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Intramolecular regulation of presynaptic scaffold protein SYD-2/liprin- α



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ABSTRACT

SYD-2/liprin- α is a multi-domain protein that associates with and recruits multiple active zone molecules to form presynaptic specializations. Given SYD-2's critical role in synapse formation, its synaptogenic ability is likely tightly regulated. However, mechanisms that regulate SYD-2 function are poorly understood. In this study, we provide evidence that SYD-2's function may be regulated by interactions between its coiled-coil (CC) domains and sterile α -motif (SAM) domains. We show that the N-terminal CC domains are necessary and sufficient to assemble functional synapses while C-terminal SAM domains are not, suggesting that the CC domains are responsible for the synaptogenic activity of SYD-2. Surprisingly, *syd-2* alleles with single amino acid mutations in the SAM domain show strong loss of function phenotypes, suggesting that SAM domains also play an important role in SYD-2's function. A previously characterized *syd-2* gain-of-function mutation within the CC domains is epistatic to the loss-of-function mutations in the SAM domain. In addition, yeast two-hybrid analysis showed interactions between the CC and SAM domains. Thus, the data is consistent with a model where the SAM domains regulate the CC domain-dependent synaptogenic activity of SYD-2's activity may be modulated to regulate synapse formation during development.

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Introduction

 $syd-2/liprin-\alpha$ plays a crucial role in organizing the active zone during development of presynaptic sites (Stryker and Johnson, 2007) and was first identified as a protein that binds to LAR (leukocyte antigen related) family of transmembrane receptor proteins with tyrosine phosphatase activity (LAR-RPTP) (Serra-Pagès et al., 1998). SYD-2 protein has a predicted N-terminal coiled-coil region and three SAM domains at the C-terminus that comprise the liprin homology (LH) domain (Fig. 1A). Besides LAR-RPTP, a number of proteins implicated in the function and development of synapses also bind to SYD-2. For example, liprin binding proteins RIM/unc-10, CAST/ERC/elks-1, and CASK are presynaptic proteins involved in neurotransmitter release (Ko et al., 2003b; Olsen et al., 2005; Schoch et al., 2002). GRIP and GIT1 are required for proper targeting of postsynaptic AMPA receptors (Ko et al., 2003a; Wyszynski et al., 2002). The fly ortholog of SYD-2, Dliprin- α , is one of the first active zone components to be recruited to new developing presynapses prior to other components such as Bruchpilot, Drosophila ortholog of ELKS-1 (Fouquet et al., 2009). In

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addition, SYD-2 recruitment is unaffected by loss of many active zone molecules such as ELKS-1, UNC-10, GIT-1, SAD-1, UNC-26 and UNC-2. Conversely, loss of *syd-2* affects the localization of almost all active zone components (Patel et al., 2006). SYD-2/liprin- α has also been shown to bind and activate the kinesin motor KIF1A/UNC-104 necessary for synaptic vesicle transport (Shin et al., 2003; Wagner et al., 2009). Mutations in *dliprin-\alpha* or *syd-2*, the *Drosophila* and *Caenorhabditis. elegans* homolog respectively, result in morphological defects in active zones and/or loss of synapses (Kaufmann et al., 2002; Patel et al., 2006; Zhen and Jin, 1999).

With a diverse range of proteins that interact with SYD-2 and genetic data to support its role in active zone assembly, SYD-2 may recruit and link multiple synaptic components into a functional active zone complex that forms the core presynaptic vesicle release machinery (Dresbach et al., 2001; Spangler and Hoogenraad, 2007). A recent study showed that a key step in synapse assembly requires the self-oligomerization of SYD-2 mediated by its coiled-coil domains (Taru and Jin, 2011). To coordinate the assembly of spatially defined synapses, synapse formation has to be precisely regulated. Recent research has identified a variety of proteins that promote and initiate this process (Sigrist and Schmitz, 2010; Ziv and Garner, 2004). However, molecular mechanisms that regulate synaptogenesis are not well understood. Since SYD-2 is a critical active zone scaffold component, a

