nature neuroscience

NAB-1 instructs synapse assembly by linking adhesion molecules and F-actin to active zone proteins

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During synaptogenesis, macromolecular protein complexes assemble at the pre- and postsynaptic membrane. Extensive literature identifies many transmembrane molecules sufficient to induce synapse formation and several intracellular scaffolding molecules responsible for assembling active zones and recruiting synaptic vesicles. However, little is known about the molecular mechanisms coupling membrane receptors to active zone molecules during development. Using *Caenorhabditis elegans*, we identify an F-actin network present at nascent presynaptic terminals and required for presynaptic assembly. We unravel a sequence of events whereby specificity-determining adhesion molecules define the location of developing synapses and locally assemble F-actin. Next, the adaptor protein NAB-1 (neurabin) binds to F-actin and recruits active zone proteins SYD-1 and SYD-2 (liprin-α) by forming a tripartite complex. NAB-1 localizes transiently to synapses during development and is required for presynaptic assembly. Altogether, we identify a role for the actin cytoskeleton during presynaptic development and characterize a molecular pathway whereby NAB-1 links synaptic partner recognition to active zone assembly.

Synapse formation is often initiated by membrane contacts between appropriate synaptic partner cells, which leads to intracellular assembly of the active zone and recruitment of synaptic vesicles beneath the presynaptic membrane. Numerous transmembrane molecules have been implicated in specifying synaptic connections[1]. Many pairs of these homotypic or heterotypic synaptic adhesion molecules, including cadherins, ephrin-B–EphB, neurexin–neuroligin, synCAMs, netrinG–netrinG ligands (NGL2) and leukocyte common antigen–related (LAR)–NGL3, can induce synapse formation through trans-synaptic interactions[2,3]. But although their synapse-inducing activities are robust *in vitro*, it is difficult to demonstrate their roles in synaptogenesis *in vivo*, possibly due to functional redundancy. For example, binding between neurexin and neuroligin triggers synapse assembly on both pre- and postsynaptic cells in dissociated cultured neurons[4,5]. However, the importance of this interaction for synapse formation *in vivo* seems to be restricted to certain systems. In *Drosophila*, mutations in the neurexin homolog, *dnrx*, cause reduced synapse number and defective active zone formation[6]. In the mammalian cerebellum, neurexin interacts trans-synaptically with the glutamate receptor subunit GluRD2 through cerebellin 1 precursor protein to mediate synapse formation[7]. Among the four neuroligins, neuroligin 2 found at inhibitory synapses is important for postsynaptic development[8]. It is still unclear whether these trans-synaptic interactions are important for synaptic target selection. In at least one case, heterologous binding between two immunoglobulin superfamily proteins, SYG-1 (NEPH1) and SYG-2 (nephrin) is critical for selective synapse formation in *C. elegans* HSN (hermaphrodite-specific neuron) neurons[9]. SYG-1 localizes to presynaptic sites before synapse formation and is necessary and sufficient for presynaptic development *in vivo*[10].

Although diverse membrane receptors induce synapse formation, it is thought that a common presynaptic assembly program constructs active zones and clusters synaptic vesicles. In mammals, many scaffolding and cytomatrix proteins are found at presynaptic terminals. For instance, one MAGUK family member, CASK, localizes to active zones and binds to neurexin and calcium channels[11]. In vertebrates, Piccolo and Bassoon are large, multi-domain presynaptic cytomatrix proteins with long stretches of coiled-coil domains[12]. Although both serve as excellent active zone markers, recent genetic analysis suggests that they are probably not essential for synaptic transmission but might function redundantly in maintaining synaptic vesicles[13].

Forward genetic approaches in worms and flies have identified three molecules as core active zone assembly genes, SYD-2 (liprin-α), SYD-1 (*Drosophila* SYD-1) and ELKS-1 (Bruchpilot (Brp)). SYD-2 mutants show complete loss of synaptic vesicles and active zone proteins in HSN synapses, and active zones are abnormal in size and shape at inhibitory synapses in worms and at *Drosophila* neuromuscular junctions[14–16]. In flies and worms, *syd-1* and *dsyd-1* mutants, respectively, also show profound presynaptic assembly defects[14,17]. Brp was first shown to be required for active zone formation and calcium channel localization at *Drosophila* neuromuscular junctions[18,19]. The *C. elegans* homolog of Brp, ELKS-1, is also involved in presynaptic development; however, its role is only revealed in sensitized backgrounds[20,21].

Of the three molecules, genetic epistasis analyses suggest that SYD-2 (liprin-α) is the most important scaffold molecule, whereas SYD-1 and ELKS-1 promote the activity of SYD-2 (refs. 20,21). Many biochemical interactions between SYD-2 and other presynaptic proteins, including UNC-10 (RIM), GIT and ELKS-1 (Brp) support the notion that SYD-2 serves as the 'hub' for active zone assembly[17,22].

Actin networks decorate presynaptic terminals by forming a ring-like structure surrounding synaptic vesicles and active zones[23].

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Received 21 March 2011; accepted 28 October 2011; published online 8 January 2012; doi:10.1038/nn.2991

Although filamentous actin (F-actin) is not required for synaptic transmission, actin dynamics have been shown to participate in regulatory mechanisms modulating synapse efficacy[24], and F-actin seems to be critical for synapse development[25]. Latrunculin A treatment of young synapses in hippocampal cultures leads to marked reduction of synapse numbers; conversely, mature synapses are resistant to actin depolymerization. Furthermore, regulators of actin dynamics such as the Rac guanine nucleotide exchange factor (GEF) Trio have been shown to be critical for growth of presynaptic terminals[26].

The molecular mechanisms linking the presynaptic actin network and the presynaptic assembly program remain unknown. NAB-1 (neurabin) is an actin-binding protein that localizes to both pre- and postsynaptic specializations[27–29]. In dendritic filopodia, neurabin regulates filopodia motility through its actin binding activity[30–32]. In *C. elegans*, *nab-1* mutants have reduced synapse density due to presynaptic defects[33]. In inhibitory neurons, NAB-1 seems to act in polarized trafficking of presynaptic components into axons through its interaction with SAD-1, an active zone serine/threonine kinase[34].

Despite the wealth of knowledge on synapse-inducing membrane receptors and active zone assembly molecules, little is known of how these two processes are coupled during development. Here we show that NAB-1 is required early during synapse formation to link the presynaptic actin network to active zone assembly proteins through its interaction with actin and to SYD-1 and SYD-2 (liprin-α) proteins. NAB-1 functions downstream of a specificity-determining transmembrane molecule, SYG-1, and upstream of active zone assembly genes. Hence, our data suggest that NAB-1 serves as an adaptor protein that links synaptogenic signals from transmembrane adhesion molecules to intracellular recruitment of active zones to specific subcellular domains.

RESULTS

Assembly of nascent presynaptic sites requires F-actin

To understand the processes that underlie synapse formation *in vivo*, we studied the synapses formed by two bilaterally symmetrical motor neurons in *C. elegans*, HSN, that are involved in egg-laying behavior. HSN cell bodies are situated posterior to the vulva, and each extends a single neuronal process anteriorly into the head. As this axon passes the vulva organ, HSN forms a cluster of 12–20 *en passant* synapses along a short stretch of the axon onto the vulval muscles and VC neurons (**Fig. 1a**). We visualized these presynaptic specializations in HSN by expressing fluorescently tagged proteins using the *unc-86* cell type–specific promoter[10]. Because of the high density of HSN synapses, it is difficult to assign synaptic vesicle fluorescence signals to individual active zones. However, their highly stereotyped subcellular localization enabled us to unambiguously identify these synapses in wild-type and mutant worms.

F-actin can be found on both sides of the synaptic cleft[30,35]. At presynaptic terminals, F-actin surrounds the synaptic bouton and is important for formation and modulation of synapses *in vitro*[36–38]. To understand the role of F-actin during synapse formation *in vivo*, we examined the distribution of stable F-actin using the utrophin calponin homology domain (utCH) fused to GFP. This F-actin probe has been shown to specifically bind subsets of stable F-actin[39]. Of note, stable F-actin labeled by GFP::utCH localized to the synaptic region of HSN, whereas GFP::actin labeled the entire HSN neuron, consistent with the notion that the utCH binds to certain fractions of F-actin[39] (**Fig. 1b**,**c**). The synaptic localization of the utCH probe was abolished when we introduced a mutation, L54R, that is postulated to destabilize the actin-binding domain on the basis of structure prediction studies, indicating that synaptic enrichment of the utCH probe is due to actin binding[40,41] (**Fig. 1d**). To verify the synaptic localization, we co-expressed GFP::utCH and mCherry::RAB-3, a fluorescently labeled synaptic vesicle–associated protein, to visualize synapses in HSN, and indeed, both proteins targeted the same region (**Fig. 1e**). Thus, a network of stable F-actin is present at HSN synapses *in vivo*.

In HSN, initiation of synaptogenesis and synaptic specificity is determined by the presence of a transmembrane immunoglobulin-superfamily protein, SYG-1 (NEPH1)[10]. In the absence of SYG-1, synapses fail to form in the synaptic region at the vulva and instead form ectopic synapses anterior to the vulva. In *syg-1* mutants, the F-actin network was lost from the synaptic region (**Fig. 1f**). Furthermore, localization of F-actin was unaffected by loss of SYD-1 or SYD-2 (**Fig. 1g**,**h**), two key active-zone scaffolding proteins required for recruiting most other presynaptic proteins to assemble HSN synapses[14], suggesting that the presynaptic F-actin network is most likely independent of the active zone structure.

Together with previous work, the data above are consistent with a hypothesis for HSN synaptogenesis whereby spatial placement of nascent synapses is determined by recruitment of SYG-1 along regions of the HSN axon where it contacts guidepost primary epithelial cells that express SYG-2 (nephrin), the trans-binding partner of SYG-1 (**Fig. 1i**)[9]. SYG-1 then recruits and assembles a stable F-actin network in the defined synaptic region. Consistent with this hypothesis, we observed that SYG-1 localizes together with utCH-labeled F-actin (**Fig. 1j**). To determine whether SYG-1 is sufficient to assemble the F-actin network, we ectopically expressed SYG-2 in more ventrally and laterally located secondary vulval epithelial cells using the *egl-17* promoter in *syg-2* mutants and asked whether aberrantly recruited SYG-1 would assemble an ectopic F-actin network (**Fig. 1k**)[9]. As in *syg-1* mutants, F-actin localization was lost from HSN synapses of *syg-2* mutants (**Fig. 1l**). In *syg-2* mutants carrying the transgene Ex[P*egl-17*::*syg-2*], F-actin accumulated in regions of the HSN axon contacting SYG-2–expressing secondary epithelial cells (**Fig. 1m**). These results demonstrate that local axonal SYG-1 is necessary and sufficient to assemble a stable F-actin network along the HSN axon.

