CASK and FARP coordinately control pre- and post-synaptic function

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Abstract

Synaptic proteins are immobilized at synapses via interactions with scaffolding proteins. Scaffolding proteins for glutamatergic and GABAergic synapses have been extensively studied; however, far less is known about how scaffolds shape the function of cholinergic synapses. Here we analyze the role of two *C. elegans* post-synaptic scaffolding proteins (LIN-2/CASK and FRM-3/FARP) at cholinergic neuromuscular junctions. Constitutive knockouts or muscle specific inactivation of *lin-2* and *frm-3* dramatically reduced spontaneous and evoked post-synaptic currents. These synaptic defects resulted from decreased abundance of post-synaptic ionotropic acetylcholine receptors (AChRs) and decreased presynaptic ACh release. Thus, our findings show that post-synaptic LIN-2/FRM-3 complexes coordinately control pre- and post-synaptic function, thereby promoting cholinergic synaptic transmission.

Introduction

Synaptic connections mediate fast intercellular communication in the nervous system. Synapses are elaborate structures comprising hundreds of pre- and post-synaptic proteins, each highly concentrated at the sites of intercellular contact. Although synaptic proteins are highly enriched at these contacts, they nonetheless must retain the ability to undergo dynamic processes (e.g. insertion, removal, intermolecular contacts, and post-translational modifications). The abundance of a protein at a synapse often dictates signaling properties of that synapse. For example, the location and abundance of the synaptic vesicle priming protein (UNC-13) controls the probability and kinetics of release (Hu et al., 2013; Liu et al., 2019). Similarly, the abundance of post-synaptic receptors dictates the size of synaptic currents (Kauer et al., 1988; Luscher et al., 1999). Beyond recruiting rate limiting synaptic components, synaptic transmission also relies upon the precise coordination of pre- and post-synaptic specializations. For example, the site of synaptic vesicle (SV) fusion in presynaptic cells (termed Active Zones) must be precisely aligned with clustered receptors in the contacting post-synaptic cell. Slight shifts in trans-synaptic alignment results in significant alterations in the ongoing signaling at that synapse (Biederer et al., 2017; Chen et al., 2018; Haas et al., 2018). For these reasons, there is significant interest in determining how rate limiting synaptic components are localized and coordinated trans-synaptically.

Post-synaptic receptors are immobilized at synapses via their interactions with scaffolding proteins. A great deal is known about the scaffolds responsible for recruiting glutamate and GABA receptors; however, much less is known about how nicotinic receptors are localized to synapses. To become functional, assembled ionotropic AChRs must be transported to the plasma membrane and clustered at post-synaptic elements, where they are metabolically stabilized. Various synaptic components, such as cytoskeleton actin filaments (Shoop et al., 2000), protein tyrosine kinases (Sanes and Lichtman, 1999; Francis et al.,

2005), rapsyn (Burns et al., 1997), Claudin-like protein HPO-30 (Sharma et al., 2018), and postsynaptic density protein 95 (PSD95) (Conroy et al., 2003), have been implicated in clustering and stabilizing ionotropic AChRs. Cholinergic synaptic transmission is severely impaired by inactivating any of these synaptic components. In particular, PSD95, the most abundant synaptic scaffolding protein which belongs to the MAGUK family (membrane-associate guanylate kinase) containing PDZ, SH3 (Src homology 3), and GK (guanylate kinase) domains, has been shown to function as a central organizer in synapses. Given that over 1000 proteins are concentrated in postsynaptic densities, there is great interest in identifying additional proteins that regulate the abundance of post-synaptic ionotropic AChRs.

CASK (a MAGUK family scaffold protein) regulates synaptic function (Dimitratos et al., 1997; Butz et al., 1998; Hsueh et al., 1998; Chen and Featherstone, 2011). Apart from the PDZ, SH3, and GK domains found in all MAGUK proteins, CASK also contains an N-terminal Ca^{2+}/calmodulin-dependent protein kinase (CaM) domain (Figure 1A). CASK orthologs are found in invertebrates and vertebrates, suggesting that CASK's synaptic function is strongly conserved across phylogeny. In mammals, CASK is primarily expressed in the brain and localizes in synaptic regions (Hata et al., 1996; Hsueh et al., 1998; Stevenson et al., 2000). Similar to other scaffolds, CASK participates in multiple interactions in different signalling processes, and has been implicated in synaptic protein targeting, synaptic organization, and transcriptional regulation (Hata et al., 1996; Hsueh et al., 2000). Human CASK has also been linked to neurological disorders such as autism and brain malformation (Dimitratos et al., 1998; Najm et al., 2008). In *C. elegans*, LIN-2/CASK together with its binding partner FRM-3 (ortholog of the mammalian scaffold FARP), promote GABAergic synaptic transmission by immobilizing $GABA_A$ receptors at the NMJ (Tong et al., 2015; Zhou et al., 2020). Inconsistent findings have been reported regarding the functions of CASK

in regulating glutamate receptors (Atasoy et al., 2007; Chen and Featherstone, 2011). Despite these advances in both vertebrates and invertebrates, the functions of CASK and its binding proteins at cholinergic synapses have yet to be explored.

Here we show that LIN-2/CASK and FRM-3/FARP promote the function of cholinergic synapses by recruiting postsynaptic ionotropic AChRs and by increasing presynaptic ACh release. The presynaptic defects in *lin-2* and *frm-3* mutants arise from the loss of LIN-2/CASK and FRM-3/FARP in postsynaptic muscles, indicating that these post-synaptic scaffolds mediate a retrograde synaptic signal controlling ACh release. The SH3 domain in LIN-2/CASK plays important roles by directly interacting with ionotropic AChRs. Our results provide a detailed picture of how LIN-2/FRM-3 complexes coordinately control pre- and post-synaptic function at cholinergic synapses.

Results

Loss of function of *lin-2* or *frm-3* decreases locomotion speed

To assess LIN-2 and FRM-3's impact on behavior, we measured locomotion speed in *lin-2* and *frm-3* mutants. The *lin-2* gene encodes two isoforms, LIN-2A and LIN-2B, which share the PDZ, SH3, and GK domains while only LIN-2A contains the CaMK homology domain (Hoskins et al., 1996). The *lin-2(e1309)* mutation deletes an N-terminal region of LIN-2A but has no effect on LIN-2B (Figure 1A). The *frm-3* gene encodes three isoforms, two of which (FRM-3A and B) possess a FERM domain. The *gk585* allele is a deletion disrupting both *frm-3*a and b (Figure 1C). Locomotion speed was significantly decreased in *lin-2(e1309)* mutants and *frm-3(gk585)* mutants (Figure 1E, F), suggesting that LIN-2A and FRM-3 are required for the function of the locomotion circuit. No additional decrease in locomotion speed was observed in *lin-2(e1309)*; *frm-3(gk585)* double mutants, indicating that LIN-2 and FRM-3 act together to promote locomotion.

Because the *lin-2(e1309)* and *frm-3(gk585)* may not be null mutants, we used CRISPR to introduce early nonsense mutations in *lin-2(syb1019)* and *frm-3(syb1036)*. The *lin-2(syb1019)* nonsense mutation is in an exon shared by LIN-2A and B (Figure 1A, B); consequently, both isoforms are inactivated by this allele. The *lin-2(syb1019)* mutants exhibited similar vulva development and egg laying defects to those seen in *lin-2*(*e1309)* mutants (data not shown). Similarly, the *frm-3(syb1036)* nonsense mutation is in an exon shared by the FRM-3A and B isoforms (Figure 1C, D). Hereafter, we refer to *syb1019* and *syb1036* as *lin-2* and *frm-3* null mutants respectively. The locomotion speed defects in *lin-2* and *frm-3* null mutants were similar to those seen in *lin-2(e1309)* and *frm-3(gk585)* mutants (Figure 1F). Together, these results reveal that LIN-2/CASK and FRM-3/FARP play important roles in locomotion behavior.

Cholinergic synaptic transmission is severely impaired in *lin-2* and *frm-3* mutants

We next asked if LIN-2/CASK and FRM-3/FARP are required for cholinergic synaptic transmission. *C. elegans* body wall muscles receive synaptic inputs from both cholinergic and GABAergic motor neurons (White et al., 1986). Prior studies demonstrated that the synaptic abundance and mobility of UNC-49/$GABA_A$ receptors ($GABA_AR$) at the NMJs are significantly disrupted in *lin-2(e1309)* and *frm-3(gk585)* mutants, leading to decreased amplitude of the miniature inhibitory postsynaptic currents (mIPSCs) (Tong et al., 2015; Zhou et al., 2020). By recording the miniature and stimulus-evoked excitatory postsynaptic currents (mEPSCs and evoked EPSCs) in *lin-2(e1309)* and *frm-3(gk585)* mutants, we found that the cholinergic excitatory synaptic transmission was also significantly impaired, with the mEPSC amplitude, and evoked EPSC amplitude and charge transfer being markedly decreased (Figure 2A, B, E, F). The mEPSC frequencies were also significantly decreased in *lin-2(e1309)* and *frm-3(gk585)* mutants (60% and 30% of WT; Figure 2B). The

lin-2(e1309); *frm-3 (gk585)* double mutants exhibited similar synaptic transmission defects to those found in *frm-3(gk585)* single mutants, including mEPSCs and evoked EPSCs, indicating that LIN-2/CASK and FRM-3/FARP function together at the ACh NMJs. Detection of spontaneous mEPSCs is impaired when their amplitudes are decreased; consequently, the decreased mEPSC frequency in *lin-2*(*e1309)* and *frm-3(gk585)* mutants could be caused by the smaller mEPSC amplitude.

Compared to *lin-2(e1309)* mutants, *lin-2* null mutants exhibited more severe defects in cholinergic synaptic transmission, including larger decreases in mEPSC frequency and amplitude, and evoked EPSC amplitude and charge transfer (Figure 2A, B, E, F). The mEPSC frequency in *frm-3* null mutants was also significantly lower than in *frm-3(gk585)* mutants. Moreover, mIPSC frequencies, which were unaltered in *lin-2(e1309)* and *frm-3(gk585)* mutants, were dramatically decreased in both *lin-2* and *frm-3* null mutants (Figure 2C, D). These results demonstrate that synaptic transmission was more severely impaired in the *lin-2* and *frm-3* null mutants, further establishing that these scaffolds play a pivotal role in the function of cholinergic synapses. It should be noted that LIN-2's impact on cholinergic transmission does not require its other binding partners LIN-7/Velis and LIN-10/Mint (Butz et al., 1998; Kaech et al., 1998), as the *lin-7* and *lin-10* mutants exhibited normal mEPSCs and evoked EPSCs (Figure S1).

The dramatically decreased mEPSC frequency in *lin-2* and *frm-3* null mutants suggest that presynaptic ACh release may be impaired in these mutants. To more directly test this idea, we analyzed quantal content (the total number of released SVs during an evoked EPSC). Quantal content was calculated as the ratio of the charge transfer in an evoked EPSC to the mean mEPSC charge transfer in the same animal. We found that quantal content was markedly decreased in both *lin-2* and *frm-3* null mutants, indicating that LIN-2 and FRM-3 are required for presynaptic ACh release (Figure 2G, H). In addition, the severe defects in

mEPSC amplitude suggest that *lin-2* and *frm-3* mutants also have impaired postsynaptic ionotropic AChRs function (e.g., decreased synaptic abundance). Taken together, our results revealed that LIN-2/CASK and FRM-3/FARP play essential roles in cholinergic synaptic transmission, likely via both pre- and post-synaptic mechanisms.

Muscle specific knockouts of *lin-2* and *frm-3* cause cholinergic transmission defects

LIN-2/CASK and FRM-3/FARP are expressed in both motor neurons and postsynaptic body wall muscles (Tong et al., 2015; Zhou et al., 2020). To determine which cells require LIN-2/CASK and FRM-3/FARP function, we generated *lin-2(nu743* FLEX*)* and *frm-3(nu751* FLEX*)* alleles that allow inactivation of these genes by the CRE recombinase. Using CRISPR, we introduced a stop cassette into *lin-2* and *frm-3* introns (in the opposite orientation). For both genes, the STOP cassette was inserted into an intron shared by both isoforms (Figure 3A). The STOP cassette is bounded by FLEX recombination signals mediating CRE induced inversions. In this manner, CRE expression inverts the stop cassette thereby blocking expression of LIN-2(A and B) and FRM-3(A and B). Neuron specific knockouts (KO) were made by expressing P*sbt-1*::Cre (termed Neuron^{Cre}) while body wall muscle KO were made by expressing P*myo-3*::Cre (termed Muscle^{Cre}).

