α-Catulin CTN-1 is required for BK channel subcellular localization in C. elegans body-wall muscle cells

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The BK channel, a voltage- and Ca2+-gated large-conductance potassium channel with many important functions, is often localized at specific subcellular domains. Although proper subcellular localization is likely a prerequisite for the channel to perform its physiological functions, little is known about the molecular basis of localization. Here, we show that CTN-1, a homologue of mammalian α-catulin, is required for subcellular localization of SLO-1, the Caenorhabditis elegans BK channel α-subunit, in body-wall muscle cells. CTN-1 was identified in a genetic screen for mutants that suppressed a lethargic phenotype caused by slo-1(gf) mutants, SLO-1, a protein with two putative membrane-spanning domains, contributes to SLO-1 subcellular localization in mammals (Knaus et al., 1999, 2000; Uebele et al., 2000; Weiger et al., 2000; Brenner et al., 2000a; Levy et al., 2008), and Slob and dSLIP1 in Drosophila (Xia et al., 1998; Zhou et al., 1999).

BK channels are often localized/enriched at specific subcellular domains. For example, in neurons, BK channels are enriched at the presynaptic nerve terminal (Robitaille et al., 1993; Knaus et al., 1996; Zhou et al., 1999; Hu et al., 2001; Misonou et al., 2006), where they colocalize with voltage-gated Ca2+-channels (VGCCs) (Roberts et al., 1990; Robitaille et al., 1993; Isa and Hudspez, 1994; Yazejian et al., 2000) to regulate neurotransmitter release (Robitaille et al., 1993; Hu et al., 2001; Wang et al., 2001; Raffaelli et al., 2004; Liu et al., 2007; Wang, 2008). In epithelial cells, BK channels are localized to the apical membrane, where they may regulate potassium secretion and cell volume (Segal and Reuss, 1990; Pacha et al., 1991; Takeuchi et al., 1992; Hirsch et al., 1993; James and Okada, 1994; Huang et al., 1999). In mouse inner hair cells of the cochlea, BK channels are localized to the apical membrane (Pyott et al., 2004) but their role in mammalian auditory function is unclear (Pyott et al., 2007). Proper subcellular localization of the channel is likely important to its physiological functions. However, this has not been experimentally demonstrated. The molecular basis of BK channel subcellular localization is also poorly understood. Although a recent study reported that ISLO-1, a protein with two putative membrane-spanning domains, contributes to SLO-1 subcellular localization in Caenorhabditis elegans (Kim et al., 2009), no homologues could be identified in mammals.

The C. elegans BK channel α-subunit SLO-1 is enriched at synaptic regions in the nervous system and clusters in the vicinity of dense bodies in body-wall muscle cells (Wang et al., 2001). In a genetic screen for suppressors of a lethargic phenotype caused by expressing a slo-1(gf) transgene, we identified CTN-1 as a protein indispensable for BK channel function in C. elegans body-wall muscle cells, presumably because of its function in channel subcellular localization. This finding may serve as a starting point for elucidating the molecular basis of BK channel subcellular localization in mammals.

Results

ctn-1 Mutants were isolated as suppressors of a lethargic phenotype caused by slo-1(gf)

To identify novel molecules related to BK channel function in vivo, we screened for mutants that suppressed the lethargic
phenotype of a worm strain expressing SLO-1(gf) under the control of slo-1 promoter (Pslo-1). SLO-1(gf) was created by mutating SLO-1 glutamate 350 to glutamine (E350Q). E350 is the same residue that was changed to lysine in a previously described slo-1(gf) mutant (Davies et al., 2003), and the equivalent of mouse Slo1 E321, which contributes to one of the two negative rings at the entrance to the intracellular vestibule of the channel (Brelidze et al., 2003). Worms expressing Pslo-1::SLO-1(E350Q) exhibited distorted locomotion waveform and greatly decreased locomotion speed (Supplementary Movies 1 and 2). From a screening of 24 000 haploid genomes, we isolated 25 mutants. Twelve of the isolated mutants belong to one gene, which was mapped to a 107-kb interval on chromosome I (2562–2669 kb) through single-nucleotide polymorphism (SNP)-based mapping (Davis et al., 2005). We then tested whether cosmids or PCR-amplified genomic DNA fragments of predicted genes within this interval could reinstate the lethargic phenotype of slo-1(gf) when they were expressed in one of the mutants that harboured the slo-1(gf) transgene. We found that two PCR-amplified overlapping genomic DNA fragments (total ~15 kb) corresponding to the predicted ctn-1 gene ( locus Y23H5A.5, http://www.wormbase.org) and 3 kb sequence upstream of its initiation site completely restored the lethargic phenotype of slo-1(gf) (not shown). The ctn-1 encodes a homologue of mammalian α-catulin (Janssens et al., 1999). Although several splice variants of ctn-1 have been identified (http://www.wormbase.org), we found that ctn-1d (Y23H5A.5d) is the predominant isoform based on reverse transcription PCR (RT-PCR). The predicted translational product of this isoform shares 41% identity with mammalian α-catulin (Janssens et al., 1999) in amino-acid sequence (Figure 1). Sequencing of five randomly picked ctn-1 mutants revealed molecular lesions of this gene in all of them (Figure 1). The ctn-1 (zw1), which is a putative null resulting from a premature stop codon, was used for all subsequent analyses.

