

Distinct active zone protein machineries mediate Ca^{2+} channel clustering and vesicle priming at hippocampal synapses

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Action potentials trigger neurotransmitter release at the presynaptic active zone with spatiotemporal precision. This is supported by protein machinery that mediates synaptic vesicle priming and clustering of Ca_v2 Ca^{2+} channels nearby. One model posits that scaffolding proteins directly tether vesicles to Ca_v2 s; however, here we find that at mouse hippocampal synapses, Ca_v2 clustering and vesicle priming are executed by separate machineries. Ca_v2 nanoclusters are positioned at variable distances from those of the priming protein Munc13. The active zone organizer RIM anchors both proteins but distinct interaction motifs independently execute these functions. In transfected cells, Liprin- α and RIM form co-assemblies that are separate from Ca_v2 -organizing complexes. At synapses, Liprin- $\alpha1$ –Liprin- $\alpha4$ knockout impairs vesicle priming but not Ca_v2 clustering. The cell adhesion protein PTP σ recruits Liprin- α , RIM and Munc13 into priming complexes without co-clustering Ca_v2 s. We conclude that active zones consist of distinct machineries to organize Ca_v2 s and prime vesicles, and Liprin- α and PTP σ specifically support priming site assembly.

The active zone is a protein scaffold that enables neurotransmitter release. It mediates synaptic vesicle priming to add vesicles to the readily releasable pool and the clustering of voltage-gated Ca^{2+} channels of the Ca_v2 family^{1,2}. Work over the past decades has generated essential insights into how active zone functions are executed. Synaptic vesicle priming depends on the recruitment of Munc13 by the active zone organizer RIM, and Munc13 supports the subsequent assembly of the exocytic SNARE complex^{3–5}. The clustering of Ca_v2 s is also orchestrated by RIM in conjunction with RIM-BP through tripartite interactions^{6–10}. At least three additional protein families further sustain active zone functions. First, ELKS is important for Ca^{2+} influx and for the generation of primed vesicles^{11–14}. Second, Piccolo and Bassoon are long-range tethers, and Bassoon contributes to Ca_v2 clustering via RIM-BP^{15–17}. Third, Liprin- α recruits presynaptic material and may act upstream in active zone assembly^{18–23}. This groundwork has led to mechanistic models for

vesicle priming and Ca_v2 clustering but it is still unclear how the active zone is organized to maintain these two functions simultaneously.

One relatively simple model suggests that vesicle priming and Ca_v2 clustering are executed by a single complex containing all active zone proteins. This is rooted in the observation that most active zone proteins interact with one another and contribute to both functions^{3,6,8–10,13,14,17,24,25}. Furthermore, Ca_v2 s might directly tether vesicles within nerve terminals^{26–28}. The findings that vesicles are recruited to reconstituted condensates consisting of RIM, RIM-BP and Ca_v2 (ref. 29) and that artificially tethering the RIM priming activity to Ca_v2 s effectively restores synaptic release³⁰ provide additional support for a ‘single complex’ model. However, active zone organization might be more complex. The coupling distance between Ca_v2 s and releasable vesicles is variable and sometimes up to 100 nm^{31–33}, modeling indicates that the exocytic reaction is best explained by positioning Ca_v2 s in the

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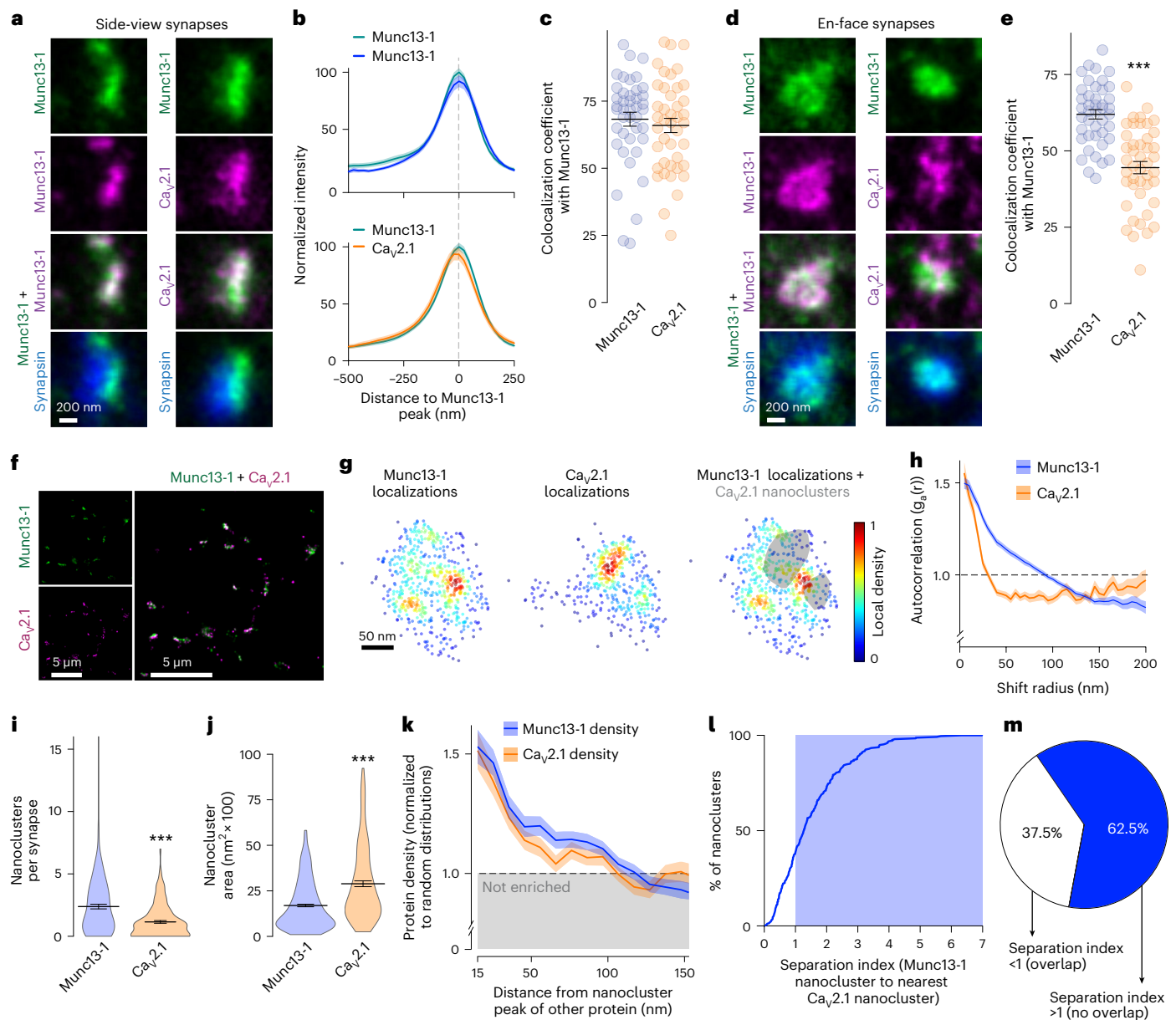


Fig. 1 | Munc13-1 and Ca_v2.1 nanoclustering. **a–c**, Example STED images of side-view synapses (**a**), quantification of line profiles (**b**) and Pearson's colocalization coefficients (**c**). Cultured hippocampal neurons stained with antibodies against Synapsin and Munc13-1, and a second Munc13-1 antibody or a Ca_v2.1 antibody. Side-view synapses were identified by the presence of an elongated Munc13-1 object (shared Munc13-1 antibody) at the edge of the Synapsin-positive vesicle cloud. A line profile (750 nm × 250 nm) was positioned perpendicular to the Munc13-1 center, and signals were aligned to the Munc13-1 peak in the averaged line profiles (**b**); Munc13-1, 42 synapses/3 cultures; Ca_v2.1, 42/3. **d, e**, Same as **a–c**, but for en-face synapses identified by the presence of a Munc13-1 area (shared Munc13-1 antibody) surrounded by Synapsin and without line profiles. Munc13-1, 43/3; Ca_v2.1, 44/3. **f**, Zoomed-out, rendered DNA Exchange-PAINT images after Munc13-1 and Ca_v2.1 staining. **g**, Zoomed-in view of the density of protein localizations in an example en-face synapse imaged with DNA Exchange-PAINT showing Munc13-1, Ca_v2.1 and Munc13-1 with shaded Ca_v2.1 nanoclusters identified with a density-based clustering algorithm. **h**, Autocorrelation of

Munc13-1 and Ca_v2.1 synaptic densities measured as the average probability of detecting localizations for the same protein at increasing distances from any given localization. Munc13-1, 147 synapses/3 cultures; Ca_v2.1, 147/3. **i**, Quantification of the number of Munc13-1 and Ca_v2.1 nanoclusters per synapse. Nanoclusters were identified using a density-based clustering algorithm; 147/3. **j**, Quantification of nanocluster area; Munc13-1, 350 nanoclusters/3 cultures; Ca_v2.1, 169/3. **k**, Density of Ca_v2.1 localizations at various distances from the peak of Munc13-1 nanoclusters and vice versa, normalized to a spatially homogenous distribution. Ca_v2.1 from 350/3, Munc13-1 from 169/3. **l, m**, Histogram showing the separation index of Munc13-1 nanoclusters to the nearest Ca_v2.1 nanocluster (**l**) and pie chart (**m**) showing the percentage of clusters that show any overlap (separation index of <1) or no overlap (>1); *n* as in **j**. Data are mean ± s.e.m.; ****P* < 0.001 compared to Munc13-1 determined by two-sided Student's *t*-test (in **e**) or two-sided Mann–Whitney *U*-tests (in **c**, **i** and **j**). For additional DNA Exchange-PAINT analyses, see Extended Data Fig. 1. For exact *P* values for this and all following figures, see Supplementary Table 1.

perimeter of release sites or by excluding them from these sites^{34,35} and the topography of Ca_v2s and vesicles changes as synapses mature³⁶. Some studies detect clusters of single active zone proteins that are separated from one another^{9,37,38}, suggesting assembly by self-clustering of individual proteins. Altogether, it has remained challenging to define

how the multiple active zone functions are related to the suborganization of its molecular components.

Liprin-α proteins might organize active zone assembly^{20–23,38}. They interact with RIM and ELKS^{39,40} and with LAR-type receptor protein tyrosine phosphatases (LAR-PTPs)^{41–43}. As LAR-PTPs are

transmembrane proteins, this provides for a plausible molecular axis to anchor active zone machineries to the presynaptic plasma membrane. In invertebrate and human-derived neurons, loss of function of Liprin- α results in loss of presynaptic material^{20,21,23,44}, and LAR-RPTPs probably function in the same pathway^{22,45}. At the fly neuromuscular junction, Liprin- α recruits a specific Munc13 isoform³⁸, and Liprin- α 2 and Liprin- α 3 also support Munc13 targeting at hippocampal synapses^{18,19}. In aggregate, this indicates that the Liprin- α –LAR-RPTP pathway may organize active zones.