We next examined synaptic transmission following *lin-2* and *frm-3* muscle and neuron KO. Compared to control *lin-2(nu743)* and *frm-3(nu751)* mutants (lacking Cre expression), the frequency and amplitude of mEPSCs, and the amplitude and charge transfer of evoked EPSCs, were all severely reduced by Muscle^{Cre}, but were not changed by Neuron^{Cre} (Figure 3B-I), demonstrating that LIN-2/CASK and FRM-3/FARP both promote cholinergic synaptic transmission by acting in body wall muscles. Both *lin-2* and *frm-3* Muscle^{Cre} mutants exhibited significantly slower mEPSC decay than control mutants (Figure 3B, G), which likely results from a greater contribution of levamisole-sensitive acetylcholine

receptors (see below) to the synaptic current. These observations further suggest a defect in postsynaptic AChRs in *lin-2* and *frm-3* mutants.

Notably, the mEPSCs and evoked EPSCs in the Muscle^{Cre} mutants are comparable with those in the *lin-2* and *frm-3* null mutants (Figure 2), indicating that the cholinergic synaptic transmission defects result from the loss of LIN-2 and FRM-3 postsynaptic function. Quantal content was significantly decreased by Muscle^{Cre} (but not by Neuron^{Cre}) indicating that muscle specific LIN-2 and FRM-3 knockouts decrease presynaptic ACh release (Figure 3E, I). These results demonstrate that postsynaptic LIN-2 and FRM-3 mediate a retrograde pathway that promotes presynaptic function.

LIN-2/CASK and FRM-3/FARP regulate the synaptic abundance of postsynaptic AChRs

The dramatic decrease in mEPSC amplitude in *lin-2* and *frm-3* null mutants suggests that the ionotropic AChRs are impaired in postsynaptic body wall muscles. There are two types of ionotropic AChRs expressed on body wall muscles in *C. elegans*, nicotine-sensitive homo-pentameri receptors (nAChRs) consisting of five ACR-16/CHRNA7 subunits, and levamisole-sensitive hetero-pentamer receptors (LAChRs) containing alternative α subunits (UNC-38, UNC-63, and LEV-8) and non-α subunits (UNC-29 and LEV-1) (Richmond and Jorgensen, 1999; Brown et al., 2006). These two types of receptors can be distinguished by their differential desensitization in response to long-time exposure of acetylcholine (Liu et al., 2009). Moreover, because of the difference in channel conductance, activation of nAChRs elicits large and rapidly decaying responses which contribute ~ 85% of the amplitudes of mEPSCs and evoked EPSCs, and LAChRs produce small synaptic responses with slow decay when activated, contributing ~15% of synaptic current (Zhang et al., 1996; Ullian et al., 1997; Babu et al., 2011).

To estimate the synaptic abundance of nAChRs, we imaged ACR-16::RFP (expressed by the *myo-3* promoter in a single copy transgene). ACR-16::RFP exhibits a punctate distribution in the nerve cords, consistent with previous observation (Babu et al., 2011). Compared to wild-type animals, the *lin-2* and *frm-3* null mutants exhibited significantly reduced ACR-16::RFP puncta fluorescence in the dorsal nerve cord (Figure 4A, B). These observations are consistent with the decreased mEPSC and evoked EPSC amplitude (Figure 2). We next asked if loss of LIN-2 and FRM-3 alters the synaptic abundance of endogenously expressed LAChRs, by analyzing the fluorescence intensity produced by an *unc-29* allele containing an RFP tag, *unc-29*(*kr208*) (Tu et al., 2015). Our data showed that the UNC-29::RFP puncta fluorescence was also remarkably decreased in both *lin-2* and *frm-3* null mutants (Figure 4C, D). There were no additional decrease in ACR-16::RFP and UNC-29::RFP in the *lin-2;frm-3* double mutants (Figure 4B, D). Together, our results demonstrate that LIN-2 and FRM-3 act in the same genetic pathway regulating the synaptic abundance of ionotropic AChRs in body wall muscles, consistent with results reported in a recent study (Zhou et al., 2021) .

To examine the function of endogenous nAChRs and LAChRs on the muscle surface, we measured acetylcholine- and levamisole-activated synaptic responses. A pulse application of ACh (0.5mM, 100ms) onto body wall muscle elicited a large and robust current in wild-type animals. In *lin-2* and *frm-3* null mutants, ACh-activated currents were indistinguishable from that in wild type (Figure 4E, F). Puffing levamisole (100μM, 100ms) produced a small but long-lasting current in wild-type animals, and the levamisole-evoked currents were also unaltered in *lin-2* and *frm-3* null mutants (Figure 4G, H). These results indicate that inactivating LIN-2 and FRM-3 did not alter the total expression level or surface delivery of nAChRs and LAChRs in muscles. Instead, inactivating LIN-2 and FRM-3 prevent nAChRs and LAChRs clustering or destabilize receptor clusters at synapses, resulting in decreased

ACR-16::RFP and UNC-29::RFP puncta fluorescence and decreased mEPSC and evoked EPSC amplitudes. Together, our results demonstrate that LIN-2 and FRM-3 regulate the synaptic abundance of AChRs in body wall muscles, leading to defects in cholinergic synaptic transmission.

LIN-2 and FRM-3 impact on post-synaptic AChR clustering is similar to their role at GABAergic synapses (Tong et al., 2015; Zhou et al., 2020). In *lin-2(e1309)* and *frm-3(gk585)* mutants, UNC-49::GFP/$GABA_A$ puncta intensity and mIPSC amplitude were significantly decreased while currents evoked by exogenous GABA puffs were unchanged. Because the null mutants of *lin-2* and *frm-3* displayed stronger transmission defects, we reexamined the effects of *lin-2* and *frm-3* knockouts on $GABA_AR$. We found that GABA-activated currents were significantly reduced (by 50%) in *lin-2* null mutants but were unchanged in *frm-3* null mutants (Figure S2). These results indicate that LIN-2 may also control total surface $GABA_AR$ levels in muscles.

Synaptic morphology is normal in *lin-2* and *frm-3* mutants

The decreased quantal content in *lin-2* and *frm-3* mutants raised the question of how, LIN-2 and FRM-3 regulate presynaptic function. By performing electron microscopy, we examined the synaptic ultrastructure of cholinergic motor neurons. Morphometric analyses showed that the terminal size and total number of SVs in in *lin-2* and *frm-3* mutants were indistinguishable from that in wild-type control (Figure 5A, B). Like the cholinergic synapses, the GABAergic synapses also exhibited normal terminal size and number of SVs (data not shown). These results indicate that LIN-2 and FRM-3 do not regulate the morphology of presynaptic terminals.

SV abundance and priming are not impaired in *lin-2* and *frm-3* mutants

To examine potential regulation of LIN-2 and FRM-3 in presynaptic terminals, we further examined SV abundance and priming in *lin-2* and *frm-3* mutants. SV abundance was analyzed by imaging synaptic vesicle markers UNC-57::mCherry (expressed by the *unc-129* promoter in a single copy transgene) and UNC-17::mKate2 (mKate2 is inserted into endogenous *unc-17* locus by CRISPR) (Pereira et al., 2019). Both UNC-57::mCherry and UNC-17::mKate2 display punctate distribution in dorsal nerve cord in wild-type animals (Figure 6A, C). Our data showed that UNC-57::mCherry in *lin-2* mutants and UNC-17::mKate2 in *frm-3* mutants have comparable puncta fluorescence compared to wild-type controls (Figure 6B, D), indicating that presynaptic SV abundance is normal in these two mutants. These results are consistent with the observation in EM in which total number of SVs is unaltered in *lin-2* and *frm-3* mutants (Figure 5B).

We next asked if SV priming is altered in *lin-2* and *frm-3* mutants. We assessed the readily releasable pool (RRP) of SVs by measuring the total charge transfer produced by a pulsed application of hypertonic sucrose (0.5M, 2s). This assay has been widely used to estimate the RRP in both vertebrates and invertebrates (Rosenmund and Stevens, 1996; Richmond et al., 1999; Li et al., 2019; Liu et al., 2019). Compared to wild type, the total sucrose-evoked charge transfer was decreased in *lin-2* and *frm-3* mutants (Figure 6E, F). Given that mEPSC amplitude was decreased in these mutants, we calculated the quantal content of the RRP (defined as the ratio of the sucrose evoked charge to the mean mEPSC charge). We found that the RRP quantal content was unchanged in *lin-2* and *frm-3* mutants, although a trend for increased quantal content was observed in both mutants. Together, our results indicate that SV abundance and priming are unchanged in *lin-2* and *frm-3* mutants, and postsynaptic LIN-2 and FRM-3 likely regulate some aspect of the presynaptic SV fusion process, thereby promoting cholinergic transmission.

The SH3 domain in LIN-2/CASK binds to both ACR-16 and UNC-29

The decreased nAChRs and LAChRs in *lin-2* and *frm-3* mutants indicates that LIN-2/CASK and FRM-3/FARP may physically bind to these receptors and regulate their synaptic abundance. To test this, we performed yeast two-hybrid (Y2H) assays. Our data showed that LIN-2A could bind to the second cytoplasmic loop of both ACR-16 and UNC-29 (Figure 7A-C), with stronger interaction with the ACR-16 loop than the UNC-29 loop. These results are consistent with the findings that *lin-2* or *frm-3* knockout led to a decrease of puncta fluorescence of AChRs (Figure 4). However, no direct interactions were detected between FRM-3 and the cytoplasmic loop of ACR-16 or UNC-29. By contrast, a recent study reported that ACR-16 binds both LIN-2 and FRM-3 (Zhou et al., 2021). This discrepancy could result from use of different binding assays (i.e. Y2H versus GST pull down). Nevertheless, since FRM-3 physically binds to LIN-2, FRM-3's impact on AChRs is likely mediated by LIN-2/FRM-3/ACR-16 and LIN-2/FRM-3/UNC-29 ternary complexes.

LIN-2 contains PDZ and SH3 domains that to be known to mediate protein-protein interactions. To examine which domain(s) in LIN-2 mediates the interaction with ACR-16 and UNC-29, we performed a Y2H assay to test the interaction of LIN-2's PDZ and SH3 domains with ACR-16. Our results showed that the SH3 but not the PDZ domain directly binds to a cytoplasmic loop of ACR-16 (Figure 7A, B). Interestingly, the SH3 domain also mediates the interaction between LIN-2 and UNC-29 (Figure 7A, D). To test the role of other LIN-2 domains (e.g., CaM kinase, L27, and GK; Figure 1), we expressed a truncated LIN-2 protein lacking the SH3 domain (LIN-2ΔSH3), and found that LIN-2ΔSH3 did not detectably interact with ACR-16 and UNC-29 (Figure 7A, B). In contrast, LIN-2 lacking the PDZ domain (LIN-2ΔPDZ) still exhibited binding activity to both receptors (Figure 7A, B), demonstrating that the SH3 is the only domain mediating interaction with ACR-16 and UNC-29. Together, our results reveal that the LIN-2/FRM-3 complexes regulate the synaptic

abundance of ionotropic AChRs, likely through binding and stabilizing of AChRs on the postsynaptic cell surface.

The PDZ domain in LIN-2/CASK and the FERM domain in FRM-3/FARP are required for LIN-2/FRM-3 interaction

To understand how the LIN-2/FRM-3 complex is formed, we examined what domains in LIN-2 and FRM-3 mediate their interaction. In LIN-2, we focused on the central PDZ and SH3 domains. Consistent with our previous findings, strong interaction was observed between full-length LIN-2 and FRM-3 (Figure 7A, F) (Tong et al., 2015). No interaction was detected between the SH3 domain and FRM-3, indicating that SH3 does not mediate the LIN-2/FRM-3 interaction (Figure 7A, F). This was confirmed by the LIN-2ΔSH3, which still exhibited strong binding to FRM-3 (Figure 7A, F). Like the SH3 domain, the PDZ domain alone did not exhibit binding to FRM-3 neither. However, the LIN-2ΔPDZ failed to bind to FRM-3 (Figure 7A, F). This indicates that the PDZ domain is required for LIN-2/FRM-3 interaction, although it is insufficient to mediate the interaction between LIN-2 and FRM-3. In FRM-3, the FERM domain exhibited strong binding to both the full-length LIN-2 and the LIN-2ΔSH3, but not the SH3, PDZ, and LIN-2ΔPDZ (Figure 7A, G), same binding pattern as full-length FRM-3. Taken together, our results demonstrate that LIN-2 binds to the FERM domain in FRM-3, and this interaction requires the PDZ domain in LIN-2.

Cholinergic transmission is decreased by deleting the SH3 or the PDZ domain in LIN-2/CASK

The interaction between LIN-2 SH3 and AChRs prompted us to investigate the functional importance of this domain. We deleted SH3 by CRISPR and analyzed synaptic transmission (Figure 8A; *syb1921*, ΔSH3). Our data showed that cholinergic transmission

was dramatically decreased in ΔSH3 mutants, including mEPSC frequency and amplitude, evoked EPSC amplitude and charge transfer, mEPSC charge, and quantal content (Figure 8B-F). These results indicate that the SH3 domain is indispensable for LIN-2's function, and the cholinergic synaptic transmission is determined by its interaction with AChRs. As the PDZ domain is required for LIN-2/FRM-3 interaction, we also tested whether it participates in the regulation of cholinergic synaptic transmission. Interestingly, the lack of the PDZ domain (*syb1937*, ΔPDZ; Figure 8A) caused similarly stronger decrease in cholinergic transmission, demonstrating that the PDZ domain also plays essential roles in LIN-2. Thus, our data suggest that the function of LIN-2/CASK in cholinergic synaptic transmission requires its PDZ domain recruiting FRM-3/FARP to form LIN-2/FRM-3 complex which binds to AChRs via the LIN-2 SH3 domain.

