D).

We also examined miniature EJPs (mEJPs), i.e. the postsynaptic response to a spontaneous single-vesicle release. The mEJP amplitudes, also known as quantal size, of Katanin 60^{17A} mutants were not significantly different from those of the wild type (Fig. 5A,C,E). The mEJP frequency in Katanin 60^{17A} mutants, however, was significantly decreased by 37% compared with wild type (P<0.01). The decreased mEJP frequency was rescued by heterozygous (P<0.05) and homozygous UAS-Katanin 60 insertions (P<0.001; Fig. 5A-C,F). Because the evoked EJP amplitudes were decreased, whereas the mEJP amplitudes were normal in Katanin mutants, quantal content (QC; the number of vesicles released per evoked event), calculated by dividing the corrected EJP amplitude by the mEJP amplitude, was reduced to 51% of the wild-type level (P<0.01) (Fig. 5G). A single copy of the UAS-Katanin 60 insertion did not rescue this phenotype, whereas two copies of the UAS-Katanin 60 insertion fully rescued the reduced QC in Katanin 60^{17A} mutants (Fig. 5G). The decreases in EJP amplitude, mEJP frequency and QC observed in Katanin mutants were all fully rescued by homozygous UAS-Katanin 60 insertions (Fig. 5D,F,G), indicating that the neurotransmission defects are specifically caused by Katanin 60 mutations.

**Histone deacetylase 6 (HDAC6) antagonizes Katanin 60 in regulating synaptic growth**

A previous study on cultured mammalian cells reported that acetylated MTs are favored for severing by katanin (Sudo and Baas, 2010). HDAC6 deacetylates MTs in both mammals and Drosophila (Hubbert et al., 2002; Matsuyama et al., 2002; Sudo and Baas, 2010; Xiong et al., 2013). We confirmed the effects of HDAC6 on the MT acetylation by western analysis (Fig. 6J). To test whether the severing activity of Katanin is affected by MT acetylation status we examined genetic interactions between Katanin 60 and HDAC6 at NMJ synapses. Presynaptic overexpression of Katanin 60 by elav-Gal4 led to more satellite boutons compared with controls (P<0.001; Fig. 6A,B,I), whereas overexpression of HDAC6 resulted in near-normal NMJ synapses (Fig. 6A,C,I). However, co-overexpression of HDAC6 fully rescued the excess satellite boutons caused by Katanin 60 overexpression (P<0.001; Fig. 6D,I). Postsynaptic overexpression of Katanin 60 driven by C57-Gal4 also led to more satellite boutons (P<0.001), although to a lower extent than with presynaptic overexpression of Katanin 60 (Fig. 6B,F,I). Consistently, postsynaptic co-overexpression of HDAC6 also completely rescued the excess of satellite boutons caused by Katanin 60 overexpression, whereas postsynaptic overexpression of HDAC6 alone did not affect synapse development (Fig. 6G-I). Together, these results demonstrate that HDAC6 antagonizes Katanin 60 in regulating NMJ development.

**Negative regulation of dendritic arborization by Katanin 60**

Microtubules play a crucial role in axon growth and dendritic morphogenesis (Conde and Cáceres, 2009; Jan and Jan, 2010; Jin et al., 2009; Sherwood et al., 2004). Immunostaining of third instar Katanin 60 larvae, however, showed normal innervation of muscles by motor neurons, suggesting that motor neuron axon growth is largely normal. Furthermore, the axons of class IV dendritic arborization (da) neurons in the ventral nerve cord of Katanin 60-null or -overexpressing larvae showed no apparent abnormalities compared with wild-type larvae (data not shown).

