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 connections1. Many pairs of these homotypic or heterotypic synaptic adhesion molecules, including cadherins, ephrin-B–EphB, neurexin–neuroligin, synCAMs, netrin-G–netrinG ligands (NGL2) and leukocyte common antigen–related (LAR)–NGL3, can induce synapse formation through trans-synaptic interactions2,3. But although their synapse-inducing activities are robust and sufficient for presynaptic development, localization to presynaptic sites before synapse formation and is necessary and sufficient for presynaptic development in vivo4,5. 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 neurons4,5. 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 formation6. In the mammalian cerebellum, neurexin interacts trans-synaptically with the glutamate receptor subunit GluRD2 through cerebellin 1 precursor protein to mediate synapse formation7. Among the four neurelinins, neuroligin 2 found at inhibitory synapses is important for postsynaptic development8. 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) neurons9. SYG-1 localizes to presynaptic sites before synapse formation and is necessary and sufficient for presynaptic development in vivo10.

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 channels11. In vertebrates, Piccolo and Bassoon are large, multi-domain presynaptic cytomatrix proteins with long stretches of coiled-coil domains12. 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 vesicles13.

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 junctions14,15. In flies and worms, syd-1 and dsyd-1 mutants, respectively, also show profound presynaptic assembly defects16,17. Brp was first shown to be required for active zone formation and calcium channel localization at Drosophila neuromuscular junctions18,19. The C. elegans homolog of Brp, ELKS-1, is also involved in presynaptic development; however, its role is only revealed in sensitized backgrounds20,21.

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 assembly17,22.

Actin networks decorate presynaptic terminals by forming a ring-like structure surrounding synaptic vesicles and active zones23.

References

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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 \textit{C. elegans}, \textit{nab-1} mutants have reduced synapse density due to presynaptic defects\(^{33}\). In inhibitory neurons, \textit{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\(^{34}\). The local F-actin network seems to be critical for growth of presynaptic terminals\(^{26}\). SAD-1 is required for actin dynamics that mediate synapse number\(^{32}\), and its activity is critical for the growth of presynaptic terminals\(^{26}\). F-actin is required for the growth of axons\(^{30,35}\).

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 \textit{syg-1} along regions of the HSN axon where it contacts specific neuronal sites. This hypothesis is consistent with previous work\(^{36-38}\). The synaptic localization of the \textit{utCH} probe is due to actin binding\(^{40,41}\) (Fig. 1d). To verify the synaptic localization, we co-expressed GFP:: \textit{utCH} and mCherry:: \textit{RAB-3}, a fluorescently labeled synaptic vesicle–associated protein, to visualize nasapses in HS N, and indeed, both proteins targeted the same region (Fig. 1e). Thus, a network of stable F-actin is present at HSN synapses \textit{in vivo}.

In HSN, initiation of synaptogenesis and synaptic specificity is determined by the presence of a transmembrane immunoglobulin-superfamily protein, \textit{syg-1} (NEPH1)\(^{10}\). In the absence of \textit{syg-1}, synapses fail to form in the synaptic region at the vulva and instead form ectopic synapses anterior to the vulva. In \textit{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.

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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. First, NAB-1 localized to synapses in HSN together with RAB-3 and F-actin. 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). This deficiency in vesicle recruitment was reflected behaviorally by defects in egg-laying ability, as nab-1 mutants laid significantly more late-stage eggs and fewer early-stage eggs than wild-type worms. Both defects were enhanced by simultaneous loss of ELKS-1, an active zone scaffolding protein whose loss alone resulted in no apparent phenotype. In C. elegans, NAB-1 has been shown to localize presynaptically and function in regulating synapse density and neuronal polarity. 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. 2j). 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. 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. Furthermore, NAB-1’s subcellular localization in these cells was largely abolished.
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).

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).

To show that F-actin binding is required for synaptic localization of NAB-1, we made a chimeric NAB-1 protein by replacing its PDZ with that of a chimeric protein by replacing its PDZ with that of RAB-3 labeled synaptic vesicles (b). 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 loss 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.

Figure 2 NAB-1 localizes to presynaptic sites and nab-1 mutants show synaptic 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 loss 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.
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. 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, 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 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.

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::moeABD transgene is 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.

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.
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. 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, 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 components, including ELKS-1, GIT, UNC-10 (RIM), calcium channels and synaptic vesicle markers to synapses.
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. 6f). This reduction in SYD-2 localization is similar to that in syd-1 mutants (Fig. 6e) and is not enhanced by loss of ELKS-1 in nab-1(ok943);elsk-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);elsk-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 interactions15. 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). 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–j). 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 formation43. 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. 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. 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. 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.