Discussion

In this study, we investigated the mechanisms by which LIN-2/CASK and FRM-3/FARP regulate cholinergic synaptic transmission at the *C. elegans* NMJ. Our results lead to several previously unknown findings. First, the null mutants of *lin-2* and *frm-3* exhibit severe defects in cholinergic synaptic transmission. Second, the cholinergic transmission defects are caused by the loss of function of LIN-2 and FRM-3 in postsynaptic body wall muscle but not presynaptic neurons. Third, LIN-2 and FRM-3 regulate the synaptic abundance of ionotropic AChRs on muscle membrane. Fourth, postsynaptic LIN-2 and FRM-3 mediate a retrograde pathway promoting presynaptic ACh release. Fifth, the SH3 domain in LIN-2 physically binds to ionotropic AChRs, and deleting this domain causes strong transmission defect. These findings reveal interesting and important regulatory mechanisms for synaptic transmission at cholinergic synapses. Below we discuss the significance of these findings.

CASK and FARP regulate cholinergic synaptic transmission

Since its discovery as a neurexin-binding protein (Hata et al., 1996), CASK has been found to be broadly involved in the regulation of cell-cell junction organization. The high homology of the C-terminal domains in CASK (e.g., PDZ, SH3, and GK) with other MAGUK proteins such as PSD95 has suggested that CASK may function similarly by acting as a scaffolding protein, while the presence of the unique N-terminal CaM kinase domain endows CASK additional functions in various cellular pathways (Lu et al., 2003). In addition to neurexin, CASK also binds to many other membrane proteins, such as Mint-1, Velis, Syndecan, FARP, glycophorins, and SynCaM through its central PDZ domain (Hata et al., 1998; Martinez-Estrada et al., 2001; Hsueh, 2006; Mukherjee et al., 2008). The CASK homolog in *C. elegans* LIN-2 is required for vulva development and proper localization of the EGF receptor LET-23 (Horvitz and Sulston, 1980; Hoskins et al., 1996).

Despite its functions in membrane organization, the physiological importance of CASK in synapses has been controversial. Knockout of CASK in mice causes lethal phenotype but does not affect the development of neurons. Cortical neurons lacking CASK display normal ultrastructural morphology and evoked EPSCs, whereas spontaneous glutamate release is increased but spontaneous GABA release is decreased (Atasoy et al., 2007). In *Drosophila*, loss of CAKI, the CASK ortholog, leads to increased frequency of MEPPs without changes in MEPP amplitude (Zordan et al., 2005). However, a later study in *Drosophila* found reduced spontaneous and evoked neurotransmitter release, as well as decreased postsynaptic glutamate receptors in CASK knockout mutants (Chen and Featherstone, 2011).

FARP was first recognized as a synaptic component that is required for dendrite growth in spinal motor neurons (Zhuang et al., 2009). Later studies revealed that FARP forms a synaptic complex with SynCAM1 through its FERM domain and functions as a synaptic

organizer in cultured dissociated hippocampal neurons (Cheadle and Biederer, 2012). It is enriched at postsynaptic sites and increases synapse number and modulates spine morphology. At *C. elegans* NMJs, FRM-3/FARP and its binding protein LIN-2/CASK promote post-synaptic localization of $GABA_AR$, most likely by immobilizing receptors at post-synaptic specializations (Tong et al., 2015; Zhou et al., 2020). Here we describe LIN-2/CASK and FRM-3/FARP function at cholinergic synapses, finding that they regulate postsynaptic abundance of ionotropic AChRs, and mediating a retrograde pathway promoting presynaptic SV fusion.

Several lines of evidence suggest that LIN-2/CASK and FRM-3/FARP form a scaffold complex regulating synaptic transmission at the *C. elegans* NMJ. First, these two scaffolds physically bind each other (Tong et al., 2015; Zhou et al., 2020). Second, synaptic transmission defects in *lin-2* and *frm-3* mutants are comparable, and no additional decrease in the *lin-2; frm-3* double mutants (Figure 1), demonstrating that they act in the same genetic pathway. Third, both scaffolds function in postsynaptic body wall muscle regulating the synaptic abundance of ionotropic AChRs (Figure 4). Fourth, presynaptic quantal content is similarly decreased in both *lin-2* and *frm-3* mutants. Prior studies in *Drosophila* NMJ have proposed that neurexin may also be involved in the CASK and protein 4.1 complex (CASK/neurexin/4.1), which regulates glutamate receptor clustering (Chen and Featherstone, 2011). However, it seems that the LIN-2/FRM-3 complex does not require neurexin for clustering and stabilizing ionotropic AChRs at the worm NMJ, as the worm mutants lacking NRX-1/neurexin exhibit normal mEPSCs and evoked EPSCs (Hu et al., 2012; Tong et al., 2015). A recent study showed that the loss of γ-neurexin causes a moderate decrease in evoked EPSCs at the NMJ; however, the mEPSCs, including frequency and amplitude, are unchanged, indicating that the ionotropic AChRs are functionally normal (Kurshan et al., 2018).

Functional roles of LIN-2A and LIN-2B isoforms in synaptic transmission

The *C. elegans lin-2* locus expresses two isoforms: a long isoform called LIN-2A which contains the N-terminal CaM kinase domain, and a short isoform which does not have the CaM kinase domain (Figure 1) (Hoskins et al., 1996). It is believed that the *lin-2 e1309* mutants express only the LIN-2B isoform, as the deletion removes the whole promoter region for LIN-2A and part of the CaM kinase domain (Figure 1A). Our data show that LIN-2A plays a more dominant role in cholinergic transmission, accounting for 70% of the evoked neurotransmitter release. The more severe decrease in cholinergic synaptic transmission including both mEPSCs and evoked EPSCs in the *lin-2* null mutants demonstrates that the LIN-2B isoform also plays important roles in synaptic function. In GABAergic synapses, it seems the LIN-2B isoform plays similarly important role as LIN-2A, as the mIPSC amplitude was decreased by 20% in the *e1309* mutants and was decreased by further 20% in the null mutants, different from the observations in mEPSC amplitude (30% decrease in *e1309* and 40% decrease in null). Moreover, the mIPSC frequency was severely decreased in the null mutants but was not changed in the *e1309* mutants. These results suggest that LIN-2A and LIN-2B may function redundantly in GABAergic synapses (Figure 2D). A *lin-2b* specific knockout mutant may help to confirm this notion in future studies.

Our data suggest that both LIN-2A and LIN-2B are involved in the regulation of AChRs clustering and stabilization in cholinergic synapses, consistent with results reported in a recent study (Zhou et al., 2021). Knockout of either LIN-2A (*e1309* mutant; data not shown) or both LIN-2A and LIN-2B causes decreased mEPSC amplitude without altering puffing ACh- or Levamisole-activated currents (Figure 1 and Figure 4), indicating normal AChRs trafficking and surface delivery. However, LIN-2A and LIN-2B appear to function differently in regulating $GABA_A$Rs. The decreased mIPSC amplitude but normal puffing

GABA-activated currents in the *e1309* mutants (Tong et al., 2015), demonstrate that LIN-2A functions only for $GABA_A$Rs clustering and stabilization. In contrast, the *lin-2* null mutants exhibited a 50% reduction of puffing GABA-activated currents (Figure S2), indicating that the LIN-2B isoform is more likely involved in the delivery or retention of $GABA_A$Rs on the muscle surface. These results suggest that the two LIN-2 isoforms have differential functions in cholinergic and GABAergic synapses. Our data do not distinguish the two isoforms of FRM-3, as both isoforms are affected in the *gk585* mutants and the null mutants.

LIN-2/CASK and FRM-3/FARP mediate a retrograde pathway

The decreased quantal content in *lin-2* and *frm-3* muscle cKO mutants reveal that the muscle LIN-2 and FRM-3 regulate presynaptic SV fusion through a retrograde pathway, representing previously unknown functions of LIN-2/CASK and FRM-3/FARP in synapses. These novel functions of LIN-2 and FRM-3 raise the question of what are the molecular targets of the retrograde pathway in presynaptic terminals. This retrograde pathway does not require presynaptic LIN-2 and FRM-3, as the neuronal KO of *lin-2* and *frm-3* do not produce synaptic transmission defects, and the *lin-2* and *frm-3* null mutants do not exhibit further decrease in quantal content compared to the muscle KO (Figure 1 and Figure 3). The unchanged SV abundance and RRP size in *lin-2* and *frm-3* mutants suggest that the postsynaptic LIN-2/FRM-3 may directly or indirectly target the release machinery in the fusion process, which consists of many synaptic proteins such as the SNAREs, Munc13s, Munc18s, Ca^{2+} channels, RIM, and tomosyn (Sudhof, 2013).

In fact, prior studies have revealed that members in the MAGUK protein family trigger retrograde pathways that regulate presynaptic functions in excitatory glutamatergic synapses. For example, postsynaptic expression of PSD-95 in hippocampal neurons enhances the maturation of presynaptic terminals, with presynaptic proteins (e.g., synaptophysin and

SV2) and mEPSC frequency being increased (El-Husseini et al., 2000). In cultured hippocampal slices, postsynaptic PSD-95 and neuroligin complex regulates the probability of presynaptic neurotransmitter release via their interaction with presynaptic neurexin (Futai et al., 2007). This retrograde modulation may involve CASK which is a binding partner of neurexin. Since the CASK-binding protein Mint1 directly interacts with Munc18, it is possible that this retrograde pathway targets the presynaptic fusion machinery. In addition to PSD-95, postsynaptic expression of synapse-associated protein 97 (SAP97) is also involved in a retrograde pathway increasing presynaptic protein content and active zone size (Regalado et al., 2006). Furthermore, a recent study reported that FARP triggers a retrograde pathway regulating presynaptic active zone composition via SynCAM in hippocampal neurons (Cheadle and Biederer, 2012). These observations, together with our findings that postsynaptic LIN-2/CASK and FRM-3/FARP regulate presynaptic SV fusion, indicate that MAGUK proteins and their binding proteins are broadly involved in the regulation of presynaptic functions by triggering retrograde signaling pathways.

We previously described a retrograde pathway at the worm NMJ that is mediated by the cell adhesion molecules neurexin and neuroligin (Hu et al., 2012). Activation of this retrograde pathway decreases presynaptic quantal content. This raised the possibility that the LIN-2/FRM-3 mediated retrograde pathway may also go through neurexin and neuroligin to regulate presynaptic SV fusion. Several lines of evidence support this possibility. First, neurexin physically binds to LIN-2/CASK and neuroligin, thereby forming LIN-2/FRM-3/neurexin/neuroligin complex bridging pre- and post-synaptic terminals. Second, the level of neuroligin in CASK knockout mice is increased (Atasoy et al., 2007). Third, quantal content is significantly increased in mutants lacking neuroligin (Hu et al., 2012), indicating an inhibitory role of neuroligin on presynaptic SV fusion. Future studies will investigate these possibilities, as well as other potential targets of the LIN-2/FRM-3 retrograde pathway. It will

be interesting to determine if this retrograde effect of LIN-2 and FRM-3 stimulating presynaptic release is conserved at other synapses.

Contributions to the trans-synaptic alignment in cholinergic synapses

Recent studies have revealed that presynaptic release sites and postsynaptic neurotransmitter receptor clusters are aligned with each other (Tang et al., 2016), and this alignment plays critical roles in synaptic transmission and plasticity, enabling efficient and precise information exchange between neurons. Increasing or decreasing the trans-synaptic alignment may alter response amplitude. The underlying mechanisms that maintain trans-synaptic alignment, however, are complicated, due to the large number of molecular elements that are involved in both pre- and post-synaptic compartments, as well as in the synaptic cleft. It is therefore important to identify key organizers at each synaptic compartment. The almost abolished nAChRs-mediated synaptic responses and dramatically decreased synaptic abundance of nAChRs in *lin-2* and *frm-3* null mutants indicate that LIN-2/CASK and FRM-3/FARP are key components in the organization of postsynaptic AChRs in cholinergic synapses. Moreover, some known binding partners of LIN-2/CASK such as neurexin and Liprin, are required for trans-synaptic alignment; however, cholinergic synaptic transmission in mutants lacking these components does not exhibit a strong change as in *lin-2* and *frm-3* mutants (Stigloher et al., 2011; Hu et al., 2012). Our results therefore highlight the critical roles of LIN-2/CASK and FRM-3/FARP in regulating cholinergic synaptic transmission, likely by acting as central organizers of trans-synaptic alignment in cholinergic synapses.

