Neural integrity is maintained by dystrophin in *C. elegans*

Shan Zhou and Lihsia Chen

Department of Genetics, Cell Biology, and Development, Developmental Biology Center, University of Minnesota, Minneapolis, MN 55455

The dystrophin protein complex (DPC), composed of dystrophin and associated proteins, is essential for maintaining muscle membrane integrity. The link between mutations in dystrophin and the devastating muscle failure of Duchenne’s muscular dystrophy (DMD) has been well established. Less well appreciated are the accompanying cognitive impairment and neuropsychiatric disorders also presented in many DMD patients, which suggest a wider role for dystrophin in membrane–cytoskeleton function. This study provides genetic evidence of a novel role for DYS-1/dystrophin in maintaining neural organization in *Caenorhabditis elegans*. This neuronal function is distinct from the established role of DYS-1/dystrophin in maintaining muscle integrity and regulating locomotion. SAX-7, an L1 cell adhesion molecule (CAM) homologue, and STN-2/γ-syntrophin also function to maintain neural integrity in *C. elegans*. This study provides biochemical data that show that SAX-7 associates with DYS-1 in an STN-2/γ-syntrophin–dependent manner. These results reveal a recruitment of L1 CAMs to the DPC to ensure neural integrity is maintained.

Introduction

It has been widely established that the actin-binding protein dystrophin is critical for muscle membrane cytoskeletal integrity. Loss-of-function mutations in dystrophin result in the X-linked catastrophic muscle-wasting disorder Duchenne’s muscle dystrophy (DMD). Dystrophin is localized to the sarcolemma in skeletal muscles, where it associates with multiple transmembrane proteins, which include dystroglycan, to form the large membrane-spanning specialized adhesion complex known as the dystrophin protein complex (DPC; Hoffman et al., 1987; Koenig et al., 1988; Zubrzycka-Gaarn et al., 1988; Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya et al., 1992). Dystrophin binds F-actin inside the cell. Through extracellular interactions with laminin by dystroglycan, the DPC connects the muscle actin cytoskeleton to the ECM and, as such, is hypothesized to provide mechanical protection against the physical stresses of repetitive muscle contractions. Interruption of this linkage results in impaired membrane integrity and degeneration of muscle tissue (Mokri and Engel, 1975; Deconinck and Dan, 2007; Ervasti, 2007). Mutations in other DPC components, such as the sarcoglycans, also result in progressive muscle dystrophies, highlighting the importance of the DPC in maintaining muscle membrane integrity (Sandonà and Betto, 2009). Interestingly, a significant number of DMD patients also exhibit cognitive deficits, the underlying pathology of which is not clear (Bresolin et al., 1994; Mehler, 2000; Wicksell et al., 2004). The prevalence of neural defects accompanying many dystrophin mutations suggests a wider role for dystrophin in humans, thus underscoring the importance of identifying their functions in the nervous system.

Like muscles, the nervous system is also subjected to mechanical forces exerted externally as well as within the organism itself. Neuronal circuitries established during development must withstand the physical stresses of brain growth and synaptic activity-dependent neuronal remodeling as well as the mechanical impacts of body movements and environmental insults (Bénard and Hobert, 2009). Adhesion protein complexes are known to participate in maintaining neural integrity against these physical forces. For example, impaired N-cadherin function causes defects in maintaining the positions of neurons in zebrafish (Lele et al., 2002; Masai et al., 2003). The identification of molecules that participate in maintaining neural architecture...
has been relatively successful in *Caenorhabditis elegans* because of the simplicity of its nervous system and the ease of performing genetic manipulations. One protein that is required to maintain the *C. elegans* neural organization is SAX-7, a homologue of the L1 family of cell adhesion molecules (CAMs; Bénard and Hobert, 2009; Chen and Zhou, 2010).

Vertebrate L1CAMs, which include L1, neuronal CAM (NrCAM), neurofascin, and CHL1, are known to function in nervous system development and function (Hortsch, 2003). Mutations in human NrCAM can result in autism, whereas impaired L1 function causes several different X-linked neural disorders, which include corpus callosum hypoplasia, mental retardation, adducted thumbs, spastic paraplegia, and hydrocephalus (Rosenthal et al., 1992; Van Camp et al., 1993; Jouet et al., 1994; Fransen et al., 1995; Marui et al., 2009). As transmembrane proteins, L1CAMs have been shown to form cell–cell and cell–ECM adhesion via diverse extracellular interactions as well as associate with the membrane actin cytoskeleton by binding cytoskeletal linkers, such as ankyrin. This cytoskeletal anchorage via ankyrin has been shown to be important for L1CAM function (Needham et al., 2001; Buhusi et al., 2008). Ankyrin interaction is similarly important for SAX-7 function (Zhou et al., 2008). Additional binding sites to cytoskeletal linkers that are conserved in L1CAM cytoplasmic tails also contribute to SAX-7 function, underscoring the importance of cytoskeletal anchorage to L1CAMs. One of these sites, a PDZ (PSD95, Dlg, ZO1)-binding (PB) motif, was shown to mediate interaction with STN-2/γ-syntrophin (Zhou et al., 2008), a putative component of the DPC. This finding raises the possibility for dystrophin to function in conjunction with L1CAMs in the nervous system.

In this study, we present genetic data that the sole *C. elegans* dystrophin gene, *dys-1*, plays a role in maintaining neural organization. This novel role, which requires *dys-1* function in neurons, is distinguishable from its requirement in body-wall muscle to maintain muscle integrity and regulate membrane excitability for proper locomotion (Bessou et al., 1998; Gieseler et al., 2000). We provide biochemical data that SAX-7 associates with DYS-1/dystrophin in an STN-2/γ-syntrophin–dependent fashion. Linkage to DYS-1/dystrophin likely provides SAX-7 anchorage to the actin cytoskeleton, thereby regulating SAX-7–mediated cell adhesions. This study provides insight into the common molecular mechanisms that may underlie diverse cognitive conditions, particularly those of the DMD and L1CAM disorders.