Finally, to determine whether the F-actin network is required during presynaptic assembly, we disrupted F-actin by injecting latrunculin A into the vulval region of worms and assessed whether perturbing F-actin would result in presynaptic assembly defects by visualizing mCherry::RAB-3–labeled synapses. Synapse formation begins as the growing HSN axon crosses the vulva during the late third larval (L3) stage and early L4 stage, and presynaptic material continues to accumulate throughout the L4 stage (**Fig. 1n**). These synapses become functional after the L4 molt. We performed latrunculin A injections at two stages: early L4 stage, during the onset of synapse formation; and mid-late L4 stage, later in development. Latrunculin A treatment at both stages resulted in a decrease in fluorescence intensity of utCH-labeled F-actin as compared to that in dimethylsulfoxide (DMSO) vehicle-treated worms, suggesting that latrunculin A disrupted the F-actin network *in vivo* (**Fig. 1n** and **Supplementary Fig. 1a–d**). However, RAB-3 fluorescence intensity was substantially decreased only by early perturbation of F-actin, with no significant effects on synapses when latrunculin A was injected late. This observation implies that the local F-actin network is required for presynaptic assembly early at the onset of HSN synapse formation. Similar effects of latrunculin have been reported on *in vitro* cultured hippocampal neuron synapses, suggestive of a requirement for F-actin during early stages of presynaptic development[36]. SYG-1 localization was unaffected by latrunculin A treatment; thus SYG-1 functions upstream of and independently of the F-actin network (**Supplementary Fig. 1f**).

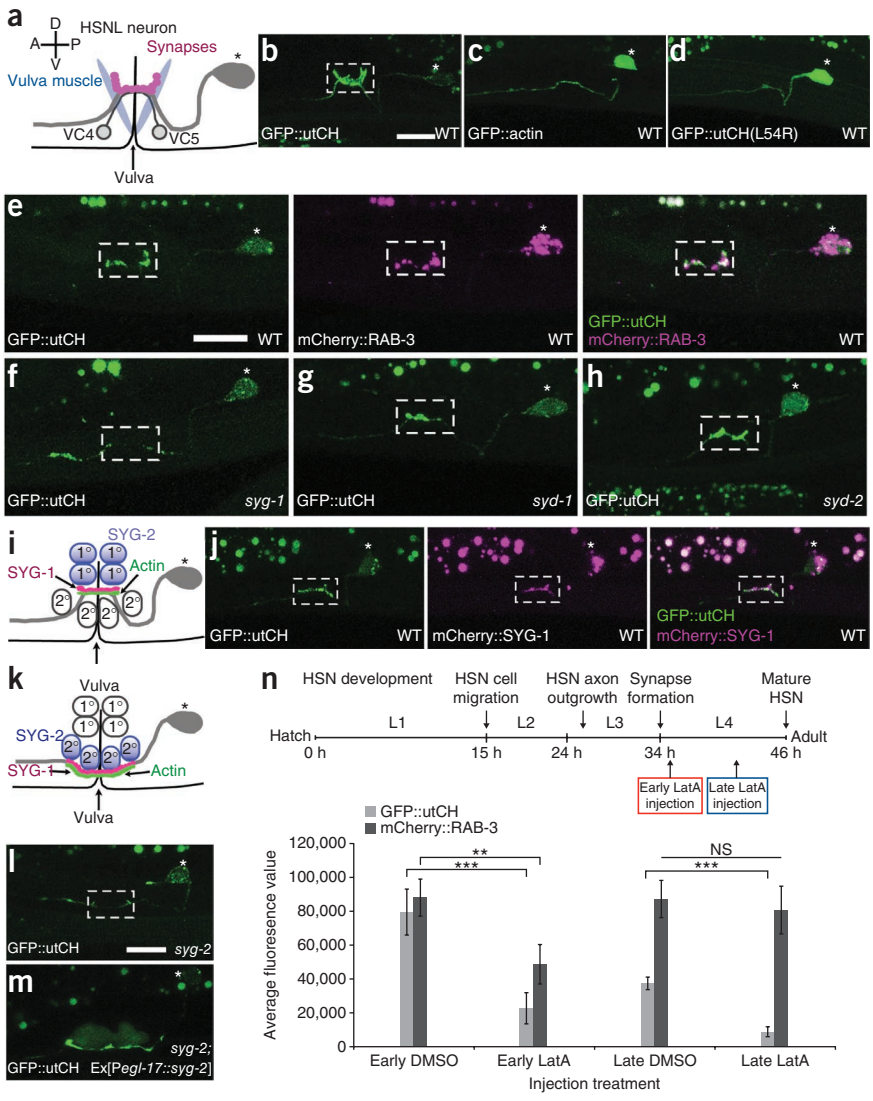

Figure 1 F-actin localizes to HSN synapses and is required for presynapse assembly. (**a**) Left HSN neuron (HSNL) schematic. Asterisk, cell body (throughout all images); presynapses form onto the vulval muscles across the vulval slit. D, dorsal; V, ventral; A, anterior; P, posterior. (**b**) GFP::utCH labels stable F-actin that localizes to presynaptic specializations (dashed white boxes throughout). (**c**) GFP::actin labels the entire HSN neuron. (**d**) L54R mutation in utCH disrupts actin binding and abolishes its synaptic localization. (**e**) GFP::utCH labels F-actin in the region with mCherry::RAB-3–labeled synapses. (**f**–**h**) utCH labeled F-actin is lost from synapses in *syg-1* mutants (**f**) but is unaffected in *syd-1* or *syd-2* mutants (**g**,**h**). (**i**) Schematic of primary (1°) vulval epithelial cells expressing SYG-2 (blue), which recruits trans-synaptic binding partner SYG-1 (pink) and F-actin (green) along the axon. (**j**) SYG-1 and F-actin are enriched at synapses. (**k**) Diagram showing ectopic SYG-2 expression in secondary (2°) vulval cells in *syg-2* mutants. (**l**,**m**) F-actin fails to localize to synapses in *syg-2* mutants (**l**), but synaptic vesicles accumulate ectopically in regions contacting SYG-2–expressing secondary vulval cells (**m**). (**n**) Top, timeline of HSN development relative to larval stages L1–L4 at 20 °C. Bottom, average fluorescence intensity, in arbitrary units, for GFP::utCH and mCherry::RAB-3. Early latrunculin A (LatA) treatment reduced utCH and RAB-3 fluorescence by 71% and 45%, respectively, as compared to DMSO controls. Late LatA reduced utCH fluorescence by 76% but did not affect RAB-3 fluorescence. Error bars, s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$; NS, $P > 0.05$; two-tailed Student's *t*-test. For each treatment, GFP::utCH and mCherry::RAB-3 fluorescence were quantified from the same 15 worms. WT, wild type. All scale bars, 10 µm; bars shared by **b**–**d**, by **e**–**j** and by **l**,**m**.

NAB-1 is required for HSN presynaptic assembly

As the F-actin network is important for presynaptic assembly, there might be presynaptic actin-binding molecules involved in this process. We performed a candidate screen to identify presynaptic proteins containing actin-binding domains and showing defects in presynaptic assembly in HSN. We identified NAB-1, a neural tissue-specific actin binding protein, as a good candidate[28]. First, NAB-1 localized to synapses in HSN together with RAB-3 and F-actin (**Fig. 2a**,**b**). Second, two mutant alleles of *nab-1* led to a marked loss of synaptic vesicles from synapses, labeled by synaptobrevin (SNB-1) fused to yellow fluorescent protein (YFP) (**Fig. 2c**–**i**). This deficiency in synaptic vesicle recruitment was reflected behaviorally by defects in egg-laying behavior, as *nab-1* mutants laid significantly more late-stage eggs and fewer early-stage eggs than wild-type worms (**Fig. 2j**). Both defects were enhanced by simultaneous loss of ELKS-1, an active zone scaffolding protein whose loss alone resulted in no apparent phenotype (**Fig. 2f**,**i** and **Supplementary Fig. 2a**,**b**). Loss of synaptic vesicle recruitment in *nab-1;elks-1* double mutants was as severe as that in *syd-1* mutants, suggesting that ELKS-1 functions redundantly with NAB-1 for presynaptic development (**Fig. 2g**–**i**).

In mammals, neurabin has been described as regulating dendritic spine dynamics[30,31]. In *C. elegans*, NAB-1 has been shown to localize presynaptically and function in regulating synapse density and neuronal polarity[33,34]. To determine where NAB-1 functions, we performed cell-autonomous rescue experiments by expressing NAB-1 in HSN alone, which was sufficient to rescue both synaptic vesicle recruitment and egg-laying defects in *nab-1;elks-1* double mutants (**Fig. 2i**,**j**). Thus, NAB-1 is a putative actin-binding protein required for synapse assembly and functions cell-autonomously in HSN.