Materials and Methods

Strains

Animals were cultivated at room temperature on nematode growth medium (NGM) agar plates seeded with OP50 bacteria. On the day before experiments L4 larval stage animals were transferred to fresh plates seeded with OP50 bacteria for all the electrophysiological, imaging, and behavioural experiments. The following strains were used:

Wild type, N2 bristol

CB1309 *lin-2(e1309)*

PHX1019 *lin-2(syb1019)*

KP7338 *frm-3(gk585)*

PHX1036 *frm-3(syb1036)*

ZTH1032 *lin-2(e1309);frm-3(gk585)*

MT106 *lin-7(n106)*

KP7637 *lin-10(n1508)*

KP10507 *lin-2(nu743, flex ON)*

KP10609 *lin-2(nu743, flex ON)*; *nuSi501[Psbt-1::NLS-CRE::SL2::NLS-BFP]*

KP10621 *lin-2(nu743, flex ON)*; *nuSi491[Pmyo-3::CRE]*

KP10590 *frm-3(nu751 FLEX ON)*

KP10676 *frm-3(nu751 FLEX ON)*; *nuSi501[Psbt-1::NLS-CRE::SL2::NLS-BFP]*

KP10677 *frm-3(nu751 FLEX ON)*; *nuSi491[Pmyo-3::CRE]*

TXJ0502 *nuSi0002[Pmyo-3::ACR-16::RFP]*

TXJ0825 *lin-2(syb1019)*; *nuSi0002[Pmyo-3::ACR-16::RFP]*

TXJ0822 *frm-3(gk585)*; *nuSi0002[Pmyo-3::ACR-16::RFP]*

EN208 *kr208 [Punc-29::UNC-29::tagRFP]*

TXJ0569 *lin-2(syb1019)*; *kr208[Punc-29::UNC-29::tagRFP]*

TXJ0816 *frm-3(gk585)*; *kr208[Punc-29::UNC-29::tagRFP]*

KP9809 *nu586[UNC-2::GFP11x7]*; *nuSi250[Punc-129::splitGFP1-10::SL2::UNC-57-mCherry::SL2::mTagBFP2]*

TXJ0818 *lin-2(syb1019)*; *nu586[UNC-2::GFP11x7]*; *nuSi250[Punc-129::splitGFP1-10::SL2::UNC-57-mCherry::SL2::mTagBFP2]*

TXJ1283 *frm-3(gk585)*; *nu586[UNC-2::GFP11x7]*; *nuSi250[Punc-129::splitGFP1-10::SL2::UNC-57-mCherry::SL2::mTagBFP2]*

KP9639 *nu586[UNC-2::GFP11x7]; nuSi251[Punc-47::splitGFP1-10::SL2::UNC-57-mCherry::SL2::mTagBFP2]*

TXJ0821 *lin-2(syb1019); nu586[UNC-2::GFP11x7]; nuSi251[Punc-47::splitGFP1-10::SL2::UNC-57-mCherry::SL2::mTagBFP2]*

TXJ0814 *frm-3(gk585); nu586[UNC-2::GFP11x7]; nuSi251[Punc-47::splitGFP1-10::SL2::UNC-57-mCherry::SL2::mTagBFP2]*

PHX1921 *lin-2 ΔSH3 (syb1921)*

PHX1937 *lin-2 ΔPDZ (syb1937)*

Generation of lin-2 and frm-3 mutants

lin-2(syb1019) contains an opal stop codon at the first common region of LIN-2A (E420) or LIN-2B (E79). Forward genotype primer: tagtatccagcccgacgagt, reverse primer: gatgtgtggctaacgggtga. *frm-3(syb1036)* contains an amber stop codon at the first common region of FRM-3A (V36) or FRM-3B (V48). Forward genotype rimer: ccaaccaggtgcggatcata, reverse primer: cgagaagttgctcacctggt. *lin-2 ΔSH3 (syb1921)* contains a deletion from F637 to A718 for LIN-2A, or F296 to A377 for LIN-2B. *lin-2 ΔPDZ (syb1937)* contains a deletion from R545 to T620 for LIN-2A, or R204 to T279 for LIN-2B.

Transgenes and germline transformation

Transgenic strains were isolated by microinjection of various plasmids using either Pmyo-2::NLS-GFP (KP#1106) or Pmyo-2::NLS-mCherry (KP#1480) as the co-injection marker. The single copy insertion lines were generated using miniMos transposon (Frokjaer-Jensen et al., 2014).

CRISPR alleles

CRISPR alleles were isolated as described (Arribere et al., 2014). Briefly, *unc-58* was used as a co-CRISPR selection to identify edited animals. Animals were injected with two guide RNAs (gRNAs) and two repair templates, one introducing an *unc-58* gain of function mutation and a second modifying a gene of interest. Progeny exhibiting the *unc-58(gf)* uncoordinated phenotype were screened for successful editing of the second locus by PCR. Split GFP and split sfCherry constructs are described in (Feng et al., 2017).
MiniMOS inserts in which Pmyo-3 drives expression of either GFP 1-10 (nuTi144) or sfCherry 1-10 SL2 GFP 1-10 (nuTi458) were created using the protocol as described by (Frokjaer-Jensen et al., 2014).

Tissue specific *frm-3* and *lin-2* knockout was performed by introducing a stop cassette into introns in the ON configuration (i.e. in the opposite orientation of the target gene) using CRISPR, creating the *frm-3(nu751, flex ON)* and *lin-2(nu743, flex ON)* alleles. In *frm-3(nu751, flex ON)*, the stop cassette was inserted into an intron shared by *frm-3a* and *b* (intron 3 of *frm-3a*). Similarly, the stop cassette in *lin-2(nu743, flex ON)* was inserted into an intron shared by both *lin-2* isoforms (intron 3 of *lin-2b*). The stop cassette consists of a synthetic exon (containing a consensus splice acceptor sequence and stop codons in all reading frames) followed by a 3' UTR and transcriptional terminator taken from the *flp-28*

gene (the 564bp sequence just 3' to the *flp-28* stop codon). The stop cassette is flanked by FLEX sites (which are modified loxP sites that mediate CRE induced inversions) (Schnutgen and Ghyselinck, 2007). In this manner, orientation of the stop cassette within the intron is controlled by CRE expression. Expression of the targeted gene is reduced when the stop cassette is in the OFF configuration (i.e. the same orientation as the targeted gene) but is unaffected in the ON configuration (opposite orientation). The endogenous *flp-28* gene is located in an intron of W07E11.1 (in the opposite orientation). Consequently, we reasoned that the *flp-28* transcriptional terminator would interfere with *frm-3* and *lin-2* expression in an orientation selective manner. A similar strategy was previously described for conditional gene knockouts in *Drosophila* (Fisher et al., 2017).

Locomotion and behavioral assays

Young adult animals were washed with a drop of PBS and then transferred to fresh NGM plates with no bacterial lawn (30 worms per plate). Worm movement recordings (under room temperature 22°C) were started 10 min after the worms were transferred. A 2 min digital video of each plate was captured at 3.75 Hz frame rate by WormLab System (MBF Bioscience). Average speed and tracks were generated for each animal using WormLab software.

Fluorescence imaging

Worms were immobilized by 30 g/l 2,3-Butanedione monoxime (Sigma) and mounted on 2% agar on glass slides. Fluorescence images were captured by 100x (NA=1.4) objective on an Olympus microscope (BX53). The mean fluorescence intensities of reference FluoSphere microspheres (Thermo Fisher Scientific) were measured during each experiment, and were used to control for illumination intensities changes. Multidimensional data were

reconstructed as maximum intensity projections using Metamorph software (Molecular Devices). Line scans were analyzed in Igor Pro (WaveMetrics) using a custom script. The intensity of peak fluorescence of each marker was analyzed.

Electrophysiology

Electrophysiology was conducted on dissected *C. elegans* as previously described (Hu et al., 2012). Worms were superfused in an extracellular solution containing 127 mM NaCl, 5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 20 mM glucose, 1 mM $CaCl_2$, and 4 mM $MgCl_2$, bubbled with 5% CO_2, 95% O_2 at 22°C. Whole-cell recordings were carried out at -60 mV for all EPSCs, including mEPSCs, evoked EPSCs, and sucrose-evoked responses. The holding potential was switched to 0mV to record mIPSCs. The internal solution contained 105 mM CH_3O_3SCs, 10 mM CsCl, 15 mM CsF, 4mM $MgCl_2$, 5mM EGTA, 0.25mM $CaCl_2$, 10mM HEPES, and 4mM Na_2ATP, adjusted to pH 7.2 using CsOH. Stimulus-evoked EPSCs were obtained by placing a borosilicate pipette (5-10 μm) near the ventral nerve cord (one muscle distance from the recording pipette) and applying a 0.4 ms, 85μA square pulse. Sucrose-evoked release was triggered by a 2s application of 0.5M sucrose dissolved in normal bath solution. For drug-activated current recordings, ACh, Levamisole, or GABA was pressure injected onto body wall muscle. The pipette containing hypertonic sucrose or different drugs was placed at the end of the patched muscle cell. All recordings were performed at room temperature (22°C).

Electron microscopy

Samples were prepared using high-pressure freeze fixation (Weimer et al., 2006). ~30 young adult hermaphrodites were placed in each specimen chamber containing *E. coli* and were frozen at -180°C under high pressure (Leica SPF HPM 100). Frozen specimens then

underwent freeze substitution (Leica Reichert AFS) during which the samples were held at −90°C for 107 h in 0.1% tannic acid and 2% OsO_4 in anhydrous acetone. The temperature was then increased at 5°C/h to −20°C, kept at −20°C for 14 h, and increased by 10°C/h to 20°C. After fixation, samples were infiltrated with 50% Epon/acetone for 4 h, 90% Epon/acetone for 18 h, and 100% Epon for 5 hr. Finally, samples were embedded in Epon and incubated for 48 h at 65°C. Ultrathin (40 nm) serial sections were cut using a Leica Ultracut 6 and collected on formvar-covered, carbon-coated copper grids (EMS, FCF2010-Cu). Poststaining was performed using 2.5% aqueous uranyl acetate for 4 min, followed by Reynolds lead citrate for 2 min. Images were acquired using a JEOL JEM-1220 transmission electron microscope operating at 80 kV using a Gatan digital camera at a magnification of 100k. Images were collected from the ventral nerve cord region anterior to the vulva for all genotypes. Cholinergic synapses were identified on the basis of their typical morphology (White et al., 1986). A synapse was defined as a series of sections (profiles) containing a dense projection as well as two flanking sections on both sides without dense projections. Image acquisition and analysis using NIH ImageJ/Fiji software were performed blinded for genotype.

Yeast-two-hybrid

The yeast two-hybrid assay was performed with the Matchmaker Gold Yeast Two-Hybrid System. Briefly, LIN-2A, LIN-2A-SH3, LIN-2A-PDZ, LIN-2A-ΔSH3, LIN-2A-ΔPDZ, FRM-3 and FERM were cloned into pGBKT7 as a bait vector. In addition, ACR-16-TM3-4(aa315-472) and UNC-29-TM3-4(aa318-445) were cloned into pGADT7 as a prey vector. The plasmid pairs were simultaneously transformed into the Y2HGold yeast cells. Transformants selected from the SD-Leu-Trp plates were restreaked onto SD-Leu-Trp-His-Ade plates to test interactions. Meanwhile, the pGBKT7-53 and pGADT7-T plasmids pair

were used as positive controls, and pGBKT7-Lam and pGADT7-T plasmids pair were used as negative controls. False-positive from autoactivation was ruled out by co-transformation of pGBKT7-LIN-2A construct with pGADT7 empty vector alone.

Plasmids

KP2561	pGADT7-ACR-16-TM3-4(aa315-472)
PXJ461	pGADT7-UNC-29-TM3-4(aa318-445)
KP1890	pGBKT7-LIN-2A
PXJ278	pGBKT7-LIN-2A-SH3
PXJ279	pGBKT7-LIN-2A-PDZ
PXJ463	pGBKT7-LIN-2A-ΔSH3
PXJ464	pGBKT7-LIN-2A-ΔPDZ
PXJ413	pGBKT7-FRM-3
KP1950	pGBKT7-FERM

Data acquisition and statistical analysis

All electrophysiological data were obtained using a HEKA EPC10 double amplifier (HEKA Elektronik) filtered at 2 kHz, and analyzed with open-source scripts developed by Eugene Mosharov (http://sulzerlab.org/Quanta_Analysis_8_20.ipf) in Igor Pro 7 (Wavemetrics). All imaging data were analyzed in ImageJ software. Each set of data represents the mean ± SEM of an indicated number (n) of animals. To analyze mEPSCs and mIPSCs, a 4pA peak threshold was preset, above which release events are clearly distinguished from background noise. The analyzed results were re-checked by eye to ensure that the release events were accurately selected.

Statistical analysis

All data were statistically analyzed in Prism 8 software. Normality distribution of the data was determined by the D'Agostino-Pearson normality test. When the data followed a normal distribution, an unpaired student's t-test (two-tailed) or one-way ANOVA was used to evaluate the statistical significance. In other cases, a Mann-Whitney test or one-way ANOVA following Kruskal-Wallis test was used.