Figure 1  The ctn-1 encodes a homologue of mammalian α-catulin. Shown is the alignment between predicted amino-acid sequences of CTN-1 and human α-catulin (41% identity). The molecular lesions of five ctn-1 alleles were determined. Four alleles have mutations leading to premature stop codon (marked with *) and one allele (marked with an arrow) disrupts a splice donor site leading to a frame shift after amino-acid Y92 and then a stop codon (CFNGQPIMCM STOP).
**ctn-1 was expressed in muscle cells and neurons**

To understand how CTN-1 contributes to SLO-1 function **in vivo**, we first analysed the expression pattern of *ctn-1* and compared it with that of *slo-1*. Two independent transgenic strains were created that expressed GFP under the control of the *ctn-1* promoter (Pctn-1) and *slo-1* promoter (Pslo-1), respectively. The expression pattern of *ctn-1* largely overlapped with that of *slo-1*. Specifically, both *ctn-1* and *slo-1* were expressed in many neurons and several types of muscles, including body-wall muscle, vulval muscle and stomatointestinal muscle. However, *slo-1* appeared to be expressed in more neurons in the head than *ctn-1*, whereas *ctn-1* was expressed in pharyngeal muscle cells and some other unidentified cells that did not express *slo-1* (Figure 2).

The identified *ctn-1* splice variants (http://www.wormbase.org) include 9 to 13 exons. All of the splice variants share the first 8 exons but each of them has a unique exon afterward. To determine where these splice variants might be expressed, we created transgenic strains expressing *ctn-1* genomic DNA with GFP-coding sequence inserted into each of the unique exons separately (Figure 3A). As GFP was fused to full-length CTN-1, GFP epifluorescence could reflect both the expression and subcellular localization patterns of the *CTN-1* isoforms. We detected strong *ctn-1d* expression in both neurons and muscle cells, obvious *ctn-1c* expression in muscle cells but weak *ctn-1c* expression in neurons, and no *ctn-1a* or *ctn-1b* expression (Figure 3B). As *CTN-1d::GFP* appeared as puncta in neuronal processes but not in the soma, it is difficult to tell whether it is expressed in all or a subset of motoneurons in the ventral nerve cord. We then tested whether the expression of wild-type *ctn-1c* in a *ctn-1::slo-1(gf)* double mutant could reinstate the lethargic phenotype as *ctn-1d* did. However, such an effect of *ctn-1c* was not observed, suggesting CTN-1c is either unrelated or unimportant to SLO-1 function **in vivo**. Therefore, *CTN-1d* appeared to be the most important isoform with respect to SLO-1 function, and was used in subsequent experiments. *CTN-1d* is referred to as CTN-1 hereafter.

**CTN-1 was required for SLO-1 function in body-wall muscle cells**

As the expression patterns of *ctn-1* and *slo-1* appeared to overlap in body-wall muscle cells and some neurons, the suppression of *slo-1(gf)* lethargic phenotype by *ctn-1(lf)* could be due to CTN-1 deficiency in muscle cells, neurons or both. To answer this question, we tested whether targeted expression of wild-type CTN-1 in body-wall muscle cells or neurons could reinstate the lethargic phenotype in the *slo-1(gf);ctn-1(lf)* double mutant. Quantitative analyses of locomotion speed showed that *ctn-1(lf)* partially but significantly suppressed the inhibitory effect of *slo-1(gf)* on locomotion, which could be reversed by expressing wild-type CTN-1 under the control of the muscle-specific *myo-3* promoter (Pmyo-3) (Okkema et al., 1993) but not the pan-neuronal *rab-3* promoter (Prab-3) (Nonet et al., 1997) (Figure 4A; Supplementary Movies 3–7). These observations suggest that the suppression of *slo-1(gf)* phenotype by *ctn-1(lf)* resulted mainly from CTN-1 deficiency in muscle cells.