NAB-1 localizes to HSN synapses through actin binding

Mammalian neurabin has a functionally confirmed N-terminal actin-binding domain (ABD), followed by a structurally predicted PDZ domain, a coiled-coil region and a sterile-α motif (SAM) domain (**Fig. 3a**)[28,31]. *C. elegans* NAB-1 has well conserved PDZ and SAM domains when aligned with human neurabin. However, NAB-1's ABD is poorly conserved at the level of amino acid sequence. As there is great diversity in ABD structures, we tested whether NAB-1 binds actin. We expressed a cyan fluorescent protein (CFP)–NAB-1 fusion in heterologous HEK-293T and NIH-3T3 cells and found that fluorescence was enriched at subcellular F-actin structures, including subcortical regions beneath the plasma membrane and stress fibers (**Supplementary Fig. 3**). Furthermore, NAB-1's subcellular localization in these cells was largely abolished

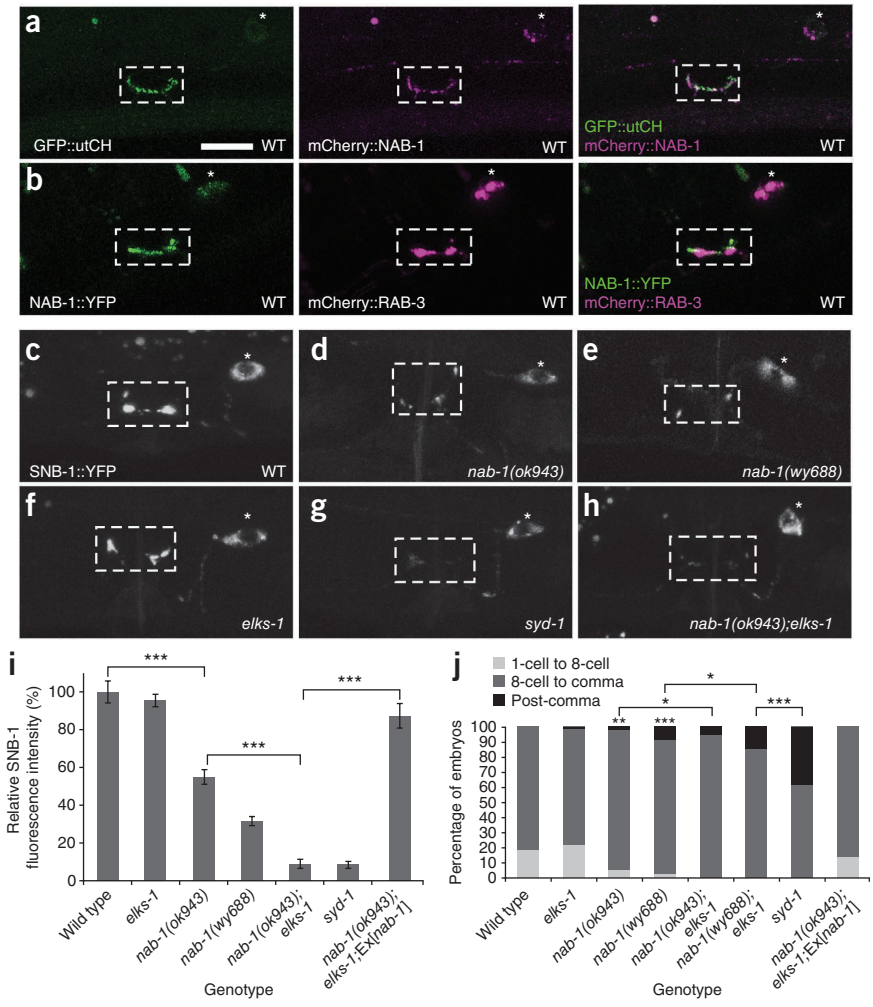

Figure 2 NAB-1 localizes to presynaptic sites and *nab-1* mutants show presynaptic assembly defects. (**a**,**b**) NAB-1 is an active zone protein that is enriched at synapses together with utCH-labeled F-actin (**a**) and RAB-3 labeled synaptic vesicles (**b**). (**c**) A wild-type (WT) HSN neuron with synapses labeled by SNB-1::YFP. (**d**,**e**) *nab-1(ok943)* (**d**) and *nab-1(wy688)* (**e**) mutants show partial loss of SNB-1 fluorescence. (**f**) This defect is not observed in *elks-1(tm1233)* mutants. (**g**,**h**) However, *nab-1(ok943);elks-1* double mutants (**h**) show almost complete lost of SNB-1 staining, like *syd-1* mutants (**g**). Scale bar, 10 μm. (**i**) Quantification of SNB-1 fluorescence intensity at synapses, normalized to WT controls. *nab-1(ok943)* (hypomorph) and *nab-1(wy688)* (functional null) had a 45 ± 4% and 68 ± 3% reduction in SNB-1 fluorescence, respectively, which was enhanced in *nab-1 (ok943);elks-1* mutants; these showed a 90 ± 2% loss of synaptic vesicles. This defect in *nab-1(ok943);elks-1* was completely rescued by cell-specific expression of *nab-1* in HSN. $n > 20$ each; error bars, s.e.m. $^{***}P < 0.001$, two-tailed Student's *t*-test. (**j**) Proportion of eggs classified in three developmental stages, scored double blind in the egg-laying behavior assay. Both *nab-1* mutants were significantly defective in egg-laying behavior compared to WT controls. $n > 50$ per group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Fisher's exact test.

with latrunculin A treatment. So even though there is low conservation of NAB-1's ABD, it interacts with F-actin.

To determine whether NAB-1's actin binding ability is related to its function in mediating synapse assembly, we performed structure-function studies by making NAB-1 truncation constructs that deleted either a single or combinations of various functional domains (**Fig. 3a**). These transgenes were expressed in HSN to assess their ability to localize to synapses as well as capacity to rescue synapse assembly defects in *nab-1;elks-1* mutants (**Fig. 3a**,**b**). NAB-1 constructs lacking individually either the PDZ, coiled-coil or SAM domains localized to punctate structures along the synaptic region like the full-length protein, but NAB-1's localization became diffused throughout the HSN axon upon loss of the ABD (**Fig. 3c**–**g**). Thus, the ABD is necessary for proper localization of NAB-1, although it is insufficient to confer synaptic localization (**Fig. 3h**). Nevertheless, the N-terminal region comprising the ABD and PDZ showed partial synaptic localization (**Fig. 3i**), and this localization was not conferred by the PDZ alone, as the isolated PDZ did not localize (**Fig. 3j**). Furthermore, the C-terminal half including the coiled-coil and SAM domains failed to localize and was diffused along the HSN neuron (**Fig. 3k**). Together, the data suggest that the ABD is required but not sufficient for localizing NAB-1 to presynaptic specializations.

When we assessed the ability of these truncated NAB-1 transgenes to rescue synapse assembly, all truncated forms of NAB-1 that failed to localize to synapses showed poor ability to rescue synaptic vesicle recruitment defects in *nab-1;elks-1* mutants, consistent with the notion that NAB-1 functions at presynaptic terminals (**Fig. 3b**). Similarly, NAB-1 missing its PDZ or SAM domain showed a poor ability to rescue (**Fig. 3b**). As these constructs are able to localize, this suggests that both the PDZ and SAM domain are required functionally for synapse assembly. Most worms expressing NAB-1 lacking the coiled-coil region showed complete rescue, implying that the coiled-coils are relatively dispensable for synapse formation (**Fig. 3b**).

To show that F-actin binding is required for synaptic localization of NAB-1, we made a chimeric NAB-1 protein by replacing its ABD with the ABD of moesin. Moesin links the cortical actin cytoskeleton to the membrane in filopodia and microvilli; its C-terminal ABD is commonly used as an *in vivo* F-actin probe[42]. Expressing GFP-tagged moesin ABD in HSN results in partial enrichment at synapses (**Fig. 3l**). The chimeric protein (moeABD::NAB-1(ΔABD)) showed a synaptic localization pattern that was indistinguishable from that of full-length NAB-1 (**Fig. 3m**). This is in contrast to NAB-1(ΔABD), which showed no synaptic enrichment (**Fig. 3g**). Furthermore, moeABD::NAB-1(ΔABD) rescued synaptic defects in *nab-1;elks-1* mutants (**Fig. 3n**,**o**). As there is no sequence homology between the NAB-1 ABD and moesin ABD, this experiment strongly argues that NAB-1 localizes and functions at synapses through binding to F-actin.

Together, the data suggest that NAB-1 localizes to synapses through actin binding and requires a combination of domains to localize correctly. Furthermore, the PDZ, SAM and ABD are functionally required for synapse assembly.