Data Availability

Data generated or analysed during this study are included in this published article or available upon request.

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Conflict of Interest Statement

The authors declare no competing interests.

Figure legend

Figure 1. LIN-2 and FRM-3 are required to maintain locomotory behaviour. (A) Genomic structure of *lin-2* and *frm-3* locus, mutant allele characterization, and protein architecture for LIN-2 and FRM-3. The whole promoter region of *lin-2a* and part of the kinase domain of LIN-2A is deleted in the *e1309* mutants. The FERM domain in both *frm-3a* and *frm-3b* is deleted in the *gk585* mutants. *Syb1019* and *syb1036* are stop codons in *lin-2* and *frm-3* that inactivate the expression of *lin-2a* and *lin-2b*, and *frm-3a* and *frm-3b*. (E, F) Locomotion speed is reduced by the loss of LIN-2 and FRM-3. Representative trajectories of locomotion in wild type (E) and mean locomotion speed in wild type, *lin-2(e1309)*, *frm-3(gk585)*, *lin-2(e1309);frm-3(gk585)*, *lin-2(syb1019)*, and *frm-3(syb1036)* mutants. Data are mean ± SEM (**, $p < 0.01$, ***, $p < 0.001$ when compared to wild type; n.s., non-significant; one-way ANOVA). The number of worms analyzed for each genotype is indicated in the bar.

Figure 2. Cholinergic synaptic transmission is dramatically decreased in *lin-2* and *frm-3* mutants. (A, B) Representative traces of mEPSCs and quantification of mEPSC frequency and amplitude from the indicated genotypes, including wild type, *lin-2(e1091)*, *frm-3(gk585)*, *lin-2(e1091);frm-3(gk585)*, *lin-2*^{null}, and *frm-3*^{null} mutants. (C, D) Example traces of mIPSCs and summary of mIPSC frequency and amplitude from the same genotypes in A. (E, F) Evoked EPSC traces and quantification of EPSC amplitude and charge transfer. (G) The charge transfer in a single animal is calculated by averaging all individual events in a mEPSC trace. (H) Quantal content in wild type, *lin-2*^{null}, and *frm-3*^{null} mutants. Quantal content is obtained by calculating the ratio of evoked EPSC charge transfer to the averaged mEPSC charge transfer in the same animal. Data are mean ± SEM (*, $p < 0.05$, ***, $p < 0.001$ when compared to wild type; n.s., non-significant; one-way ANOVA). The number of worms analyzed for each genotype is indicated in the bar.

Figure S1. Cholinergic synaptic transmission is normal in *lin-7* and *lin-10* mutants. mEPSCs and evoked EPSCs were recorded in lin-7 and lin-10 mutants. Representative traces of mEPSCs and evoked EPSCs (A, C), and mean mEPSC frequency and amplitude, and evoked EPSC amplitude and charge transfer are shown. Data are mean ± SEM. The number of worms analyzed for each genotype is indicated in the bar.

Figure 3. Conditional knockouts of *lin-2* and *frm-3* in muscle reduce cholinergic transmission. (A) The STOP cassette is inserted into the common intron region in *lin-2a* and *lin-2b*, and in *frm-3a* and *frm-3b*. The presence of CRE inverts the STOP cassette to inactivate expression of *lin-2* and *frm-3* isoforms. (B) Example traces of mEPSCs from control (no expression of CRE), neuron KO (expressing CRE in all neurons driven by the *sbt-1* promoter, $\text{Neuron}^{\text{Cre}}$), and muscle KO (expressing CRE in body wall muscles driven by the *myo-3* promoter $\text{Muscle}^{\text{Cre}}$) of *lin-2*. (C) Quantification of mEPSC frequency, amplitude, decay, and charge in B. (D) Evoked EPSC traces from control, *lin-2* neuron KO and muscle KO. (E) Quantification of evoked EPSC amplitude, charge transfer, and quantal content. (F, G) mEPSC traces and averaged mEPSC frequency, amplitude, decay and charge from control, *frm-3* neuron KO, and *frm-3* muscle KO. (H, I) Evoked EPSCs and summary of EPSC amplitude, charge transfer, and quantal content from control and *frm-3* conditional KO mutants. Data are mean ± SEM (**, $p < 0.01$, ***, $p < 0.001$ when compared to control; one-way ANOVA). The number of worms analyzed for each genotype is indicated in the bar.

Figure 4. LIN-2 and FRM-3 regulate the synaptic abundance but not surface expression level of AChRs. (A-D) (A-D) ACR-16::GFP and UNC-29::RFP synaptic abundance were decreased in $lin\text{-}2^{null}$ and $frm\text{-}3^{null}$ mutants. Representative images (A, C, scale bar 5 μm) and

mean puncta intensity (B, D) are shown. (E-H) ACh- and Levamisole-activated currents were unaltered in *lin-2*^{null} and *frm-3*^{null} mutants. Representative traces (E, G) and mean current amplitude (F, H) are shown. Data are mean ± SEM (***, $p < 0.001$ when compared to control; one-way ANOVA). The number of worms analyzed for each genotype is indicated in the bar.

Figure S2. GABA-evoked currents are reduced in *lin-2*^{null} but not *frm-3*^{null} mutants. GABA-activated currents were reduced by 50% in *lin-2*^{null}, whereas were not altered in *frm-3*^{null} mutants. Representative traces (A) and mean current amplitude (B) are shown. Data are mean ± SEM (***, $p < 0.001$ when compared to control; one-way ANOVA). The number of worms analyzed for each genotype is indicated in the bar.

Figure 5. Ultrastructural analysis of *lin-2* and *frm-3* mutants. (A) Representative micrographs of wild-type control, *lin-2* mutant, and *frm-3* mutant synaptic profiles. Arrows indicate the dense projection, which is also labelled by the red line. The terminal area is labelled by the purple line. Docked SVs on the dense projection are labelled by the green circle. Scale bar = 200 nm. (**B**) Quantification of synaptic terminal area (measured in mm^2) and total number of SVs. Data are mean ± SEM.

Figure 6. Presynaptic SV abundance and priming are normal in *lin-2* and *frm-3* mutants. (A-D) Expression levels of UNC-57::mCherry and UNC-17::mKate2, two SV markers, were unchanged in *lin-2*^{null} and *frm-3*^{null} mutants. Representative images (A, C, scale bar 10 µm) and mean puncta intensity (B, D) are shown. The wild type is normalized to 1. (E, F) SV priming was measured by recording hypertonic sucrose-evoked currents. Representative traces (E) and mean current amplitude (F) were shown. The currents were

reduced due to the defects in AChRs. Quantal content was normal in both *lin-2* and *frm-3* mutants. Data are mean ± SEM (*, $p < 0.05$, n.s., non-significant when compared to wild type; unpaired student's t-test for B and D, one-way ANOVA with for F). The number of worms analyzed for each genotype is indicated in the bar.

Figure 7. LIN-2/CASK binds to both ACR-16 and UNC-29 through SH3 domain. (A) Summary of interactions by Yeast two-hybrid. Strong interaction （++); weak interaction (+), and no interactions (-) were indicated. (B) LIN-2A's SH3 domain binds the ACR-16's second intracellular loop (LoopII) in a Yeast two-hybrid assay. Y2HGold cells carrying indicated plasmids (Left) growing on selective media (-Trp/-Leu/-His/-Ade) is shown (Right). (C) LIN-2A's SH3 domain binds the UNC-29's second intracellular loop (LoopII) in the Yeast two-hybrid assay. (D-E) FRM-3 do not bind the ACR-16's second intracellular loop (LoopII) (D) and UNC-29's second intracellular loop (LoopII) (E) in the Yeast two-hybrid assay. (F-G) LIN-2A binds FRM-3 (F) and its FERM domain (G) requiring its PDZ domain, but not SH3 domain.

Figure 8. The SH3 and PDZ domains are indispensable for LIN-2 function. (A) The SH3 domain and the PDZ domain are deleted in the endogenous *lin-2* gene by crispr. (B-F) Cholinergic synaptic transmission was severely reduced in *lin-2* ΔSH3 and ΔPDZ mutants. Representative traces of mEPSCs (E) and evoked EPSCs (F), and quantification of mEPSC frequency and amplitude (C), evoked EPSC amplitude (E), charge transfer, and quantal content (F) are shown. Data are mean ± SEM (***, $p < 0.001$ when compared to wild type; one-way ANOVA). The number of worms analyzed for each genotype is indicated in the bar.

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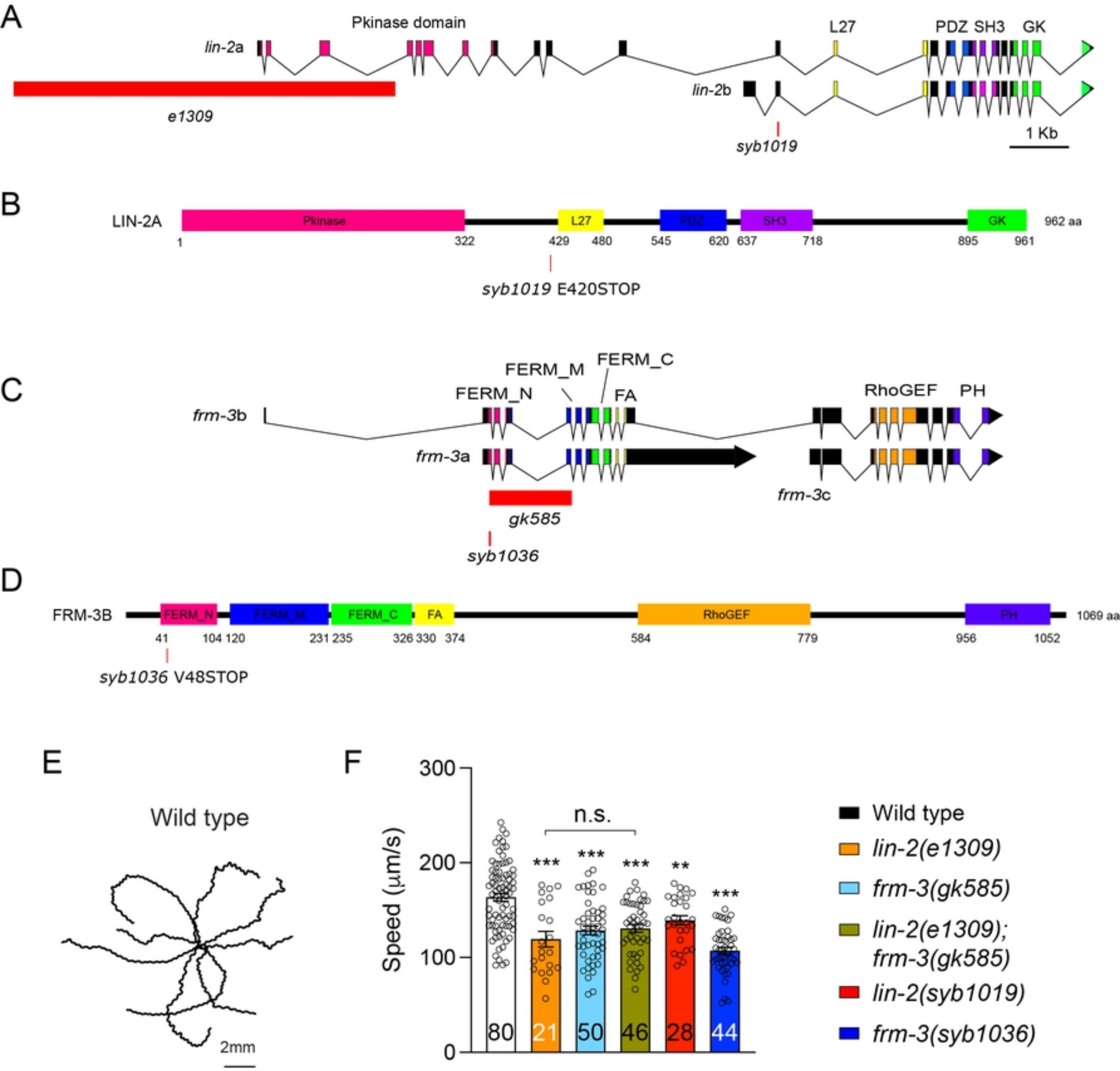

A
Pkinase domain
L27
PDZ SH3 GK
lin-2a
lin-2b
e1309
syb1019
1 Kb
B
LIN-2A
Pkinase
L27
PDZ
SH3
GK
962 aa
1
322
429
480
545
620
637
718
895
961
syb1019 E420STOP
C
FERM_M
FERM_C
FERM_N
FA
RhoGEF
PH
frm-3b
frm-3a
frm-3c
gk585
syb1036
D
FRM-3B
FERM_N
FERM_M
FERM_C
FA
RhoGEF
PH
1069 aa
41
104 120
231 235
326 330
374
584
779
956
1052
syb1036 V48STOP
E
Wild type
2mm
F
Speed (µm/s)
300
200
100
0
n.s.
***
***
***
**
***
80
21
50
46
28
44
Wild type
lin-2(e1309)
frm-3(gk585)
lin-2(e1309);
frm-3(gk585)
lin-2(syb1019)
frm-3(syb1036)