### Results

**dys-1 participates in positional maintenance of neurons**

We previously identified STN-2/γ-syntrophin as a SAX-7 interactor that regulates SAX-7 activity in maintaining placement of neurons and their axons (Zhou et al., 2008). Although the role of STN-2/γ-syntrophin has not been characterized, mammalian syntrophins are known components of the DPC that link diverse cytoplasmic and membrane proteins, including signaling proteins as well as water and sodium channels, to the DPC (Brenman et al., 1996; Hasegawa et al., 1999; Lumeng et al., 1999; Adams et al., 2001; Gavillet et al., 2006; Hirn et al., 2008). Consistent with STN-2/γ-syntrophin functioning together with DYS-1/dystrophin, *stn-2(tm1869)* mutant animals exhibit similar abnormal movements with exaggerated head bending (Fig. 1), which was previously described for *dys-1* mutant animals (Bessou et al., 1998). Indeed, *dys-1* and *stn-2* mutant animals show the characteristic head bend (Fig. 1 A), and they move with a reduced number of body bends per minute as compared with wild-type animals (Fig. 1 B). As a putative DPC component, we hypothesized that STN-2/γ-syntrophin may link SAX-7 to the DPC and that this association may be important for SAX-7 function. To test this hypothesis, we assayed whether DYS-1/dystrophin participates in the positional maintenance of neurons (see Materials and methods). Analysis of *dys-1* mutant animals revealed displaced cholinergic neurons in >20% of *dys-1(cx18)* and *dys-1(cx40)* adult animals (Fig. 2). Displacement of the affected neurons, which are embryonically derived (Sulston et al., 1983), is less prevalent in younger *dys-1* animals and is first detected in a small proportion of L3-staged larvae and an increasing proportion of L4-staged larvae (Fig. 2 C). This apparent progressive displacement is similarly observed in *sax-7* mutant animals (Wang et al., 2005) and is consistent with a role for *dys-1* in maintaining the position of neurons.

To further investigate a role for *dys-1* in neuronal position maintenance, we assessed whether *dys-1(cx18)* or *dys-1(cx40)* genetically interacts with *sax-7*. Genetic or pharmacological suppression of movements in *sax-7* animals was previously shown to suppress the neuronal displacement phenotype (Sasakura et al., 2005; Pocock et al., 2008). Thus, in these genetic assays, we intentionally used *dys-1(+/−)* heterozygosity to circumvent the possibility that the prolonged muscle excitation displayed by *dys-1* homozygous animals (Bessou et al., 1998; Kim et al., 2004, 2009) may nonspecifically enhance neuronal displacement in *sax-7* mutant backgrounds. Moreover, because homozygous *sax-7(eq1)*–null animals exhibit >95% penetrance of displaced neurons (Fig. 2, D and E; Wang et al., 2005), we assessed the consequences of *dys-1(+/−)* heterozygosity on neuronal displacement in *sax-7(eq1)/+* animals. Displaced cholinergic and γ-aminobutyric acid (GABA) neurons located along the ventral nerve cord (VNC) were observed in 10% *dys-1(+/−);sax-7(eq1)/+* trans-heterozygous animals, whereas neuronal displacement was not detected in either *dys-1(+/−)* or *sax-7(eq1)/+* single heterozygous mutant animals (Fig. 2, D and E). This genetic interaction between *dys-1* and *sax-7* is significant and is also illustrated by an enhancement of displaced neurons by *dys-1(+/−)* in animals that are homozygous for the hypomorphic *sax-7* allele eq2 (Wang et al., 2005). Indeed, displaced cholinergic neurons were detected in 37% of *dys-1(+/−);sax-7(eq2)* as compared with 4% of *sax-7(eq2)* single mutant animals (Fig. 2 D). The penetrance of GABA neuron displacement is also increased in *dys-1(+/−);sax-7(eq2)* as compared with *sax-7(eq2)* single mutant animals (Fig. 2 E). Collectively, these results reveal a role for *dys-1* in maintaining neuron positions.

Aging *dys-1* animals are reported to exhibit some muscle degeneration, particularly in a sensitized *hli-1* mutant background (Gieseler et al., 2000; Grisoni et al., 2003). Thus, it is possible that...
**dys-1**/+ in a sensitized sax-7 background could result in muscle degeneration or abnormal muscle morphology, which may indirectly cause altered positioning of the adjacent VNC neurons and tissues along the ventral midline. Immunostaining of β-integrin and phalloidin staining of actin did not reveal the presence of muscle degeneration or obvious defects in muscle morphology and sarcomeric organization in young adult **dys-1** or sax-7 animals or in **dys-1**/+ animals in sax-7 mutant backgrounds (Fig. S1). Furthermore, young adult **dys-1** or sax-7 animals do not exhibit vulval displacement (Fig. S2), suggesting that this positional maintenance role for **dys-1** and sax-7 is specific for neurons.

**dys-1** and **stn-2** are required in neurons, but not body-wall muscles, to maintain neuronal positioning

We previously showed that sax-7 is required in neurons as well as the adjacent hypodermis and body-wall muscles for positional maintenance of the VNC cholinergic and GABA neurons (Wang et al., 2005). **dys-1** is predominantly expressed in the VNC neurons as well as body-wall and vulval muscles, as determined by P_{dys-1}:gfp transcriptional reporters (Bessou et al., 1998; Dupuy et al., 2007; Hunt-Newbury et al., 2007). To determine the site of function for **dys-1**, we assayed for rescue of the displaced neuron phenotype in **dys-1** animals expressing full-length DYS-1/dystrophin in either neurons or muscles using tissue-specific promoters. Only DYS-1/dystrophin expressed in neurons rescued the neuronal displacement phenotype in **dys-1** homozygous and **dys-1**/+;sax-7/+ animals (Fig. 3). Similarly, only neuronally expressed DYS-1/dystrophin suppressed the genetic enhancement displayed in **dys-1**/+;sax-7(eq2) animals, whereas DYS-1/dystrophin expressed in body-wall muscles did not (Fig. 3).