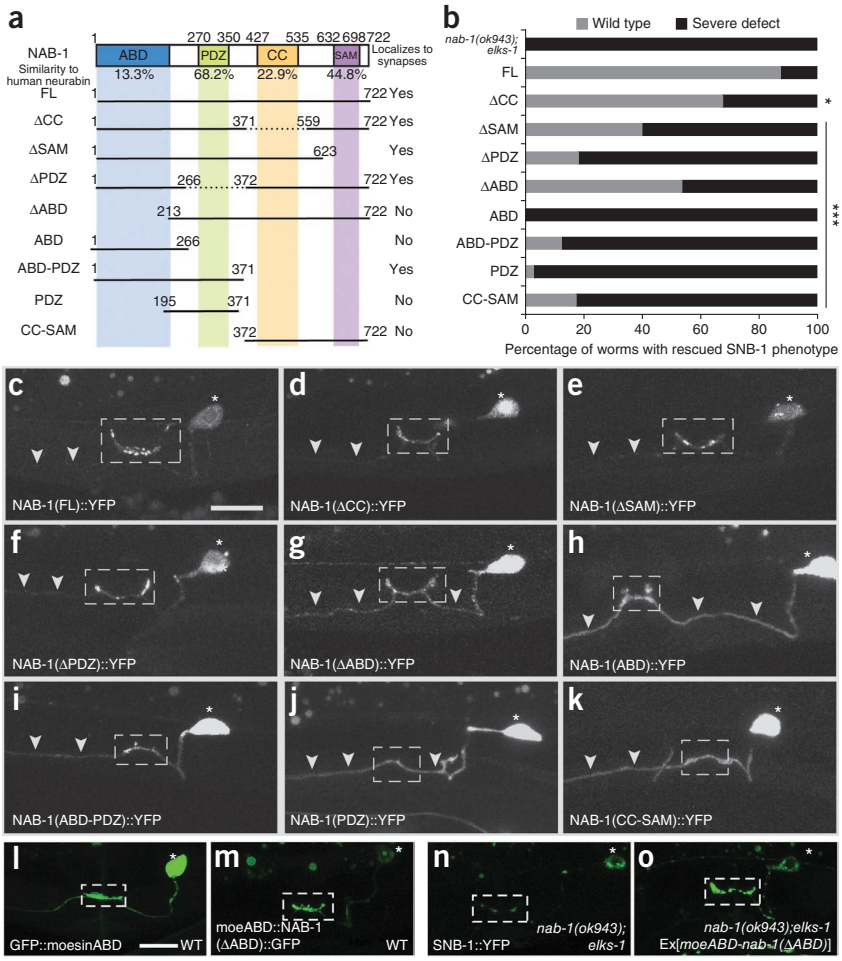

Figure 3 NAB-1 is an actin-binding protein that localizes to presynapses by binding to F-actin. (**a**) Diagram of NAB-1 protein highlighting its protein domains, including an N-terminal actin-binding domain (ABD), a PDZ domain, a coiled-coil (CC) region and a C-terminal SAM domain. *C. elegans* amino acid sequence similarity of each domain to human neurabin is shown as a percentage. Below is a list of NAB-1 truncation transgenes made for structure-function studies, with a summary of each construct's ability to localize to presynaptic sites in HSN. (**b**) Proportion of worms showing wild type or severe defects in synaptic vesicle recruitment with the expression of each NAB-1 truncation transgene. $n > 50$ per group, $^{*}P < 0.05$, $^{***}P < 0.001$, Fisher's exact test as compared to full-length transgene rescue. (**c**–**k**) Localization patterns of each YFP-tagged NAB-1 transgene. All constructs lacking the ABD failed to localize to synapses. Arrowheads point to axon regions that should normally be devoid of NAB-1 labeling. (**l**) The GFP::moesinABD F-actin probe labels the entire HSN neuron. (**m**–**o**) Making a chimeric protein by swapping in NAB-1's ABD for the moesin ABD (moeABD::NAB-1(ΔABD)) was sufficient to localize the protein (**m**) and rescue synaptic vesicle recruitment, marked by SNB-1::YFP (**n**,**o**), in *nab-1(ok943);elks-1* mutants. WT, wild type. All scale bars, 10 μm; bars shared by **c**–**k** and by **l**–**o**.

NAB-1 functions early in development downstream of SYG-1

If NAB-1's synaptic localization requires actin binding, we expect NAB-1 to be dependent on SYG-1 because SYG-1 is required to recruit a stable F-actin network at synapses. Previous work has shown that SYG-1 localizes to developing synapses during the L4 stage but becomes diffused along the axon in adults owing to loss of SYG-2 expression in primary vulval epithelial cells[9]. We expressed NAB-1::YFP and mCherry::SYG-1 in HSN and observed that both proteins localized to synapses during the L4 stage and both became more diffusely localized in adult worms while synapses were still present (**Fig. 4a**–**d**), suggesting that NAB-1 requires SYG-1 for proper localization. This was verified when we observed a loss of NAB-1 recruitment to synapses in *syg-1* mutants and upon disruption of F-actin by latrunculin (**Fig. 4e**,**f** and **Supplementary Fig. 1g**). Given that F-actin is necessary for NAB-1 localization, F-actin assembly should be independent of

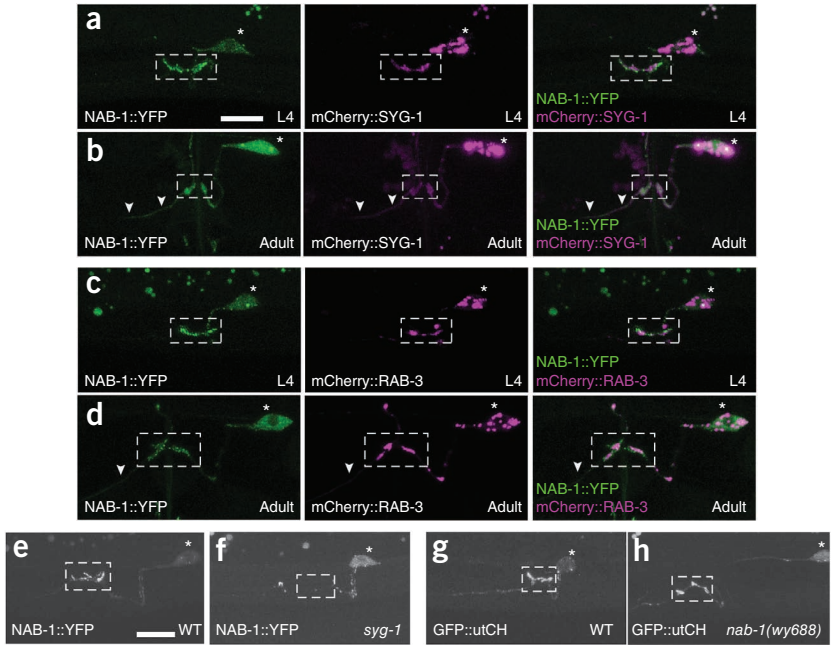

Figure 4 NAB-1 requires SYG-1 for synaptic localization and functions downstream of F-actin. (**a**,**b**) NAB-1::YFP and mCherry::SYG-1 are targeted to synapses in the early L4 larval stage (**a**), but become diffuse along the entire neuron (arrowheads throughout figure) in later adult stages (**b**). (**c**,**d**) This is clearly shown when we examine NAB-1::YFP together with mCherry::RAB-3. NAB-1 is recruited to developing synapses in the L4 stage (**c**), but is diffused in adult stages when RAB-3 labeled synapses have matured (**d**). (**e**,**f**) NAB-1::YFP synaptic localization is lost in *syg-1* mutants. (**g**,**h**) In *nab-1* mutants, F-actin localization is unaffected. Scale bars, 10 μm; bars shared by **a**–**d** and by **e**–**h**.

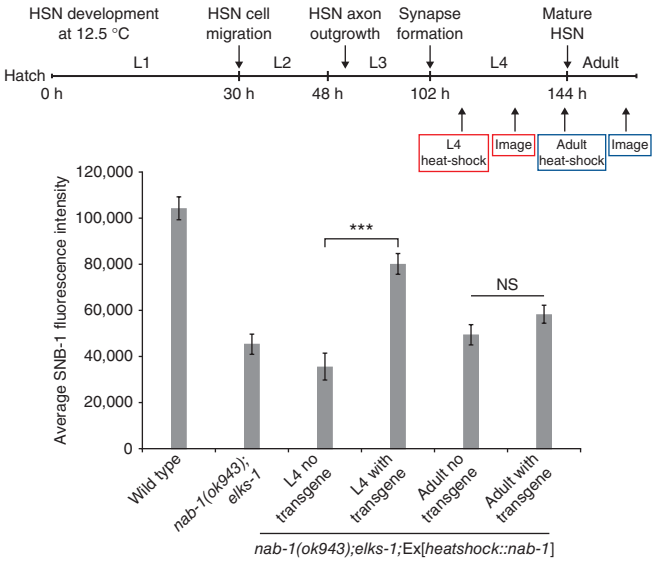

Figure 5 NAB-1 functions in the L4 stage early during synaptogenesis. Timeline shows the development of HSN at 12.5 °C and the time point when heat-shock treatment was given to induce expression of NAB-1 driven by the heat-shock promoter in *nab-1(ok943);elks-1* double mutants. The graph plots the average intensity of SNB-1::YFP fluorescence, in arbitrary units. Heat-shocked L4 worms carrying the transgene showed significant rescue of synaptic vesicle recruitment as compared to *nab-1(ok943);elks-1* worms and to heat-shocked L4 mutant worms with no transgene. No significant differences were observed with heat-shock treatment in adults. Each bar represents an average from 20 worms ± s.e.m. ***$P < 0.001$; NS, $P > 0.05$; two-tailed Student's *t*-test.

NAB-1. Consistently, synaptic localization of F-actin was unperturbed in *nab-1(wy688)* mutants (**Fig. 4g**,**h**). Thus, NAB-1 functions downstream of SYG-1 and the F-actin network.

Both localization and perturbation experiments suggest that SYG-1 and F-actin function during the initial phase of presynaptic formation. NAB-1's transient presynaptic localization also hints that it functions during early stages. To test when NAB-1 is required during synapse development, we induced expression of NAB-1 driven by a heat-shock promoter in *nab-1;elks-1* worms. We heat-shocked either during the early L4 stage, when synapses are developing, or in the young adult stage, when synapses are mature, and assayed for rescue of synaptic vesicle recruitment in HSN (**Fig. 5**). Worms carrying the transgene that receive heat-shock treatment during the L4 stage showed significantly more SNB-1::YFP fluorescence at synapses than control heat-shocked *nab-1;elks-1* worms without the transgene (**Fig. 5**). This rescue was not observed when adult worms were heat-shocked. Therefore, NAB-1 is required early during synaptogenesis, which is consistent with the temporal localization pattern of NAB-1.

NAB-1 recruits active zone molecules to instruct assembly

Previous work has established a hierarchical order in which various active zone molecules are recruited and assembled during development of HSN synapses[14,20]. Two core active zone scaffold molecules, SYD-1 and SYD-2, are required to recruit multiple presynaptic components, including ELKS-1, GIT, UNC-10 (RIM), calcium channels and synaptic vesicle markers to synapses. When we asked whether NAB-1 was affected by loss of SYD-2 and SYD-1, notably, we found that NAB-1's synaptic localization was unaffected in *syd-1;syd-2* double mutants (**Fig. 6a**). This is noteworthy in contrast with the severe loss of multiple active zone and synaptic vesicle proteins in *syd-2* or *syd-1* mutants[14], which demonstrates that NAB-1's recruitment to synapses is independent of SYD-1 and SYD-2 and that NAB-1 probably functions upstream of these scaffold proteins. In addition, *nab-1* mutants show defects in proper localization of active zone