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Fig. 1. Structure-function dissection of SYD-2 shows that N-terminal CC domains are sufficient for SYD-2 recruitment and synapse assembly. (A) Schematic of SYD-2 protein domains highlighting the mutations found in the different mutant alleles (black arrows are loss-of-function and red arrows are gain-of-function). (B) Schematic depicting the HSNL neuron. Asterisk denotes position of HSNL cell body and dashed box outlines location of presynapses that are labeled by SNB-1::YFP. Confocal images showing SNB-1 labeling in HSNL in (C) wild-type and (D) syd-2(ju37) mutant allele where presynapses are lost. Scale bar represents 10 μ m. (E) Cell-autonomous expression of full-length syd-2 cDNA in HSNL rescue synapse formation, similar to (F) the CC domains but not (G) expression of the SAM domains. (H) Quantification of total SNB-1::YFP fluorescence at HSNL presynapses normalized to wild-type values in syd-2(ju37) animals rescued by either full-length SYD-2, CC or SAM domains alone. Expression of CC domains in syd-1(ju82) and syd-1(ju82); syd-2(ju37) mutants also rescues. Error bars represent SEM. ***p-value < 0.001, Student's t-test. (I) Egg-laying assay results showing proportion of eggs laid categorized into three different stages. Functional rescue of egg-laying behavior is compared to syd-2(ju37) mutants. SYD-2 full-length and CC domains rescue egg-laying behavior significantly with ***p-value < 0.001, Fisher's exact test. (J) Punctate localization of SYD-2::GFP at synapses that are recapitulated by the (K) CC domains. (L) The SAM domains fail to localize and are diffused along the entire neuron.

fundamental question regarding the control of synapse formation is what regulates the availability of SYD-2 to self-assemble and associate with its binding partners to promote assembly.

Using *C. elegans* as a genetic model to study synapse assembly, we further characterize an interaction between the N-terminal coiled-

coils and C-terminal SAM domains of SYD-2 that had previously been identified (Wagner et al., 2009). We provide evidence using SYD-2 structure–function studies to show that the CC domains are sufficient for forming functional synapses while the SAM domains form a regulatory domain that is capable of recruiting negative regulators of

synaptogenesis. Our results implicate a model where an interaction between the CC and SAM domains is crucial for modulating the synaptogenic function of SYD-2. This interaction could be intramolecular as this form of inhibition is a widespread regulatory strategy to allow for precise modulation of protein activities (Pufall and Graves, 2002). Together, the data show the distinct functions of the two domains of SYD-2 and highlight the importance of regulating active zone scaffold molecules during synapse formation.

Results

SYD-2 coiled-coil domains are sufficient to assemble functional synapses

syd-2 is a multi-domain protein (Fig. 1A) and has previously been shown to affect presynaptic assembly in multiple classes of C. elegans neurons (Patel et al., 2006; Zhen and Jin, 1999). In the egg-laying motor neuron HSNL, en passant synapses form along a short stretch of the axon onto vulva muscles and VC4 and VC5 interneurons. These synapses can be visualized by expressing synaptic vesicle protein synaptobrevin (SNB-1) fused to YFP using a HSN cell-specific promoter (Fig. 1B). Loss-of-function (LOF) mutations in syd-2 results in complete loss of synaptic vesicles due to the failure in recruitment of many active zone proteins to presynaptic sites in HSNL (Figs. 1C and D) (Patel et al., 2006). The loss of synaptic function in the HSN neurons also results in defective egg-laying ability due to decreased excitability of vulva muscles (Patel et al., 2006; White et al., 1986). Presynaptic assembly and egg-laying behavior are completely rescued in syd-2(ju37) null mutants by cell-autonomous expression of SYD-2 in HSN using the unc-86 promoter (Figs. 1E, H and I).