DYS-1/dystrophin expressed in body-wall muscles did, however, rescue the abnormal movement and head-bending phenotype, which is characteristic of **dys-1** animals (Fig. 1),
Figure 2. Mutations in dys-1 cause defects in maintaining neuronal positions. (A and B) Schematics and corresponding micrographs of VNC cholinergic neurons, as visualized in a young adult wild-type (WT) and dys-1(cx18) animal expressing the Punc-129::gfp marker (A) and VNC GABA neurons, as visualized in a young adult wild-type and a dys-1(cx18)/+,sax-7(eq1)/+ animal expressing UNC-47::GFP (B). The brackets in A mark the vulval muscles, which also express unc-129::gfp. The green dots and lines represent the neuronal cell bodies and axonal processes, respectively. (C–E) The cholinergic neurons DB5, DA5, and DB6 and GABA neurons VD11, VD12, DD6, and VD13 are stereotypically positioned in wild-type animals (see Materials and methods; Fig. S4). The relative positions of the cholinergic neurons are altered in >20% dys-1 adult animals, a phenotype that is less prevalent in dys-1 larvae (C), suggesting a positional maintenance role for dys-1. The quantification of young adult animals exhibiting displaced cholinergic (D) and GABA (E) neurons in dys-1 and sax-7 mutant backgrounds reveals a genetic interaction between dys-1 and sax-7. Error bars show the standard error of the proportions of three sample sets in which n = 100 in each set. The p-values in C–E show the statistical significance as assessed by the Z test between the indicated strains. Bars, 10 µm.
DYS-1/dystrophin maintains neural integrity

Zhou and Chen

Both dys-1 and stn-2 are required in neurons to maintain neuronal positioning, a function that is independent of their role in body-wall muscles.

Figure 3. dys-1 is required in neurons, but not muscle, to maintain neuronal position. (A and B) Tissue-specific expression assays reveal that dys-1 functions in neurons, but not body-wall muscles, to maintain the position of cholinergic (A) and GABA (B) neurons. Neuronal and muscle expressions of dys-1 were driven by the unc-119 and myo-3 promoters, respectively. Three independent transgenic lines were analyzed for each construct. The error bars show the standard error of the proportions of three sample sets in which n = 100 in each set. Statistical significance was assessed by the Z test. WT, wild type.

indicating that the expressed DYS-1/dystrophin in muscles is functional. In fact, transgenic dys-1 animals that expressed DYS-1/dystrophin in body-wall muscles are indistinguishable from wild-type animals, exhibiting no obvious phenotype other than displaced neurons. In contrast, dys-1 animals expressing DYS-1/dystrophin in neurons still displayed the characteristic locomotory and head-bending phenotype (Fig. 1). This result is consistent with a previous study that revealed a requirement for DYS-1 in muscles, but not in neurons, for wild-type movements (Bessou et al., 1998). Thus, dys-1 has two separable roles; i.e., dys-1 functions in neurons to maintain neuronal positioning and acts in body-wall muscles to control locomotory behavior.

stn-2 is also expressed in VNC motor neurons and body-wall muscles (Zhou et al., 2008). Because dys-1 is required only in neurons to maintain neuronal positioning, we anticipated that stn-2 would also be required in neurons, but not muscles, for this function. Indeed, neuronal expression of STN-2/γ-syntrophin rescues the displaced VNC cholinergic and GABA neurons in stn-2/+ animals in either sax-7(eq1)+ or a homozygous sax-7(eq2) sensitized background (Fig. 4). In contrast, muscle expression of STN-2/γ-syntrophin failed to rescue the neuronal displacement (Fig. 4). Like dys-1, expression of STN-2/γ-syntrophin in muscles, but not in neurons, rescues the abnormal locomotion and head bending exhibited by stn-2 animals (Fig. 1). Similar to dys-1, both stn-2 animals and stn-2/+ animals in either a sax-7(eq1)+ or a homozygous sax-7(eq2) sensitized background do not exhibit muscle degeneration or obvious muscle abnormalities (Fig. S1), which may indirectly cause displacement of neurons. Thus, both dys-1 and stn-2 are required in neurons to maintain neuronal positioning, a function that is independent of their role in body-wall muscles.

STN-2 interacts with DYS-1 by yeast two-hybrid (Y2H) assays

Vertebrate syntrophins, including γ-syntrophins, can bind dystrophins via their C-terminal syntrophin unique (SU) region (Ahn and Kunkel, 1995; Ahn et al., 1996; Castelló et al., 1996). The 62–amino acid SU region of STN-2 shares 55 and 62% similarity with the SU region of human γ1- and γ2-syntrophin, respectively. This conservation of the SU region in STN-2 indicates a possible interaction between STN-2 and DYS-1.

To investigate whether STN-2 interacts with DYS-1, a Y2H assay was performed. In this assay, rather than using full-length DYS-1, which is predicted to be a 417-kD protein, we tested the DYS-1 C-terminal end, which contains homologous sequences to mammalian dystrophins that are required for interacting with syntrophins (Bessou et al., 1998; Gieseler et al., 1999a). Yeast transformed with the full-length stn-2 and C-terminal dys-1 clones showed robust cell growth on the L/T/H and L/T/H/A selective media, indicating a positive interaction between STN-2 and DYS-1 (Fig. 5 A). An interaction between STN-1/αβ-syntrophin and DYS-1 was assessed as a negative control. As expected, STN-1/αβ-syntrophin and DYS-1 did not interact (Fig. 5 B), which was similar to results of a previous Y2H assay (Grisoni et al., 2003). We also did not observe an interaction between STN-2 and the dystrophin-like molecule DYS-B-1/dystrobrevin (Fig. 5 C). To determine that the negative controls STN-1 and DYS-B-1 were expressed and functional in the Y2H
of STN-2 to the cell cortex where the neurofascin::SAX-7 chimera is localized (Fig. 6 B). This result is consistent with the previously described interaction of STN-2/α-syntrophin with the SAX-7CT (Zhou et al., 2008). If STN-2/α-syntrophin functions as an adaptor to link SAX-7 to DYS-1/dystrophin, SAX-7 should similarly recruit DYS-1/dystrophin to the cell cortex in an STN-2/α-syntrophin–dependent manner. To test this prediction, HEK293T cells were transfected with dys-1::myc and neurofascin::SAX-7 clones. As with the Y2H assay, the dys-1::myc clone encodes only the DYS-1 C-terminal region, which carries the syntrophin-interacting domain. DYS-1::Myc is cytoplasmic and does not colocalize with neurofascin::SAX-7 at the plasma membrane (Fig. 6 C). However, when FLAG::STN-2 is coexpressed together with DYS-1::Myc and neurofascin::SAX-7, DYS-1::Myc is largely redistributed from the cytoplasm to the cell cortex (Fig. 6 D). These results strongly suggest that DYS-1/dystrophin associates with SAX-7 only when STN-2/α-syntrophin is present.