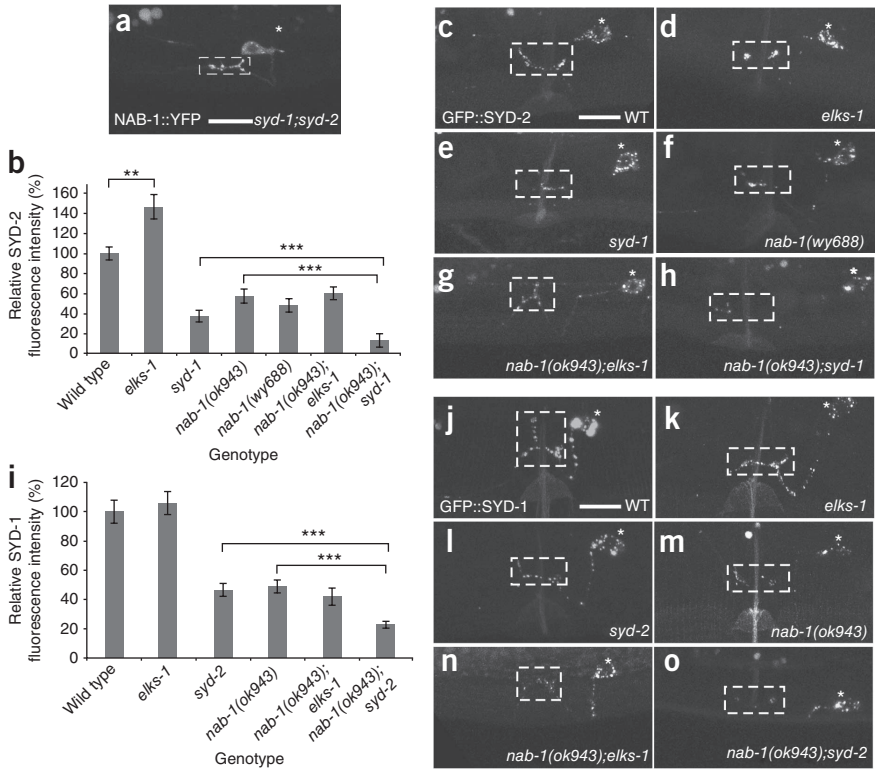

Figure 6 NAB-1 functions upstream and is required to recruit both active zone scaffolding molecules SYD-1 and SYD-2. (**a**) NAB-1::YFP localizes to synapses in *syd-1;syd-2* double mutants. (**b**) Quantification of SYD-2::GFP fluorescence, normalized to wild type (WT). (**c**,**d**) Images of GFP::SYD-2 localization in HSN. Scaffolding molecule SYD-2 localizes to punctate structures along the synaptic region in WT (**c**) and *elks-1* mutants (**d**). (**e**) *syd-1* mutants showed 62 ± 6% lower SYD-2 recruitment than WT. (**f**,**g**) Similarly, *nab-1* mutants showed a partial loss of SYD-2 (43 ± 7% and 52 ± 6% less in *ok943* and *wy688*, respectively; **f**), and *nab-1;elks-1* showed a 40 ± 7% reduction (**g**). (**h**) *nab-1;syd-1* double mutants showed almost complete lost of SYD-2 (87 ± 7% reduction in fluorescence). (**i**) Quantification of SYD-1::GFP fluorescence, normalized to WT. *syd-2*, *nab-1(ok943)* and *nab-1(ok943);elks-1* mutants all showed a ~50% reduction in SYD-1 recruitment to synapses. This lost was enhanced in *nab-1;syd-2* mutants, with a 77 ± 3% loss of SYD-1 at synapses. (**j**–**o**) GFP::SYD-1 localization in HSN. In **b**,**i**, $n = 20$ worms each; error bars, ± s.e.m. **$P < 0.01$, ***$P < 0.001$, two-tailed Student's *t*-test. All scale bars, 10 μm; bars shared by **c**–**h** and by **j**–**o**.

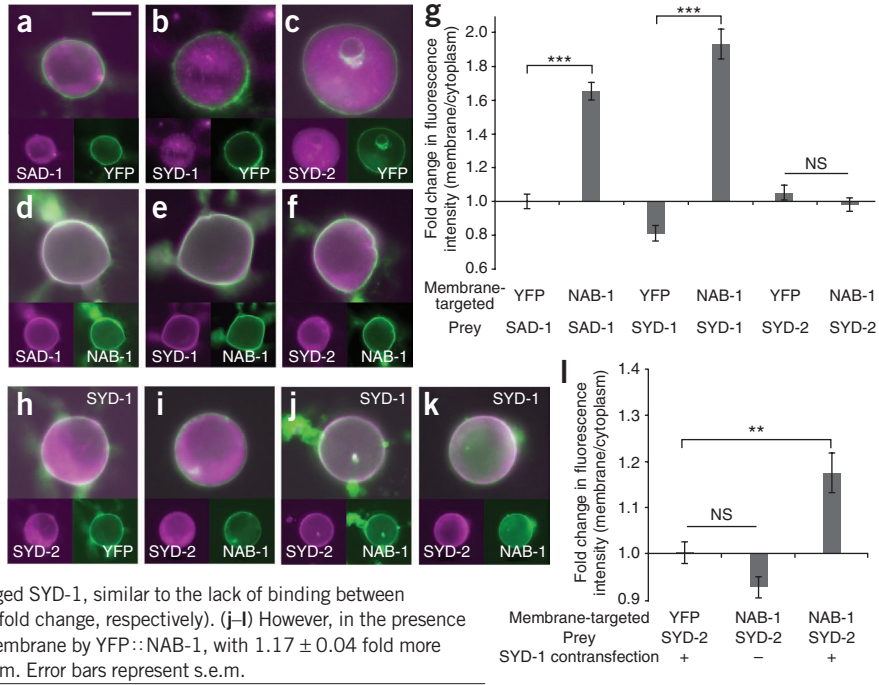

Figure 7 NAB-1 recruits downstream active zone molecules by interacting with SYD-1 and SYD-2. Potential NAB-1 protein interactions were tested in a heterologous system using HEK-293T cells. (**a**–**c**) CFP-tagged proteins remain diffused in the cytoplasm of cells expressing control YFP fused to a membrane-targeting sequence. (**d**) When YFP::NAB-1 fused to a membrane-targeting sequence was expressed together with CFP::SAD-1, SAD-1 was recruited to the membrane (1.65 ± 0.05 fold more fluorescence at the membrane than in the cytoplasm) and co-localized with NAB-1. SAD-1 was used as a positive control, as the protein binds to NAB-1. (**e**) Protein interaction was observed between CFP::SYD-1 and NAB-1 (1.93 ± 0.09 fold more fluorescence intensity at the membrane). (**f**) No significant difference in binding was observed between SYD-2 and NAB-1 (0.98 ± 0.04 fold change). (**g**) Quantification of fold change in CFP fluorescence pixel intensity at the membrane compared to cytoplasm. Average ratio from 20 cells each, ± s.e.m. ***$P < 0.001$; NS, $P > 0.05$, two-tailed Student's *t*-test. (**h**,**i**) No binding was observed between SYD-2 and YFP when cotransfected with untagged SYD-1, similar to the lack of binding between SYD-2 and NAB-1 alone (1.00 ± 0.02 and 0.93 ± 0.02 fold change, respectively). (**j**–**l**) However, in the presence of untagged SYD-1, CFP::SYD-2 was recruited to the membrane by YFP::NAB-1, with 1.17 ± 0.04 fold more fluorescence intensity at the membrane. Scale bar, 10 μm. Error bars represent s.e.m.

proteins such as ELKS-1, GIT and SAD-1 similar to that in *syd-1* and *syd-2* mutants (**Supplementary Fig. 2c**,**d**)[14].

To understand the mechanism by which NAB-1 interacts with SYD-1 or SYD-2 to execute the presynaptic assembly program, we examined whether recruitment of SYD-1 and SYD-2 is affected in *nab-1* mutants (**Fig. 6**). We found that the fluorescence intensity of GFP::SYD-2 at synapses in both *nab-1* mutants was decreased, suggesting that SYD-2 recruitment is partially dependent on NAB-1 (**Fig. 6b**,**f**). This reduction in SYD-2 localization is similar to that in *syd-1* mutants (**Fig. 6b**,**e**) and is not enhanced by loss of ELKS-1 in *nab-1(ok943);elks-1* mutants (**Fig. 6b**,**g**). However, we observed almost complete loss of GFP::SYD-2 fluorescence in *nab-1(ok943);syd-1* double mutants (**Fig. 6b**,**h**), suggesting a model whereby SYD-1 and NAB-1 both recruit SYD-2 in parallel pathways to HSN synapses.

Likewise, we observed similar data for SYD-1 recruitment when we quantified the fluorescence intensity of GFP::SYD-1 in the various mutants. *syd-2*, *nab-1(ok943)* and *nab-1(ok943);elks-1* mutants showed a partial reduction in SYD-1 recruitment to synapses (**Fig. 6i**–**o**). In *nab-1(ok943);syd-2* double mutants, we observed almost complete loss of SYD-1 protein (**Fig. 6n**). Thus, both SYD-2 and NAB-1 are required to recruit SYD-1 to synapses in HSN.

To address how NAB-1 recruits SYD-1 and SYD-2 to synapses, we used an *in vitro*, single-cell protein-protein interaction assay to assess potential interactions[21]. We expressed NAB-1::YFP fused to a membrane-targeting sequence together with a potential interacting protein tagged with CFP in HEK-293T cells (**Fig. 7**). If the protein binds to NAB-1, it should localize to the plasma membrane. An active zone serine/threonine kinase, SAD-1, has previously been found to bind NAB-1 (ref. 34) and showed synapse assembly defects in HSN (**Supplementary Fig. 4**). To validate the *in vitro* binding assay, we used SAD-1::CFP as a positive control and observed recruitment of SAD-1 to the membrane when transfected along with membrane-targeted NAB-1::YFP (**Fig. 7a**,**d**,**g**). When we expressed SYD-1 together with membrane-tethered NAB-1, we observed marked recruitment of SYD-1 to the plasma membrane (**Fig. 7b**,**e**,**g**). Structure-function analyses showed that SYD-1 interacts with the N-terminal regions of NAB-1 (**Supplementary Fig. 5a**–**g**). SYD-2 by itself remained in the cytoplasm of cells expressing NAB-1 (**Fig. 7c**,**e**,**g**). However, we observed significant recruitment of SYD-2 to membrane-tethered NAB-1 when SYD-1 was co-expressed (**Fig. 7h**–**l**). These results suggest that whereas SYD-2 and NAB-1 do not show strong interactions alone, they can form a complex in the presence of SYD-1 (**Supplementary Fig. 5h**–**j**).

Taken together, our genetic experiments suggest that NAB-1 recruits both SYD-1 and SYD-2 to presynaptic sites. The interaction data identify SYD-1 as a new binding partner for NAB-1, and together these two proteins may form a complex to recruit SYD-2 to nascent presynaptic sites to initiate downstream presynaptic assembly.