SYD-2 comprised multiple CC motifs at its N-terminus and three homotypic SAM domains at its C-terminus (Fig. 1A). A number of active zone proteins such as ELKS-1, UNC-10 and GIT-1 have been shown to bind SYD-2/liprin- α at its CC regions, while PTP-3 and GRIP interact with SYD-2/liprin- α 's SAM domains (Ackley et al., 2005; Dai et al., 2006; Ko et al., 2003a, 2003b; Wyszynski et al., 2002). To further understand the importance of these interactions and their role in presynaptic assembly, we expressed truncated SYD-2 cDNA constructs of either the CC domains or SAM domains in HSNL. The SYD-2 CC domains localized to punctate structures along the synaptic domain similar to full-length SYD-2 (Figs. 1] and K) and were sufficient to rescue both synaptic vesicle recruitment and egg-laying function (Figs. 1F, H and I). Conversely, the SAM domains failed to rescue both phenotypes and localized diffusely along the entire axon (Figs. 1G and L). It has previously been shown that SYD-1 functions as a positive regulator upstream of SYD-2 (Hallam et al., 2002; Patel et al., 2006). In syd-1 mutants, synapses also fail to form in HSNL, but this defect can be bypassed by the over-expression of SYD-2. Likewise, the CC domains of SYD-2 are sufficient to overcome the deficiency in syd-1, to rescue synapse formation and egg-laying function in both syd-1 single mutants and syd-1;syd-2 double mutants (Figs. 1H and I). These results strongly suggest that SYD-2 CC domains are necessary and sufficient to form functional presynaptic specializations in HSNL. Our analysis corroborates with data published from a recent paper in which it was shown that the first 719 amino acids containing both liprin- α homology domains are necessary for HSN synapse assembly (Taru and Jin, 2011).

Mutations in SYD-2 SAM domains result in severe loss of synapses in HSNL

Although the SAM domains of SYD-2 do not seem to be required for localization or for synapse assembly, mutations in the SAM domains result in loss of synapses in HSNL. We identified two LOF mutant alleles *syd-2(ky292)* and *syd-2(wy11)* (Fig. 1A) using forward genetic screens. These two alleles show an almost complete loss of SNB-1::YFP fluorescence at the synaptic region (Figs. 2A–C) which can be rescued by HSN-specific expression of wild-type full-length SYD-2 (Figs. 2D, E). These mutants have synaptic defects in HSN that are almost as severe as syd-2 null alleles syd-2(wy5) and syd-2(ju37) (Fig. 2F). Loss of SYD-2 function in these alleles is caused by missense point mutations in the third SAM domain that result in changes in coding sequence: *syd-2(ky292)* has a Leu1086Phe (L1086F) change while syd-2(wy11) has a Gln1046Pro change (Fig. 1A). Leu1086 is a highly conserved residue while Gln1046 is not as conserved across the phylogenic tree from C. elegans to Homo sapiens (data not shown). Even though most of the synaptic vesicles are lost in *syd-2(ky292)*, ky292 animals have significantly more synaptic vesicles than null allele *syd-2(ju37)* (Student's t-test, p < 0.01) and retain more egg-laying function as compared to *syd-2(wy5)* and *syd-2(ju37)* mutants (Fisher's exact probability test, p < 0.01) (Fig. 2G). This result suggests that the ky292 mutation is a strong hypomorph. Furthermore, YFP-tagged SYD-2(L1086F), containing the ky292 mutation, localizes normally to the presynaptic sites in HSN, suggesting that the L1086F mutation does not affect the synaptic localization of SYD-2 (Fig. 3A). Together, the data suggest that the L1086F mutation produces a properly folded and localized SYD-2 protein but severely reduces the synaptogenic ability of SYD-2 by affecting the normal function of the SAM domains.

Intragenic suppression of SAM domain mutations by a gain-of-function mutation in the coiled-coils