Here, we show that Ca_v2 and Munc13 each form nanoclusters that are positioned at variable distances from one another at hippocampal synapses. RIM organizes Ca_v2s and Munc13, but it executes these functions through distinct protein complexes. In heterologous cells, Liprin- α recruits RIM into molecular condensates that co-exist with separate Ca_v2-organizing complexes. At synapses, quadruple knockout of Liprin- α 1 to Liprin- α 4 reduces RIM and Munc13 levels at the active zone and diminishes synaptic vesicle priming, but Ca_v2 clustering remains intact. In transfected cells and at synapses, PTPo tethers Liprin- α , RIM and Munc13, but Ca_v2 is not co-recruited into these complexes. We conclude that Ca_v2 clusters and vesicle priming sites are organized independently and that Liprin- α and PTPo at the active zone specifically support priming site assembly. Our work argues against a single-complex model and instead identifies at least two distinct active zone machineries with independent assembly mechanisms.

Results

Variable segregation of Munc13 and Ca_v2 nanoclusters

To dissect active zone organization, we first determined the subsynaptic localization of two of its essential components with immunostaining: Munc13-1, a protein that defines release sites by priming synaptic vesicles^{4,46,47}, and Ca_v2.1, a voltage-gated Ca²⁺ channel that is critical at most central synapses^{48–50}. We imaged the samples with stimulated emission depletion (STED) microscopy and identified synapses via Munc13-1 and the vesicle-associated protein Synapsin using an established workflow^{18,30,48,51}. At side-view synapses, Munc13-1 and Ca_v2.1 were restricted to the edge of the synaptic vesicle cloud and colocalized well, in line with their targeting of the presynaptic plasma membrane (Fig. 1a–c). To estimate their lateral distribution, we analyzed en-face synapses (Fig. 1d,e). The colocalization between Munc13-1 and Ca_v2.1 was partial and lower than that of the signals of two Munc13 antibodies. This confirms that Munc13-1 and Ca_v2.1 are both at the active zone (Fig. 1a–c) but they may have distinct nanoscale lateral distributions (Fig. 1d,e).

We next assessed the localization of these two proteins with single-molecule resolution using DNA Exchange-PAINT^{52–54}. En-face synapses containing Munc13-1 and Ca_v2.1 were selected and autocorrelation functions were used to assess their distributions within single synapses. These analyses revealed that local densities were

heterogeneous (Fig. 1f–h), which indicates nanoclustering⁵⁵. We then detected nanoclusters for each protein using density-based spatial clustering analyses⁵². Munc13-1 nanoclusters were present at a higher density than Ca_v2.1 nanoclusters and were smaller (Fig. 1i,j), with estimated cluster radii of ~23 nm for Munc13-1 and ~30 nm for Ca_v2.1. We next measured the enrichment of these proteins, assessing the density of one protein relative to the nanoclusters of the other. Ca_v2.1 density was high within the average radius of Munc13-1 nanoclusters, and vice versa (Fig. 1k), indicating some colocalization. However, a robust fraction of each protein was enriched at distances beyond the nanoclusters of the other protein. Overall, 43% of Munc13-1 nanoclusters were enriched with Ca_v2.1, and 61% of Ca_v2.1 nanoclusters were enriched with Munc13-1 (Extended Data Fig. 1a). For each protein, roughly half of the nanoclusters had either a density of the other protein that was indistinguishable from a randomized distribution or were even de-enriched of it, suggesting independent clustering. The distances of Munc13-1 nanoclusters to the closest Ca_v2.1 nanocluster were variable and often exceeded nanocluster size (Extended Data Fig. 1b). We next calculated a ‘separation index’. This index reveals nanocluster spatial relationships; it is <1 when a nanocluster overlaps with the closest nanocluster of the other protein and >1 when there is no overlap. Consistent with the enrichment measurements, 63% of Munc13-1 nanoclusters did not overlap with any Ca_v2.1 nanoclusters (Fig. 1l,m) and 47% of Ca_v2.1 nanoclusters showed no overlap with those of Munc13-1 (Extended Data Fig. 1c,d). We conclude that Munc13-1 and Ca_v2.1 have partially distinct distributions at the active zone, arguing against the model that these proteins are co-organized into single complexes.

Independent assembly of Ca_v2 clusters and priming machinery

The differences in the organization of Ca_v2 and Munc13 suggest separable pathways for the assembly of the underlying machineries. To assess this hypothesis, we studied whether assemblies for vesicle priming can be generated without recruiting Ca_v2 and vice versa, or whether these two pathways are inseparable.

Simultaneous deletion of RIM and ELKS (that is, RIM1 α β , RIM2 α β γ , ELKS1 α and ELKS2 α) results in active zone disassembly, loss of most Munc13 and impaired Ca_v2 clustering^{24,30}. We used this model to test whether restoring Ca_v2.1 clustering also restores Munc13-1 recruitment and vice versa. We cultured hippocampal neurons from RIM + ELKS conditional knockout mice and used lentiviral expression of Cre or an inactive, truncated Cre to generate quadruple RIM + ELKS knockout (cQKO^{R+E}) or control (control^{R+E}) neurons, respectively (Fig. 2a). In cQKO^{R+E} neurons, we used lentiviral expression of either RIM1 α (to restore both functions³⁰) or one of the two following complementary RIM fragments (Fig. 2b). The first, PDZ-C2A-P, consists of the PDZ domain that binds to Ca_v2, three PxxP motifs that interact with RIM-BP to cluster Ca_v2s and the C2A domain^{6–9,56}. The second, Zn-C2B, contains

Fig. 2 | Distinct RIM domains mediate Ca_v2 clustering and priming machinery assembly.

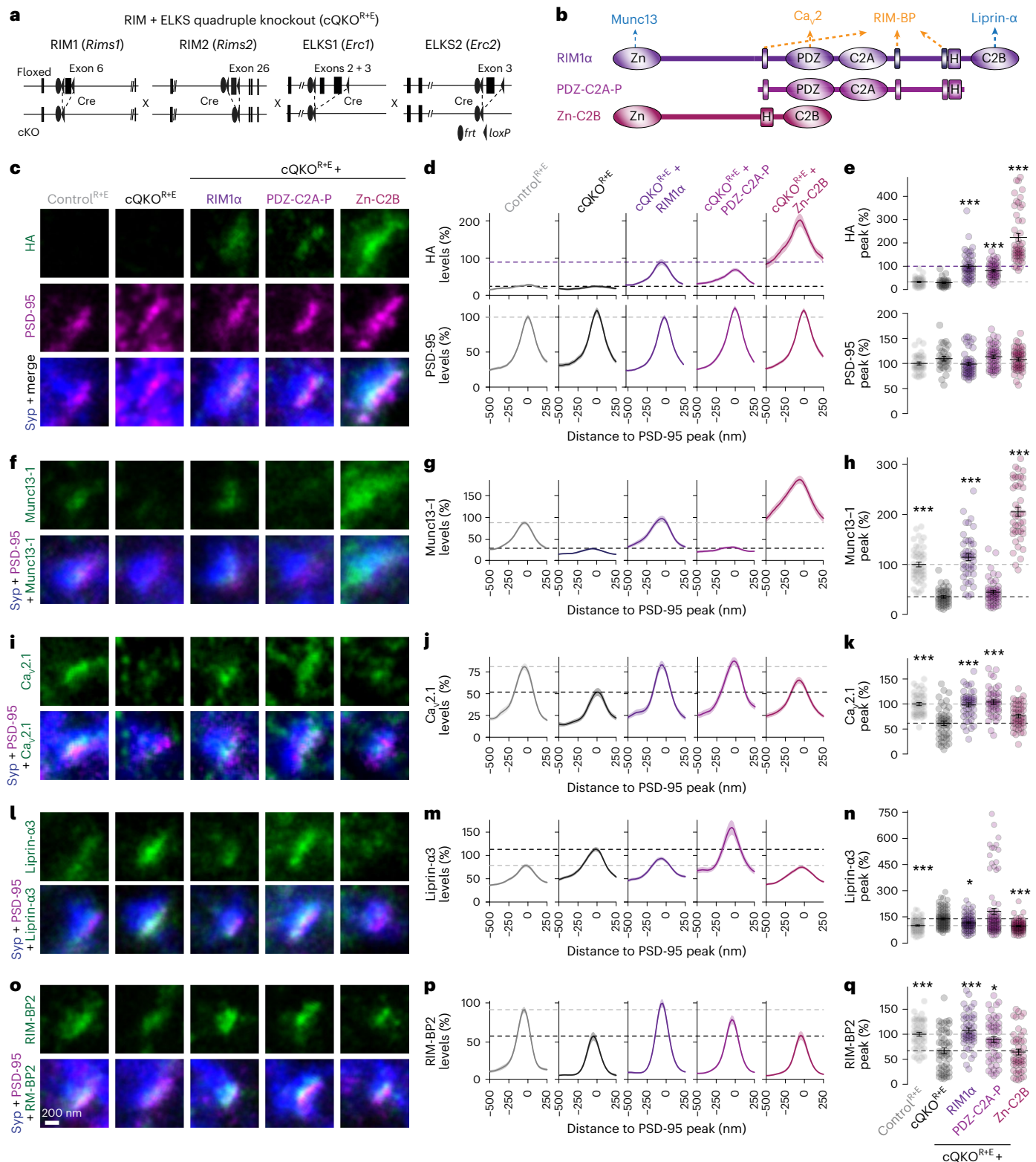
a, b, Schematics of the conditional RIM + ELKS knockout mice (from previous studies^{24,30,60}) (**a**) and of HA-tagged rescue constructs with key protein interactions (**b**). Cultured RIM + ELKS knockout neurons (cQKO^{R+E}; lentiviral expression of Cre) without or with expression of rescue constructs and corresponding control neurons (control^{R+E}; lentiviral expression of recombination-deficient Cre) were analyzed; H, HA tag; Zn, zinc finger domain; PDZ, PDZ domain; C2A and C2B, C2 domains; P, proline-rich motif. **c–q**, Example STED images, average profiles and quantification of the peak intensities of HA and PSD-95 (**c–e**), Munc13-1 (**f–h**), Ca_v2.1 (**i–k**), Liprin- α 3 (**l–n**) and RIM-BP2 (**o–q**) at excitatory side-view synapses identified by Synaptophysin (Synp) and PSD-95. Neurons were stained for a protein of interest (RIM1 α with HA, Munc13-1, Ca_v2.1, Liprin- α 3 or RIM-BP2; imaged in STED), PSD-95 (imaged in STED), and Synaptophysin (imaged in confocal). Signals were aligned via PSD-95 (in **d, g, j, m** and **p**) and the maximum value of each profile was plotted for statistical analyses (in **e, h, k, n** and **q**). Dotted lines mark levels of control^{R+E}