Figure 1

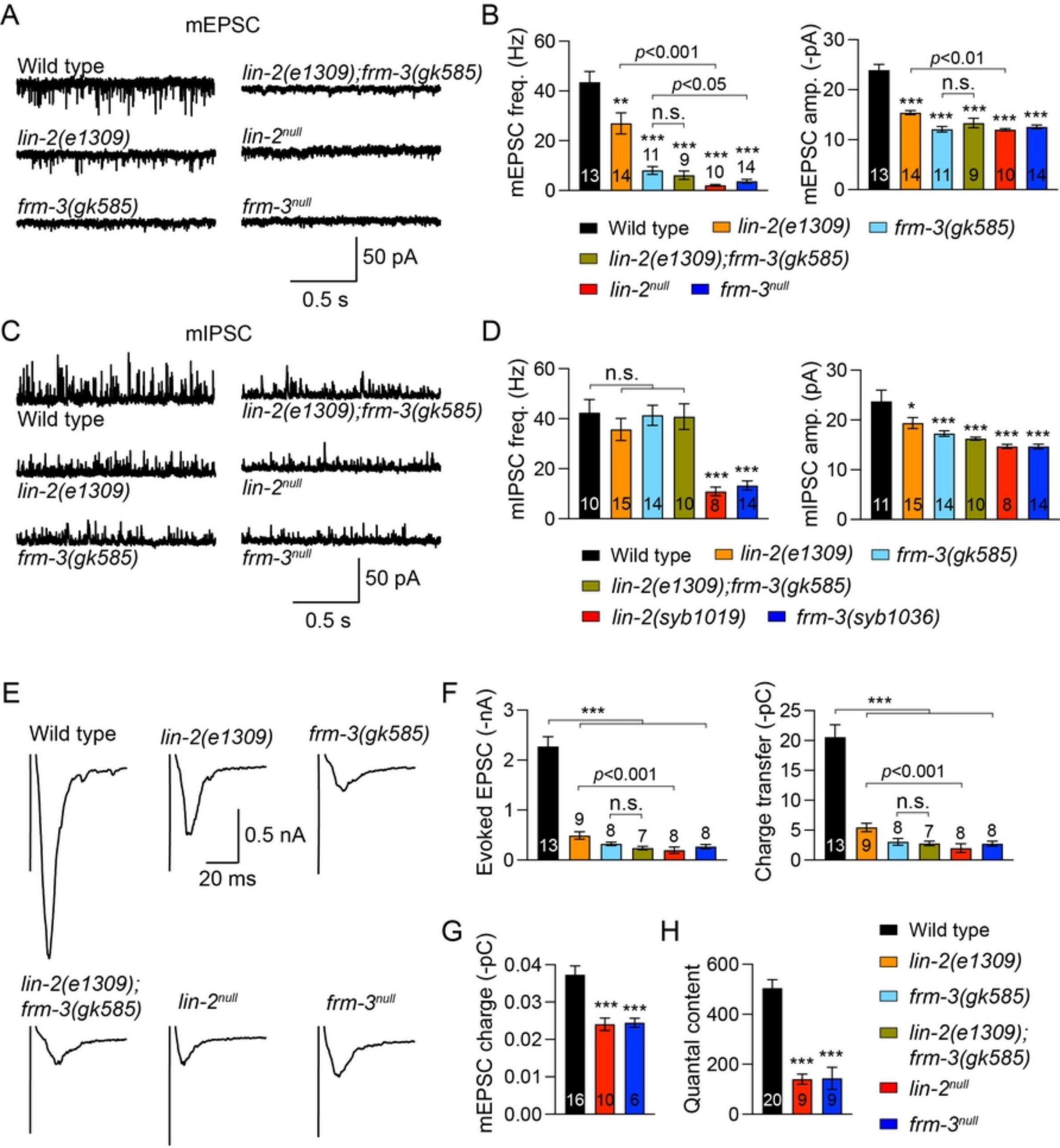

A
mEPSC
Wild type
lin-2(e1309);frm-3(gk585)
lin-2(e1309)
lin-2null
frm-3(gk585)
frm-3null
50 pA
0.5 s
B
mEPSC freq. (Hz)
p<0.001
p<0.05
n.s.
mEPSC amp. (-pA)
p<0.01
n.s.
Wild type
lin-2(e1309)
frm-3(gk585)
lin-2(e1309);frm-3(gk585)
lin-2null
frm-3null
C
mIPSC
Wild type
lin-2(e1309);frm-3(gk585)
lin-2(e1309)
lin-2null
frm-3(gk585)
frm-3null
50 pA
0.5 s
D
mIPSC freq. (Hz)
n.s.
mIPSC amp. (pA)
Wild type
lin-2(e1309)
frm-3(gk585)
lin-2(e1309);frm-3(gk585)
lin-2(syb1019)
frm-3(syb1036)
E
Wild type
lin-2(e1309)
frm-3(gk585)
0.5 nA
20 ms
lin-2(e1309);
frm-3(gk585)
lin-2null
frm-3null
F
Evoked EPSC (-nA)
p<0.001
n.s.
Charge transfer (-pC)
p<0.001
n.s.
G
mEPSC charge (-pC)
H
Quantal content
Wild type
lin-2(e1309)
frm-3(gk585)
lin-2(e1309);
frm-3(gk585)
lin-2null
frm-3null

Figure 2

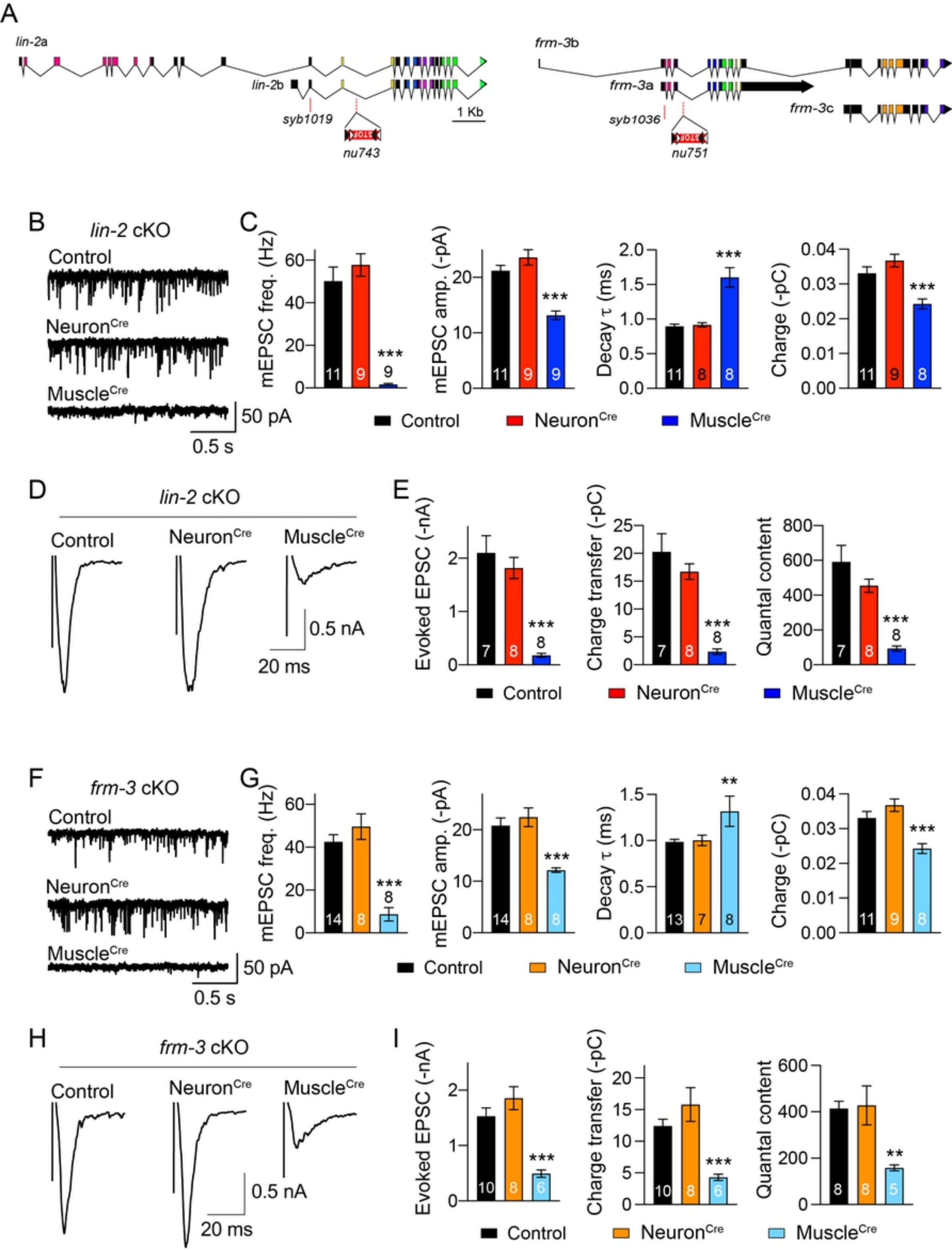

A
lin-2a
lin-2b
syb1019
nu743
1 Kb
frm-3b
frm-3a
frm-3c
syb1036
nu751
B
lin-2 cKO
Control
NeuronCre
MuscleCre
50 pA
0.5 s
C
mEPSC freq. (Hz)
mEPSC amp. (-pA)
Decay τ (ms)
Charge (-pC)
Control
NeuronCre
MuscleCre
D
lin-2 cKO
Control
NeuronCre
MuscleCre
0.5 nA
20 ms
E
Evoked EPSC (-nA)
Charge transfer (-pC)
Quantal content
Control
NeuronCre
MuscleCre
F
frm-3 cKO
Control
NeuronCre
MuscleCre
50 pA
0.5 s
G
mEPSC freq. (Hz)
mEPSC amp. (-pA)
Decay τ (ms)
Charge (-pC)
Control
NeuronCre
MuscleCre
H
frm-3 cKO
Control
NeuronCre
MuscleCre
0.5 nA
20 ms
I
Evoked EPSC (-nA)
Charge transfer (-pC)
Quantal content
Control
NeuronCre
MuscleCre