Both *slo-1(lf)* and *ctn-1(lf)* mutants appeared to be grossly distinct from the wild type in head movement behaviours. To further examine functional relationship between CTN-1 and SLO-1, we quantified the head-bending angle of the wild type and mutant animals using an automated tracking and analysis system. Consistent with a previous report (Kim et al., 2009), we observed a significant increase in head-bending angle in *slo-1(lf)*. This phenotype was shared by *ctn-1(lf)*, and that its severity was not additive in the *ctn-1(lf);slo-1(lf)* double mutant (Figure 4B and C), suggesting that CTN-1 and SLO-1 likely function together. The head-bending phenotype of *ctn-1(lf)* could be rescued by expressing wild-type CTN-1 in muscle cells but not in neurons (Figure 4B and C), suggesting that the mutant phenotype was mainly caused by CTN-1 dysfunction in muscle cells.

**SLO-1 is an important negative regulator of neurotransmitter release at the C. elegans neuromuscular junction (NMJ)** (Wang et al., 2001; Liu et al., 2007). The identification of *ctn-1* expression in ventral cord motoneurons (Figure 2) raised the possibility that CTN-1 might be needed for SLO-1 function in regulating neurotransmitter release. To investigate this possibility, we analysed the effect of *ctn-1(lf)* on miniature and evoked postsynaptic currents (mPSCs and ePSCs) recorded from body-wall muscle cells at two different extracellular Ca2+ concentrations (5 and 0.25 mM). The higher [Ca2+]i
more suitable for determining whether slo-1(gf) reduces ePSC amplitude and whether this effect may be reversed by ctn-1(lf), whereas the lower [Ca\(^{2+}\)] is more suitable for testing whether ctn-1(lf) could increase ePSC amplitude as slo-1(lf) does (Liu et al., 2007).

ePSCs were evoked by photoactivation of motoneurons expressing channelrhodopsin-2 under the control of the unc-17 promoter (Liewald et al., 2008; Liu et al., 2009). At 5 mM [Ca\(^{2+}\)], slo-1(gf) significantly decreased ePSC amplitude and mPSC frequency without affecting mPSC amplitude compared with the wild type, and these effects of slo-1(gf) were not suppressed by ctn-1(lf) (Figure 5). At 0.25 mM [Ca\(^{2+}\)], slo-1(lf) significantly increased ePSC amplitude and mPSCs frequency without affecting mPSC amplitude, and these effects of slo-1(lf) were not shared by ctn-1(lf) (Figure 5). These observations suggest that CTN-1 is not required for the function of SLO-1 in regulating neurotransmitter release at the NMJ.

CTN-1 was required for SLO-1 subcellular localization in body-wall muscle cells

CTN-1 could contribute to SLO-1 function in body-wall muscle cells through several potential mechanisms. We first tested whether CTN-1 has a function in SLO-1 subcellular localization. We previously showed that SLO-1 is enriched at dense body areas in body-wall muscle cells and in the synapse-rich nerve ring in the nervous system, and that these subcellular localization patterns may be recapitulated by an SLO-1::GFP fusion protein (Wang et al., 2001). We created two transgenic strains expressing integrated SLO-1::GFP and Pmyo-3::SLO-1::GFP for analysing SLO-1 subcellular localization in the nerve ring and body-wall muscle cells, respectively. These two transgenes were then separately crossed into ctn-1(lf) mutant. Although SLO-1::GFP appeared as puncta at locations matching dense bodies in body-wall muscle cells of the wild type, the fluorescent puncta were almost absent in ctn-1(lf) (Figure 6A and B). In contrast, SLO-1::GFP localization in the nerve ring appeared indistinguishable between the wild-type and ctn-1(lf) mutant (Figure 6A). We then asked whether ctn-1(lf) would affect the subcellular localization of two other proteins in body-wall muscle cells, including INX-11 and vinculin. INX-11 is an innexin that may form gap junctions or hemichannels, whereas vinculin is a membrane-cytoskeletal protein. Both proteins are expressed in body-wall muscle cells and localized to dense body regions (Francis and Waterston, 1985; Barstead and Waterston, 1989; Altun et al., 2009). We found that both GFP-tagged INX-11 and native vinculin were normally localized in the ctn-1(lf) mutant (Supplementary Figure S2). These observations suggest that CTN-1 may be specifically required for SLO-1 subcellular localization in body-wall muscle cells.