To further confirm that DYS-1/dystrophin and STN-2/α-syntrophin form a complex with SAX-7 only when STN-2/α-syntrophin is present.

**Figure 4.**  

*stn-2* is required in neurons, but not muscle, to maintain neuronal position. (A–D) Tissue-specific expression assays reveal that *stn-2* functions in neurons, but not body-wall muscles, to maintain the position of cholinergic (A and B) and GABA (C and D) neurons. Moreover, this function requires the STN-2 PDZ domain. Three independent transgenic lines were analyzed for each construct in which neuronal and muscle expressions of STN-2 were driven by the *unc-119* and *myo-3* promoters, respectively. The error bars show the standard error of the proportions of three sample sets in which n = 100 in each set. Statistical significance was assessed by the Z test. WT, wild type.

assays, a known interaction between them (Grisoni et al., 2003) was tested, which was positive as expected (Fig. 5 D). These negative controls underscore the specificity of the interaction between STN-2 and DYS-1. Thus, the two *C. elegans* syntrophins differentially interact with the dystrophin family of proteins; STN-2/α-syntrophin interacts specifically with DYS-1/dystrophin, whereas STN-1/β-syntrophin interacts with DYB-1/dystrobrevin. Consistent with these molecular interactions, STN-1/αβ-syntrophin and DYB-1/dystrobrevin do not participate in neuronal positional maintenance (Fig. S3; Zhou et al., 2008).

**STN-2 acts as an adaptor to link SAX-7 to the DYS-1 cytoskeleton**

STN-2/α-syntrophin can interact with both SAX-7 (Zhou et al., 2008) and DYS-1/dystrophin (Fig. 5 A), which was consistent with our hypothesis that STN-2/α-syntrophin couples SAX-7 to DYS-1/dystrophin. To further test this hypothesis, we performed a protein recruitment assay using cultured cells, which was previously used to evaluate interactions between ankyrin and L1CAMs (Zhang et al., 1998; Chen et al., 2001; Zhou et al., 2008). FLAG::STN-2 is primarily localized in the cytoplasm when expressed in HEK293 cells (Fig. 6 A). However, cotransfection with a SAX-7 construct, which is composed of rat neurofascin extracellular and transmembrane domains fused to the SAX-7 cytoplasmic tail (SAX-7CT), results in the redistribution of STN-2 to the cell cortex where the neurofascin::SAX-7 chimera is localized (Fig. 6 B). This result is consistent with the previously described interaction of STN-2/α-syntrophin with the SAX-7CT (Zhou et al., 2008). If STN-2/α-syntrophin functions as an adaptor to link SAX-7 to DYS-1/dystrophin, SAX-7 should similarly recruit DYS-1/dystrophin to the cell cortex in an STN-2/α-syntrophin–dependent manner. To test this prediction, HEK293T cells were transfected with dys-1::myc and neurofascin::SAX-7 clones. As with the Y2H assay, the dys-1::myc clone encodes only the DYS-1 C-terminal region, which carries the syntrophin-interacting domain. DYS-1::Myc is cytoplasmic and does not colocalize with neurofascin::SAX-7 at the plasma membrane (Fig. 6 C). However, when FLAG::STN-2 is coexpressed together with DYS-1::Myc and neurofascin::SAX-7, DYS-1::Myc is largely redistributed from the cytoplasm to the cell cortex (Fig. 6 D). These results strongly suggest that DYS-1/dystrophin associates with SAX-7 only when STN-2/α-syntrophin is present.
Dys-1/dystrophin maintains neural integrity

• Zhou and Chen suggested otherwise (Gieseler et al., 1999a). This difference may reflect dissimilar experimental approaches and conditions that could suggest a weak interaction between STN-1/αβ-syntrophin and DYS-1/dystrophin. For example, the intrinsic ability for GST to dimerize (Walker et al., 1993; Dirr et al., 1994; Lim et al., 1994) could have resulted in the oligomerization of the GST–STN-1 fusion protein that was used in the in vitro binding assay, which, in turn, may have enhanced a weak interaction between STN-1 and DYS-1. Similar oligomerization and influence of activity on diverse fusion proteins induced by GST have been reported (Haldeman et al., 1997; Mernagh et al., 1997; Niedziela-Majka et al., 1998).