The above genetic data suggest that the SAM domains are important for SYD-2 function, yet the structure function data suggest that they are dispensable for protein interactions necessary for establishing active zone formation and synaptic vesicle recruitment. A hypothesis that reconciles these two paradoxical observations is that the SAM domains are negative regulators of the synaptogenic activity of SYD-2. According to this hypothesis, the mutations in the SAM domain lead to constitutive inactivation of SYD-2 function carried out by the CC domains. To determine whether there are interactions between the CC and SAM domains, we took advantage of a previously identified gain-of-function (GOF) allele, syd-2(ju487) (Dai et al., 2006), which has a Arg184Cys missense mutation in the CC domain (Fig. 1A). This allele rescue the synaptogenesis defects in syd-1 mutants and has increased ability to recruit ELKS-1 to the presynaptic active zone (Dai et al., 2006). We generated syd-2 cDNA containing both syd-2(ju487) GOF and syd-2(ky292) or syd-2(wy11) LOF mutations, SYD-2(R184C, L1086F) and SYD-2(R184C, Q1046P). Cell-specific expression of either of these cDNAs in HSNL was able to strongly rescue the SNB-1::YFP phenotype and defects in egg-laving behavior in syd-2(ju37) mutants (Figs. 3D, F–H). In addition, YFP-tagged SYD-2(R184C, L1086F) localized to presynaptic sites in HSN is comparable to wildtype SYD-2 (Fig. 3B), suggesting that the R184C mutation does not affect the expression or stability of SYD-2 and produces a functional protein. In contrast, the SYD-2(L1086F) transgene when expressed at similar levels as SYD-2(R184C, L1086F), resulted in subtle rescue of the SNB-1::YFP phenotype, suggesting that SYD-2(L1086F) is a strong hypomorph and overexpression of this mutated protein can only achieve a minimal amount of activity (Figs. 3C, G and H). Expression of SYD-2(Q1046P) showed no significant rescue (Figs. 3E, G and H). The fact that SYD-2(R184C, L1086F) and SYD-2(R184C, Q1046P) achieved a much higher level of rescue compared with SYD-2(L1086F) or SYD-2(Q1046P), argues that the GOF R184C mutation in the CC domain is epistatic to, and suppresses the LOF mutations in the SAM domain. Consistently, SYD-2(R184C, L1086F) also achieved more complete rescue of the egg-laying behavior compared to SYD-2(L1086F). Together, these data support our hypothesis that the CC domains are essential for presynaptic assembly and the SAM domains are potential negative regulators of this function of the CC domains. The L1086F and Q1046P mutations increase the "inhibitory effects" of the SAM domains while the R184C mutation is epistatic and thus suppresses the inhibition from the SAM domains and render the SYD-2 molecule functionally active.



Fig. 2. Multiple syd-2 alleles show severe loss of synapses in HSNL. Confocal images of SNB-1::YFP in (A) syd-2(wy5), an early nonsense mutation allele, (B) syd-2(ky292) and (C) syd-2(wy11) alleles are single missense mutations in the third SAM domain. All three alleles display similar severity of phenotype. Scale bar represents 10 μ m. Expression of full-length SYD-2 in HSN rescues synapse assembly in (D) syd-2(ky292) and (E) syd-2(wy11) mutants. (F) Quantification of total SNB-1::YFP fluorescence normalized to wild-type values in the various syd-2 mutants. syd-2(ju487) is a GOF allele. Error bars represent SEM. *p-value < 0.05 and ***p-value < 0.001, Student's t-test. (G) Quantification of egg-laying defects in the different syd-2 alleles compared to wild-type. The various syd-2 alleles all have significant defects in egg-laying behavior, ***p-value < 0.001, Fisher's exact test. syd-2(ky292) is a strong hypomorph compared to the null alleles syd-2(ju37) and syd-2(wy5) (p-value < 0.001).

Direct binding between the coiled-coil domains and SAM domains of SYD-2

This model is supported by the previous report that the N-terminal CC region and C-terminal SAM domain region of SYD-2 interact with each other in a yeast two-hybrid assay (Wagner et al., 2009). To further test our model, we set out to determine if the missense mutations we have identified change the affinity between the two domains, we performed similar yeast two-hybrid experiments using SYD-2 coiledcoils containing the GOF R184C mutation and SAM domains containing the LOF L1086F mutation. The first 695 amino acids of SYD-2 containing the CC domains have an intrinsic ability to bind to one another and oligomerize (Taru and Jin, 2011; Wagner et al., 2009). In the presence of the GOF R184C mutation, there is a significant increase in binding affinity between the coiled-coil domains (Fig. 3I). This increase in interaction was not observed between a wild-type CC protein and GOF mutant CC protein indicating that there is a potential direct interaction between the CC regions containing the mutation. This provides additional data to support the hypothesis that the GOF R184C mutation causes an increase in SYD-2 assembly activity by enhancing its ability to bind to itself or other scaffold molecules (Dai et al., 2006; Patel and Shen, 2009).

The interaction between the SYD-2 CC domains and SAM domains containing the LOF L1086F mutation showed no significant change as compared to wild-type SAM domains suggesting no difference in apparent binding affinities between the two domains of SYD-2 (Fig. 31).

Our hypothesis would have predicted an increase in affinity: on the other hand, several possibilities might explain this result. First, the binding between the wild-type coiled-coils and SAM domains is already quite strong. Therefore any subtle changes in binding affinities might not be detected. Second, the increased inhibitory effect of the L1086F mutation might not be through a tighter binding between the SAM domains and the CC domains. For example, it is conceivable that the L1086F increases the ability of the SAM domains to recruit a negative regulator of the presynaptic assembly and hence causes reduced assembly.