The disappearance of SLO-1::GFP puncta in body-wall muscle cells of ctn-1(lf) mutants could be due to decreased...
gene transcription or decreased protein synthesis/stability. To determine whether CTN-1 controls slo-1 transcription in muscle cells, we compared the expression of a P\textit{slo-1}::GFP transcriptional fusion between the wild-type and \textit{ctn-1(lf)} mutant. GFP expression in body-wall muscle cells was similar between the two groups (Figure 6C). To determine whether CTN-1 has an effect on SLO-1 protein level, we compared the total SLO-1::GFP protein level between the wild-type and \textit{ctn-1(lf)} mutant by western blot using a GFP antibody but found no difference (Figure 6D). These observations suggest that the apparent SLO-1 mislocalization observed in \textit{ctn-1(lf)} mutant did not result from a deficiency in \textit{slo-1} transcription or SLO-1 protein synthesis/stability.

CTN-1 physically interacted with SLO-1 both in vivo and in vitro

To determine whether CTN-1 mediates SLO-1 subcellular localization through a local effect, we analysed subcellular localization pattern of a CTN-1::EGFP fusion protein in body-wall muscle cells. The fusion protein was expected to recapitulate the subcellular localization pattern of wild-type CTN-1 because it reinstated the lethargic phenotype when expressed in the \textit{slo-1(gf);ctn-1(lf)} double mutant (not shown). CTN-1 is required for BK channel localization

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We found that CTN-1::EGFP was enriched at dense body regions (Supplementary Figure S4A), which was independent of SLO-1 (Supplementary Figure S4B). Furthermore, we found that CTN-1::mStrawberry, which was also fully functional, colocalized with SLO-1::GFP at dense body regions (Figure 6E). These observations suggest that CTN-1 likely mediates SLO-1 subcellular localization through a local effect.

The colocalization data shown in Figure 6E did not have enough resolution to suggest whether CTN-1 physically interacts with SLO-1. To address this question, we performed bimolecular fluorescence complementation (BiFC) assays (Chen et al., 2007; Shyu et al., 2008), which tells not only whether but also where two proteins interact in vivo. In this assay, the nonfluorescent amino- and carboxyl-terminal portions of yellow fluorescent protein (YFPα and YFPc) are fused separately to a pair of proteins of interest. The fluorophore of YFP may be reconstituted if these two proteins are physically very close (Shyu et al., 2008). We inserted YFPα into the linker region between the two RCK domains of SLO-1 and fused YFPc to the carboxyl terminus of CTN-1, and coexpressed these two fusion proteins in body-wall muscle cells under the control of Pmyo-3. Fluorescent puncta were observed in body-wall muscle cells coexpressing SLO-1::YFPα and CTN-1::YFPc (Figure 7A), suggesting that SLO-1 and CTN-1 are physically very close in the muscle cells. SLO-1 may be divided into two major structural components, including the amino-terminal portion (1–352 aa) that contains seven membrane-spanning domains and the channel pore domain, and the cytoplasmic carboxyl terminal portion (353–1140) that contains two RCK domains (Wang et al., 2001; Jiang et al., 2002; Salkoff et al., 2006; Yusifov et al., 2008; Yuan et al., 2010). To determine which part of SLO-1 is important for the interaction with CTN-1, we tested whether the amino- or carboxyl-terminal portion of SLO-1 is required for BiFC with CTN-1. In these experiments, YFPα was either fused to the carboxyl terminus of SLO-1(1–370) or inserted into the linker region between the two RCK domains of SLO-1(371–1140). We found that SLO-1(371–1140) but not SLO-1(1–370) allowed BiFC with CTN-1 (Figure 7A). These observations suggest that CTN-1 is physically very close to SLO-1, and likely interacts with the carboxyl terminal portion of SLO-1 to mediate SLO-1 subcellular localization.

To obtain independent evidence showing the interaction between CTN-1 and SLO-1, we performed coimmunoprecipitation experiments with HA-tagged CTN-1, and Myc-tagged full-length SLO-1 or SLO-1 amino- and carboxyl-terminal portions. We found that either full-length SLO-1 or SLO-1(371–1140) coimmunoprecipitated with CTN-1 whereas SLO-1(1–370) did not (Figure 7B). These observations reinforced the notion that the cytoplasmic carboxyl terminal portion of SLO-1 is important to the interaction with CTN-1.
CTN-1 did not modulate SLO-1 current properties

The physical closeness between CTN-1 and SLO-1, as revealed by the biochemical assays, suggested that CTN-1 might be able to directly modulate SLO-1 functional properties. We examined this possibility by coexpressing CTN-1 with SLO-1 in *Xenopus* oocytes, and analysing SLO-1 currents recorded from inside–out patches at three different cytoplasmic Ca$^{2+}$ concentrations (10 $\mu$M, 100 $\mu$M and 1 mM). CTN-1 did not show a significant effect on SLO-1 conductance–voltage relationship (Supplementary Figure S5). SLO-1 current amplitude and kinetics also appeared to be unaltered by CTN-1 (not quantified). These observations indirectly suggest that proper subcellular localization of SLO-1 is critical to its function in body-wall muscle cells.