The Drosophila da neurons provide an ideal system for studying dendritic morphogenesis. Four classes of da neurons can be distinguished according to their dendritic branching patterns and complexity, ranging from class I neurons with simple dendritic...
arbors to class IV neurons with the most highly branched dendritic trees (Grueber et al., 2002; Jan and Jan, 2010). To investigate a possible role of Katanin in dendritic morphogenesis, we examined the dendritic elaboration of class IV neurons labeled by plasma membrane-bound GFP using the UAS-Gal4 system (ppk-Gal4 > UAS-mCD8-GFP). We quantified three dendritic features: dendrite field area, total dendritic length and number of termini. In Katanin 6017A mutants the total dendritic length and the number of termini were significantly increased (Fig. 7A,B,G-I), whereas the dendrite field area of Katanin 6017A mutants showed no significant difference from the wild type. By contrast, overexpression of Katanin 60 significantly reduced all three parameters (P<0.001; Fig. 7D,G-I).

Importantly, the dendritic phenotypes of Katanin 6017A mutants were fully rescued by Katanin 60 overexpression from the UAS-Katanin 60 insertion (Fig. 7C,G-I). The rescue was confirmed using an independent non-targeted UAS-Katanin 60 line.

To understand the effect of Katanin 60 on sensory neuron dendrites, we stained GFP-labeled class IV da neurons with anti-Futsch. Overexpression of Katanin 60 significantly reduced the Futsch staining in cell bodies (P<0.001), axons (P<0.01) and dendrites (P<0.001) of class IV da neurons compared with the wild type.
staining in the wild type (Fig. 8A,C), whereas *Katanin 60* knockdown by RNAi resulted in normal levels of Futsch staining in the three compartments of class IV da neurons (Fig. 8B,F).

To further understand the interaction between *Katanin 60* and HDAC6 in dendritic morphogenesis, we co-overexpressed *Katanin 60* and HDAC6. Overexpression of HDAC6 had no effect on dendritic elaboration (Fig. 7E,G-I). Unexpectedly, co-overexpression of *HDAC6* did not rescue the reduced dendritic elaboration caused by *Katanin 60* overexpression (Fig. 7D-I). Consistently, the reduced Futsch staining in dendrites, as well as in axons, caused by *Katanin 60* overexpression was not rescued by co-overexpression of HDAC6, though the reduced intensity of Futsch in the soma was fully rescued (Fig. 8C,E,F). The failure of HDAC6 co-overexpression to rescue the reduced dendritic elaboration may be caused by MT-independent toxicity conferred by *Katanin 60* overexpression. Alternatively, the antagonistic interaction between *Katanin 60* and HDAC6 may not be effectively executed in dendrites, for unknown reasons. In any case, the genetic interaction between *Katanin 60* and HDAC6 in regulating Futsch-positive MT levels in the neuronal soma is consistent with that in regulating NMJ development (Fig. 6) and MT network formation in muscle cells (see below).

**HDAC6 antagonizes Katanin in regulating MT network formation in muscle cells**

To define further the effects of HDAC6 on the severing ability of Katanin, we analyzed genetic interactions between Katanin 60 and HDAC6 by quantifying the intensities of perinuclear MT staining and the length of MT fibers in larval muscle cells, which are large and allow clear visualization of MT networks (Jin et al., 2009; Xiong et al., 2013; Yao et al., 2011). *Katanin 60* mutant muscle cells had an unevenly distributed MT network and a significantly increased perinuclear MT intensity compared with wild-type cells (*P*<0.01; Fig. 9A,B,M). Overexpression of *Katanin 60* in muscles, however, dramatically decreased perinuclear MT density (*P*<0.001) with sparser and shorter MT fibers compared with wild-type muscles (Fig. 9A,C,M). Loss and gain of function of HDAC6 did not obviously alter the perinuclear MT network (Fig. 9A,D,E,M). As expected, co-overexpression of HDAC6 significantly rescued the decrease in MT density caused by *Katanin 60* overexpression (Fig. 9C,G,M). Conversely, the *HDAC6* null mutation enhanced the severing ability of overexpressed *Katanin 60* because MT fibers were ~50% shorter than in *Katanin 60*-overexpressing muscles (*P*<0.001; Fig. 9C,F,N). We also analyzed genetic interactions between HDAC6 and *spastin*; however, we found no effect of HDAC6 on the MT-severing ability of *Spastin* (Fig. 9I-N). Notably, overexpression of *Katanin 60* produced longer MT fragments compared with that of *Spastin* (Fig. 9C,J), consistent with the effects of their respective mammalian counterparts on MTs in fibroblasts (Yu et al., 2008). Together, our genetic analyses revealed an antagonistic interaction between HDAC6 and Katanin, supporting the finding that Katanin but not Spastin exhibits enhanced severing of acetylated MTs.