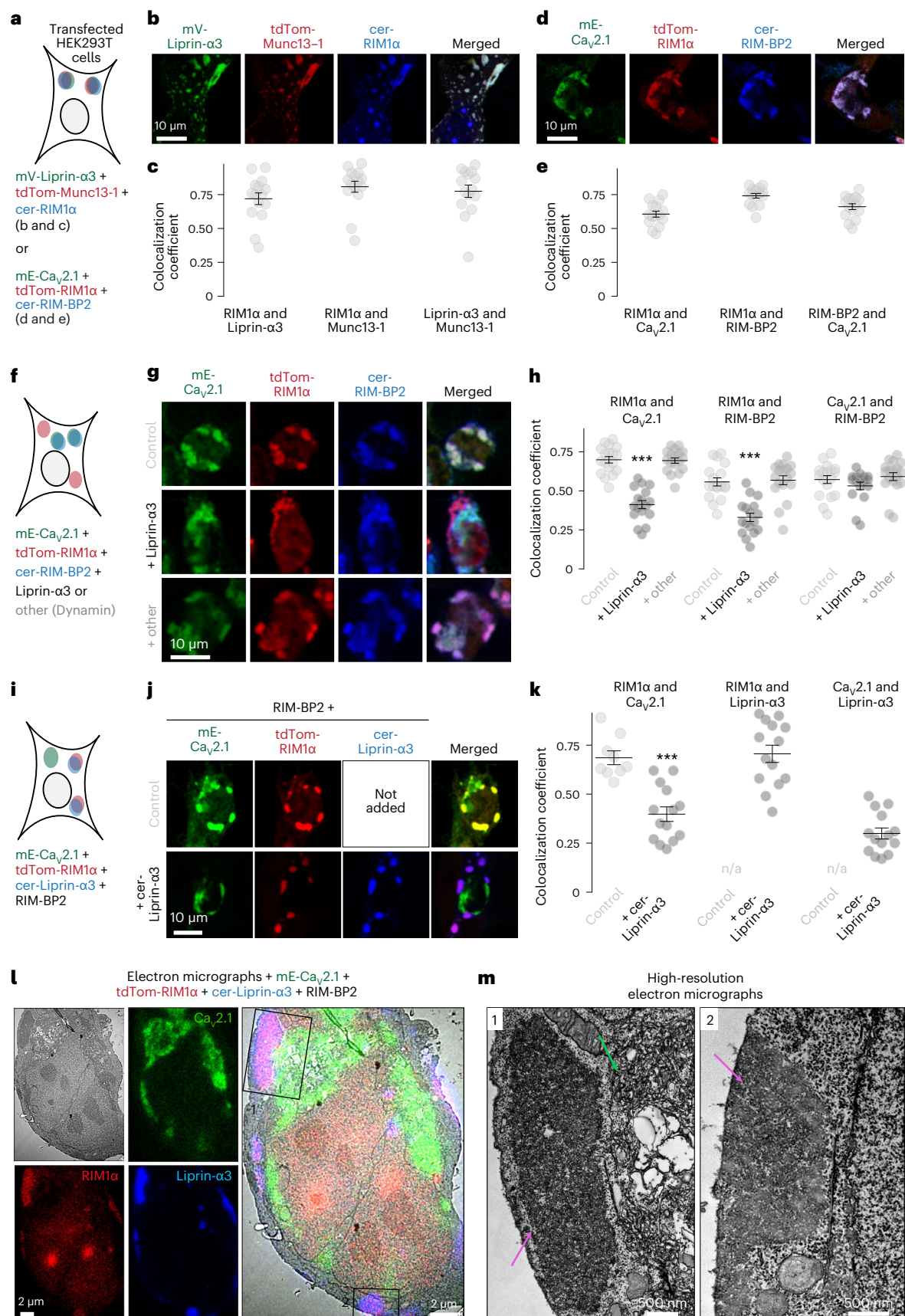
(gray), cQKO^{R+E} (black) and/or cQKO^{R+E} + RIM1 α (purple). Line profiles and peak intensities were normalized to the average control^{R+E} or cQKO^{R+E} + RIM1 α (for HA) signal per culture; in **c–e**, control^{R+E}, 36 synapses/3 cultures; cQKO^{R+E}, 29/3; cQKO^{R+E} + RIM1 α , 47/3; cQKO^{R+E} + PDZ-C2A-P, 47/3; and cQKO^{R+E} + Zn-C2B, 48/3; in **f–h**, control^{R+E}, 48/3; cQKO^{R+E}, 44/3; cQKO^{R+E} + RIM1 α , 45/3; cQKO^{R+E} + PDZ-C2A-P, 49/3; and cQKO^{R+E} + Zn-C2B, 43/3; in **i–k**, control^{R+E}, 45/3; cQKO^{R+E}, 47/3; cQKO^{R+E} + RIM1 α , 45/3; cQKO^{R+E} + PDZ-C2A-P, 44/3; and cQKO^{R+E} + Zn-C2B, 46/3; in **l–n**, control^{R+E}, 92/6; cQKO^{R+E}, 93/6; cQKO^{R+E} + RIM1 α , 87/6; cQKO^{R+E} + PDZ-C2A-P, 92/6; and cQKO^{R+E} + Zn-C2B, 86/6; in **o–q**, control^{R+E}, 50/3; cQKO^{R+E}, 51/3; cQKO^{R+E} + RIM1 α , 47/3; cQKO^{R+E} + PDZ-C2A-P, 51/3; and cQKO^{R+E} + Zn-C2B, 47/3. The scale bar in **o** applies to all STED images. Data are mean $\pm$ s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001 compared to cQKO^{R+E} determined by Kruskal–Wallis and Holm multiple comparisons post hoc tests (in **e, h, n** and **q**) or one-way ANOVA and Tukey–Kramer post hoc tests (in **k**). For expression of RIM constructs, confocal microscopic analyses and inhibitory synapse STED analyses, see Extended Data Figs. 2 and 3.

the zinc finger that binds to Munc13 and Rab3 (refs. 3,57,58) and the C2B domain that binds to Liprin- α and PIP₂ (refs. 40,59). Each construct also contained endogenous linkers and an HA tag for detection.

We then used antibody staining followed by sequential confocal and STED imaging. For STED analyses, we selected side-view synapses and assessed line profiles perpendicular to the PSD-95 signal to evaluate localization and levels of the tested proteins. RIM1 fragments were localized apposed to PSD-95 (Fig. 2c–e) at levels similar to (PDZ-C2A-P)

or above (Zn-C2B) those for RIM1 α , establishing their targeting to the active zone. As described before^{24,30,60}, the active zone was disrupted in cQKO^{R+E} neurons with very low levels of Munc13-1 and robust reductions in Ca_v2.1 and RIM-BP2. The postsynaptic marker PSD-95 was unaltered, and Liprin- α 3 was increased by ~30%, in agreement with previous work³⁰ and with upstream functions^{20,21,23}. RIM1 α expression in cQKO^{R+E} neurons restored the levels of all measured proteins to those in control^{R+E} synapses. Although PDZ-C2A-P restored Ca_v2.1 and RIM-BP2,





it failed to recover Munc13-1. Conversely, Zn-C2B restored Munc13-1 and Liprin- α 3 but not Ca $_v$ 2.1 or RIM-BP2 (Fig. 2f–q). Similar results were obtained when we quantified confocal images, and these analyses

also established that overall excitatory and inhibitory synapse numbers were not affected (Extended Data Fig. 2). STED analyses of inhibitory synapses also matched with these outcomes (Extended Data Fig. 3).

Fig. 3 | Liprin- α and Ca_v2.1-organizing complexes compete for RIM.

a–c, Overview of experiments in transfected HEK293T cells (**a**), example confocal images (**b**) and quantification (**c**) of HEK293T cells transfected with cerulean-RIM1 α (cer-RIM1 α), tdTomato-Munc13-1 (tdTom-Munc13-1) and mVenus-Liprin- α 3 (mV-Liprin- α 3). Pearson's colocalization coefficient is shown in **c**; 15 images/3 transfections. **d,e**, As in **b** and **c**, but for HEK293T cells transfected with cerulean-RIM-BP2 (cer-RIM-BP2), tdTomato-RIM1 α (tdTom-RIM1 α) and mEOS-Ca_v2.1 (mE-Ca_v2.1); 15/3. **f–h**, Overview of the experiment (**f**), example confocal images (**g**) and quantification (**h**) of HEK293T cells transfected with cer-RIM-BP2, tdTom-RIM1 α and mE-Ca_v2.1, and without (control) or with either Liprin- α 3 (+ Liprin- α 3) or Dynamin 1 (+ other). Pearson's colocalization coefficient is shown in **h**; control, 18/4; + Liprin- α 3, 17/4; + other, 18/4. **i–k**, Overview of the experiment (**i**), example confocal images (**j**) and quantification (**k**) of HEK293T cells transfected with untagged RIM-BP2, tdTom-RIM1 α and mE-Ca_v2.1, and without (control) or with cerulean-Liprin- α 3 (+ cer-Liprin- α 3).

This experiment provides a proof-of-principle that Munc13-containing and Ca_v2-containing complexes can be assembled independently of one another and supports the hypothesis that two distinct pathways may control these active zone subassemblies.

Liprin- α and Ca_v2 complexes compete for RIM

If independent assembly pathways exist, it should be possible to reconstitute and assess them in a reduced environment. We transfected HEK293T cells with either fluorophore-tagged Liprin- α 3, RIM1 α and Munc13-1, or fluorophore-tagged Ca_v2.1, RIM1 α and RIM-BP2 (Fig. 3a–e). Consistent with previous work, this resulted in the formation of condensates that reflect either priming complexes (RIM1 α , Munc13-1 and Liprin- α ; Fig. 3b,c)^{18,61} or Ca_v2 clustering complexes (Ca_v2.1, RIM1 α and RIM-BP2; Fig. 3d,e)^{6,8}. Next, we reasoned that if only one assembly pathway exists, adding priming components to condensates of RIM, RIM-BP and Ca_v2 should lead to co-condensation. Instead, when Liprin- α 3 was added ('+Liprin- α 3'), a fraction of RIM1 α was removed from Ca_v2.1- and RIM-BP2-containing condensates (Fig. 3f–h). Addition of another protein that has no known interactions with RIM (Dynamin 1; '+other') did not have the same effect. We repeated this experiment with tagged Liprin- α 3 instead of tagged RIM-BP2 and obtained similar results (Fig. 3i–k). We next used correlative light-electron microscopy (Fig. 3l,m) and found that RIM1 α and Liprin- α 3 localized to electron-dense condensates that were not surrounded by lipid bilayers, similar to the structures containing RIM1 α , Munc13-1 and Liprin- α that we previously characterized¹⁸. Ca_v2.1 was localized distinctly; it was present in the same cell but in separate electron-dense structures that contained membranous organelles (Fig. 3m, zoom-in 1) and it was mostly intracellular. Together with previous work^{18,61}, these experiments indicate that multiple distinct active zone complexes, akin to Ca_v2 clustering machineries and priming complexes, can be assembled in transfected cells and compete for RIM1 α .