To investigate whether SAX-7 could interact with DYS-1/dystrophin, anti-Myc IPs were performed on cell lysates containing both DYS-1::Myc and neurofascin::SAX-7. Neurofascin::SAX-7 did not coimmunoprecipitate (Fig. 7, E [lane 2] and F [lane 3]), which was consistent with a lack of association between SAX-7 and DYS-1/dystrophin, as suggested in the protein recruitment assay. However, in anti-Myc IPs on lysates of cells in which FLAG::STN-2 was cotransfected with both DYS-1::Myc and neurofascin::SAX-7CT, neurofascin::SAX-7CT coimmunoprecipitated along with FLAG::STN-2 (Fig. 7, E and F, lanes 1), which was consistent with SAX-7 being present in a complex with DYS-1 and STN-2. Collectively, these results were expected based on our previous results that STN-2/γ-syntrophin, but not STN-1/αβ-syntrophin, interacts with SAX-7 via the PDZ domain (Zhou et al., 2008). Neurofascin::SAX-7 was also not detected in IPs of cell lysates containing a mutated FLAG::STN-2 that lacks the PDZ domain (STN-2::ΔPDZ) and neurofascin::SAX-7 (Fig. 7 B, lane 2), suggesting that the PDZ domain is required for STN-2/γ-syntrophin to interact with SAX-7. The importance of the STN-2 PDZ domain is underscored by the result that expression of STN-2::ΔPDZ cannot rescue the neuronal displacement phenotype in sax-7(eq1)/+;stn-2/+ animals or suppress the phenotypic enhancement in sax-7(eq2)/sax-7(eq2);stn-2/+ animals (Fig. 4).

The interaction between STN-2/γ-syntrophin and DYS-1/dystrophin was tested next. Reciprocal co-IPs were observed with FLAG::STN-2 and DYS-1::Myc (Fig. 7, C and D, lane 2) but not with FLAG::STN-1 and DYS-1::Myc (Fig. 7, C and D, lane 4). It is interesting that both our Y2H and co-IP experiments, as well as an independent Y2H assay (Grisoni et al., 2003), consistently showed a lack of interaction between STN-1/αβ-syntrophin and the C-terminal end of DYS-1, whereas a previously published in vitro binding assay using a purified GST–STN-1 fusion protein suggested otherwise (Gieseler et al., 1999a). This difference may reflect dissimilar experimental approaches and conditions that could suggest a weak interaction between STN-1/αβ-syntrophin and DYS-1/dystrophin. For example, the intrinsic ability for GST to dimerize (Walker et al., 1993; Dirr et al., 1994; Lim et al., 1994) could have resulted in the oligomerization of the GST–STN-1 fusion protein that was used in the in vitro binding assay, which, in turn, may have enhanced a weak interaction between STN-1 and DYS-1. Similar oligomerization and influence of activity on diverse fusion proteins induced by GST have been reported (Haldeman et al., 1997; Mernagh et al., 1997; Niedziela-Majka et al., 1998).

To investigate whether SAX-7 could interact with DYS-1/dystrophin, anti-Myc IPs were performed on cell lysates containing both DYS-1::Myc and neurofascin::SAX-7. Neurofascin::SAX-7 did not coimmunoprecipitate (Fig. 7, E [lane 2] and F [lane 3]), which was consistent with a lack of association between SAX-7 and DYS-1/dystrophin, as suggested in the protein recruitment assay. However, in anti-Myc IPs on lysates of cells in which FLAG::STN-2 was cotransfected with both DYS-1::Myc and neurofascin::SAX-7CT, neurofascin::SAX-7CT coimmunoprecipitated along with FLAG::STN-2 (Fig. 7, E and F, lanes 1), which was consistent with SAX-7 being present in a complex with DYS-1 and STN-2. Collectively, these
A distinct neuronal DPC functions to maintain neural architecture

Mutations in dys-1 cause progressive muscle degeneration in sensitized genetic backgrounds (Gieseler et al., 2000; Mariol et al., 2007), indicating a conserved role for dystrophin in maintaining muscle integrity. dys-1 mutant animals also exhibit a distinctive movement phenotype (i.e., exaggerated bending of the head and anterior body and prolonged muscle contraction), which is shown, in part, to be caused by abnormal cholinergic transmission (Bessou et al., 1998; Giugia et al., 1999). This phenotype can be rescued by wild-type DYS-1 expressed in muscle but not neurons (Bessou et al., 1998). Mutations in genes encoding other components of the C. elegans DPC, such as smo-2γ-syntrophin, smo-1alβ-syntrophin, and dyb-1/dystrobrevin, also lead to locomotory phenotypes that are indistinguishable from that of dys-1, suggesting that these DPC components function in muscles for proper cholinergic transmission.

Discussion

This study uncovers a novel neuronal role for DYS-1/dystrophin in maintaining the structure and organization of the C. elegans nervous system in collaboration with the SAX-7/L1CAM. This role is distinct from its previously established muscle function in maintaining muscle integrity and regulating locomotion. The presented genetic and biochemical data provide evidence that SAX-7 is linked to DYS-1/dystrophin via STN-2/γ-syntrophin and that this tripartite protein complex is required in neurons (Fig. 8). Dystrophin and the associated actin cytoskeleton likely provide anchorage to SAX-7, thereby regulating SAX-7 activity in maintaining neuronal positions.

Figure 6. Protein recruitment assays in HEK293 cells suggest that SAX-7 associates with DYS-1 only in the presence of STN-2. (A) FLAG::STN-2 is primarily localized in the cytoplasm, as detected by immunostaining with an anti-FLAG antibody. (B) Cotransfection with neurofascin::SAX-7CT (labeled SAX-7) causes FLAG::STN-2 to be redistributed to the cell cortex where SAX-7 is localized. (C) DYS-1::Myc, which contains the DYS-1 C-terminal amino acid residues 3,402–3,674, is primarily localized in the cytoplasm, as detected by anti-Myc antibody in cells transfected with both DYS-1::Myc and neurofascin::SAX-7CT. (D) When STN-2 is cotransfected, DYS-1::Myc is redistributed to the cell cortex where SAX-7 is localized, thus suggesting that SAX-7 can associate with DYS-1 via STN-2. Bar, 15 µm.
DYS-1/dystrophin maintains neural integrity

Zhou and Chen (Fig. S3; Zhou et al., 2008), although both proteins are also extensively expressed in the nervous system (Gieseler et al., 2001; Grisoni et al., 2003). It is not known what function STN-1/αβ-syntrophin or DYS-1/δystrobrevin mediates in neurons or whether these roles involve DYS-1/dystrophin. Thus, at least in neurons, the DPCs are likely variable in composition and function.