HDAC6 has many substrates such as tubulin, cortactin and heat shock protein 90 (Valenzuela-Fernández et al., 2008). To investigate whether the tubulin deacetylase activity of HDAC6 was responsible for the protection of MTs from severing by Katanin 60, we tested genetic interactions when Katanin 60 and wild-type or mutated HDAC6 disrupting the deacetylase activities were co-overexpressed. The effect on MT severing was examined for two HDAC6 mutants with mutations in either of the two deacetylase domains (DD), *HDAC6* disrupting the deacetylase activities were co-overexpressed. The effect on MT severing was examined for two HDAC6 mutants with mutations in either of the two deacetylase domains (DD), H237A mutant rescued the MT defects caused by *Katanin 60* overexpression as well as wild-type HDAC6, whereas the H664A mutant, expressed at similar levels as the H237A mutant (Xiong et al., 2013), did not
the formation of excess satellite boutons remains to be elucidated. With observations in more stable MT loops in the NMJ terminals (Fig. 3), contrasting et al., 2001). We report here that mutations of at the postsynaptic structures of excitatory synapses in the mammalian MTs have been shown to regulate the formation of dendritic spines, from Katanin severing. Fig. S3), confirming that reducing MT acetylation can protect MTs not affect synaptic endocytosis. How mutations in null mutants (supplementary material Fig. S4). Stimulation (data not shown) indicate that Katanin 60 may regulate NMJ synapse development primarily in presynaptic neurons. Consistent with a presynaptic role of Katanin 60, spastin also acts presynaptically to regulate synaptic growth (Sherwood et al., 2004). Thus both Katanin 60 loss- and gain-of-function mutants were mutually rescued to wild type (Fig. 7). The effects of Katanin 60 on dendritic elaboration are different from those of two other MT-severing proteins, Spastin and kat-60 L1. Both loss-of-function and gain-of-function of Spastin lead to reduced dendritic growth (Jinushi-Nakao et al., 2007; Ye et al., 2011), consistent with a previous report that mammalian spastin levels are important for neurite growth (Riano et al., 2009). In kat-60 L1 mutants the dendritic field area and the number of the dendritic termini are significantly reduced, but the

**DISCUSSION**

**Katanin 60 regulates NMJ synapse development**

MTs have been shown to regulate the formation of dendritic spines, the postsynaptic structures of excitatory synapses in the mammalian brain (Penzes et al., 2009; Jaworski et al., 2009) and synapse growth at Drosophila NMJ terminals (Conde and Cáceres, 2009; Jin et al., 2009; Nahm et al., 2013; Wang et al., 2007; Yao et al., 2011; Zhang et al., 2001). We report here that mutations of Katanin 60 resulted in more stable MT loops in the NMJ terminals (Fig. 3), contrasting with observations in spastin mutants in which MT loops were reduced and MTs depleted at the distal boutons (Sherwood et al., 2004). Consistently, Katanin 60 and spastin regulate distinct aspects of NMJ growth, as Katanin 60 and spastin double mutants showed a combined phenotype of the single mutants, i.e. more satellite boutons as in Katanin 60 null mutants and more branches as in spastin null mutants (supplementary material Fig. S4).