Fig. 4 | Ablation of Liprin- α disrupts priming machinery but not Ca_v2 clustering.

a, Schematics of the *Ppfia1–Ppfia4* alleles (*Ppfia1* and *Ppfia4* are newly generated; *Ppfia2* and *Ppfia3* are previously described^{18,19}) that constitute the Liprin- α mutant mice. These mice were used to generate cultured hippocampal neurons with quadruple knockout of Liprin- α 1 to Liprin- α 4 (cQKO^{L1–L4}; lentiviral Cre expression) or corresponding controls (control^{L1–L4}; lentiviral expression of recombination-deficient Cre and of Liprin- α 3). **b**, Western blot in cultured neurons. **c–n**, Example STED images, average line profiles and quantification of the peak intensities of Munc13-1 and PSD-95 (**c–e**), RIM (**f–h**), Ca_v2.1 (**i–k**) and RIM-BP2 (**l–n**) at excitatory side-view synapses identified by Synaptophysin (Syn) and PSD-95. Dotted lines in line profile plots mark the levels in control^{L1–L4} neurons; line profiles and peak intensities were normalized to the average signal in control^{L1–L4} per culture; in **c–e**, control^{L1–L4}, 62 synapses/3 cultures; cQKO^{L1–L4}, 65/3; in **f–h**, control^{L1–L4}, 65/3; cQKO^{L1–L4}, 71/3; in **i–k**, control^{L1–L4}, 63/3; cQKO^{L1–L4}, 61/3; in **l–n**, control^{L1–L4}, 54/3; cQKO^{L1–L4}, 56/3. The scale bar in **l** applies to all STED images. **o,p**, Example traces (**o**) and average

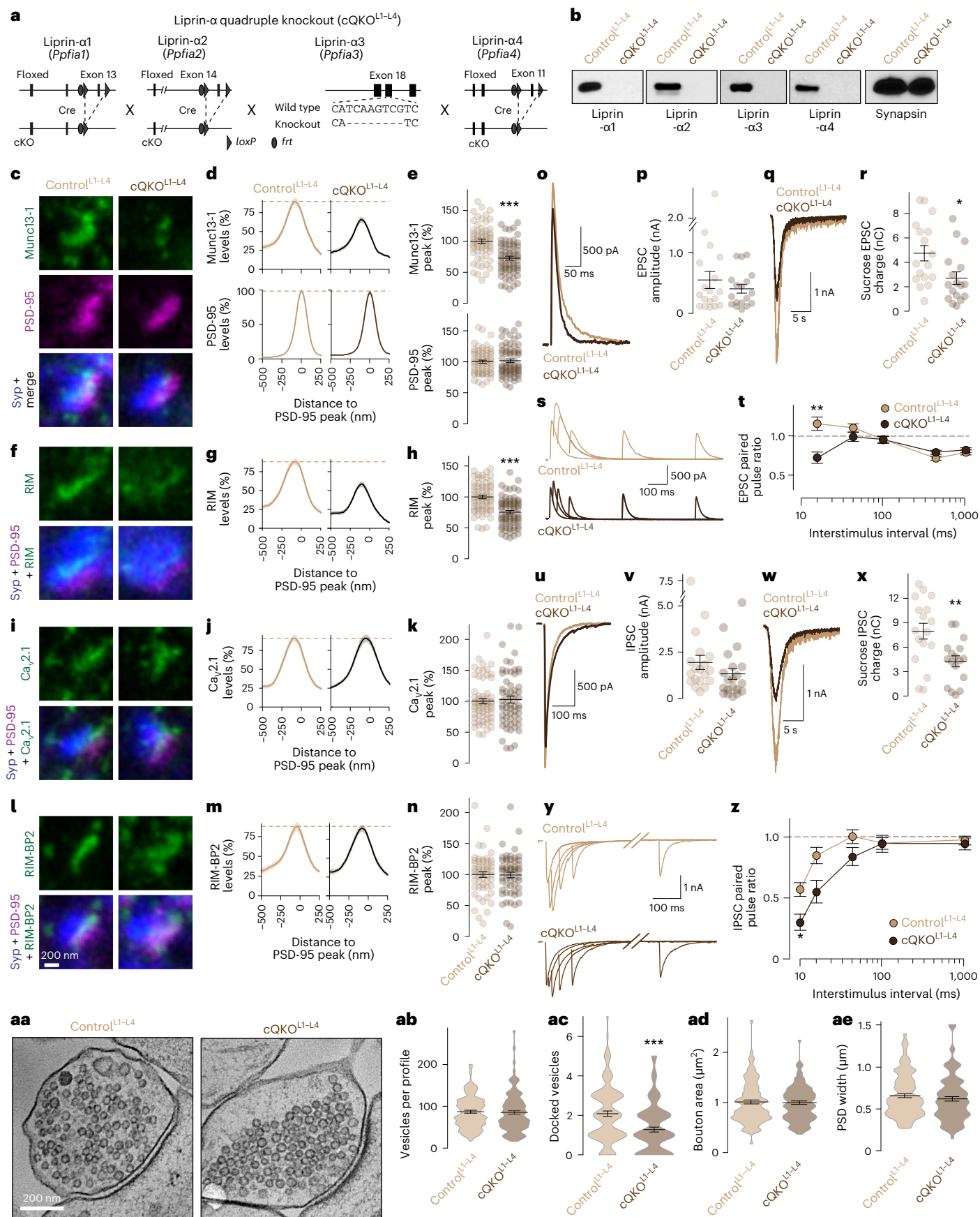
Pearson's colocalization coefficient is shown in **k**; control, 9/3; + cer-Liprin- α 3, 14/3. **l,m**, Correlative light-electron microscopy example images (**l**) of HEK293T cells transfected with mE-Ca_v2.1, tdTom-RIM1 α , cer-Liprin- α 3 and RIM-BP2. High-resolution zoomed-in images (**m**) of areas 1 and 2 indicated in **l** show protein condensates with cer-Liprin- α 3 and tdTom-RIM1 α (purple arrows) or mE-Ca_v2.1 (green arrows); an example cell is shown of a total of 7 cells/2 transfections. Data are mean $\pm$ s.e.m.; *** P < 0.001 compared to control determined by one-way ANOVA and Tukey–Kramer multiple comparisons post hoc tests (in **h**, RIM1 α and Ca_v2.1), Kruskal–Wallis and Holm multiple comparisons post hoc tests (in **h**, RIM1 α and RIM-BP2, and Ca_v2.1 and RIM-BP2) or two-sided Student's t -test (in **k**). In **a–k**, each image is 250 μ m $\times$ 250 μ m, 185 μ m $\times$ 185 μ m or 125 μ m $\times$ 125 μ m and contains multiple cells. In experiments including mE-Ca_v2.1, mEOS was used as a conventional fluorophore for visualization; the cerulean channel was imaged last to prevent photoconversion of mEOS before other fluorophores were assessed.

Liprin- α knockout selectively disrupts priming machinery

Liprin- α forms condensates with RIM and Munc13 and competes for RIM with Ca_v2-organizing complexes (Fig. 3). Hence, Liprin- α might organize priming machinery but not Ca_v2 clustering at synapses. We tested this hypothesis through the generation and characterization of knockout mice to simultaneously remove all four Liprin- α proteins (Liprin- α 1 to Liprin- α 4) encoded by four genes (*Ppfia1* to *Ppfia4*)⁶². Conditional Liprin- α 1 (Liprin- α 1^{fl/fl}) and Liprin- α 4 (Liprin- α 4^{fl/fl}) knockout mice were generated by homologous recombination (Extended Data Fig. 4). They were crossed to previously generated conditional Liprin- α 2 (Liprin- α 2^{fl/fl})¹⁸ and constitutive Liprin- α 3 (Liprin- α 3^{+/−})¹⁹ knockout mice to generate a quadruple homozygous line (Fig. 4a). We cultured hippocampal neurons from these mice and infected them with lentivirus expressing Cre to generate cQKO^{L1–L4} neurons. We chose day in vitro (DIV) seven for viral transduction, as earlier cre delivery induced neuronal death, consistent with developmental effects observed after constitutive Liprin- α ablation^{22,44}. Infection with a virus expressing inactive, truncated Cre and a virus expressing HA-tagged Liprin- α 3 was used to generate control^{L1–L4} neurons (Fig. 4a,b). We previously established that lentiviral expression of Liprin- α 3 in constitutive Liprin- α 3 knockout neurons results in normal Liprin- α 3 active zone levels and we did not observe gain-of-function effects^{18,19}.

We first assessed active zone assembly at cQKO^{L1–L4} synapses using STED and confocal microscopy. At excitatory synapses, there was a significant reduction of RIM and Munc13-1 but intact levels of Ca_v2.1, RIM-BP2 and PSD-95 (Fig. 4c–n and Extended Data Fig. 5a–e). Consistently, there was a high colocalization between Liprin- α 3 and Munc13-1 when analyzed in STED microscopy both at side-view and en-face synapses (Extended Data Fig. 5f–j). The selective reduction in RIM and Munc13-1 was also observed at inhibitory synapses (Extended Data Figs. 5a–e and 6a–l). These results suggest that at hippocampal synapses, Liprin- α ablation selectively disrupts priming machinery.

amplitudes (**p**) of single action potential-evoked NMDAR-mediated EPSCs; control^{L1–L4}, 18 cells/3 cultures; cQKO^{L1–L4}, 18/3. **q,r**, Example traces (**q**) and average AMPAR-mediated charge (**r**) in response to 500 mM sucrose; 17/3 each. **s,t**, Example traces (**s**) and average NMDA-EPSC paired pulse ratios (**t**) at various interstimulus intervals; 18/3 each. **u–z**, Same as **o–t**, but for IPSCs; in **u** and **v**, control^{L1–L4}, 19/3; cQKO^{L1–L4}, 20/3; in **w** and **x**, 17/3 each; in **y** and **z**, 19/3 each. **aa–ae**, Example electron micrographs of synapse cross-sections (**aa**) and quantification of the number of synaptic vesicles per cross-section (**ab**), number of docked vesicles per active zone cross-section (**ac**), bouton area (**ad**) and postsynaptic density width (**ae**); control^{L1–L4} 103 synapses with 105 active zones/2 cultures; cQKO^{L1–L4}, 104/109/2. Data are mean $\pm$ s.e.m.; * P < 0.005, *** P < 0.001 compared to cQKO^{L1–L4} determined by two-sided Student's t -tests (**e** for Munc13-1, **h**, **n** and **x**), two-sided Mann–Whitney U -tests (**e** for PSD-95, **k**, **p**, **r**, **v** and **ab–ae**) or two-way ANOVA and Dunnett's post hoc tests (**t** and **z**). For validation of the mutant alleles, confocal analyses, inhibitory synapse STED analyses and analyses of spontaneous synaptic transmission, see Extended Data Figs. 4–6.



We used whole-cell electrophysiological recordings to assess synaptic transmission. The frequency of spontaneous miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs, respectively) was decreased in cQKO^{L1-L4} neurons but their amplitudes were unchanged (Extended Data Fig. 6m–t). We evoked synaptic responses with a focal stimulation electrode and recorded resulting EPSCs or IPSCs in separate batches of cells (Fig. 4o–z). NMDAR-EPSCs were assessed because electrical stimulation induces extensive network activity when AMPARs are not blocked. Overall, evoked EPSCs and IPSCs were similar between genotypes (Fig. 4o,p,u,v). Synaptic strength is determined by the size of the readily releasable pool generated by Munc13-mediated vesicle priming and by vesicular release probability⁶³. The readily releasable pool, estimated by superfusion with hypertonic sucrose, was reduced by ~45% at both excitatory and inhibitory cQKO^{L1-L4} synapses (Fig. 4q,r,w,x). Vesicular release probability was assessed by measuring the ratio of two consecutive stimuli at short intervals. Decreases in these ratios at both synapse types in cQKO^{L1-L4} neurons indicated an increase in vesicular release probability (Fig. 4s,t,y,z). Overall, these results suggest a decrease in the readily releasable pool matching with reductions in RIM and Munc13 (Fig. 4c–h). This is accompanied by an increase in vesicular release probability to account for normal single evoked PSCs.