Negative regulator of synapse assembly, RSY-1, binds strongly to loss-of-function SYD-2 SAM domains

A good candidate for such a protein is RSY-1, a negative regulator of SYD-2-dependent synapse assembly (Patel and Shen, 2009). RSY-1 disrupts the interaction between SYD-2 and SYD-1 with ELKS-1. This in turn affects the ability for the synapse assembly positive regulator SYD-1 to promote synaptogenesis through SYD-2. In addition, RSY-1 has been shown to bind the SAM domains of SYD-2 (Patel and Shen, 2009).

Using a previously established single-cell in situ protein-protein interaction assay (Patel and Shen, 2009) that assesses the physical association of two proteins by the recruitment of a cytosolic prey protein to the plasma membrane through a membrane-targeted bait protein, we tested the molecular consequence of introducing the LOF



Fig. 3. Interaction observed between the N-terminal CC domains and C-terminal SAM domains. (A) SYD-2(L1086F) protein containing the mutation found in *syd-2(ky292)* LOF allele still localizes to presynapses. (B) Expression of SYD-2(L1086F) does not rescue synapse formation as assayed by SNB-1::YFP fluorescence, but (C) SYD-2(R184C, L1086F) containing an extra *syd-2(ju487)* GOF mutation is functional and rescues synapse assembly. Similarly, (D) SYD-2(Q1046P) the mutation in *syd-2(wy11)* fails to rescue and (E) SYD-2(R184C, Q1046P) rescues synapse assembly. Scale bar represents 10 µm. This effect is quantified in (F). Error bars represent SEM. *p-value < 0.05 and ***p-value < 0.001, Student's t-test. Two independent transgenic lines were analyzed for each SYD-2 (R184C, L1086F) compared to SYD-2(L1086F). ***p-value < 0.001, Fisher's exact test. (H) Score table showing the strength of interactions between the various SYD-2 CC and SAM domain constructs. *There is a significant increase in affinity between SYD-2(R184C) CC homodimerization as compared to SYD-2 C homodimerization (p-value < 0.001, Student's t-test). p53 and Large T serve as positive controls or negative controls for unspecific interactions. Data represents score for 2–4 independent experiments. For scoring, see Experimental methods.

L1086F mutation into the SAM domains and how this changes its interaction with RSY-1. We detected no interaction between membranetargeted RSY-1 Δ SR (truncated version of RSY-1 that lacks the nuclear localization sequence) and full-length SYD-2 or the C-terminal of SYD-2 comprising all three SAM domains (Figs. 4A–D and G). Previous work has shown that RSY-1 is able to interact with SAM domains 1–2 suggesting that the interaction surface for RSY-1 is contained within the first two SAM domains (Patel and Shen, 2009). The full complement



Fig. 4. Negative regulator of synaptogenesis, rsy-1, associates strongly with SYD-2(L1086F) SAM domains. (A) mcherry::SYD-2 is localized to the cytoplasm when coexpressed with either a membrane-targeted GFP or (B) a membrane-targeted GFP::RSY-1\DeltaSR (lower left inset image) in Hek293T cells, suggesting no or weak transient binding. Merged images are in the lower right corner of each panel. (C and D) Similarly, mcherry::SYD-2SAM1-3 is cytoplasmic. (E and F) mcherry::SYD-2SAM1-3(L1086F) is recruited to the plasma membrane only in the presence of RSY-1\DeltaSR suggesting an increase in binding affinity. Scale bar represents 10 µm. (G) Quantification of prey enrichment at the membrane by calculating the ratio of (pixel intensity of fluorescence at the membrane/pixel intensity in cytoplasm) and averaging across at least 20 cells. Membrane-targeted GFP is used as a negative control for non-specific binding. Error bars represent SEM. ***p-value < 0.001, Student's t-test.

of all three SAM domains may display weaker interaction due to the folding of the tandem SAM domains that are known have homotypic interactions (Thanos et al., 1999). However, in the presence of the LOF L1086F mutation, cytosolic SYD-2 SAM domains translocate to the plasma membrane, indicating that there is a significant increase in binding affinity to RSY-1 Δ SR (Figs. 4E–G). These results suggest that wildtype SYD-2 SAM domains have a weak and potentially transient association with RSY-1 but with the LOF L1086F mutation this interaction is strengthened and may increase negative regulation of SYD-2 activity.