Satellite boutons have been well documented in mutants with defects in endocytosis (Dickman et al., 2006), bone morphogenetic protein (BMP) signaling (O’Connor-Giles et al., 2008) and actin cytoskeleton dynamics (Coyle et al., 2004; Rodal et al., 2008; Ball et al., 2010). Because endocytosis attenuates BMP signaling and BMP signaling promotes F-actin formation by activating the expression of Trio, a guanine nucleotide exchange factor for Rac1, endocytosis-BMP-actin acts as an emerging pathway in regulating NMJ growth and bouton formation (Ball et al., 2010; O’Connor-Giles et al., 2008; Shi et al., 2013). The normal profile of synaptic vesicles at active zones (Fig. 4D,E), the normal amplitudes of mEJPs (Fig. 5) and the normal FM1-43 dye uptake and rundown under high-frequency stimulation (data not shown) indicate that Katanin 60 probably does not affect synaptic endocytosis. How mutations in Katanin 60 lead to the formation of excess satellite boutons remains to be elucidated.

Given that Katanin 60 NMJ phenotypes were effectively rescued by leaky expression from the targeted UAS-Katanin 60 insertion and the high sensitivity of neurons to Katanin 60 protein levels (Figs 2, 6), we were unable to address the tissue and/or cell type-specific requirements of Katanin 60 for NMJ growth. However, as presynaptic overexpression of Katanin 60 in neurons produced pronounced NMJ abnormalities, whereas postsynaptic overexpression of Katanin 60 in muscles only caused a mild NMJ phenotype (Fig. 6A,B,F,I), Katanin 60 may regulate NMJ synapse growth primarily in presynaptic neurons. Consistent with a presynaptic role of Katanin 60, spastin also acts presynaptically to regulate synaptic growth (Sherwood et al., 2004). Thus both spastin and Katanin 60 normally suppress bouton formation at Drosophila NMJ terminals largely from the presynaptic neuron. In addition to similar NMJ morphological phenotypes, the neurotransmission efficiency (i.e. quantal content) was also reduced at NMJs of both Katanin 60 and spastin mutants, again supporting a presynaptic function for both.

**Katanin 60 negatively regulates dendritic elaboration**

We demonstrate for the first time that Katanin 60 negatively regulates dendritic elaboration; Katanin 60 mutations promote dendritic overgrowth, whereas overexpression of Katanin 60 loss- and gain-of-function inhibits dendritic growth of class IV da neurons (Fig. 7). Furthermore, the opposite dendritic phenotypes of Katanin 60 loss- and gain-of-function mutants were mutually rescued to wild type (Fig. 7). The effects of Katanin 60 on dendritic elaboration are different from those of two other MT-severing proteins, Spastin and kat-60 L1. Both loss-of-function and gain-of-function of Spastin lead to reduced dendritic growth (Jinushi-Nakao et al., 2007; Ye et al., 2011), consistent with a previous report that mammalian spastin levels are important for neurite growth (Riano et al., 2009). In kat-60 L1 mutants the dendritic field area and the number of the dendritic termini are significantly reduced, but the
Regulation of Katanin 60 activity and expression

Neurons contain high levels of Katanin compared with other cell types; without effective control the amount of Katanin 60 would theoretically be able to sever all MTs (McNally and Vale, 1993; Qiang et al., 2006). How are the MT-severing activity and expression of Katanin 60 regulated? It has been shown recently that Qiang et al., 2006). How are the activities of Katanin 60 differentially regulated (see below). The acetylation level of MTs and binding of MTs by various MAPs also differentially affect the severing activities of spastin and katanin 60 (see below).

**Effect of its overexpression on dendritic elaboration has not been documented (Stewart et al., 2012). Furthermore, kat-60 L1 preferentially severs MTs in the proximal dendrites of sensory neurons before dendritic detachment from the soma during metamorphosis (Lee et al., 2009). The apparent differential effects of different MT-severing proteins indicate that they regulate dendritic growth through distinct mechanisms. For example, the differences between katanin 60 and spastin may be due to their different subcellular localizations in the neuron. Spastin is enriched at growth cones, synapses and axonal branching points where extensive cytoskeletal remodeling occurs (Trotta et al., 2004; Yu et al., 2008), whereas katanin 60 is present in all regions of neurons (Karabay et al., 2004). The acetylation level of MTs and binding of MTs by various MAPs also differentially affect the severing activities of spastin and katanin 60 (see below).