To complement the functional and STED analyses, we performed electron microscopy on synapses of high-pressure frozen neurons (Fig. 4aa–ae). Matching with the reductions in Munc13 and the readily releasable pool, the number of docked vesicles, identified as synaptic vesicles apposed to the postsynaptic density and with no detectable gap between the vesicle and plasma membranes, was reduced in cQKO^{L1-L4} neurons. Total vesicle numbers, bouton area and PSD width were unchanged. Overall, these analyses indicate that Liprin-α selectively supports the assembly of priming machinery.

Liprin-α tethering functions necessitate N- and C-terminal regions

Liprin-α contains two N-terminal Liprin homology regions (LH1 and LH2) that bind to RIM and ELKS and that undergo oligomerization^{39,40,61}. These sequences are followed by a linker and three sterile alpha motifs that mediate binding to other presynaptic scaffolds including the cell adhesion proteins LAR-RPTPs^{41–43}. We reasoned that specific fragments of Liprin-α might be sufficient to mediate distinct assembly roles, as observed for RIM (Fig. 2). We generated lentiviruses to express complementary fragments of Liprin-α containing either the N-terminal LH sequences (Liprin-α^N) or the C-terminal sterile alpha motif domains (Liprin-α^C) and compared their rescue activity to full-length Liprin-α (Fig. 5a). All constructs were expressed effectively and targeted to the active zone (Fig. 5b–d and Extended Data Fig. 7a–d). Full-length Liprin-α restored Munc13-1 levels at excitatory and inhibitory synapses (Fig. 5e–g and Extended Data Figs. 7e–g and 8a–c). This was probably mediated by RIM, whose levels were restored when measured with confocal microscopy at excitatory synapses and with STED at inhibitory synapses, and showed strong trends towards rescue in the other conditions (Fig. 5h–j and Extended Data Figs. 7h–j and 8d–f). Neither Liprin-α^N nor Liprin-α^C

increased RIM or Munc13-1 levels (Fig. 5e–j and Extended Data Figs. 7e–j and 8). Correspondingly, the number of docked vesicles was only rescued by full-length Liprin-α (Fig. 5k–o). These data support that Liprin-α operates as a tether for synaptic vesicle priming machinery through simultaneous actions of N-terminal and C-terminal regions.

PTPσ anchors priming machinery to membranes in HEK293T cells

RIM and ELKS bind to sequences contained within the N terminus of Liprin-α^{39,40,61} and the C terminus binds to LAR-RPTPs^{41–43}. We tested whether LAR-RPTPs anchor priming machinery to plasma membranes in transfected HEK293T cells.

We used mVenus-tagged Liprin-α3 (mV-Liprin-α3) alone or in combination with one of the three LAR-RPTPs (PTPσ, PTPδ or LAR, not fluorophore-tagged; Fig. 6a). When transfected alone, mV-Liprin-α3 was mostly diffuse. Co-transfection of PTPσ, PTPδ or LAR promoted Liprin-α3 clustering^{42,43} but to different degrees (Fig. 6b–d). LAR induced fewer mV-Liprin-α3 condensates than PTPδ (LAR vs PTPδ, $P = 0.002$; Fig. 6c) and PTPσ (LAR vs PTPσ, $P < 0.001$; Fig. 6c), and these condensates were smaller than those induced by PTPσ (LAR vs PTPσ, $P = 0.023$; Fig. 6d). Only mV-Liprin-α3 condensates induced by PTPσ or PTPδ, not those induced by LAR, showed robust recovery after photobleaching (Fig. 6e–g), suggesting that they are liquid droplets similar to Liprin-α3 condensates induced by PKC phosphorylation¹⁸. PTPσ-induced Liprin-α3 condensation was independent of PKC activation (Extended Data Fig. 9a–c). This might be functionally important because the liquid state of Liprin-α supports recruitment of active zone material to synapses^{18,21}.

Given that LAR-RPTPs are transmembrane proteins, the induced Liprin-α3 condensates should be associated with membrane compartments. Using correlative light-electron microscopy (Fig. 6h,i), we found that mV-Liprin-α3 condensates were electron-dense but not surrounded by lipid bilayers, consistent with liquid droplets. Many of these structures were adjacent to the plasma membrane. There were also fluorescent condensates away from the plasma membrane, and these contained membranous organelles that might be small vesicles or tubulovesicular structures (Fig. 6i, zoom-in 6). The same intracellular membranous compartments were observed in larger plasma membrane-associated condensates (zoom-ins 1 and 5). These data suggest that PTPσ anchors Liprin-α condensates to membranes. Furthermore, stretches of plasma membrane associated with condensates (zoom-ins 1–3 and 5), as opposed to those without fluorescent signals (zoom-in 4), were separated from the apposed cell with even intercellular spacing. This organization, similar in appearance to a synaptic cleft, suggests a function of PTPσ in organizing cellular appositions.

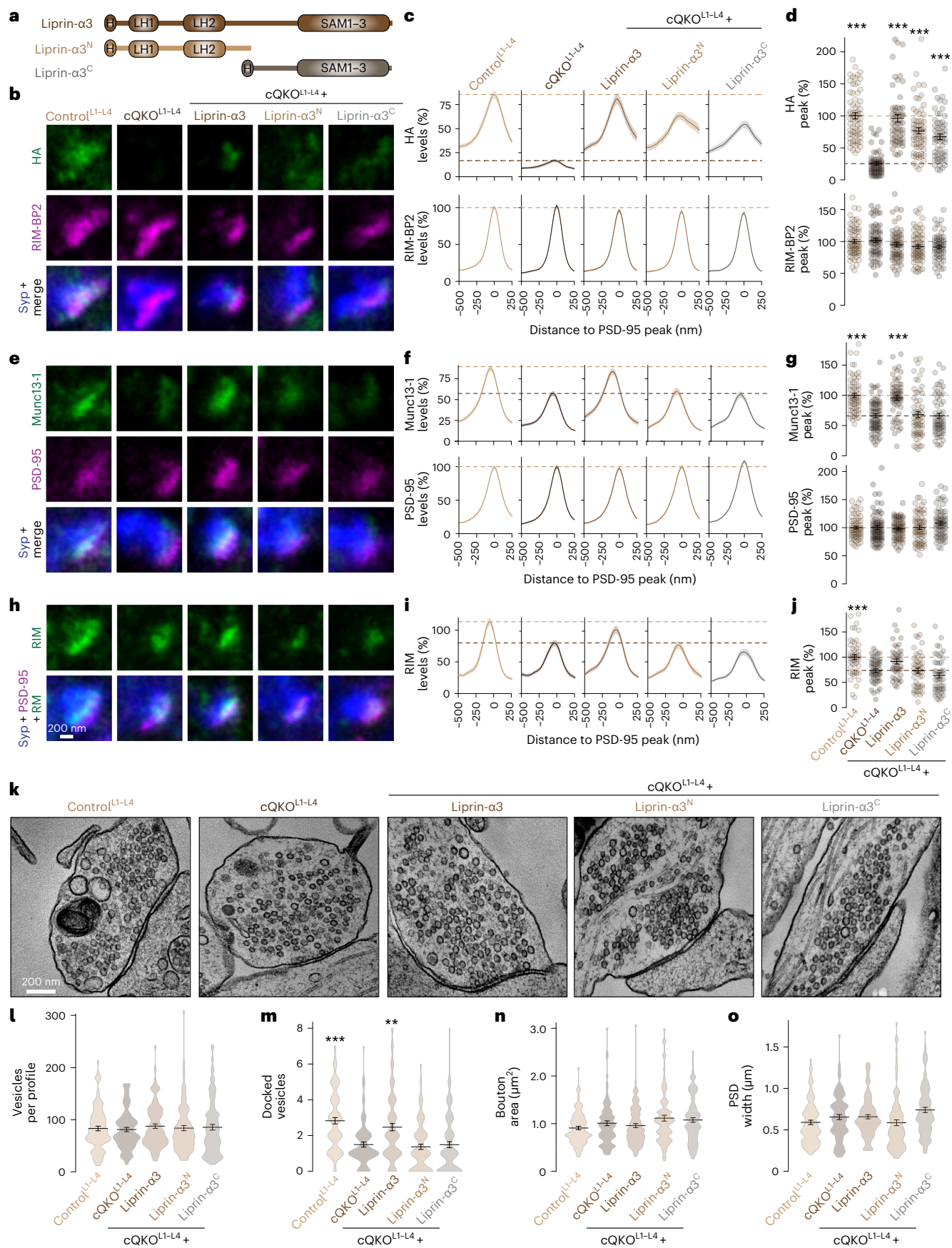
Finally, we tested whether PTPσ-induced Liprin-α3 condensates co-recruit priming machinery. We co-transfected cerulean-tagged RIM1α (cer-RIM1α) and Munc13-1, as these two proteins co-condensate in HEK293T cells in the presence of Liprin-α3 (ref. 18), and included tdTomato-tagged PTPσ (tdTom-PTPσ) with or without Liprin-α3 (Fig. 6j). In the presence of Liprin-α3, tdTom-PTPσ and cer-RIM1α colocalized into protein condensates, whereas colocalization was poor

Fig. 5 | Liprin-α operates as a tether via N-terminal and C-terminal domains.

a, Schematics of HA-tagged Liprin-α, Liprin-α^N and Liprin-α^C; H, HA tag; LH, Liprin homology region; SAM, sterile alpha motif. **b–j**, Example STED images, average line profiles and quantification of the peak intensities of HA and RIM-BP2 (**b–d**), Munc13-1 and PSD-95 (**e–g**) and RIM (**h–j**) at excitatory side-view synapses identified by Synaptophysin (Syp) and PSD-95. Dotted lines mark levels of control^{L1-L4} (light brown) and cQKO^{L1-L4} (dark brown). Note that control^{L1-L4} has an HA signal because of the lentiviral expression of HA-tagged Liprin-α. Line profiles and peak intensities were normalized to the average signal in control^{L1-L4} per culture; **b–d**, control^{L1-L4}, 67 synapses/4 cultures; cQKO^{L1-L4}, 59/4; cQKO^{L1-L4} + Liprin-α, 57/4; cQKO^{L1-L4} + Liprin-α^N, 63/4; cQKO^{L1-L4} + Liprin-α^C, 60/4; **e–g**, control^{L1-L4}, 65/4; cQKO^{L1-L4}, 70/4; cQKO^{L1-L4} + Liprin-α,

60/4; cQKO^{L1-L4} + Liprin-α^N, 63/4; cQKO^{L1-L4} + Liprin-α^C, 66/4; **h–j**, control^{L1-L4}, 50/3; cQKO^{L1-L4}, 47/3; cQKO^{L1-L4} + Liprin-α, 43/3; cQKO^{L1-L4} + Liprin-α^N, 49/3; cQKO^{L1-L4} + Liprin-α^C, 49/3. The scale bar in **h** applies to all STED images.

k–o, Example electron micrographs of synapse cross-sections (**k**) and quantification of the number of synaptic vesicles per cross-section (**l**), docked vesicles per active zone cross-section (**m**), bouton area (**n**) and postsynaptic density width (**o**); control^{L1-L4}, 91 synapses with 92 active zones/2 cultures; cQKO^{L1-L4}, 86/87/2; cQKO^{L1-L4} + Liprin-α, 95/96/2; cQKO^{L1-L4} + Liprin-α^N, 92/92/2; cQKO^{L1-L4} + Liprin-α^C, 96/96/2. Data are mean ± s.e.m.; *** $P < 0.001$ compared to cQKO^{L1-L4} determined by Kruskal–Wallis and Holm multiple comparisons post hoc tests. For confocal analyses and inhibitory synapse STED analyses, see Extended Data Figs. 7 and 8.