Therefore, the loss of synapses in the *syd-2(ky292)* mutant might result from excessive inhibition of *syd-2* due to increase RSY-1 binding to SYD-2(L1086F). To test this hypothesis, we investigated if loss of *rsy-1* would restore synapse formation *in vivo* in *syd-2(ky292)* mutants. We analyzed *syd-2(ky292);rsy-1* double mutants and failed to detect rescue of the loss of synapses in HSNL (results not shown) possibly because *rsy-1* is not the sole factor suppressing the activity of *syd-2*.

Discussion

Recent studies of molecules involved in regulating the assembly of presynaptic densities suggest that to achieve spatially precise synaptic development requires multiple mechanisms (Jin and Garner, 2008; Ziv and Garner, 2004). In this study we provide evidence for a model where the association between the N-terminal CC domains and C-terminal SAM domains of SYD-2 inhibits its activity to assemble synapses.

By performing structure–function studies, we have shown that the CC domains of SYD-2 as being functionally necessary and sufficient to promote synapse assembly are similar to the conclusions of a recent paper where they demonstrated the requirement of the coiled-coil region for synapse assembly (Taru and Jin, 2011). SYD-2's first 800 amino acids contain 8–9 CC domains that are intrinsically unstructured, but can scaffold a large number of proteins in a structurally adaptive process to form the complex presynaptic active zone matrix (Wagner et al., 2009). Despite the sufficiency of the CC region, various *syd-2* genetic lesions, including nonsense mutations in the CC domains and missense mutations in the SAM domains, cause a severe loss of

synapses in HSNL. This similarity in morphological phenotype argued strongly for the functional importance for *syd-2* SAM domains. In fact, the C-terminal SAM domains, originally termed the liprin homology (LH) domain, are highly conserved between human *liprin-\alpha* and *C. elegans syd-2* (Serra-Pagès et al., 1998).

syd-2 binds to LAR-RPTP/*ptp-3*, via its SAM domain. *ptp-3* mutants display altered synapse morphology in some *C. elegans* neurons (Ackley et al., 2005) but have no obvious phenotype in HSNL, suggesting that its role in synapse assembly in HSNL is not vital (data not shown). *syd-2* SAM domains also interacts with scaffolding proteins such as GRIP, a multi-PDZ domain protein (Wyszynski et al., 2002), and CASK, a peripheral membrane protein that is found in a ternary complex including Veli/*lin-7* and Mint-1 (Olsen et al., 2005). These interactions have been implicated in the recruitment of synaptic proteins, though neither has been implicated as essential for a functioning synapse.

Even though the SAM domains alone are unable to rescue synapse formation in our system, we cannot exclude the possibility that in the context of full-length SYD-2, protein interactions with the SAM domains play a minor role in assembling the active zone scaffold and defects are undetectable by our assays. However, it is clear that certain mutations that affect the SAM domains have negative effects on synapse assembly.

Further genetic experiments and yeast two hybrid analyses revealed an interaction between the CC domains and the SAM domains of *syd-2*. From our data, several lines of evidence argue strongly that the role of this interaction is to self-regulate SYD-2's synaptogenic activity. A previous study on the interaction between SYD-2 and UNC-104, found that full-length SYD-2 had decreased binding affinity as compared to the coiled-coils alone (Wagner et al., 2009).This finding supports our hypothesis that the SAM domains may decrease the association ability of binding sites along the CC region of SYD-2.