**MATERIALS AND METHODS**

**Drosophila stocks and husbandry**

Flies were cultured on standard cornmeal medium at 25°C unless otherwise specified. w^{1118} was used as the wild-type control. Other stocks used included...
muscle-specific C57-Gal4 (Budnik et al., 1996), pan-neuronal elav-Gal4, ubiquitous da-Gal4, deficiency Df(3R)ED5138, which removes Katanin 60 completely (all three from Bloomington Stock Center), and a GFP marker (UAS-mCD8-GFP under the control of ppk-Gal4) for class IV sensory neurons (Matthews et al., 2007). The spastin-null mutant spastin^H237A^ was from N. Sherwood (Sherwood et al., 2004) and a UAS-spastin line was from K. Brodie (Trotta et al., 2004). The HDAC6 null mutant (HDAC6^H664A^) and the UAS-HDAC6 line were from R. Jiao (Du et al., 2010), and two UAS lines expressing mutant HDAC6 with mutation H237A or H664A in the two deacetylase domains were described previously (Xiong et al., 2013). An RNAi line of mutant HDAC6 with mutation H237A or H664A in the two deacetylase domains were described previously (Matthews et al., 2007). The spastin-null mutant spastin^H237A^ was from N. Sherwood (Sherwood et al., 2004) and a UAS-spastin line was from K. Brodie (Trotta et al., 2004). The HDAC6 null mutant (HDAC6^H664A^) and the UAS-HDAC6 line were from R. Jiao (Du et al., 2010), and two UAS lines expressing mutant HDAC6 with mutation H237A or H664A in the two deacetylase domains were described previously (Xiong et al., 2013). An RNAi line of

Knockout of Katanin 60 by local hop and ends-out targeting

P-element-mediated excision was used to generate intragenic deletions in Katanin 60 following a standard protocol. The original stock EY09078 from Bloomington has a P-element insertion in the intergenic region between Mns19 and Katanin 60 (Fig. 1B). w^1^ deletion lines in which the P-insertion was excised were initially screened by PCR followed by DNA sequencing, in conjunction with immunohistochemical analyses to confirm the mutations at the protein level (Fig. 1C).

For ends-out targeting of Katanin 60, the targeting donor DNA fragment contains 5' and 3' homologous arms flanking the target gene. The 5' arm consisting of 2966 bp from 3R: 1058181 towards Katanin 60 and the 3' arm covering 3562 bp from 3R: 1049723 towards Katanin 60 were cloned into the donor vector pGX-attP (Huang et al., 2009; Huang et al., 2009). The deletion mutants including 17A were generated according to the protocol of Huang (Huang, 2008) and verified at DNA and protein levels (Fig. 1B-D).

Generation of UAS transgenic flies

For overexpression studies, a UAS-Katanin 60 construct was made by cloning the full-length cDNA into the transformation vector pUASTattB (Bischof et al., 2007) and the commonly used pUAST. pUASTattB-Katanin 60 was inserted into the second chromosome at 51D by site-directed integration using a specific stock ZH-attP-51D from the Bloomington Stock Center (BL24483), whereas pUAST-Katanin 60 was inserted into chromosomes at non-targeted sites. The targeted insertion at 51D may express Katanin 60 ectopically because of an enhancer trap effect from the nearby hibris gene (http://flystocks.bio.indiana.edu/Reports/24483.html). Two independent, non-targeted UAS insertion lines, one in the second another in the third chromosome, were obtained. For overexpression and rescue experiments, we primarily used the targeted UAS insertion at 51D; in some cases, the results were verified by non-targeted insertions.