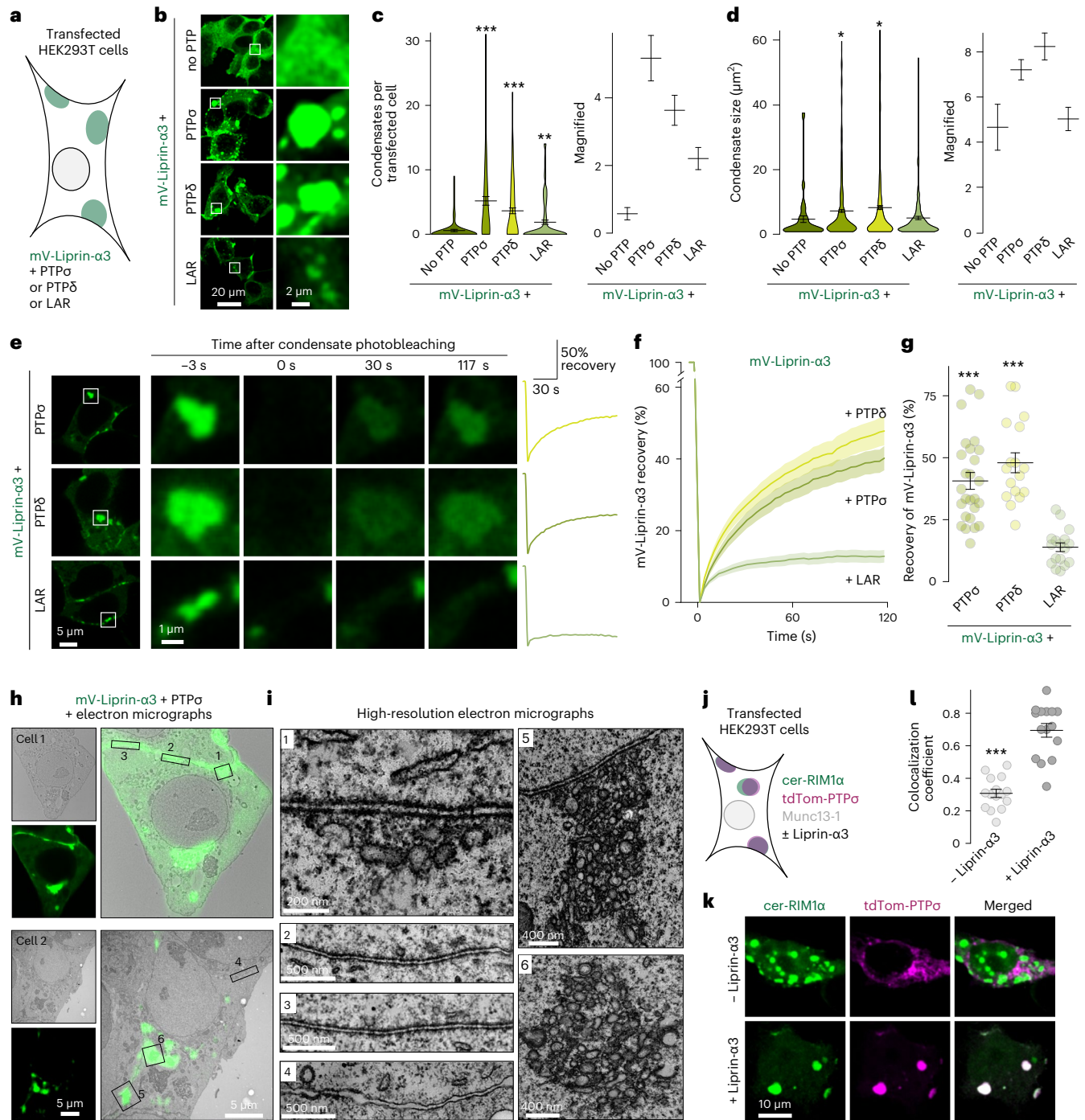
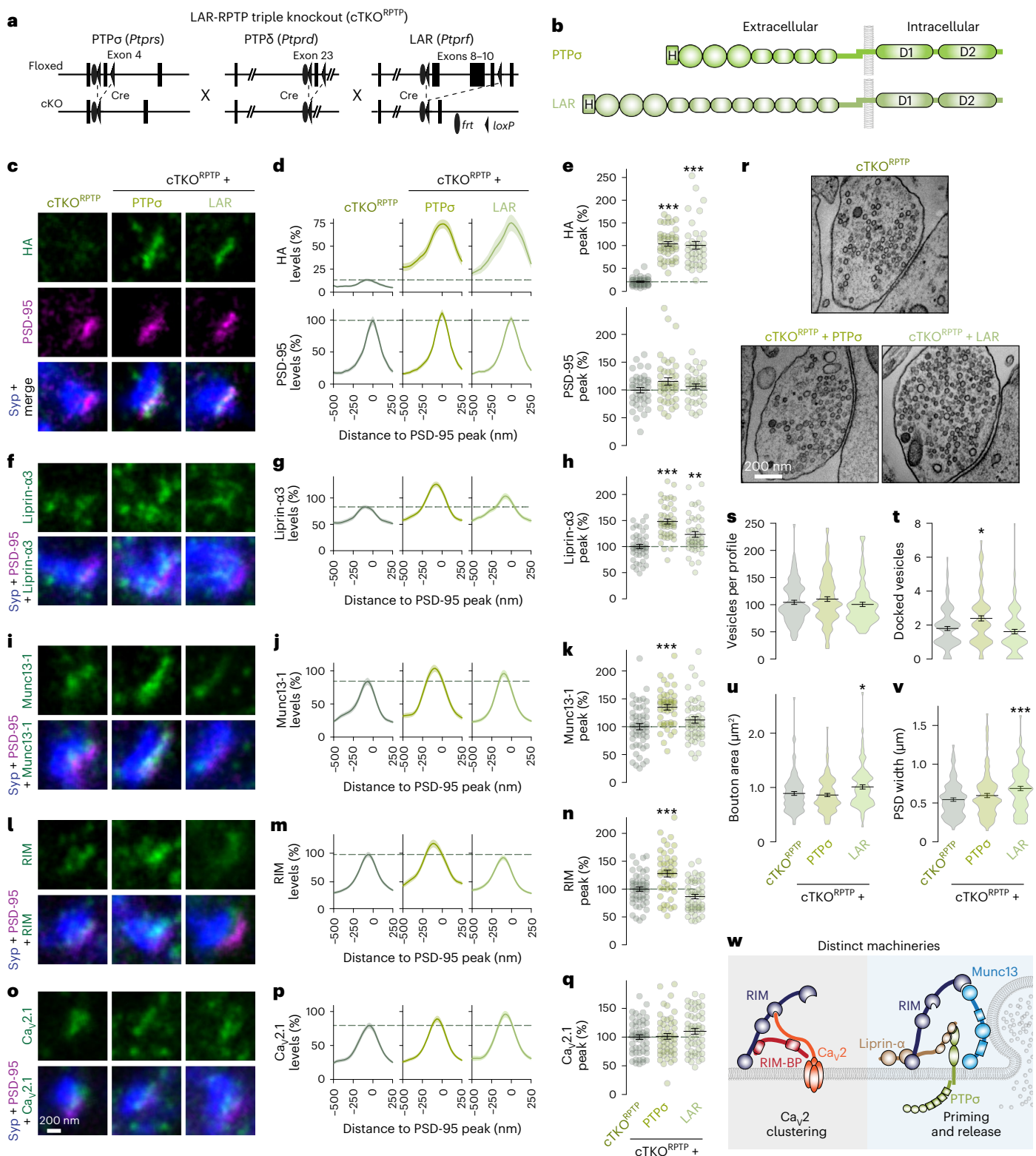


Fig. 6 | LAR-RPTPs can anchor priming machinery through Liprin-α in HEK293T cells. **a**, Overview of the experiment in HEK293T cells co-transfected with mVenus-Liprin-α3 (mV-Liprin-α3) and untagged LAR, PTPσ or PTPδ. **b–d**, Example confocal images (**b**) and quantification of number and size (**c,d**; magnified averages on the right) of mV-Liprin-α3 condensates. Images are 250 μm × 250 μm and contain multiple cells. In **c**, mV-Liprin-α3 + no PTP, 91 cells/3 transfections; mV-Liprin-α3 + PTPσ, 104/3; mV-Liprin-α3 + PTPδ, 114/3; mV-Liprin-α3 + LAR, 112/3. In **d**, mV-Liprin-α3 + no PTP, 52 condensates/3 transfections; mV-Liprin-α3 + PTPσ, 505/3; mV-Liprin-α3 + PTPδ, 414/3; mV-Liprin-α3 + LAR, 209/3. **e–g**, Example confocal images and intensity profiles of recovery after photobleaching (**e**) and quantification (**f,g**). In **e**, zoomed-in images were adjusted to visually match the fluorescence intensity of condensates before bleaching, and adjustments were identical within each condition; mV-Liprin-α3 + PTPσ, 26 condensates from different cells/4 transfections; mV-Liprin-α3 + PTPδ, 17/4; mV-Liprin-α3 + LAR, 17/4. **h,i**, Correlative light-electron microscopy example images (**h**) of HEK293T cells transfected with mV-Liprin-α3 and PTPσ. High-resolution

zoomed-in images (**i**) of areas 1 to 6 indicated in **h** show membrane-attached condensates (1–3 and 5), membranes without condensates (4) and a condensate that appears cytosolic (6); two examples are shown from 5 cells/1 transfection. **j**, Overview of the experiment in HEK293T cells co-transfected with cer-RIM1α, Munc13-1 and tdTomato-PTPσ (tdTom-PTPσ), and either with or without Liprin-α3. **k,l**, Example confocal images (**k**) and quantification of Pearson's colocalization coefficient of tdTom-PTPσ and cer-RIM1α. The distribution of cer-RIM1α in the – Liprin-α3 condition was variable, being either in condensates (shown) or widespread (not shown); colocalization with PTPσ was low in both cases and cells were included regardless of cer-RIM1α distribution; 15 images/3 transfections each; images are 250 μm × 250 μm, 185 μm × 185 μm or 145 μm × 145 μm and contain multiple co-transfected cells. Data are mean ± s.e.m.; **P* < 0.05, ****P* < 0.001 compared to mV-Liprin-α3 + no PTP (in **c** and **d**) or to mV-Liprin-α3 + LAR (in **g**) determined by Kruskal–Wallis and Holm multiple comparisons post hoc tests (in **c**, **d** and **g**) or two-sided Student's *t*-tests (in **l**). For assessment of PKC in PTPσ-induced Liprin-α3 condensates, see Extended Data Fig. 9.



in the absence Liprin- α 3 (Fig. 6k,l). Overall, we conclude that PTP σ induces liquid–liquid phase condensation of Liprin- α 3. This process drives the assembly of cellular compartments that include membranous structures that can be anchored to the plasma membrane, and RIM can be co-recruited into these condensates.