CTN-1 was normally localized in dys-1 mutant

A recent study suggested that ISLO-1 contributes to SLO-1 subcellular localization in body-wall muscle cells by interacting with the dystrophin-associated protein complex (DAPC). SLO-1 is mislocalized in mutants of either *islo-1* or *dys-1* (dystrophin) (Kim *et al*., 2009). We were able to reproduce the reported SLO-1 mislocalization in the *dys-1(cx18)* mutant (Figure 8). To determine whether CTN-1 depends on DAPC for localization, we compared CTN-1::EGFP expression and subcellular localization in body-wall muscle cells between the wild-type and *dys-1(cx18)* mutant but found no difference (Figure 8), suggesting that dystrophin is not required for CTN-1 subcellular localization in body-wall muscle cells. To address whether mutation of CTN-1 would have an effect on the assembly of the DAPC, we cloned the cDNA of z-dystrobrevin gene (*dyb-1*), which encodes a major component of the DAPC (Blake *et al*., 2002), and expressed mStrawberry-tagged DYB-1 (DYB-1::mStrawberry) in body-wall muscle cells of the wild-type and *cnl-1* mutant. We found
that DYB-1::mStrawberry was localized in dense body areas in both the wild-type and ctn-1 mutant (Supplementary Figure S6). These results suggest that CTN-1 and DAPC might be required for SLO-1 localization independently.

Discussion

This study showed that CTN-1-mediated SLO-1 subcellular localization was important to SLO-1 function in vivo. This conclusion is supported by the disruption of SLO-1 localization in body-wall muscle cells of ctn-1(lf) and the suppression of slo-1(gf)-induced lethargy by ctn-1(lf). Although there may be other proteins implicated in BK channel subcellular localization, the fact that 12 independent alleles of ctn-1 were isolated in the genetic screen suggests that CTN-1 is an important player in SLO-1 function.

What might be the physiological significance of localizing SLO-1 to dense body regions by CTN-1? The function of body-wall muscle cells is to contract and relax in response to changes in [Ca\(^{2+}\)]. EGL-19, an L-type (Ca\(_V1.1\)) VGCC (Bargmann, 1998), is also localized to dense body regions (Kim et al, 2009) and is the predominant carrier of inward currents in C. elegans body-wall muscle cells (Jospin et al, 2002). It is well established that Ca\(^{2+}\) entry through VGCCs creates local high Ca\(^{2+}\) concentration domains known as Ca\(^{2+}\) microdomains at the inner openings of the channels, where [Ca\(^{2+}\)] could be as high as over 100 \(\mu\)M (Adler et al, 1991; Llinas et al, 1992; Yazejian et al, 2000). The high [Ca\(^{2+}\)] may serve as a strong activator of colocalized SLO-1. Indeed, the activity of BK channels has been used as a measure of Ca\(^{2+}\) concentrations at the presynaptic terminal resulting from the opening of VGCCs (Yazejian et al, 2000). Thus, localization of SLO-1 to the vicinity of EGL-19 by CTN-1 potentially allows SLO-1 to be activated by Ca\(^{2+}\) entering through EGL-19, and SLO-1 could in turn down-regulate the activity of EGL-19 through its effect on the membrane potential.

The mammalian CTN-1 homologue \(\alpha\)-catulin is almost ubiquitously expressed (Janssens et al, 1999; Park et al, 2002). However, there are only a few published studies on \(\alpha\)-catulin (Janssens et al, 1999; Demacio and Ray, 2001; Park et al, 2002; Merdek et al, 2004; Wiesner et al, 2008). As \(\alpha\)-catulin shares sequence homology with \(\beta\)-catenin, which binds a variety of cell adhesion or cytoskeletal proteins, such as \(\alpha\)-catenin, \(\beta\)-catenin, zona occludence protein 1 (ZO-1) and \(\alpha\)-actin (Knudsen et al, 1995; Nieset et al, 1997; Muller et al, 2005; Nelson, 2008), CTN-1 may also bind such proteins, thus contributing to BK channel subcellular localization by serving as a cytoplasmic linker between the channel and cytoskeletons. Recently, ISLO-1 was suggested to mediate SLO-1 subcellular localization in C. elegans body-wall muscle by interacting with the DAPC (Kim et al, 2009), which raises the possibility that CTN-1 might also interact with the DAPC to localize SLO-1. However, mutation of dystrophin did not disrupt CTN-1 subcellular localization in body-wall muscle cells (Figure 8), and ctn-1(lf) did not affect the assembly of dystrophin complex (Supplementary Figure S6), suggesting that SLO-1 subcellular localization in body-wall muscle cells likely involves two independent mechanisms, and disrupting one of them is sufficient to mislocalize SLO-1.