DISCUSSION

Using *C. elegans* HSN synapses, we investigated the molecular mechanisms of presynaptic assembly. Our results are consistent with a model in which transmembrane adhesion molecule SYG-1 (NEPH1) defines a region of the axon where nascent synapses are induced to form during development. SYG-1 initiates synaptogenesis by recruiting a local F-actin network. NAB-1 (neurabin) links SYG-1 to the presynaptic assembly program by binding to the local F-actin network and functions as an adaptor for active zone molecules such as SYD-1 and SYD-2 (liprin-α) (**Supplementary Fig. 6**). Recruitment of downstream active zone molecules by NAB-1 potentially functions through the newly identified interaction between NAB-1, SYD-1 and SYD-2. Our results highlight the importance of local cytoskeletal rearrangements that can be mediated by transmembrane receptors during initiation of synaptogenesis.

Membrane receptors organize synapses through local F-actin

Recent studies on synapse formation present the notion that many molecules involved in axon guidance are also important in synapse formation[43]. This is not surprising, because postsynaptic development requires dendritic spine motility in many experimental systems,

which depends on dynamic actin-mediated motility similar to that occurring in axonal grow cone turning. Therefore, it is conceivable that molecules that affect actin dynamics might underlie both axonal growth cone guidance and dendritic filopodial motility. However, it is harder to reconcile how axon guidance molecules can pattern and form presynaptic specializations, because recruitment of synaptic vesicles and active zone components are fundamentally distinct cellular processes from growth cone turning through selective stabilization of subcellular F-actin.

The requirement of F-actin during early presynaptic development provides a conceptual framework for how guidance molecules can pattern presynaptic terminals. Actin has been found at the presynaptic terminals surrounding synaptic boutons[23]. In dissociated neuronal cultures, latrunculin A treatment blocks synaptogenesis in young cultures but has little effect after the initial phase of synapse formation, suggesting that there is an early phase of synaptogenesis that requires F-actin[36]. Indeed, heparan sulfate proteoglycans, including syndecan-2, adsorbed onto beads or expressed on the axon surface, can assemble synapses through a mechanism dependent on the dynamic reorganization of F-actin[25]. This notion is strengthened by a recent report that an actin modification pathway involving a Rac GEF, Trio, is required presynaptically for growth of neuromuscular junctions[26].

In this study, we provide several lines of evidence that the transmembrane adhesion receptor SYG-1, which is necessary and sufficient to trigger synapse formation in HSN, patterns an actin network at presynaptic terminals. First, an F-actin network labeled by the utCH domain showed notable enrichment with SYG-1 in HSN. Second, this presynaptic F-actin was lost in the *syg-1* mutants. Third, artificial targeting of SYG-1 to specific axonal domains was sufficient to recruit F-actin to ectopic sites, suggesting that SYG-1 is necessary and sufficient to build an F-actin network near nascent presynaptic clusters. Fourth, *in vivo* latrunculin treatment caused failure of synaptic vesicle clustering. Together, these results argue that SYG-1 might organize presynaptic terminals in HSN through building a F-actin network. Studies on *Drosophila* muscle fusion have convincingly showed that the SYG-1 homologs, Duf and Rst, both induce F-actin formation near the fusion pore[44]. Hence, the ability of SYG-1 family of genes to organize local F-actin networks is conserved, although downstream functions of these genes have diversified in different contexts of development.

Is this relationship between F-actin and presynaptic assembly a general phenomenon? Given that latrunculin A treatment results in synaptic assembly defects both in *C. elegans* HSN neurons and hippocampal neurons, it seems plausible that local F-actin is a critical component of presynaptic development in many neurons. Of note, another study has shown that UNC-40 (DCC) receptors can also induce local F-actin formation through Rac proteins at presynaptic specializations in *C. elegans* AIY interneurons (personal communication, A. Stavoe and D. Colon-Ramos, Yale University), further suggesting that multiple synapse organizing membrane receptors can induce F-actin assembly and might be important during presynapse formation.

NAB-1 links presynaptic F-actin to assembly proteins

If local F-actin assembly is a crucial event for presynapse development, how does F-actin lead to the construction of active zones and accumulation of synaptic vesicles? It is conceivable that adaptor proteins can couple the actin network to active zone components and therefore anchor the active zone at nascent synapses. Such an adaptor protein should have the following properties. First, it should bind to both F-actin and active zone proteins. Second, it should localize to nascent presynaptic terminals. Third, because F-actin is only required early during presynaptic development, such adaptors might also be required only during the development of synaptic structure but not the maintenance of synapses. Fourth, such adaptors should function upstream of active zone proteins to recruit them to developing synaptic terminals.

Our data suggest that NAB-1 fits the criteria to function as such an adaptor. First, NAB-1 bound to F-actin. When expressed in HEK-293T cells or NIH-3T3 fibroblasts, NAB-1 localized to F-actin structures such as cortical actin and stress fibers in a latrunculin A–dependent manner. Through structure-function analysis, we found that, like that of the mammalian homolog, the N-terminal domain of NAB-1 was responsible for actin binding. Interestingly, the 'actin-binding domain' of NAB-1 was also required for its presynaptic localization. Furthermore, an unrelated protein's actin-binding domain could substitute for the N-terminal portion of NAB-1 to localize NAB-1 to synapses (**Fig. 3l**,**m**), strongly suggesting that NAB-1 is recruited to presynaptic terminals by the local F-actin network. We also showed that NAB-1 interacts with core active zone assembly molecules, SYD-1 and SYD-2, suggesting that NAB-1 can couple actin and active zone.

Second, NAB-1 localized to developing presynaptic specializations. Notably, unlike other known active zone proteins, NAB-1's synaptic localization in HSN is transient. HSN synapses form in the L4 stage and become functional in adults when egg-laying behavior begins. In the adult stage, NAB-1's synaptic localization was lost, while active zone proteins such as SYD-2, SYD-1, ELKS-1 and GIT-1 continued to localize to synapses. This transient synaptic localization resembles that of SYG-1, and, together with the colocalization of NAB-1 and SYG-1 at developing synapses, these data argue that NAB-1 is transiently recruited to developing synapses, possibly through SYG-1 or SYG-1–assembled F-actin.

Third, consistent with its transient localization, NAB-1 is required during early stages of synapse development. This is again in distinct contrast to other structural components of the active zone that are required throughout. Therefore, among the presynaptic proteins in our knowledge, NAB-1 has a unique function during early stages of active zone formation.

Finally, NAB-1 seems to function upstream of the core active zone proteins SYD-2 and SYD-1. Whereas almost all synaptic vesicle and active zone markers disappear from HSN synapses in *syd-2* mutants[14], NAB-1's localization is unaffected in *syd-1;syd-2* double mutants. In *syd-1;nab-1* double mutants, SYD-2 fails almost completely to localize. Similarly, SYD-1 is largely absent from developing synapses in *syd-2;nab-1* double mutants. But although all of our data agree with this model, NAB-1 might not be the only adaptor protein, because loss of SYD-2 showed a quantitatively stronger phenotype than the *nab-1* single mutant. In addition, *nab-1* mutants showed qualitatively weaker phenotypes in other synapses in worms, indicating that there might be other redundant proteins (data not shown).

How does NAB-1 recruit these active zone proteins? Our *in vitro* data suggest that NAB-1 interacts with SYD-1. Although it showed no significant interaction with SYD-2 alone, in the presence of SYD-1, both SYD-1 and SYD-2 seemed to bind NAB-1. From previous data, we could not detect any interaction between SYD-1 and SYD-2 (data not shown). Therefore, NAB-1 may catalyze the recruitment of SYD-1 and SYD-2, two molecules essential for presynaptic assembly in HSN.

A recent study showed that NAB-1 and the presynaptic kinase SAD-1 function together in regulating the polarized distribution of synaptic components in the D-type inhibitory motor neurons[34]. This study observed dendritic mislocalization of synaptic vesicles in *nab-1* and *sad-1* single mutants that was not enhanced in *nab-1;sad-1* double

mutants, hinting that these proteins function in the same genetic pathway. In HSN, both *nab-1* and *sad-1* mutants had fewer synaptic vesicles than wild type. However, in the double mutants, *nab-1* and *sad-1* strongly enhanced each other and resulted in almost complete lost of synaptic vesicles, suggesting that NAB-1's assembly function is distinct from its SAD-1–related function. Previous work has shown that SAD-1 functions downstream of SYD-1 and SYD-2 (ref. 14), consistent with our model that NAB-1 is required to recruit SYD-1 and SYD-2. We found that SAD-1's localization was also dependent on NAB-1 and NAB-1's localization was unaffected in *sad-1* mutants. These data suggest that SAD-1 functions downstream of NAB-1 in our model.

Collectively, these results present strong evidence that NAB-1 serves as an adaptor protein that localizes to the nascent presynaptic terminals to link the local F-actin network to developing active zones. The model also provides a potential framework for understanding how a diverse set of extracellular cues may recruit and assemble the stereotyped intracellular presynaptic machinery crucial for synapse function.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank the Caenorhabditis Genetics Center and Japanese National Bioresource Project for strains. We also thank C. Gao and Y. Fu for technical assistance and T. Clandinin, K. Zito, K. Mizumoto, C.Y. Ou and Shen laboratory members for manuscript comments. This work was funded by the Howard Hughes Medical Institute and US National Institutes of Health grant R01 NS048392. P.H.C. is supported by the Agency for Science, Technology, and Research (Singapore).