PTP σ promotes assembly of priming machinery in neurons

We investigated whether PTP σ anchors vesicle priming machinery at synapses. Previous work, including ours, has reported that the

combined deletion of PTP σ , PTP δ and LAR during synaptogenesis and synapse maturation does not produce detectable impairment in active zone structure and function when assessed with microscopy and electrophysiology^{51,64}. This is probably because of redundant functions with other presynaptic cell adhesion proteins; for example, neuroligins⁶⁵. Furthermore, the distinct properties of PTP σ , PTP δ and LAR in clustering Liprin- α (Fig. 6) suggest diverging roles for these proteins, complicating the assessment of knockout phenotypes. To bypass this limitation, we forced synapses to operate with a single LAR-RPTP, either PTP σ or

Fig. 7 | PTP σ assembles priming machinery but does not cluster Ca $_v$ 2s.

a,b, Schematic of the conditional LAR-RPTP knockout mice (from a previous study⁵¹) (**a**) and map of rescue constructs (**b**). Cultured hippocampal neurons after knockout of PTP σ , PTP δ and LAR (cTKO^{RPTP}; through expression of cre-lentiviruses) were analyzed without or with lentiviral expression of LAR-RPTPs (**b**). Schematics of HA-tagged PTP σ and LAR are shown; H, HA tag; D1 and D2, phosphatase domains 1 and 2. **c–q**, Example STED images, average line profiles and quantification of the peak intensity of HA and PSD-95 (**c–e**), Liprin- α 3 (**f–h**), Munc13-1 (**i–k**), RIM (**l–n**) and Ca $_v$ 2.1 (**o–q**) at excitatory side-view synapses identified by Synaptophysin (Syn) and PSD-95. Dotted lines mark levels of cTKO^{RPTP}. Line profiles and peak intensities were normalized to the average signal in cTKO^{RPTP} per culture; in **c–e**, cTKO^{RPTP}, 38 synapses/3 cultures; cTKO^{RPTP} + PTP σ , 42/3; cTKO^{RPTP} + LAR, 37/3; in **f–h**, cTKO^{RPTP}, 45/3; cTKO^{RPTP} + PTP σ , 46/3; cTKO^{RPTP} + LAR, 45/3; in **i–k**, cTKO^{RPTP}, 51/3; cTKO^{RPTP} + PTP σ , 45/3; cTKO^{RPTP} + LAR,

48/3; in **l–n**, cTKO^{RPTP}, 50/3; cTKO^{RPTP} + PTP σ , 45/3; cTKO^{RPTP} + LAR, 48/3; in **o–q**, cTKO^{RPTP}, 51/3; cTKO^{RPTP} + PTP σ , 50/3; cTKO^{RPTP} + LAR, 51/3. The scale bar in **o** applies to all STED images. **r–v**, Example electron micrographs of synapse cross-sections (**r**) and quantification of the number of synaptic vesicles per cross-section (**s**), docked vesicles per active zone cross-section (**t**), bouton area (**u**) and postsynaptic density width (**v**); cTKO^{RPTP}, 97 synapses with 99 active zones/2 cultures; cTKO^{RPTP} + PTP σ , 105/112/2; cTKO^{RPTP} + LAR, 95/103/2. **w**, Working model of active zone organization with two distinct machineries: one to cluster Ca $_v$ 2 and one to prime synaptic vesicles for fusion. Data are mean $\pm$ s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001 compared to cTKO^{RPTP} determined by Kruskal–Wallis and Holm multiple comparisons post hoc tests (in **e**, **h**, **k**, **n** and **s–v**) or one-way ANOVA and Tukey–Kramer multiple comparisons post hoc tests (in **q**). For confocal analyses and inhibitory synapse STED analyses, see Extended Data Figs. 9 and 10.

LAR, by re-expressing them individually in LAR-RPTP triple knockout neurons (Fig. 7a,b)⁵¹. We lentivirally expressed Cre recombinase to produce LAR-RPTP triple knockout (cTKO^{RPTP}) neurons and added a second lentivirus to express PTP σ (cTKO^{RPTP} + PTP σ) or another lentivirus to express LAR (cTKO^{RPTP} + LAR). Both PTP σ and LAR were expressed effectively and targeted to synapses in cTKO^{RPTP} neurons (Fig. 7c–e and Extended Data Fig. 9d–f). We first measured the levels of active zone proteins with STED and confocal microscopy. The cTKO^{RPTP} + PTP σ neurons showed a ~50% increase in Liprin- α 3 and concurrent enhancement in RIM and Munc13-1 at both excitatory and inhibitory synapses, while Ca $_v$ 2.1 was unchanged (Fig. 7f–q and Extended Data Figs. 9g–r and 10a–l). By contrast, cTKO^{RPTP} + LAR neurons had only modestly (~20%) increased Liprin- α 3 but no detectable increase in RIM and Munc13-1. This indicates that the priming machinery consisting of Liprin- α , RIM and Munc13 can be specifically recruited to synapses by PTP σ .

We then assessed the effects of the same manipulations on synapse function and ultrastructure. PTP σ expression induced an increase in the pool released in response to hypertonic sucrose, consistent with the recruitment of priming machinery, whereas expression of LAR did not induce this change (Extended Data Fig. 10m–r). Correspondingly, the number of docked vesicles was increased in electron microscopic images of cTKO^{RPTP} + PTP σ synapses, whereas the other quantified parameters were unaffected (Fig. 7r–v). LAR expression induced somewhat enlarged boutons and PSDs, perhaps reflecting LAR's synaptogenic roles⁶⁶, but did not produce an increase in docking. These data support the model that PTP σ anchors priming machinery through the recruitment of Liprin- α , whereas LAR does not support these functions in the preparation assessed here.

Discussion

We dissected assembly mechanisms that underlie the nano-organization of the machinery for neurotransmitter release. We found that the active zone at hippocampal synapses is defined by two distinct subassemblies: one for clustering Ca $_v$ 2 channels and one for docking, priming and fusing synaptic vesicles. This conclusion relies on five lines of evidence. First, Ca $_v$ 2.1 and Munc13-1 can form separable nanoclusters, with approximately half of Munc13-1 nanoclusters not enriched with Ca $_v$ 2.1 and vice versa. Second, each complex is assembled by RIM, but distinct RIM sequence motifs mediate their assembly independently. Third, in transfected cells, RIM forms condensates with Ca $_v$ 2.1 and RIM-BP2, but co-expression of Liprin- α leads to a redistribution of RIM into separate condensates. Fourth, mouse gene knockout and rescue experiments establish that Liprin- α participates in the assembly of priming complexes but not in clustering Ca $_v$ 2 channels. Finally, PTP σ anchors priming machinery to plasma membranes, probably via Liprin- α , in transfected cells and at synapses. Our work establishes that vesicle priming and Ca $_v$ 2 clustering machineries are independent and that Liprin- α and PTP σ specifically support priming machinery assembly.

Previous work suggested that Ca $_v$ 2 is a core component of the machinery that primes and releases synaptic vesicles, arguing for a

single active zone protein complex. RIM binds to both Munc13 and Ca $_v$ 2 channels and might directly link docked vesicles to Ca $_v$ 2s^{2,8,67}. Fittingly, vesicles can be recruited to the surface of reconstituted liquid condensates containing RIM, RIM-BP and Ca $_v$ 2 (refs. 6,29), and artificially attaching the Munc13-binding domain of RIM to Ca $_v$ 2 channels is effective in mediating release³⁰. If a single protein complex clusters Ca $_v$ 2s and organizes vesicle priming, Ca $_v$ 2s should be at short, invariable distances from releasable vesicles. By contrast, functional studies and modeling suggest that Ca $_v$ 2 channel–vesicle distances are larger and variable^{31–36}. In line with these studies, we found that separable assembly pathways lead to distinct nano-organization of Ca $_v$ 2 and Munc13 (Fig. 7w). The model of separate machineries for vesicle priming and Ca $_v$ 2 clustering allows for flexible positioning of primed vesicles from Ca $_v$ 2 entry, enabling diverse release properties and regulation. In this model, the many parallel interactions between active zone proteins^{1,2} do not result in a 'supercluster' containing all active zone components and docked vesicles. Instead, there can be competition between different machineries for specific protein components (Fig. 3)⁶¹. This might also explain the increases in release probability after selective disruption of the priming complex by Liprin- α ablation (Fig. 4): when one assembly is impaired, the other might work more efficiently because competition for space at the plasma membrane and for components that participate in both complexes decreases.

RIM is a central active zone organizer that participates in both complexes. It has been studied in Ca $_v$ 2 clustering together with RIM-BP^{6–10,68,69}. Parallel studies discovered that RIM docks vesicles and recruits Munc13 for priming^{3,8,57,58,68}. We now find that the two machineries are separate and mechanistically extend this finding to reveal that Liprin- α and PTP σ selectively support priming complex assembly (Figs. 3–7)^{18,19,38}. Although RIM participates in both complexes, RIM-BP, Liprin- α and Munc13 primarily associate with one of the two. There may be exceptions to this separation. At hippocampal mossy fiber synapses and in autaptic cultures, for example, RIM-BP has roles in vesicle priming^{25,70}. Another active zone protein, ELKS, supports priming at some synapses and Ca $_v$ 2 entry at others^{11,13,14,71}. Ultimately, the composition of any given active zone may not always be strictly defined into two types of complexes. Vesicle docking and priming, the coupling of these vesicles to Ca $_v$ 2 channels and the vesicular fusion reaction are active processes that are accompanied by the movement of vesicles and active zone material. Presynaptic composition is also modulated over longer time scales. The model of liquid–liquid phase separation for active zone assembly^{6,18,21,29} is well suited to support these dynamics and may continuously reshape the subcomplexes described here.

One question that remains open is whether the presented findings and mechanisms for Liprin- α are generalizable across synapses. Through orthogonal approaches, we found that organizational roles are shared between excitatory and inhibitory hippocampal synapses, supporting some generalization within this preparation. Previous studies found roles for Liprin- α different from those identified

here. At invertebrate neuromuscular junctions, Liprin- α functions in the recruitment of Ca_v2 and of synaptic vesicles^{20,21,23,72}. In human IPSC-derived neurons, deletion of Liprin- α before neuronal differentiation disrupts the development of presynaptic nerve terminals⁴⁴, inducing a defect that is more severe than the one we observe in mouse neurons. These differences in phenotypic magnitude may reflect that the molecular organization of an active zone and its dependence on specific scaffolds varies across synapse types. An alternative explanation is that Liprin- α has essential developmental roles. Studies with Liprin- α ablation throughout development find a strong disruption of synapses, while Liprin- α knockout after axon outgrowth and after initial steps of synaptogenesis leads to specific deficits in active zone assembly. The roles of Liprin- α in synaptogenesis may be as a seeding scaffold or may be supporting axon growth or maturation. Our work emphasizes the role of Liprin- α in selectively organizing and maintaining priming machineries, which we probably detect because of the later deletion. This approach enabled the discovery of subassemblies and their mechanisms within the active zone.