Although ctn-1 appeared to be also expressed in many neurons, we were unable to detect a significant effect of CTN-1 in the nervous system through analyses of locomotion behaviors and postsynaptic currents at the NMJ. These observations, however, do not exclude the possibility that CTN-1 has a function in the nervous system but was not detected in this study because our analyses were biased toward detecting defects in muscle cells, motoneurons or other neurons important to locomotion. Thus, the function of CTN-1 in the nervous system remains to be further investigated.

An elucidation of the molecular basis of BK channel subcellular localization could be a key to understanding how the channel performs its various physiological functions. Given that the primary sequences of both SLO-1 and CTN-1 share high level of homology with their mammalian counterparts, it would be interesting to know whether \(\alpha\)-catulin has a similar function in localizing mammalian BK channels, and whether an analogous mechanism is used to localize the channel in other types of cells.

Materials and methods

Growth and culture of C. elegans

C. elegans hermaphrodites were grown on agar plates with a layer of OP50 Escherichia coli at room temperature (21–22°C) or inside an environmental chamber (21°C).

Mutant screening

An integrated transgenic strain expressing Pslo-1::SLO-1(E\(^{350}\)Q) was used for mutant screen. Synchronized L4-stage slo-1(gf) worms were treated with the chemical mutagen ethyl methanesulfonate (50 mM) for 4 h at room temperature. The F2 progeny were screened for animals that moved better than the original slo-1(gf) animals. Isolated mutants were grouped through complementation tests.

Behavioural assay

Locomotion velocity was determined using a technique described previously (Liu et al, 2006), whereas the head-bending angle was quantified using a newly developed worm tracking and analysing system. Specifically, a single adult hermaphrodite was transferred to an agar plate with a thin layer of OP50 E. coli. After allowing ~30 s for recovery from the transfer, snapshots of the worm were taken at 15 frames per second for 30 s using a VGA FireWire camera (XCD-V60, Sony, Tokyo, Japan) mounted on a stereomicroscope (SMZ2000, Nikon, Tokyo, Japan). The worm was constantly kept in the centre of the view field with a motorized microscope stage (OptiScan™ ES111, Prior Scientific, Inc., Rockland, MA). Both the camera and the motorized stage were controlled by a custom program running in MATLAB (The MathWorks, Inc., Natick, MA).

Cloning of ctn-1

ctn-1(czu1) was used for SNP-based genetic mapping (Davis et al, 2005). After mapping the mutation to a small interval, the candidate gene was identified by testing whether cosmids and PCR-amplified genomic DNA fragments covering this interval could reinstate the lethargic phenotype in ctn-1(lf);slo-1(gf). Full-length cDNA of the candidate gene was obtained through RT–PCR. Molecular lesions of ctn-1 were identified by sequencing the cDNA prepared from five randomly chosen mutant alleles.

Analysis of expression pattern and subcellular localization

The expression pattern of ctn-1 was determined by expressing GFP under the control of 4.3 kb ctn-1 promoter (Petn-1:::GFP, wp761), whereas that of slo-1 by expressing EGFP under the control of 5.2-kb slo-1 promoter (Petl-1::EGFP, wp758). The plasmids were separately injected into the lin-15(n765) strain using a lin-15 rescue plasmid as a transformation marker. Cells expressing the fluorescent protein were visualized and photographed with a Zeiss
Axiovert 200 M fluorescence microscope (× 40 objective) with an apotome device (Zeiss) for optical sectioning.

To determine the expression patterns of different splicing variants of ctn-1, we cloned ctn-1 genomic DNA including 4.3 kb upstream of the initiation site and 0.6 kb downstream of the last exon, and inserted GFP into a unique axon of each splicing variant. As the genomic DNA of ctn-1 was too long (~16 kb) to be cloned into one plasmid, it was cloned into two plasmids as two separate fragments with 0.6 kb overlap. Homologous recombination in vitro would result in a full-length CTN-1::GFP translational fusion. After linearization, the plasmids were cointegrated with a lin-15 rescue plasmid into the lin-15(n765) strain. Transformed worms were identified as lacking a multiviral phenotype. Recombination of the two ctn-1 gene fragments in transgenic worms was verified by PCR using a GFP primer and a primer specific to the plasmid that did not contain GFP-coding sequence. Epifluorescence of the fusion protein in transgenic animals was visualized and photographed with a Nikon TE2000-U inverted microscope (× 60 objective) and a monochrome-cooled CCD camera (F-View II, Olympus).