Figure 3

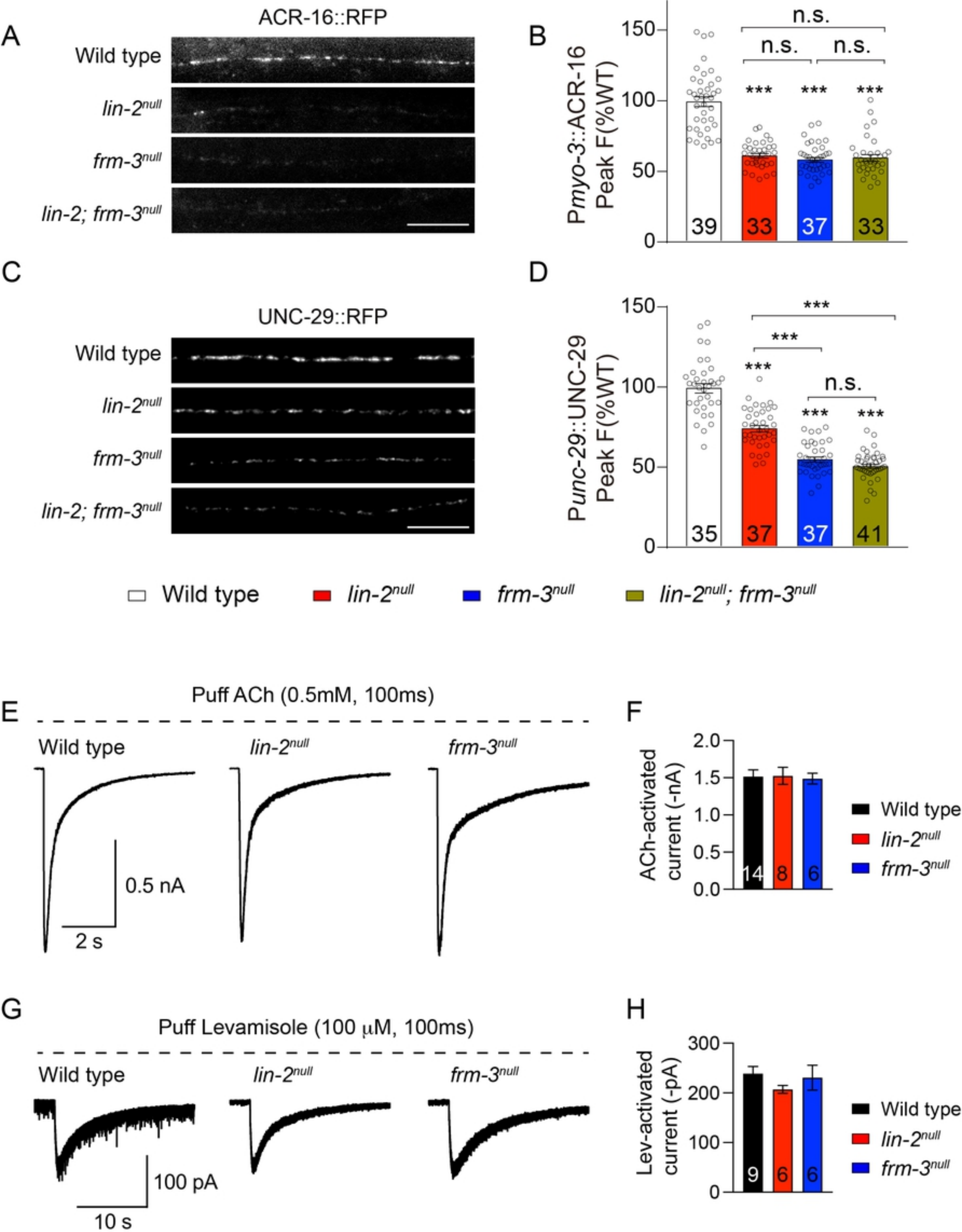

A
ACR-16::RFP
Wild type
lin-2null
frm-3null
lin-2; frm-3null
B
Pmyo-3::ACR-16 Peak F(%WT)
150
100
50
0
n.s.
n.s.
n.s.
***
***
***
39
33
37
33
C
UNC-29::RFP
Wild type
lin-2null
frm-3null
lin-2; frm-3null
D
Punc-29::UNC-29 Peak F(%WT)
150
100
50
0
***
***
***
n.s.
***
***
35
37
37
41
Wild type
lin-2null
frm-3null
lin-2null; frm-3null
E
Puff ACh (0.5mM, 100ms)
Wild type
lin-2null
frm-3null
0.5 nA
2 s
F
ACh-activated current (-nA)
2.0
1.5
1.0
0.5
0.0
14
8
6
Wild type
lin-2null
frm-3null
G
Puff Levamisole (100 μM, 100ms)
Wild type
lin-2null
frm-3null
100 pA
10 s
H
Lev-activated current (-pA)
300
200
100
0
9
6
6
Wild type
lin-2null
frm-3null

Figure 4

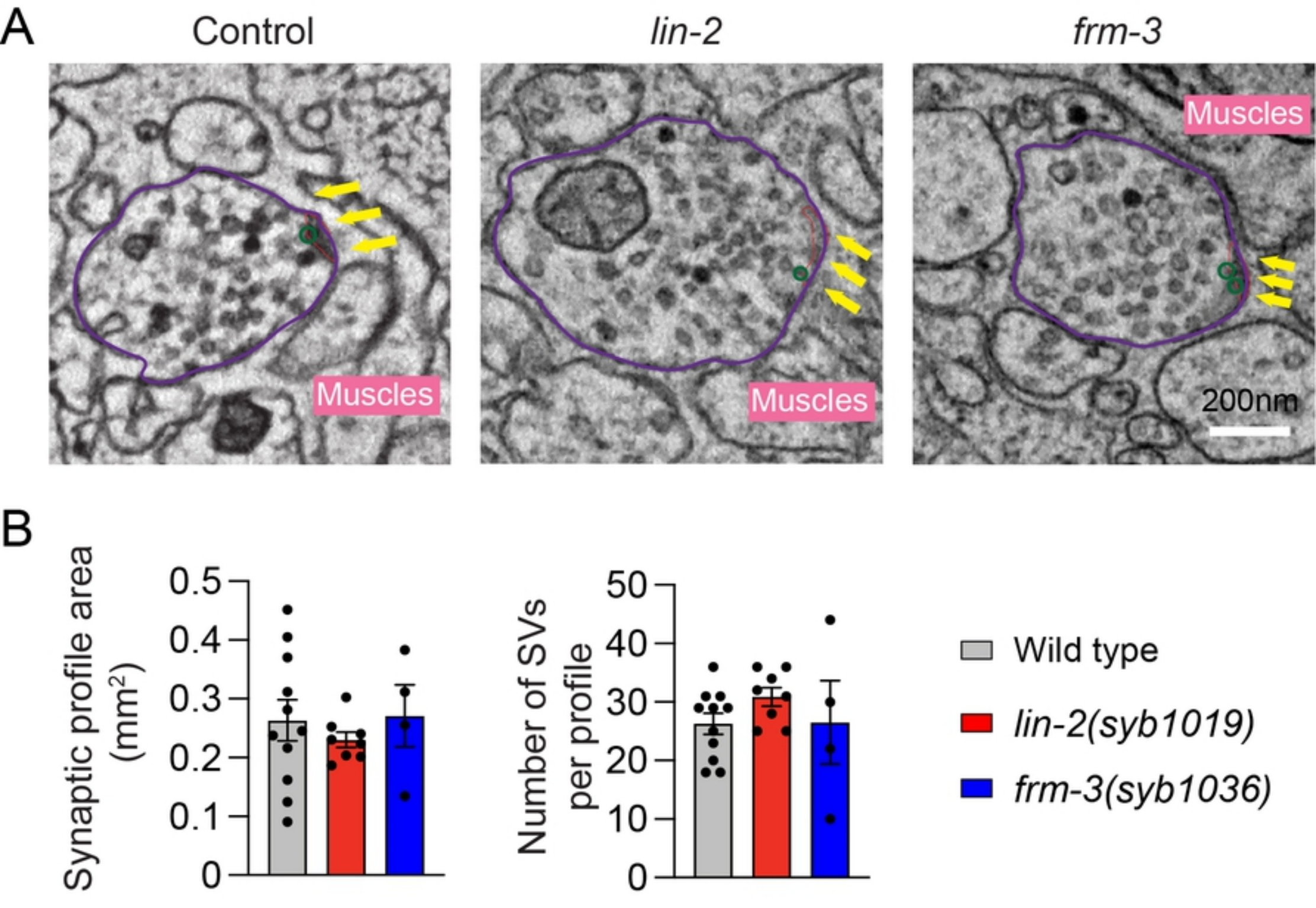

A
Control
lin-2
frm-3
Muscles
Muscles
Muscles
200nm
B
Synaptic profile area (mm²)
0.5
0.4
0.3
0.2
0.1
0
Number of SVs per profile
50
40
30
20
10
0
Wild type
lin-2(syb1019)
frm-3(syb1036)

Figure 5

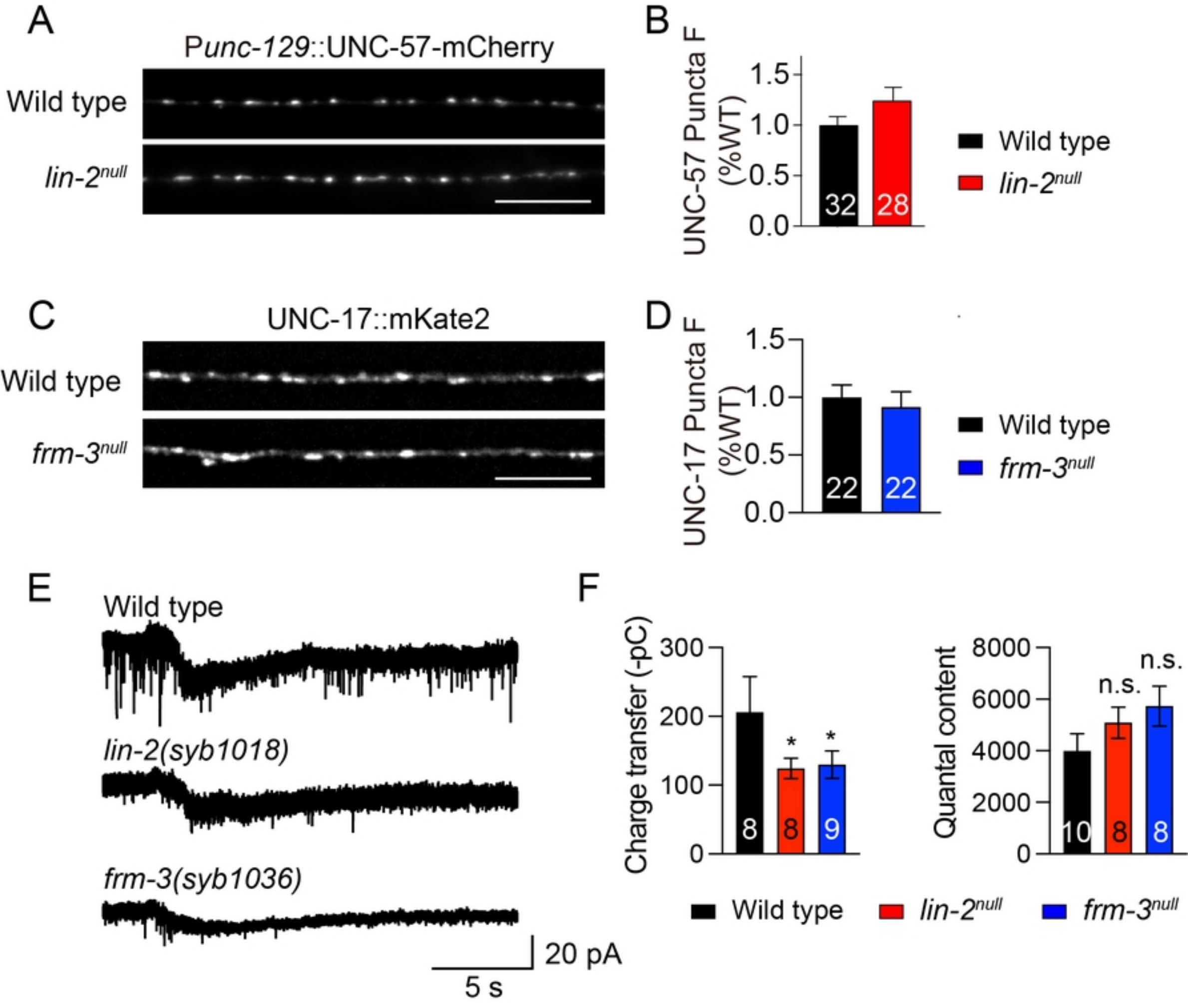

A
Punc-129::UNC-57-mCherry
Wild type
lin-2null
B
UNC-57 Puncta F (%WT)
1.5
1.0
0.5
0.0
32
28
Wild type
lin-2null
C
UNC-17::mKate2
Wild type
frm-3null
D
UNC-17 Puncta F (%WT)
1.5
1.0
0.5
0.0
22
22
Wild type
frm-3null
E
Wild type
lin-2(syb1018)
frm-3(syb1036)
20 pA
5 s
F
Charge transfer (-pC)
300
200
100
0
*
*
8
8
9
Quantal content
8000
6000
4000
2000
0
n.s.
n.s.
10
8
8
Wild type
lin-2null
frm-3null

Figure 6

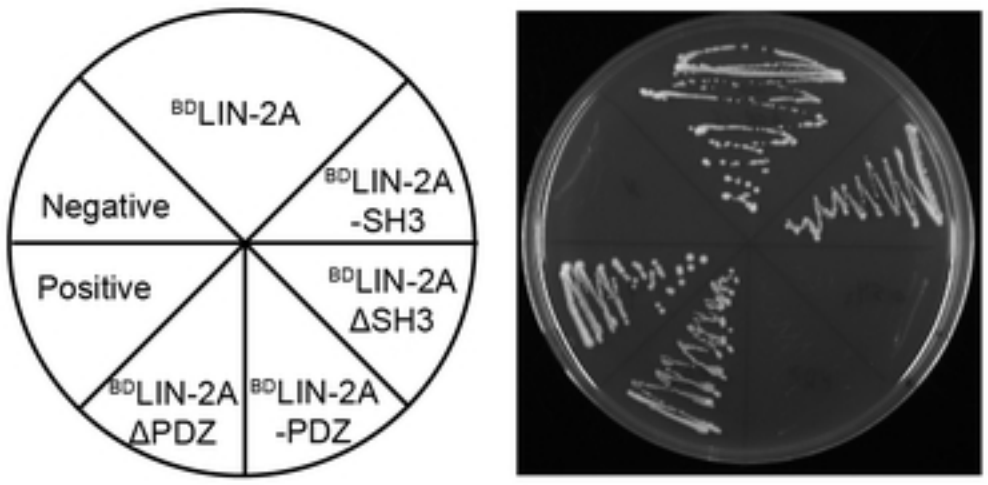
A

Yeast two-hybrid assay

	^{BD}FRM-3	^{BD}FERM	^{BD}LIN-2A	^{BD}LIN-2A-SH3	^{BD}LIN-2AΔSH3	^{BD}LIN-2A-PDZ	^{BD}LIN-2AΔPDZ
^{AD}ACR-16 LoopII	-	-	++	++	-	-	++
^{AD}UNC-29 LoopII	-	-	+	+	-	-	+
^{AD}FRM-3			++	-	++	-	-
^{AD}FERM			++	-	++	-	-