AUTHOR CONTRIBUTIONS

P.H.C. and K.S. designed experiments and wrote the paper. M.R.P. made initial observations and performed initial experiments. P.H.C. performed experiments and analyzed the data.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Williams, M.E., de Wit, J. & Ghosh, A. Molecular mechanisms of synaptic specificity in developing neural circuits. *Neuron* **68**, 9–18 (2010).
2. Tallafuss, A., Constable, J.R. & Washbourne, P. Organization of central synapses by adhesion molecules. *Eur. J. Neurosci.* **32**, 198–206 (2010).
3. Yamagata, M., Sanes, J. & Weiner, J.A. Synaptic adhesion molecules. *Curr. Opin. Cell Biol.* **15**, 621–632 (2003).
4. Scheiffele, P., Fan, J., Choih, J., Fetter, R. & Serafini, T. Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* **101**, 657–669 (2000).
5. Dean, C. *et al.* Neurexin mediates the assembly of presynaptic terminals. *Nat. Neurosci.* **6**, 708–716 (2003).
6. Li, J., Ashley, J., Budnik, V. & Bhat, M.A. Crucial role of *Drosophila* neurexin in proper active zone apposition to postsynaptic densities, synaptic growth, and synaptic transmission. *Neuron* **55**, 741–755 (2007).
7. Uemura, T. *et al.* Trans-synaptic interaction of GluRδ2 and Neurexin through Cbln1 mediates synapse formation in the cerebellum. *Cell* **141**, 1068–1079 (2010).
8. Poulopoulos, A. *et al.* Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. *Neuron* **63**, 628–642 (2009).
9. Shen, K., Fetter, R.D. & Bargmann, C.I. Synaptic specificity is generated by the synaptic guidepost protein SYG-2 and its receptor, SYG-1. *Cell* **116**, 869–881 (2004).
10. Shen, K. & Bargmann, C.I. The immunoglobulin superfamily protein SYG-1 determines the location of specific synapses in *C. elegans*. *Cell* **112**, 619–630 (2003).
11. Butz, S., Okamoto, M. & Südhof, T.C. A tripartite protein complex with the potential to couple synaptic vesicle exocytosis to cell adhesion in brain. *Cell* **94**, 773–782 (1998).
12. tom Dieck, S. *et al.* Bassoon, a novel zinc-finger CAG/glutamine-repeat protein selectively localized at the active zone of presynaptic nerve terminals. *J. Cell Biol.* **142**, 499–509 (1998).
13. Mukherjee, K. *et al.* Piccolo and bassoon maintain synaptic vesicle clustering without directly participating in vesicle exocytosis. *Proc. Natl. Acad. Sci. USA* **107**, 6504–6509 (2010).
14. Patel, M.R. *et al.* Hierarchical assembly of presynaptic components in defined *C. elegans* synapses. *Nat. Neurosci.* **9**, 1488–1498 (2006).
15. Zhen, M. & Jin, Y. The liprin protein SYD-2 regulates the differentiation of presynaptic termini in *C. elegans*. *Nature* **401**, 371–375 (1999).
16. Kaufmann, N., DeProto, J., Ranjan, R., Wan, H. & Van Vactor, D. *Drosophila* liprin-alpha and the receptor phosphatase Dlar control synapse morphogenesis. *Neuron* **34**, 27–38 (2002).
17. Owald, D. *et al.* A Syd-1 homologue regulates pre- and postsynaptic maturation in *Drosophila*. *J. Cell Biol.* **188**, 565–579 (2010).
18. Wagh, D.A. *et al.* Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. *Neuron* **49**, 833–844 (2006).
19. Kittel, R.J. *et al.* Bruchpilot promotes active zone assembly, Ca^{2+} channel clustering, and vesicle release. *Science* **312**, 1051–1054 (2006).
20. Dai, Y. *et al.* SYD-2 Liprin-alpha organizes presynaptic active zone formation through ELKS. *Nat. Neurosci.* **9**, 1479–1487 (2006).
21. Patel, M.R. & Shen, K. RSY-1 is a local inhibitor of presynaptic assembly in *C. elegans*. *Science* **323**, 1500–1503 (2009).
22. Spangler, S.A. & Hoogenraad, C.C. Liprin-alpha proteins: scaffold molecules for synapse maturation. *Biochem. Soc. Trans.* **35**, 1278–1282 (2007).
23. Sankaranarayanan, S., Atluri, P.P. & Ryan, T.A. Actin has a molecular scaffolding, not propulsive, role in presynaptic function. *Nat. Neurosci.* **6**, 127–135 (2003).
24. Morales, M., Colicos, M.A. & Goda, Y. Actin-dependent regulation of neurotransmitter release at central synapses. *Neuron* **27**, 539–550 (2000).
25. Lucido, A.L. *et al.* Rapid assembly of functional presynaptic boutons triggered by adhesive contacts. *J. Neurosci.* **29**, 12449–12466 (2009).
26. Ball, R.W. *et al.* Retrograde BMP signaling controls synaptic growth at the NMJ by regulating trio expression in motor neurons. *Neuron* **66**, 536–549 (2010).
27. Allen, P.B., Ouimet, C.C. & Greengard, P. Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. *Proc. Natl. Acad. Sci. USA* **94**, 9956–9961 (1997).
28. Nakanishi, H. *et al.* Neurabin: a novel neural tissue-specific actin filament-binding protein involved in neurite formation. *J. Cell Biol.* **139**, 951–961 (1997).
29. Satoh, A. *et al.* Neurabin-II/spinophilin. An actin filament-binding protein with one pdz domain localized at cadherin-based cell-cell adhesion sites. *J. Biol. Chem.* **273**, 3470–3475 (1998).
30. Zito, K., Knott, G., Shepherd, G.M., Shenolikar, S. & Svoboda, K. Induction of spine growth and synapse formation by regulation of the spine actin cytoskeleton. *Neuron* **44**, 321–334 (2004).
31. Terry-Lorenzo, R.T. *et al.* Neurabin/protein phosphatase-1 complex regulates dendritic spine morphogenesis and maturation. *Mol. Biol. Cell* **16**, 2349–2362 (2005).
32. Ryan, X.P. *et al.* The Rho-specific GEF Lfc interacts with neurabin and spinophilin to regulate dendritic spine morphology. *Neuron* **47**, 85–100 (2005).
33. Sieburth, D. *et al.* Systematic analysis of genes required for synapse structure and function. *Nature* **436**, 510–517 (2005).
34. Hung, W., Hwang, C., Po, M.D. & Zhen, M. Neuronal polarity is regulated by a direct interaction between a scaffolding protein, Neurabin, and a presynaptic SAD-1 kinase in *Caenorhabditis elegans*. *Development* **134**, 237–249 (2007).
35. Dai, Z. & Peng, H.B. Dynamics of synaptic vesicles in cultured spinal cord neurons in relationship to synaptogenesis. *Mol. Cell. Neurosci.* **7**, 443–452 (1996).
36. Zhang, W. & Benson, D.L. Stages of synapse development defined by dependence on F-actin. *J. Neurosci.* **21**, 5169–5181 (2001).
37. Zhang, W. & Benson, D.L. Developmentally regulated changes in cellular compartmentation and synaptic distribution of actin in hippocampal neurons. *J. Neurosci. Res.* **69**, 427–436 (2002).
38. Cingolani, L.A. & Goda, Y. Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. *Nat. Rev. Neurosci.* **9**, 344–356 (2008).
39. Burkel, B.M., Von Dassow, G. & Bement, W. Versatile fluorescent probes for actin filaments based on the actin-binding domain of utrophin. *Cell Motil. Cytoskeleton* **64**, 822–832 (2007).
40. Keep, N.H. Structural comparison of actin binding in utrophin and dystrophin. *Neurol. Sci.* **21**, S929–S937 (2000).
41. Norwood, F.L., Sutherland-Smith, A.J., Keep, N.H. & Kendrick-Jones, J. The structure of the N-terminal actin-binding domain of human dystrophin and how mutations in this domain may cause Duchenne or Becker muscular dystrophy. *Structure* **8**, 481–491 (2000).
42. Edwards, K.A., Demsky, M., Montague, R.A., Weymouth, N. & Kiehart, D.P. GFP-moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in *Drosophila*. *Dev. Biol.* **191**, 103–117 (1997).
43. Shen, K. & Cowan, C. Guidance molecules in synapse formation and plasticity. *Cold Spring Harb. Perspect. Biol.* **2**, a001842 (2010).
44. Chen, E.H. & Olson, E.N. Towards a molecular pathway for myoblast fusion in *Drosophila*. *Trends Cell Biol.* **14**, 452–460 (2004).

ONLINE METHODS

Strains. All worms strains were maintained at 20 °C on OP50 *Escherichia coli*–seeded nematode growth medium plates. N2 Bristol stain worms were used as the wild-type reference and the following mutants were used: *nab-1(ok943)I*, *nab-1(wy688 ::unc-119+)I* (targeted deletion, method described below), *syd-1(ju82)II*, *elks-1(tm1233)IV*, *syd-2(ju37)X*, *syg-1(ky652)X*, *syg-2(ky673)X*, *sad-1(ky330)X* and *unc-119(ed3)III*.

The *nab-1(ok943)* mutation is a 1,032-base-pair deletion that removes exon 7 to part of exon 8 of *nab-1*'s genomic sequence, resulting in a premature stop after the deleted region. It results in a truncated *nab-1* sequence that still contains the putative actin binding domain and PDZ. This allele is a hypomorph, as overexpression of the truncated protein can partially rescue synapse formation defects.

Mos transposon mediated targeted deletion of *NAB-1*. The targeted knockout *nab-1(wy688 ::unc-119+)* was made using the MosDEL method[45] with the Mos insertion line *ttTi6300* (requested from L. Ségalat, University Claude Bernard Lyon)[46]. Fifty *ttTi6300;unc-119(ed3)* worms were co-injected with the following plasmids: pJL43.1 (*Pglh-2 ::MOStransposase*) at 50 ng µl^{-1}, pPH32 (targeting plasmid with positive selection marker *cb-unc-119(+)*, made from *pRS413*; see Cloning for methods) at 50 ng µl^{-1}, pCFJ90 (*Pmyo-2 ::mCherry*) at 2.5 ng µl^{-1} and *Punc-122 ::rfp* at 40 ng µl^{-1}. Plates with F_1 worms carrying both fluorescent markers and rescued for *unc-119* movement defects were identified and kept at 25 °C until the plate was starved. We identified worms with the deletion by screening plates for ones that had lost both co-injected fluorescent markers with no movement defects (owing to *unc-119* rescue). The deletion was verified by PCR genotyping (forward primer, TTCACTTTCAGGTCTTCTCGGCGT; reverse primer reading from inserted *cb-unc-119(+)*, GCGCCCTAACTTTGAGCCAATTCA; reverse primer reading from deleted region of *nab-1*, TCTGCACCAACACCCA TACCTGAA) as well as sequencing. This deletion removes the entire *nab-1* gene except the portion encoding the first 246 amino acids and is a functional null.