Subcellular localization of CTN-1 was determined by fusing EGFP to its carboxyl terminus and expressing the fusion protein in body-wall muscle cells under the control of Pmyo-3 (Okkema et al., 1993) (Pmyo-3::CTN-1::EGFP, wp771). To determine whether SLO-1 protein expression and subcellular localization was altered in ctn-1(lf) mutant, a transgenic strain expressing Pmyo-3::SLO-1::GFP (wp746) or Pseo-1::SLO-1::GFP (wp5) was integrated through γ-irradiation, backcrossed with wild-type worms three times, and crossed into ctn-1(lf). To determine whether CTN-1 and SLO-1 are colocalized in muscle cells, CTN-1 was fused with mStrawberry (Pmyo-3::CTN-1::mStrawberry, wp904) and coexpressed with Pmyo-3::SLO-1::GFP. Epifluorescence of the fusion proteins in transgenic animals was visualized and photographed with a Nikon TE2000-U inverted microscope (× 60 objective) and the F-view II digital camera.

**BifC assay**

The DNA sequences encoding YFP amino and carboxyl terminals (YFpa and YFpc) were amplified by PCR from pCE-BiFC-VC155 vectors (Hiai et al., 2008), respectively, to make the following plasmids: Pmyo-3::SLO-1::YFpa (wp805), Pmyo-3::SLO-1(1–370)::YFpa (wp913), Pmyo-3::SLO-1(371–1140)::YFpa (wp914) and Pmyo-3::CTN-1::YFpc (wp772). The plasmid encoding SLO-1::YFpa was first injected into lin-15(n765) to establish independent transgenic lines, with a lin-15 plasmid cointegrated to serve as a transformation marker. A representative transgenic line thus obtained was then injected with the plasmids encoding CTN-1::YFpc and the transformation marker Pmyo-2::DsRed2 (wp568). Epifluorescence of transgenic worms was visualized and photographed as described above.

**Coimmunoprecipitation and western blot**

HA-tagged CTN-1 and Myc-tagged SLO-1 were cloned into the pIRE2-mCherry and pIRE2-EGFP vectors (Clontech), respectively, to generate the following plasmids: CTN-1::HA-IRE2-mCherry (wp847), SLO-1::Myc-IRE2-EGFP (wp857), SLO-1(1–370)::Myc-IRE2-EGFP (wp932) and SLO-1(371–1140)::Myc-IRE2-EGFP (wp933). HEK293 cells were cultured in DMEM with 10% FBS, and transiently transfected with Lipofectamine 2000 (Invitrogen). Cells were harvested 48 hours after localization, and lysed in 1% CHAPS/150 mM NaCl/1 mM CaCl2/62.5 mM Tris, pH 6.8. Sodium protease inhibitor (Roche). The supernatants of cell lysates were separated on 4–12% SDS–PAGE gels and probed with a HA antibody (Neomarkers).

To examine SLO-1::GFP protein level expressed in worms, worms of mixed stages were homogenized in a lysis buffer (2% SDS/100 mM NaCl/10% glycerol/50 mM Tris, pH 6.8). Soluble protein extracts were separated on 4–12% SDS–PAGE gels and probed with GFP (Molecular Probes) and α-tubulin (Santa Cruz Biotechnology) antibodies. Anti-mouse IgG HRP (Santa Cruz Biotechnology) was used as the secondary antibody for detection by enhanced chemiluminescence (Pierce).

**Surface biotinylation**

Biotinylation assays were performed in transiently transfected HEK293 cells using the Cell Surface Protein Isolation Kit (Pierce). Surface proteins were biotinylated 48 hours after the transfection, precipitated with neutrAvidin–agarose beads, and eluted with SDS sample buffer (1% SDS/50 mM DTT/10% glycerol/62.5 mM Tris, pH 6.8). Total lysate or biotinylated proteins were separated by 4–12% SDS–PAGE, and the blots were detected as described above.