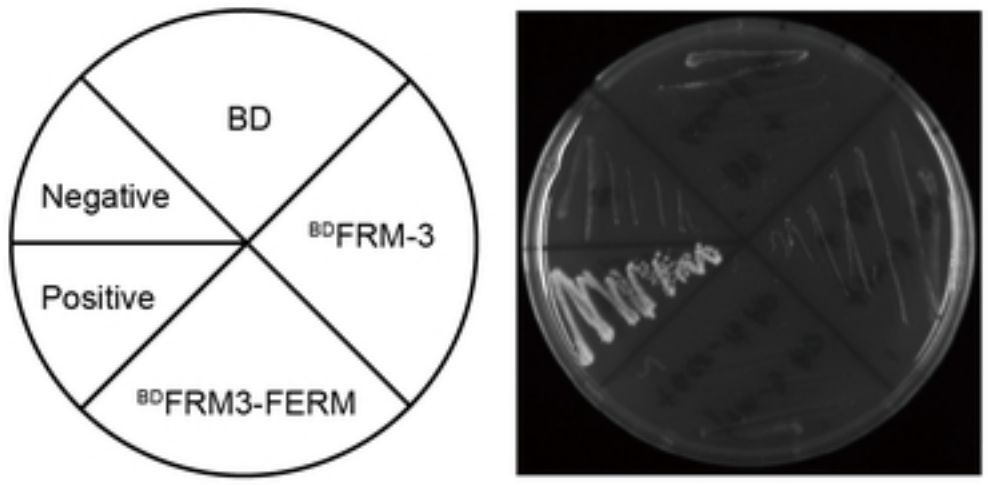
B

^{AD}ACR-16 LoopII and ^{BD}LIN-2A



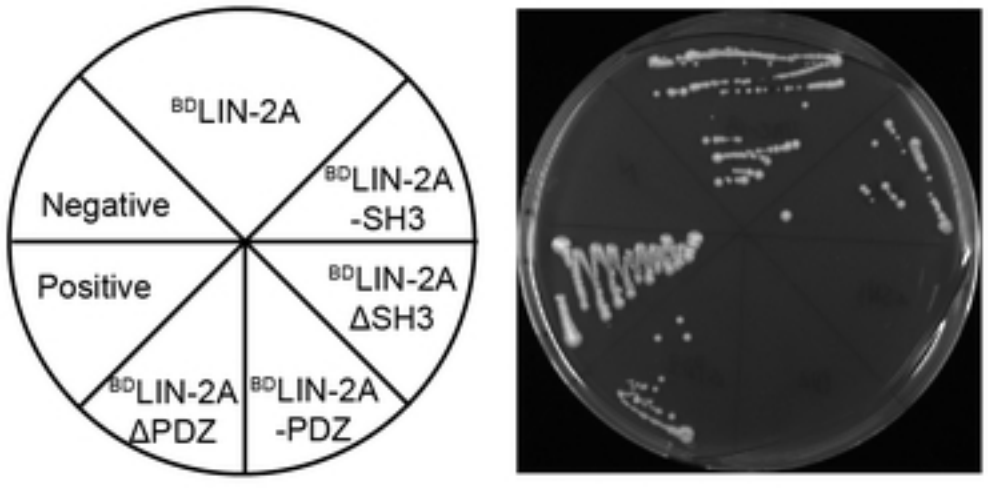
C

^{AD}ACR-16 LoopII and ^{BD}FRM-3



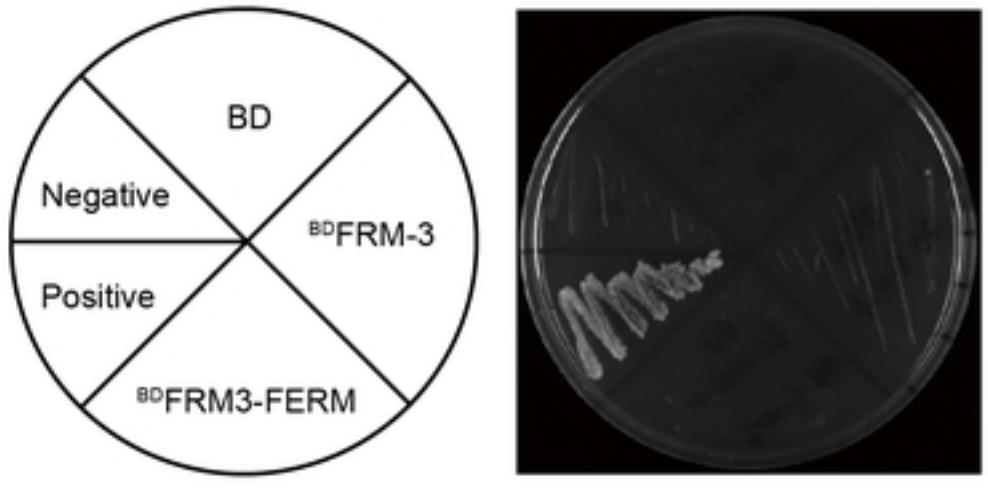
D

^{AD}UNC-29 LoopII and ^{BD}LIN-2A



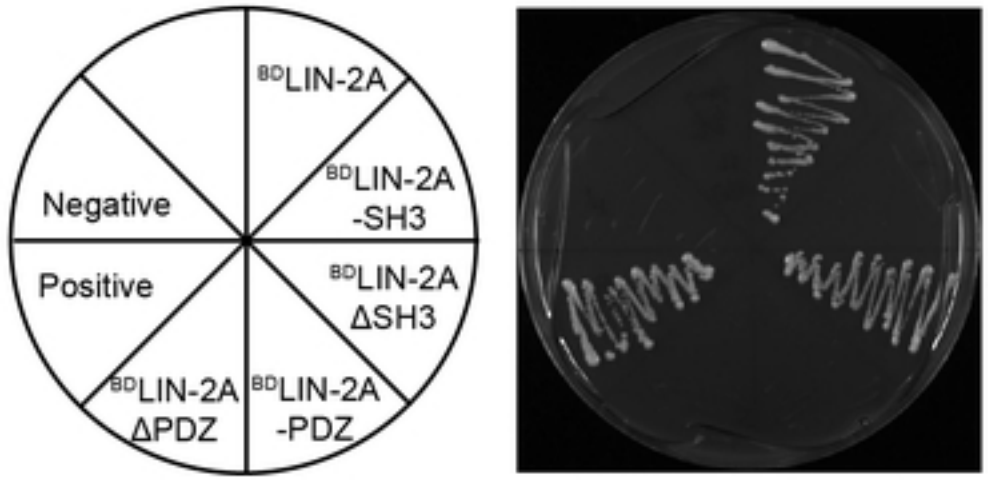
E

^{AD}UNC-29 LoopII and ^{BD}FRM-3



F

^{AD}FRM-3 and ^{BD}LIN-2A



G

^{AD}FERM and ^{BD}LIN-2A

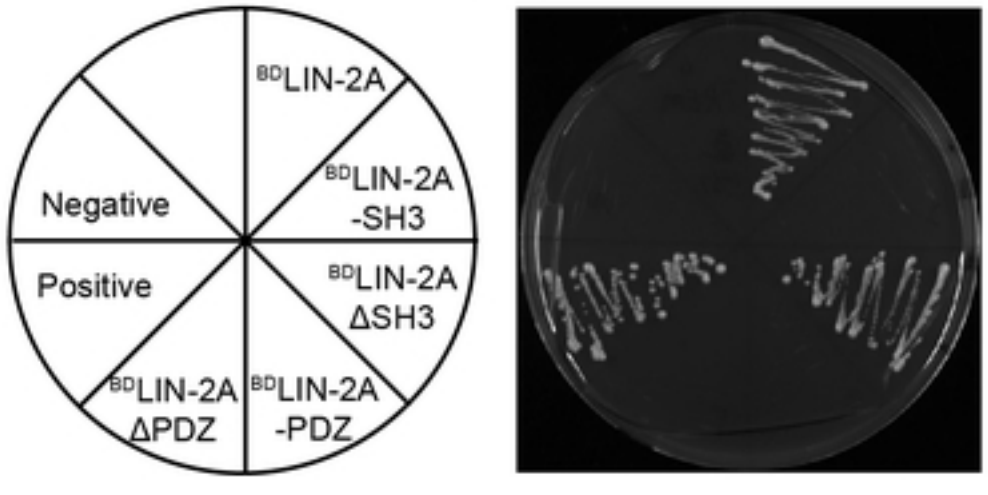


Figure 7

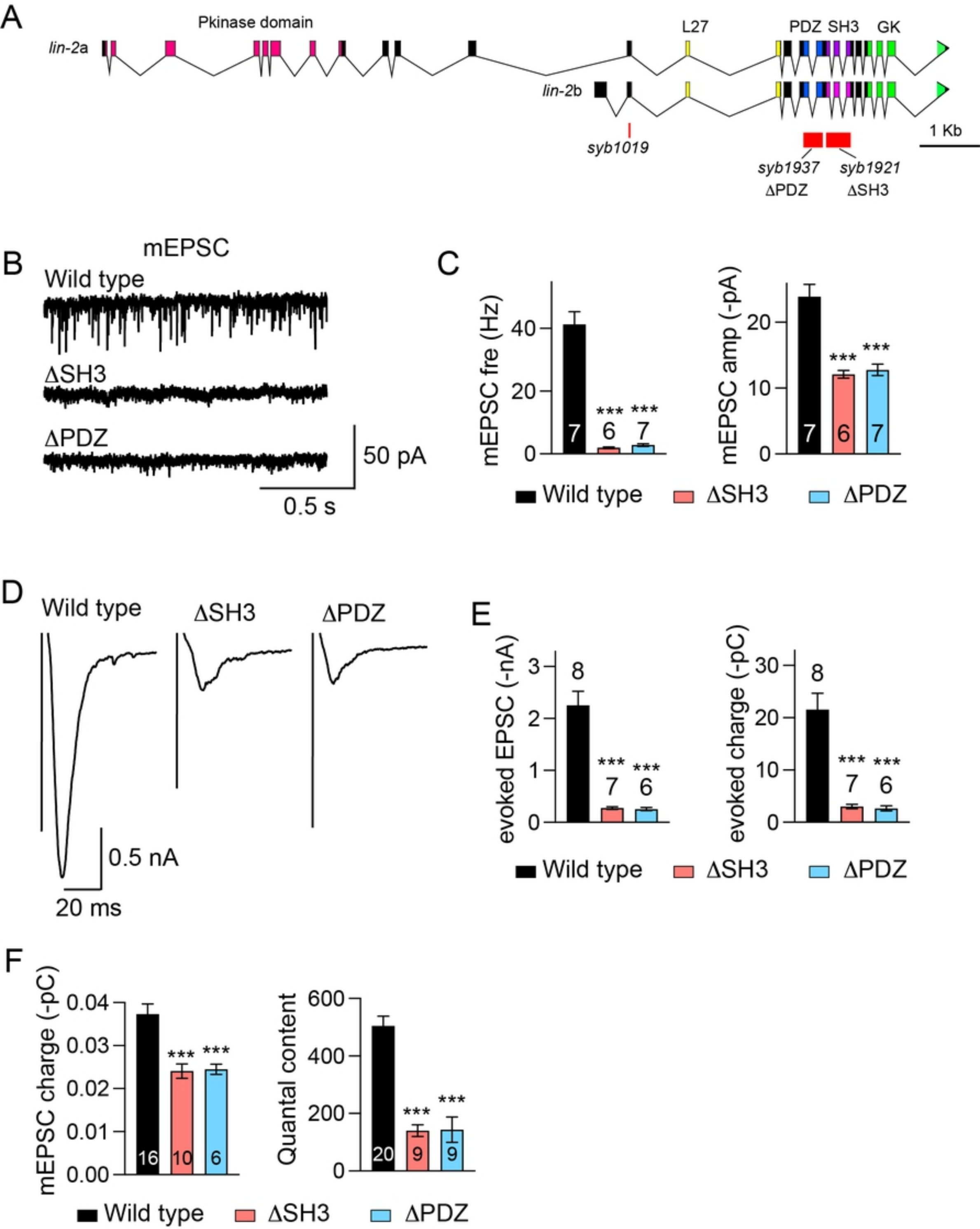

A
Pkinase domain
lin-2a
L27
PDZ SH3 GK
lin-2b
syb1019
1 Kb
syb1937
ΔPDZ
syb1921
ΔSH3
B
mEPSC
Wild type
ΔSH3
ΔPDZ
50 pA
0.5 s
C
mEPSC fre (Hz)
mEPSC amp (-pA)
Wild type
ΔSH3
ΔPDZ
D
Wild type
ΔSH3
ΔPDZ
0.5 nA
20 ms
E
evoked EPSC (-nA)
evoked charge (-pC)
Wild type
ΔSH3
ΔPDZ
F
mEPSC charge (-pC)
Quantal content
Wild type
ΔSH3
ΔPDZ

Figure 8