Immunohistochemical analyses and confocal microscopy

Western analysis was performed as described previously (Yao et al., 2011). Third instar larvae were dissected in PBS with all internal organs removed, followed by homogenization in a lysis buffer. Blots were first probed with primary antibodies: anti-Katanin 60 (1:500) against the N-terminal 264 amino acids of Katanin 60 (Zhang et al., 2007), anti-a-tubulin (1:50,000; mAb B-5-1-2; Sigma), anti-acetylated tubulin (1:5000; mAb 6-11B-1; Sigma) and anti-actin (1:50,000; mAb 1501; Chemicon), followed by incubation with HRP-coupled secondary antibodies. Protein bands were visualized by a chemiluminescence method (ECL Kit, Amersham).

Dissection and antibody staining of third instar larvae were performed as previously described (Jin et al., 2009; Yao et al., 2011). Primary antibodies included: anti-a-tubulin (1:1000; Sigma), rabbit anti-katanin 60 (1:50) (Zhang et al., 2007), Texas Red-conjugated goat anti-HRP (1:100; Jackson Laboratory), anti-Futsch [1:50; 22C10; the Developmental Studies Hybridoma Bank, University of Iowa, for fly stocks, vectors, and antibodies. We thank Drs P. W. Baas, Z. H. Liu, N. T. Sharp, N. Sherwood, the Bloomington Stock Center, the Vienna Drosophila RNAi Center and the Developmental Studies Hybridoma Bank, University of Iowa, for fly stocks, vectors, and antibodies. We thank Drs P. W. Baas, Z. H. Liu, N. T. Sharp, N. Sherwood, and Y. Ye for discussions and comments on the manuscript and Dr L. Yang at the Microscopy Facility of our institute for electron microscopy analysis of NMJ synapses.

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Quantification of perinuclear MT density and MT fiber length in muscle cells, and Futsch staining intensity in class IV da neurons

Quantification of MT density in muscles was performed as previously described (Xiong et al., 2013). The perinuclear area was defined as the circular coverage that spans 20 μm around nuclei, which were visualized with T3605 staining. Tubulin staining signals within the perinuclear area from muscle 2 of abdominal segment A4 were calculated with ImageJ; the ratio of the tubulin-positive area to the perinuclear area was calculated automatically. The length of MT fragments was measured using ImageJ in a square of 20×20 μm as shown in Fig. 9A. For quantification of Futsch intensities in the cell bodies and 20 μm proximal axons or dendrites of class IV da neurons of the abdominal segment A3, staining signals were digitalized automatically using ImageJ and normalized to that of the neighboring control class I ddaF neurons without ppk-Gal4 expression (Fig. 8).

Physiological analyses

Intracellular recordings were carried out at 18°C following a conventional procedure. Specifically, wandering third instar larvae were dissected in Ca2+-free HL3.1 saline (Feng et al., 2004; Jin et al., 2009) and recorded in HL3.1 saline containing 0.5 mM Ca2+. Excitatory junctional potentials (EJPs) were recorded from muscle 6 of abdominal segment A3, followed by miniature EJP (mEJP) recording for 180 seconds. Quantal content was calculated by dividing the corrected EJP amplitude by the mEJP amplitude according to a classical protocol (Martin, 1955). The EJP correction for nonlinear summation was performed using a reversal potential of 10 mV.

Electron microscopy

Electron microscopy of NMJ terminals was performed largely according to previously published procedures (Liu et al., 2010; Liu et al., 2011; Zhao et al., 2013). Ultrathin (70-80 nm) longitudinal sections of NMJ 6 or 7 from the abdominal segment A3 were cut with a Leica UC6 ultramicrotome. Processed samples were observed under a JEOL 1400 electron microscope.

Statistical analyses

All statistical comparisons were performed using GraphPad InStat 5 software. P-values were calculated by one-way ANOVA. Comparisons were made between a specific genotype and the wild-type control (asterisks are located above a column) or between two specific genotypes (asterisks are located above a bracket).

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Competing interests

The authors declare no competing financial interests.
Author contributions
S.J. and Y.Q.Z. designed the project. C.M., Y.X., Q.W. and S.J. performed the experiments. Y.Q.Z. and S.J. analyzed data and wrote the paper with inputs from C.M. and Y.X.

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Supplementary material
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References