**Recording of postsynaptic currents**

An integrated Punc-17::ChR2::mCherry transgene in the genetic background of wild-type worms (Liu et al., 2009) was crossed into slo-1(mdi745), ctn-1(zue1), slo-1(gf) and slo-1(gf);ctn-1(21w) strains. Both the Punc-17::ChR2::mCherry and Pseo-1::SLO-1::GFP transgenes appeared to be integrated into the X chromosome at close proximity because we were unable to identify a worm homozygous for both transgenes from several hundreds of cross progeny. Therefore, worms heterozygous for both transgenes were used for experiments involving comparisons with slo-1(gf) mutant (Figure 5), whereas worms homozygous for Punc-17::ChR2-::mCherry were used for experiments that did not involve comparisons with slo-1(gf) (Figure 5). Both mPSCs and ePSCs were recorded from body-wall muscle cells using the voltage-clamp technique with the membrane potential held at −60 mV, as described previously (Liu et al., 2005, 2007). mPSCs were evoked by applying a pulse (3 ms) of blue light using a 470×20 nm excitation filter (59222, Chroma Technology Corp.), and a light source equipped with a shutter (Lambda XL with SmartShutter, Sutter Instrument). The recording pipette solution contained (in mM) 120 KCl, 4 MgCl2, 36 sucrose, 5 EGTA and 4 Na2ATP (pH 7.2). Two external solutions with different [Ca2+] (5 and 0.25 mM) were used. The external solution with the higher [Ca2+] contained (in mM) 140 NaCl, 5 KCl, 5 CaCl2, 5 MgCl2, 11 dextrose and 5 HEPES (pH 7.2). This solution was modified by reducing CaCl2 to 0.25 mM and increasing NaCl to 145 mM to make the external solution with the lower [Ca2+].

**Xenopus oocyte expression**

Capped cRNAs were synthesized using the mMessage mMachine Kit (Ambion). Approximately 50 nl cRNA (1 ng/ml) was injected into each oocyte using a Drummond Nanoject II injector (Drummond Scientific). Inside-out patches were obtained from the oocyte 2–3 days after cRNA injection. Macroporous currents induced by voltage steps (−80 to +160 mV in 20 mV increments, 50 ms duration) were amplified with a Multiclamp 700B amplifier (Molecular Devices), and acquired with the Clampex software (version 10.2, Molecular Devices). Data were sampled at 10 kHz after filtering at 2 kHz. Compositions of the pipette solution included (in mM) 140 K gluconate, 1 Mg2+ gluconate and 5 HEPES (pH 7.2). Three cytoplasmic solutions were used that differed in free [Ca2+] (5 and 0.25 mM) were used to perfuse the cytoplasmic side of the patches. All cytoplasmic solutions contained 140 mM K gluconate and 10 mM HEPES. Besides, other components were added to adjust free [Ca2+] (1 mM Ca2+ gluconate for 1 mM [Ca2+]i, 0.1 mM Ca2+ gluconate for 100 μM [Ca2+]i and 3.39 mM Ca2+ gluconate plus 5 mM HEDTA for 10 μM free [Ca2+]i). Free [Ca2+]i was calculated using online software [http://maxchelator.stanford.edu].

**Data analysis**

**Electrophysiological data.** Amplitude and frequency of mPSCs were analysed using MiniAnalysis (Synaptosoft, Inc., Decatur, GA). A detection threshold of 10 pA was used in initial automatic analysis, followed by visual inspections to include missed smaller events (5 pA or larger) and to exclude false events resulting from baseline fluctuations. Amplitudes of ePSCs were measured with the Clampfit software (Molecular Devices). The average of the two largest ePSCs from each experiment was used for statistical analysis. Peak macroscopic currents from isolated oocyte patches were determined and used to plot the conductance–voltage relationship.

**Behavioural data.** The longest series of successive worm images showing continuous forward locomotion (10–20 s) was chosen from the 30-s recording of each worm. To find head-bending angles, a custom MATLAB program was used to detect the shape of the
worm, place 13 equally spaced markers along the midline, and distinguish the head and tail (Supplementary Figure S1A). The angle supplementary to the angle formed by two straight lines connecting the marker points 1 and 2, and the marker points 2 and 3 (Supplementary Figure S1B) was plotted over successive frames to produce a sinusoidal head-bending trace (Supplementary Figure S1D). From the head-bending trace, the maximal-bending angle was found as the full amplitude of bending between the ventral and dorsal sides (Supplementary Figure S1C). Although the maximal-bending angle metric provides an intuitive description of the data, it does not account for smaller and/or irregular oscillations. To mathematically quantify the magnitude of the head bending as a whole, the root mean square of the trace was also calculated.

Data graphing and statistical analyses. Graphing and statistical analyses were performed with Origin (version 8.0, OriginLab). One-way ANOVA (followed by Bonferroni’s post hoc test) was used for statistically controlled comparisons. P < 0.01 is considered to be statistically significant. All values are expressed as mean ± s.e. n is the number of patches or muscle cells analysed.

References


Additional data supplementary are available at The EMBO Journal Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.
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