It is not clear how neuronal displacement impacts neural functions in dys-1 mutant animals or whether dys-1 mediates additional functions in neurons other than maintaining...
of linkage to the spectrin-actin cytoskeleton (Zhou et al., 2008). We thus speculate that STN-2/\(\gamma\)-syntrophin and DYS-1/dystrophin participate in neuronal positional maintenance by coupling SAX-7 to the cortical actin cytoskeleton. Interruption of this cytoskeleton linkage by either deletion of the SAX-7 PB sequence or the STN-2 PDZ domain or by knocking out stn-2 or dys-1 function leads to defects in maintaining neuronal position, probably caused by reduced SAX-7 activity in mediating cell adhesion.

It is thus puzzling that displaced cholinergic neurons are detected in dys-1, but not in stn-2, mutant animals. This difference suggests that DYS-1/dystrophin can also mediate SAX-7 function in an STN-2–independent fashion. Proteins that may compensate for the loss of STN-2/\(\gamma\)-syntrophin are not known, but one candidate protein may be UNC-44/ankyrin. A recent study uncovered the ability of mammalian ankyrins to bind dystrophin via a cysteine-rich domain, which is located close to, but distinct from, the syntrophin-interacting domain in the C terminus of dystrophin (Ayalon et al., 2008). Interaction with dystrophin has been shown for ankyrin-G and ankyrin-B, which are required for the proper localization of dystrophin at the costameres and sarcolemma, respectively, in murine skeletal muscle (Ayalon et al., 2008). Thus, UNC-44/ankyrin may

neuronal positioning. Animals that lack dys-1 in neurons (i.e., dys-1 mutant animals expressing dys-1 only in muscles) have no obvious phenotype other than displaced neurons. Assays that assess behavior and higher order neural function (e.g., learning and memory) still need to be performed to address these questions.

**Figure 8.** A model depicting how STN-2/\(\gamma\)-syntrophin may regulate SAX-7 activity in maintaining neuronal positions, as based on genetic and biochemical data. The model speculates that STN-2/\(\gamma\)-syntrophin links SAX-7 to the DYS-1/dystrophin-based actin cytoskeleton in neurons but not muscles. This interaction is dependent on the STN-2 PDZ domain to interact with the SAX-7 PB motif and requires the STN-2 syntrophin unique (SU) domain to interact with the C-terminal end of DYS-1. SAX-7 is predicted to bind to the actin cytoskeleton by its N-terminal actin-binding domain. This linkage to the actin cytoskeleton may provide SAX-7 anchorage, thereby regulating SAX-7–mediated cell adhesion. Interruption of this anchorage leads to a defect in maintaining neuronal positions. UNC-44/ankyrin also interacts molecularly and genetically with SAX-7 (Zhou et al., 2008), but it is not known whether UNC-44/ankyrin is required in neurons and/or body-wall muscles; this unknown is depicted as “UNC-44?” in the schematic. Ig, Ig-like repeats; FNIII, fibronectin type III repeats; FB, FERM-binding sequence; and AB, ankyrin-binding sequence.
compensate for the loss of STN-2 to couple SAX-7 to the dystrophin-based cytoskeleton, thus reflecting the lack of phenotype in 

\textit{strn-2} mutant animals. This speculation would require that the regulation of SAX-7 activity by UNC-44/ankyrin occurs in neurons, which is currently not known.

The expression of UNC-44/ankyrin in neurons, body-wall muscles, and the hypodermis (Chen et al., 2001) suggests that UNC-44/ankyrin could regulate SAX-7 activity in one or more of these tissues; SAX-7 is required in all three tissues to ensure that neural integrity is maintained (Wang et al., 2005). Consistent with UNC-44/ankyrin acting as an adaptor to link SAX-7 to the spectrin-actin cytoskeleton, SPC-1/α-spectrin and UNC-70/β-spectrin are similarly expressed in multiple tissues, including neurons, body-wall muscles, and the hypodermis (Hammarlund et al., 2000; Moorthy et al., 2000; Norman and Moerman, 2002). That SAX-7 may be coupled to both the dystrophin- and spectrin-based actin cytoskeletons raises the question of how cytoskeletal linkage of SAX-7 is coordinated. Are both the dystrophin- and spectrin-based cytoskeletons required in neurons for SAX-7 activity? Is SAX-7 associated with both cytoskeletons simultaneously, or are distinct sub-populations of SAX-7 linked to each cytoskeleton? If only the dystrophin-based cytoskeleton is required in neurons, how is linkage to the spectrin-based cytoskeleton prevented? In addition to the cortical actin cytoskeleton, dystrophin also binds intermediate filaments and microtubules (Prins et al., 2009; Le Rumeur et al., 2010). Thus, SAX-7 may be simultaneously anchored to one of these alternative cytoskeletons in addition to the spectrin-actin cytoskeleton.

### Implications for mammalian L1CAMs

Mammalian dystrophin and DPC components are also expressed in neurons (Waite et al., 2009). In contrast to their functions in muscles, dystrophin and the DPC components in the mammalian nervous system have not been as extensively characterized. These neuronal DPC complexes appear to be molecularly heterogeneous (Blake and Kröger, 2000; Waite et al., 2009), suggesting multiple neuronal roles for the DPC.