There are two potential models for SYD-2 regulation. The first is a model where the SYD-2 protein physically folds upon itself to mask binding sites required for recruiting other active zone molecules for synapse formation (Fig. 5). Such a mechanism has been found for other synaptic assembly molecules. For example, the presynaptic active zone molecule, GIT-1 uses the N-terminal arf-GAP and ankyrin repeat domains interact with the C-terminal Spa2-homology domains



Fig. 5. Model for regulating SYD-2 during synapse formation. SYD-2 binds to multiple active zone molecules such as RIM, GIT-1 and ELKS-1 to facilitate synapse formation whilst in an (A) active state. ELKS-1 is able to promote the formation of an active SYD-2 molecule. Negative regulator RSY-1 promotes the inactive conformation of SYD-2 that may be due to (B) folding of the molecule upon itself (C) or by the binding of RSY-1 and other negative regulators that render SYD-2 inactive due to occlusion of SYD-2 coiled-coil regions important for interacting with other active zone molecules.

to regulate binding to paxillin, a focal adhesion protein (Totaro et al., 2007). Another multidomain molecule, SAP97 that is part of the MAGUK family, negatively inhibits its interaction with binding partners through intramolecular association between its Src homology 3 domain and GUK domain (Wu et al., 2000). Thus, it is feasible that physical association of SYD-2 SAM domains with the CC regions modulates its activity.

A second model involves the binding of regulatory proteins to the SAM domains that occludes protein interactions with the CC domains or constrains SYD-2 in a nonfunctional conformation (Fig. 5). This model seems more likely since our yeast two hybrid data failed to show a significant increase in binding of the SAM domain harboring the LOF L1086F mutation with the CC domains. Previous work has shown that a negative regulator of synapse assembly, RSY-1, disrupts the binding of SYD-2 and scaffolding molecule ELKS-1, an interaction important to promote synapse assembly in HSNL (Patel and Shen, 2009). Our in vitro biochemical experiments show that RSY-1 binds tighter to LOF L1086F mutated SYD-2 SAM domains as compared to wild-type indicating that the suppression of SYD-2's ability to form synapses in the *syd-2(ky292)* mutant may be partly due to excessive recruitment of RSY-1. In the wild-type situation, the physical association between RSY-1 and SYD-2 is probably weak or transient since RSY-1 is unable to confer membrane localization of full-length SYD-2 in our in-vitro protein-protein binding assay, but this affinity is sufficient as it may function in concert with other repressors to regulate SYD-2.

To relieve negative regulation, SYD-2 may interact with positive regulators like SYD-1 that increases the binding of SYD-2 to ELKS-1 to promote synapse assembly (Patel and Shen, 2009; Patel et al., 2006). Furthermore, coexpression of ERC2/ELKS-1 and liprin- α in hippocampal neurons has been shown to increase liprin- α localization at presynaptic active zones (Ko et al., 2003b). Thus ELKS-1 stabilizes liprin- α at active zones supporting the hypothesis that ELKS-1 functions as an activator of SYD-2. Along the same lines, we found

that the *syd-2(ju37)* GOF mutation R184C, which has higher binding affinity for ELKS-1, is able to rescue the synaptic defects caused by *syd-2(ky292)* LOF mutation (Dai et al., 2006). This might be due to increased ELKS-1 binding that overcomes excessive SYD-2 repression. This substantiates the hypothesis that ELKS-1 also positively regulates synapse assembly.

Given the complexity of the protein assemblies that are possible by the multiple interactions between SYD-2 and its binding partners, tight regulation of these interactions is necessary for proper function of SYD-2 in neurons. Intramolecular inhibition offers a potential model that presents a reversible barrier to prevent ectopic SYD-2 function while keeping it primed to respond when appropriate synaptogenic signals are present.

Experimental methods

Strains

Strains were maintained at 20 °C on NGM agar plates with OP50 *Escherichia coli* as described previously (Brenner, 1974). Wildtype animals were of Bristol variety *N*2 strain and the following mutants were used: LGII, *syd-1(ju82)*, LGX, *syd-2(ju487)*; *syd-2(ju37)*; *syd-2(ky292)*; *syd-2(wy5)*; *syd-2(wy11)*.