Transgenic lines. Utrophin label lines: *wyEx3992* [*Punc-86 ::gfp ::utrophinCH; Podr-1 ::gfp*], *wyEx4095* [*Punc-86 ::gfp ::utrophinCH; Punc-86 ::mCherry ::rab-3 ; Podr-1 ::gfp*], *wyEx4099* [*Punc-86 ::gfp ::utrophinCH; Punc-86 ::syg-1 ::mCherry ; Podr-1 ::gfp*], *wyEx4445* [*Punc-86 ::gfp ::utrophinCH; Punc-86 ::mCherry ::nab-1 ; Podr-1 ::dsred*], *wyEx3840* [*Punc-86 ::gfp ::utrophinCH; Punc-86 ::mCherry ::rab-3 ; Podr- 1 ::gfp*].

Other markers: *kyIs235* [*Punc-86 ::snb-1 ::yfp; Punc-4 ::lin-10 ::dsred; Podr-1 ::dsred*], *wyIs12* [*Punc-86 ::gfp ::syd-2; Podr-1 ::gfp*], *wyEx118* [*Punc-86 ::gfp ::syd-1; Podr-1 ::dsred*], *wyEx314* [*Punc-86 ::syd-1; Podr-1 ::dsred*], *kyEx673* [*Pegl-17 ::syg-2; Podr-1 ::gfp*], *wyEx196* [*Punc-86 ::elks-1 ::yfp; Podr-1 ::dsred*], *wyEx163* [*Punc-86 ::sad-1 ::yfp; Podr-1 ::dsred*], *wyEx146* [*Punc-86 ::git-1 ::yfp; Podr-1 ::dsred*].

Fluorescently tagged NAB-1 localization lines: *wyEx245* [*Punc-86 ::nab-1 ::YFP; Podr-1 ::dsred*], *wyEx3353* [*Punc-86 ::nab-1(1-623aa) ::YFP; Podr-1 ::gfp*], *wyEx3733* [*Punc-86 ::nab-1(1-197,370-722aa) ::YFP; Podr-1 ::gfp*], *wyEx1492* [*Punc-86 ::NAB-1(1-371,559-722aa) ::YFP; Podr-1 ::dsred*], *wyEx3550* [*Punc-86 ::nab-1(1-371aa) ::YFP; Podr-1 ::gfp*], *wyEx1363* [*Punc-86 ::nab-1(372-722aa) ::YFP; Podr-1 ::dsred*], *wyEx3364* [*Punc-86 ::nab-1(195-371aa) ::YFP; Podr-1 ::gfp*], *wyEx3845* [*Punc-86 ::nab-1(1-266aa) ::YFP; Podr-1 ::gfp*], *wyEx4602* [*Punc-86 ::nab-1(213-722aa) ::YFP; Podr-1 ::dsred*], *wyEx3905* [*Punc-86 ::nab-1 ::YFP; Punc-86 ::syg-1 ::mCherry; Podr-1 ::gfp*].

NAB-1 expression lines: *wyEx3164* [*Punc-86 ::nab-1; Podr- 1 ::gfp*], *wyEx3329* [*Punc-86 ::nab-1(1-623aa); Podr-1 ::gfp*], *wyEx3929* [*Punc-86 ::nab-1(1-197,370-722aa) ::YFP; Podr-1 ::gfp*], *wyEx3444* [*Punc-86 ::nab-1(1-371,559-722aa); Podr-1 ::gfp*], *wyEx3333* [*Punc-86 ::nab-1(1-371aa); Podr-1 ::gfp*], *wyEx3336* [*Punc-86 ::nab-1(372-722aa); Podr- 1 ::gfp*], *wyEx3907* [*Punc-86 ::nab-1(1-266aa); Podr-1 ::gfp*], *wyEx4750* [*Punc-86 ::nab-1(213-722aa); Podr-1 ::gfp*], *wyEx3297* [*Pheatshock:nab-1; Podr-1 ::gfp*].

NAB-1 chimeric protein lines: *wyEx4639* [*Punc-86 ::moesinABD ::nab-1(213-722aa) ::YFP; Podr-1 ::dsred*], *wyEx4641* [*Punc-86 ::moesinABD ::nab-1(213-722aa); Podr-1 ::gfp*].

Cloning of constructs. Expression plasmids for transgenic worm lines were made using the pSM vector, a derivative of pPD49.26 (A. Fire, Stanford). The *unc-86* promoter was cloned between SphI and XmaI and genes of interests were cloned between NheI and KpnI. Plasmids were injected into worms at 1 ng µl^{-1} together with injection markers *Podr-1 ::gpf* or *Podr-1 ::dsred* at 20 ng µl as previously described[47]. The MosDEL targeting construct, pPH32, was made using yeast homologous recombination methods from an EcoRI linearized pRS413 vector (American Type Culture Collection)[48]. The targeting construct includes a 2-kb homologous sequence that flanks the genomic region left of the Mos insertion site in *ttTi6300*, followed by the *cb-unc-119(+)* positive selection marker replicated by PCR from pCFJ151 and a 2-kb homologous region right of the *nab-1* gene. Expression plasmids for HEK293T cells were made using cytomegalovirus-promoter mCerulean-C1 and eYFP-C1 vectors from Clontech. cDNAs of genes were cloned between HindIII and KpnI. A *KRAS* membrane targeting sequence was cloned between KpnI and XbaI sites. A Flag sequence was cloned between NheI and HindIII.

Fluorescence quantification and confocal imaging. All fluorescence images of left HSN neuron (HSNL) synapses in L4 or young adults were taken with a ×63 objective on a Zeiss Axioplan 2 Imaging System or a Plan-Apochromat ×63, numerical aperture 1.4 objective on a Zeiss LSM710 confocal microscope using similar imaging parameters for the same marker across different genotypes. Total fluorescence intensity of across the synaptic region was determined using ImageJ software (NIH) by summing pixel intensity, and the average fluorescence intensity was calculated for each group ($n > 20$). A two-tailed Student's *t*-test was used to assess statistical significance. Live cell images for *in vitro* assays were obtained using a ×20 objective on a Zeiss Axioplan 2 inverted fluorescence imaging system.

Latrunculin A injections. Late L3, early L4 or mid-L4 worms were immobilized using 1 mM levamisole (Sigma) and the developmental stage as well as expression levels of markers was verified under a Zeiss Axioplan 2 upright microscope before the worm was isolated. Selected worms received microinjections of either 1 mM or 400 µM latrunculin A (Sigma) in 25% (vol/vol) DMSO (Sigma) or 25% DMSO alone, into the pseudocoelom of the worm at a site slightly posterior to the developing vulva, and then kept at 22–24 °C to recover. Three hours after injection, worms were imaged and the fluorescence intensity of markers was quantified. Fifteen worms were quantified for each treatment group, and statistical significance was determined using a two-tailed Student's *t*-test.

Egg-laying assay. Fifteen L4-stage worms were isolated and allowed to develop 28 h at 20 °C into gravid adults. The worms were transferred to a fresh plate and the stage of each egg laid was scored double blind after 1 h at 20 °C. The eggs were classified into one of three developmental stages: 1–8 cell, 8-cell to comma, or post-comma. Two independent assays were performed with at least 50 eggs quantified in total for each genotype. Fisher's exact test was performed to compare statistical significance between two groups. Day-to-day variation was observed, but each experimental round was performed together with wild-type and mutant controls for comparison.

Heat-shock rescue experiments. Worms were maintained at 12.5 °C for multiple generations before being shifted to a different temperature. Experimental worms in the early L4 larval stage or young adults were shifted to 33 °C for 2 h (ref. 49), followed by a recovery period at 20 °C for 6 h before imaging. At least 20 worms were quantified for each heat-shock treatment time point.

***In vitro* protein–protein interaction assay.** As previously described[50], Hek-293T cells were transfected with plasmid constructs driven by the cytomegalovirus promoter with TransIT-LT1 reagent (Mirus) according to the manufacturer's recommendations, and cells were incubated at 37 °C for 24 h before imaging. To quantify the recruitment of protein to 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 intensities of the two compartments ($n > 20$ cells).

Immunocytochemistry. Transfected Hek-293T and NIH-3T3 cells were fixed with 4% paraformaldehyde and permeabilized with either 0.2% Triton X-100 (Sigma) or 0.05% saponin (Sigma). Samples were blocked with 10% BSA before antibody staining. Antibodies used included rhodamine phalloidin (Invitrogen) diluted to 0.165 µM, mouse monoclonal anti-Flag M2 (Sigma) diluted 1:1,000 and goat anti-mouse Alexa 568 diluted 1:1,000.

Statistical analysis. Data are expressed as average mean ± s.e.m. All *in vitro* experiments were performed in duplicates. Statistical significance was assessed

by two-tailed Student's *t*-test when comparing between two groups and Fisher's exact test for cross-categorized frequency data.

45. Frøkjaer-Jensen, C. *et al.* Targeted gene deletions in *C. elegans* using transposon excision. *Nat. Methods* **7**, 451–453 (2010).
46. Granger, L., Martin, E. & Ségalat, L. Mos as a tool for genome-wide insertional mutagenesis in *Caenorhabditis elegans*: results of a pilot study. *Nucleic Acids Res.* **32**, e117 (2004).
47. Mello, C. & Fire, A. DNA transformation. *Methods Cell Biol.* **48**, 451–482 (1995).
48. Oldenburg, K.R., Vo, K.T., Michaelis, S. & Paddon, C. Recombination-mediated PCR-directed plasmid construction in vivo in yeast. *Nucleic Acids Res.* **25**, 451–452 (1997).
49. Stringham, E.G., Dixon, D., Jones, D. & Candido, E. Temporal and spatial expression patterns of the small heat shock (hsp16) genes in transgenic *Caenorhabditis elegans*. *Mol. Biol. Cell* **3**, 221–233 (1992).
50. Patel, M.R. & Shen, K. RSY-1 is a local inhibitor of presynaptic assembly in *C. elegans*. *Science* **323**, 1500–1503 (2009).