The importance of dystrophin in the brain is directly indicated by cognitive impairments and neuropsychiatric disorders (e.g., autism and schizophrenia) that can be presented in DMD patients (Bresolin et al., 1994; Anderson et al., 2002). Autopsy studies on DMD patients revealed brain abnormalities that include disordered connections and architectural changes (Rosman, 1970; Jagadha and Becker, 1988; Moriuchi et al., 1993; Uchino et al., 1994; Kim et al., 1995). The 

\textit{mdx} dystrophin mutant mice similarly exhibit an abnormal architecture of the brain that includes altered distribution of populations of neurons, body-wall muscles, and the hypodermis (Hammarlund et al., 2000; Moorthy et al., 2000; Norman and Moerman, 2002). That SAX-7 may be coupled to both the dystrophin- and spectrin-based actin cytoskeletons raises the question of how cytoskeletal linkage of SAX-7 is coordinated. Are both the dystrophin- and spectrin-based cytoskeletons required in neurons for SAX-7 activity? Is SAX-7 associated with both cytoskeletons simultaneously, or are distinct sub-populations of SAX-7 linked to each cytoskeleton? If only the dystrophin-based cytoskeleton is required in neurons, how is linkage to the spectrin-based cytoskeleton prevented? In addition to the cortical actin cytoskeleton, dystrophin also binds intermediate filaments and microtubules (Prins et al., 2009; Le Rumeur et al., 2010). Thus, SAX-7 may be simultaneously anchored to one of these alternative cytoskeletons in addition to the spectrin-actin cytoskeleton.

### Materials and methods

#### Strains

\textit{C. elegans} strains, provided by the Caenorhabditis Genetics Center, were grown on nematode growth medium plates at 21°C as described by Brenner (1974). N2 Bristol served as the wild-type strain. The alleles used in this study are listed by linkage groups as follows: LGI, dys-1(cx18), dys-1(cx40) (Bessou et al., 1998), and dyb-1(cx26) (Gieseler et al., 1999b); LGIV, sax-7(eq1) (Zhou et al., 2008), and sax-7(eq2) (Wang et al., 2005); and LGX and strn-2(tm1869) (Zhou et al., 2008).

#### \textit{C. elegans} expression vectors and generation of transgenic animals

Transgenic animals were generated according to standard procedures (Mello et al., 1991). To generate \( P_{unc-119}:dys-1 (\text{pLC578}) \), a 9.0-kb dys-1 genomic sequence from \text{WRM06111E10} (Geneservice) was pieced together at exon 30 with 3.9-kb dys-1 cDNA and 169-bp dys-1 3' untranslated region (obtained from yk1473g12 and yk1434h02; Y. Kohara, National Institute of Genetics, Mishima, Japan) and subcloned into the pBluescript II KS vector between NotI and XhoI sites. The 12-bp Punc-119 promoter (Maduro and Pilgrim, 1995, Maduro et al., 2000) was inserted at NotI to drive dys-1 expression in neurons. The resulting construct was injected at 190 ng/µl along with 2 ng/µl of the coinjection marker Pmyo-2::tdTomato (a gift from S. Panowski and A. Dillin, Salk Institute, La Jolla, CA) into \text{dys-1(cx18)}.

To generate \( P_{myo-2}:dys-1 (\text{pLC586}) \), a 9.0-kb dys-1 genomic sequence was inserted at the Pmyo-2 promoter with the muscle-specific myo-3 promoter (Fire et al., 1998) to drive dys-1 expression in muscles. 160 ng/µl of the resulting construct was injected into \text{dys-1(cx18)} with 40 ng/µl of the coinjection marker sur::dRed (Yochem et al., 1998).

To generate \( P_{unc-119}:stn-2::gfp (\text{pLC551}) \), the stn-2 promoter (3.82-kb sequences upstream of the start codon) and 1.5-kb dys-1 cDNA (obtained from yk788e09; Y. Kohara) were placed in frame with gfp coding sequences and the unc-54 3' untranslated region (Fire et al., 1990), all of which were subcloned into the pBluescript II KS vector between NotI and Apal. 50 ng/µl of the resulting construct was injected into \text{strn-2(tm1869)} animals along with 50 ng/µl of the coinjection marker sur::dRed (Yochem et al., 1998).

To generate \( P_{unc-119}:stn-2::gfp (\text{pCL553}) \), a 9.0-kb dys-1 genomic sequence was inserted at the Punc-119 promoter with the muscle-specific myo-3 promoter (Fire et al., 1998) to drive dys-1 expression in muscles. 160 ng/µl of the resulting construct was injected into \text{dys-1(cx18)} with 40 ng/µl of the coinjection marker sur::dRed (Yochem et al., 1998).
this construct was injected into sta-2 (tm1869) animals along with 70 ng/μl of the coinjection marker Punc:gro (Troemel et al., 1995). Punc::gfp::sta-2 (pLC590) was generated from a pclC553 derivative lacking the STN2 PDZ domain (amino acids 74–150), which was injected at 50 ng/μl into sta-2 (tm1869) animals along with 70 ng/μl of the coinjection marker Punc::gfp (Troemel et al., 1995).
Punc::gfp::sta-2::gfp (pLC554) was generated from a pclC553 derivative that replaced the sta-2 promoter with the myo-3 promoter. This construct was injected at 50 ng/μl into sta-2 (tm1869) animals along with 70 ng/μl of the coinjection marker Punc::gfp (Troemel et al., 1995).

Live animal microscopy—scoring for displaced neurons

The ols12 ( unc-47::gfp; McIntire et al., 1997) and evs78 ( Punc::gfp; Caloviita et al., 1998) integrated transgenes were crossed into strains that lack wild-type, VNC cholinergic or GABA or cholinergic neurons, respectively. Synchro-
nized young adult animals of all these strains as well as synchronized dya-1 larvae of various stages were mounted on 2% agarose pads and scored for neuronal displacement with the 40 x 1.3 NA Neofluar objective using a microscope (Axioplan 2 IIE) with the image captured with a camera (AxioCam MRm) and imaging software (AxioVision 4.5; Carl Zeiss, Inc.).

Of the VNC cholinergic neurons, the relative positions of the DB5, DA5, and DB6 neurons are most consistently altered in the examined gen-
etic strains as compared with wild type (Fig. 2 A) and, thus, we were the focus of our analysis. The ratio of the distance between DB5 and DA5 (designated D1) to the distance between DB5 and DB6 (designated D2) was calculated (Fig. S4 A). D1 and D2 are similar in wild-type animals so that the mean D1/D2 ratio is slightly more than one (Fig. S4 B). Neurons are considered displaced when the D1/D2 ratio in an animal is two or greater, as a result of the D2 value being less than half that of D1 because DA5 and DB6 are closer to each other (Fig. 2 A).