Transgenic lines

kyls235 [Punc-86::snb-1::yfp; Punc-4::lin-10::dsred; Podr-1::dsred], wyls12 [Punc-86::gfp::syd-2; Podr-1::gfp], wyEx7 [Punc-86::syd-2; Podr-1::gfp], wyEx11 [Punc-86::syd-2(1-858aa); Podr-1::gfp], wyls135 [Punc-86::gfp::syd-2(1-858aa); Podr-1::rfp], wyEx856 [Punc-86:: syd-2(556-1140aa); Podr-1::gfp], wyEx23 [Punc-86::gfp::syd-2(556-1140aa); Podr-1::gfp], wyEx2321 [Punc-86::syd-2(L1086F); Podr-1:: gfp], wyEx2323 [Punc-86::syd-2(L1086F); Podr-1::gfp], wyEx1006[Punc-86::yfp::syd-2(L1086F); Podr-1::rfp], wyEx2189 [Punc-86::syd-2(R184C; L1086F); Podr-1::gfp], wyEx3966 [Punc-86::syd-2(R184C;L1086F); Podr-1::gfp], wyEx5567 [Punc-86::syd-2(Q1046P); Podr-1::gfp], wyEx5568 [Punc-86::syd-2(Q1046P); Podr-1::gfp], wyEx5569 [Punc-86::syd-2(R184C;Q1046P); Podr-1::gfp], wyEx5570 [Punc-86::syd-2(R184C;Q1046P); Podr-1::gfp].

Cloning of constructs

Plasmids for transgenic lines were made using the pSM vector, a derivative of pPD49.26 (A. Fire). The *unc-86* promoter was cloned between SphI and XmaI and *syd-2* cDNA was cloned between NheI and KpnI. Plasmids were injected into animals at 1 ng/µl. Plasmids for expression in Hek293T cells were made using pFLAG-CMV-2 (mCherry tag) and pcDNA3.1/*myc*-His(-)A (GFP tag).

SNB-1 fluorescence quantification

Fluorescence images of HSNL synapses in young adults were taken with a $63 \times$ objective on a Zeiss Axioplan 2 Imaging System using similar imaging parameters for the same marker across different genotypes. For extrachromosomal array transgenic lines, two independent lines were analyzed. At least 20 individual animals were imaged and quantified for each group. Total fluorescence intensity of SNB-1::YFP across the synaptic region was determined using ImageJ software (NIH) by summing pixel intensity and the average fluorescence intensity was calculated for each group. All statistical comparisons were done with a two-tailed unpaired Student's t-test.

Egg-laying assay

20 L4 animals were allowed to develop overnight at 20 °C into gravid adults. Animals were transferred onto a fresh plate and the stage of each egg laid was scored double blind after an hour at 20 °C. The eggs were classified into one of three developmental stages: 1–8 cell, 8-cell comma, or post-comma. 2–3 independent assays were performed with at least 100 eggs quantified in total for each genotype. Day to day variation was observed but each experimental round was performed together with wild type and respective mutant controls for comparison. Statistical analysis between groups was done using the Fisher's exact test.

Yeast 2-hybrid analysis

Yeast transformation was performed according to the manufacturer's protocol (Matchmaker System 3, Clontech). Transformed cells were first plated on low-stringency selection medium (-LT) to identify doubletransformants. Cells grown on -LT agar were either 1) replica-plated onto -HALT agar (high stringency) immediately or 2) first replicaplated on - HLT-deficient media containing adenine (medium stringency), and then replica-plated onto – HALT agar (high stringency) plates. Colonies that grow on -HALT plates were scored based upon the following criteria: no colonies on - HALT or red or pinkish colony on - HALT, "-" (red color is a sign for adenine deficiency andreceives a score of 0); white colony on - HALT, "+" (score of 1); light blue colony on -HALT, "++" (score of 2); and dark blue colony on -HALT, "+++" (score of 3). For example, a -HALT plate (replica-plated from a -LT plate) with 1 white, 1 red, 3 light blue, and 4 dark blue colonies received an overall score of (1 + 0 +(3 * 2) + (4 * 3) / 9 = 2.1 which would correspond to "++". The maximum average score of any plate is 3 or "+++".

In vitro protein interaction assay

As previously described (Patel and Shen, 2009), Hek293T cells were co-transfected with plasmid constructs driven by the CMV promoter with TransIT-LT1 reagent (Mirus) according to the manufacturer's recommendations and cells were incubated at 37 °C for 24 h prior to imaging. Live cell images were obtained using a 20× objective on a Zeiss Axioplan 2 Inverted Fluorescence Imaging System. To quantify the recruitment of prey at the cell membrane, we used ImageJ to perform line scans across the cell membrane and into the cytoplasm. The fluorescence pixel intensity at the membrane and pixel intensity in the cytoplasm were used to obtain a ratio comparing the fold difference in intensities of the two compartments. (n > 20 cells) and statistical analysis was performed with a two-tailed unpaired Student's t-test.

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