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 uch 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 shown that the SYG-1 homologs, Duf and Rst, both induce F-actin formation near the fusion pore. 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 AII 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. 3I,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, 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. 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 loss 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 manuscript comments. This work was funded by the Howard Hughes Medical Institute and the Howard Hughes Medical Institute, the National Institutes of Health, and the Cold Spring Harbor Laboratory.

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.

Published online at http://www.nature.com/natureneuroscience/
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Mos transposon mediated targeted deletion of NAB-1. The targeted knockout nab-1(wy688 :: unc-119(+)) was made using the MosDEL method with the Mos insertion line ttT6300 (requested from L. Segalat, University Claude Bernard Lyon)28. Fifty ttT6300::unc-119(d3) worms were co-injected with the following plasmids: pL43.1 (86 ng μl−1 between NheI and KpnI). Plasmids were injected into worms at 1 ng μl−1 together with the promoter was cloned between SphI and XmaI and genes of interests were cloned between NheI and KpnI. The plasmid with positive selection marker was injected at 50 ng μl−1, pCF90 (Pnpyo-2 :: mCherry) at 2.5 ng μl−1 and Punc-122 :: rfp at 40 ng μl−1. Plates with F1 progeny were scored for fluorescent markers and rescued for unc-119 movement defects and identified and kept at 25 °C until the plate was starved. We identified worms with the deletion by screening for movement defects (owing to defects in the truncated protein can partially rescue synapse formation defects.

Fluorescence quantification and confocal imaging. All fluorescence images of left HS 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.

Fluorescently tagged NAB-1 localization lines: wyEx245 [Punc-86 :: nab-1(1-623aa); Podr-1 :: gfp]; wyEx3353 [Punc-86 :: nab-1(1-623aa); Podr-1 :: dsred]; wyEx3733 [Punc-86 :: nab-1(1-197,370-722aa); YFP; Podr-1 :: gfp]; wyEx1492 [Punc-86 :: Nab-1(1-197,370-722aa); YFP; Podr-1 :: dsred]; wyEx3550 [Punc-86 :: nab-1(372-722aa); YFP; Podr-1 :: dsred]; wyEx3364 [Punc-86 :: nab-1(195-372aa); YFP; Podr-1 :: gfp]; wyEx3845 [Punc-86 :: nab-1(1-666aa); YFP; Podr-1 :: gfp]; wyEx4602 [Punc-86 :: nab-1(213-722aa); YFP; Podr-1 :: dsred]; wyEx3905 [Punc-86 :: nab-1(675-1199); YFP; Podr-1 :: gfp]; wyEx3229 [Punc-86 :: nab-1(1-666aa); 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(371-559-722aa); YFP; Podr-1 :: gfp]; wyEx3333 [Punc-86 :: nab-1(371-722aa); YFP; Podr-1 :: dsred]; wyEx3336 [Punc-86 :: nab-1(372-722aa); YFP; Podr-1 :: gfp]; wyEx3907 [Punc-86 :: nab-1(1-666aa); YFP; Podr-1 :: gfp]; wyEx4750 [Punc-86 :: nab-1(213-722aa); YFP; Podr-1 :: gfp]; wyEx3229 [Pheathshock::nab-1; Podr-1 :: gfp].

Cloning of constructs. Expression plasmids for transgenic worm lines were made using the pSMS vector, a derivative of pPD49.26 (A. Fire, Stanford). The unc-86 promoter was cloned between Sph and XmaI and genes of interests were cloned between Nhel and KpnI. Plasmids were injected into worms at 1 ng μl−1 together with injection markers Podr-1 :: gfp or Podr-1 :: dsred at 20 ng μl−1 as previously described27. The MosDEL targeting construct, pPH32, was made using yeast homologous recombination methods from an EcoRI linearized pRS413 vector (American Type Culture Collection)26. The targeting construct includes a 2.5-kb homologous sequence that flanks the genomic region left of the Mos insertion site in ttT6300, followed by the cb-unc-119(+(-) positive selection marker replicated by PCR from pCF151 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 P3KII. A KRA5 membrane targeting sequence was cloned between K3P1 and XbaI sites. A Flag sequence was cloned after Nhel and HindIII.

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 vivo protein–protein interaction assay. As previously described50, 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 phallolidin (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.

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