Of the VNC GABA neurons, the relative positions of VD1, VD2, DD6, and VH13 neurons are most consistently altered in the examined gen-
etic strains as compared with wild type (Fig. 2 B) and, thus, were the focus of our analysis. The ratio of the distance between VD1 and VD2 (designated D1) to the distance between VD12 and VH13 (designated D2) was calculated (Fig. S4 C). In wild-type animals, D1 is generally more than three times larger than D2 so that the mean D1/D2 ratio is 3.5 (Fig. S4 D). Neurons are considered displaced when the D1/D2 ratio in an animal is one or less, as a result of the D1 value being equal to or less than that of D2 because VD12 and DD6 is closer to VD11 (Fig. 2 B).

Live animal microscopy—determining vulval position

Synchronized L4 wild-type, stn-1::gfp (Troemel et al., 1997) and stn-2::gfp::stn-1::gfp (pLC565), the str-1 cDNA (obtained from yak0919) that was cloned into p3xFLAG-CMV7.1 (Sigma-Aldrich) between NolI and KpnI; Punc::FLAG::sta-2 (pLC558), a pclC566 derivative lacking the PDZ domain (amino acids 74–150); Punc::dys-1::Myc::pLC574, a dys-1 cDNA fragment encoding amino acids 3402–3674, was cloned into pdCNA3.1/Myc::His-B (Invitrogen) between KpnI and BamHI.

Cell culture and transfection

HEK293T cells were cultured in DME (Mediatech, Inc.) with 10% newborn bovine serum and treated with transfection reagent Lipofectamine 2000; Invitrogen) according to the standard procedure provided by the manufacturer during DNA transfection. 40 h after transfection, cells were collected and washed with PBS in preparation for either immunofluorescence or co-IP assays.

Protein recruitment assay in HEK293 cells

The assay was performed as previously described (Zhang et al., 1998). Cells were fixed with formaldehyde, washed, and incubated with anti-FLAG (M2; Sigma-Aldrich), anti-Myc (9E10; Covance), or anti-SAX-7 (6991; Chen et al., 2001) antibodies to visualize FLAG::STN-2, DYS-1::Myc, or SAX-7::M2 antibody at 4°C for 1 h. Immune complexes were precipitated with 20 µl of protein A/G beads (Santa Cruz Biotechnology, Inc.) at 4°C for 4 h. The beads were washed three times with NTE buffer and then the immu-
ne complexes were eluted by heating the beads to 95°C for 5 min in SDS sample buffer containing DTT. Blots were probed as described in the next section.

Co-IP assays

Cell lysates were prepared in NETN buffer containing 1 mM NaF, 2.5 mM β-glycero-phosphate, and a protease inhibitor cocktail (Roche). 200–500 μl of cell lysate was incubated with NETN buffer (0.05 M Tris, pH 7.8, 0.1-M NaCl, 1 mM EDTA, and 0.5% NP-40) containing the FLAG or Myc antibody at 4°C for 1 h. Immune complexes were precipitated with 20 μl of protein A/G beads (Santa Cruz Biotechnology, Inc.) at 4°C for 4 h. The beads were washed three times with NETN lysis buffer, and then the immu-
ne complexes were eluted by heating the beads to 95°C for 5 min in SDS sample buffer containing DTT. Blots were probed as described in the next section.

Western blot analysis and reagents

Cell lysates were prepared in NETN buffer containing 1 mM NaF, 2.5 mM β-glycero-phosphate, and a protease inhibitor cocktail. Cell lysates were resolved by SDS-PAGE and electrophoretically transferred to the nitrocell-
ulose membrane. Membranes were blocked in TBS-T (0.15-M NaCl, 20 mM Tris, pH 8, and 0.05% Tween 20) containing 5% bovine albumin (Sigma-Aldrich) for 1 h. Blots were probed with primary antibodies followed by horseradish peroxidase–conjugated anti-mouse (Mouse TrueBlot Utitre) or anti-rabbit secondary antibody (Jackson ImmunoResearch)
Wicksell, R.K., M. Kihlgren, L. Melin, and O. Eeg-Olofsson. 2004. Specific cognitive deficits are common in children with Duchenne muscular dystrophy. Dev. Med. Child Neurol. 46:154–159. doi:10.1111/j.1469-8749.2004.tb00466.x

Wu, J.Y., K.C. Kuban, E. Allred, F. Shapiro, and B.T. Darras. 2005. Association of Duchenne muscular dystrophy with autism spectrum disorder. J. Child Neurol. 20:790–795. doi:10.1177/0883073805020010201

Yochem, J., T. Gu, and M. Han. 1998. A new marker for mosaic analysis in Caenorhabditis elegans indicates a fusion between hyp6 and hyp7, two major components of the hypodermis. Genetics. 149:1323–1334.

Zhang, X., J.Q. Davis, S. Carpenter, and V. Bennett. 1998. Structural requirements for association of neurofascin with ankryin. J. Biol. Chem. 273:30785–30794. doi:10.1074/jbc.273.46.30785

Zhou, S., K. Opperman, X. Wang, and L. Chen. 2008. unc-44 Ankryin and stn-2 gamma-syntrophin regulate sax-7 L1CAM function in maintaining neuronal positioning in Caenorhabditis elegans. Genetics. 180:1429–1443. doi:10.1534/genetics.108.091272

Zubrzycka-Gaarn, E.E., D.E. Bulman, G. Karpati, A.H. Burghes, B. Belfall, H.J. Klastun, J. Talbot, R.S. Hodges, P.N. Ray, and R.G. Worton. 1988. The Duchenne muscular dystrophy gene product is localized in sarcolemma of human skeletal muscle. Nature. 333:466–469. doi:10.1038/333466a0