An additional question that arises from the multi-complex model (Fig. 7w) is how the different active zone protein machineries are tethered to one another. The two complexes must be close enough for Ca^{2+} sensors on primed vesicles to detect Ca^{2+} entry for exocytosis. Different parts of a single RIM molecule could simultaneously associate with separate machineries for vesicle priming and Ca_v2 clustering. This is possible because the distance between the RIM zinc finger, which binds to Munc13 and vesicular Rab3, and the RIM PDZ domain, which binds to the C terminus of Ca_v2 or ELKS, can be several tens of nanometers long if the linker between them is unfolded. The Ca_v2 intracellular C terminus can bridge a similar distance as well. Other proteins, for example, ELKS or RIM-BP, could similarly connect the two machineries. An alternate possibility is that one RIM molecule can only participate in priming or in Ca_v2 clustering. This scenario is supported by previous work and by the observation that Liprin- α competes for RIM with Ca_v2 -organizing complexes in HEK293T cells (Fig. 3)⁶¹. It is also strengthened by mutually exclusive biochemical interactions: Ca_v2 and ELKS, for example, bind to the same binding pocket of the RIM PDZ domain^{8,73}, and the specific binding partner could determine which machinery a given RIM protein associates with.

The relative positioning of active zone protein complexes could also be determined by their interactions with transmembrane proteins. The main candidates are neurexins, LAR-RPTPs and Ca^{2+} channels themselves^{48,51,64,65,74}, and interactions of these proteins with extracellular material or other membrane proteins could control the spatial organization. Several recent findings on synaptic cell adhesion are suggestive of this model. Work on neurexins revealed that these proteins modulate synapse properties^{74,75}, fitting with a role in the positioning of specific complexes within an active zone. Furthermore, knocking out one protein family rarely has strong synapse assembly defects^{48,51,64,74}. Instead, there is redundancy, as illustrated by studies on neurexins and LAR-RPTPs⁶⁵. On their own, neurexins probably shape synapse properties by organizing Ca_v2 complexes⁷⁵. LAR-RPTPs specifically support the assembly of priming complexes (Figs. 6 and 7). Removing both proteins at the same time disrupts both complexes and induces strong synapse assembly defects⁶⁵. We propose that the substructuring of the active zone that we describe here accounts for the resilience of synapse and active zone assembly that is often observed in loss-of-function studies^{10,24,30,60,65,72,74}.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41593-024-01720-5>.

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Methods

Mouse lines

The quadruple floxed mice for RIM1 (RRID: IMSR_JAX:015832, targeting the *Rims1* gene to remove RIM1 α and RIM1 β)⁷⁶, RIM2 (RRID: IMSR_JAX:015833, targeting the *Rims2* gene to remove RIM2 α , RIM2 β and RIM2 γ)⁸, ELKS1 α (RRID: IMSR_JAX:015830, targeting the *Erc1* gene to remove ELKS1 α A and ELKS1 α B)¹⁴ and ELKS2 α (RRID: IMSR_JAX:015831, targeting the *Erc2* gene to remove ELKS2 α A and ELKS2 α B)⁷¹ have been previously described^{24,30}. The triple floxed mice for PTP σ (RRID: IMSR_CMMR:ABCA, targeting the *Ptprs* gene to remove PTP σ), PTP δ (RRID: IMSR_EM:11805, targeting the *Ptprd* gene to remove PTP δ) and LAR (RRID: IMSR_EUMMCR:8210, targeting the *Ptprf* gene to remove LAR) have been previously described⁵¹. The Liprin- α quadruple knockout mice were newly generated. Embryonic stem cells containing the Liprin- α 1 (*Ppfia1*) or Liprin- α 4 (*Ppfia4*) mutant alleles were obtained from the European Mouse Mutant Cell Repository and we acknowledge the EUCOMM Consortium as the source of this material. The specific cells used were *Ppfia1*^{tm1a(EUCOMM)Hmgu} (clone A02; RRID: IMSR_EUMMCR:25506) and *Ppfia4*^{tm1a(EUCOMM)Hmgu} (clone D06; RRID: IMSR_EUMMCR:3103) in which exon 13 (Liprin- α 1) or 16 (Liprin- α 4) were flanked by loxP sites. The mutant alleles were generated by homologous recombination in embryonic stem cells by the International Knockout Mouse Consortium. The embryonic stem cells were expanded and injected into C57BL/6N-A/a blastocysts at the Transgenic Mouse Core (Dana-Farber/Harvard Cancer Center, Harvard Medical School) to generate chimeric founders. After germline transmission, the original knockin mice were crossed to mice that express Flp-recombinase under a β -actin promoter⁷⁷ to remove the LacZ/Neomycin cassette to generate the conditional ‘floxed’ alleles. To analyze survival, we compared the obtained offspring ratio from heterozygote matings at postnatal day 21 (P21) to the expected Mendelian genotype distribution. The Liprin- α 1 and Liprin- α 4 floxed mice were crossed to previously generated Liprin- α 2 floxed mice and constitutive Liprin- α 3 knockout mice^{18,19} to generate the quadruple mutant mice that were maintained as quadruple homozygotes. Mice were genotyped either in the laboratory or by Transnetx. For in-lab genotyping, oligonucleotide primers AGCAGAAGCTGGGTCTCC and GTGACCACAGGTGTTGGAG (547 and 360 bp bands for the floxed and wild-type alleles, respectively) were used for Liprin- α 1; an additional reaction was used occasionally (with CCCTGTCTCTTACAAGAAACC and GTGACCACAGGTGTTGGAG, 174 bp band for wild type; no band for floxed). The conditional floxed Liprin- α 4 mice were genotyped with oligonucleotide primers GCTATCTCCAGCAGGTAGGAC and CACAGTGCCTGGTGTTCACG (383 and 240 bp bands for the floxed and wild-type alleles, respectively); genotyping reactions for Liprin- α 2 and Liprin- α 3 were performed as previously described^{18,19}. Mouse lines were maintained on a mixed background (containing at least C57BL/6 and 129/Sv) and at a standard 12 h dark/light cycle in a room set to 20–24 °C and 35–70% humidity (set point 50%). Animal experiments were approved by the Harvard University Animal Care and Use Committee.

Primary neuronal cultures

Primary hippocampal cultures were prepared as previously established^{18,19,24,30,48,51}. Hippocampi were dissected from newborn pups (at P0 to P1 within ~36 h or birth) of either sex and were digested and dissociated. Dissociated cells were plated onto glass coverslips (or Mat-Tek plates for DNA Exchange-PAINT) in ‘plating medium’ (minimum essential medium (MEM) supplemented with 0.5% glucose, 0.02% NaHCO₃, 0.1 mg ml⁻¹ transferrin, 10% fetal select bovine serum, 2 mM L-glutamine and 25 mg ml⁻¹ insulin). Approximately 24 h after plating, the medium was exchanged with ‘growth medium’ (MEM with 0.5% glucose, 0.02% NaHCO₃, 0.1 mg ml⁻¹ transferrin, 5% fetal select bovine serum, 2% B-27 supplement and 0.5 mM L-glutamine). At approximately 48–60 h after plating, cytosine β -D-arabinofuranoside (AraC) was added at a final concentration of 2–6 μ M. The cells were kept in an incubator at 37 °C until DIV 15–17.

Production of lentiviruses and transduction of primary neurons

Lentiviruses were produced in HEK293T cells (purchased from ATCC as a mycoplasma-free cell line; cat. no. CRL-3216; RRID: CVCL_0063) maintained in Dulbecco’s MEM (DMEM) supplemented with 10% bovine serum and 1% penicillin–streptomycin (HEK medium). HEK293T cells were transfected with the Ca²⁺ phosphate method with a combination of the packaging plasmids REV, RRE and VSV-G plus a lentiviral plasmid encoding Cre recombinase (lab plasmid code p009), an inactive, truncated version of Cre (p010) or a protein used for rescue at a molar ratio of 1:1:1:1 and with a total amount of ~4 μ g DNA per T25 flask. At 20–30 h after transfection, the medium was changed to ‘growth medium’, and the supernatant was collected 48–60 h after transfection. Viral transductions were done either with freshly collected viral supernatant or with snap-frozen supernatant stored at ~80 °C. Neuronal cultures were transduced at DIV 5 (for RIM + ELKS), DIV 6 (for LAR-RPTP) or DIV 7 (for Liprin- α) with lentiviruses expressing GFP-tagged Cre recombinase (p010) or the inactive version (p011) under a human Synapsin promoter^{14,30}, and expression was monitored by the presence of GFP in the nucleus. Only cultures in which no neurons without nuclear green fluorescence were readily detected were used for experiments. In trial experiments, earlier addition of Cre lentivirus in cultures of LAR-RPTP triple or Liprin- α quadruple mutant alleles resulted in neuronal death. Transduction for expression of rescue proteins was done at DIV 1 or DIV 2 with lentivirus-expressing versions of Liprin- α 3, RIM1 α , LAR or PTP σ under a human Synapsin promoter, or with a lentivirus without an insert in the multiple cloning site as a control (pFSW control; p008, as previously described^{24,30}). pFSW RIM1 α -HA (rat, p592) has been previously established^{30,59}. The numbering used below and the sequence of p592 correspond to Uniprot ID [Q9JIR4](#) but without the alternatively spliced exons N₈₃–W₁₀₅, H₁₀₈₄–R₁₁₆₉, and A₁₂₀₇–T₁₃₇₈, and with two variants (H263D and A453P; see NCBI Reference Sequence [NM_052829.3](#)). pFSW RIM1 α -HA PDZ-C2A-P (rat, p1050) was produced from p592 and contains amino acids M₅₀₉ through L_{1,446}. pFSW RIM1 α -HA Zn-C2B (rat, p1051) was produced from p592 and contains amino acids M₁ through S₅₀₈ and S_{1,380} through S_{1,615}. An HA tag with short exogenous linkers was present between E_{1,379} and S_{1,380} for RIM1 α and PDZ-C2A-P (a position that did not alter RIM1 α function or localization in previous studies^{30,59}, sequence ...E_{1,379}-EAAAG-YPYDVDPYA-AGAP-S_{1,380}...), and at S508 for Zn-C2B (sequence ...S₅₀₈-YPYDVDPYA-AGAP-S_{1,380}...). pFSW HA-Liprin- α 3 (rat, p526) corresponds in sequence and numbering to NCBI Reference Sequence [NP_001257914.1](#) with the addition of an N-terminal HA tag and a short linker (sequence M-YPYDVDPYA-GAPS-C₃...C_{1,192}) and has been previously described^{18,19}. pFSW HA-Liprin- α 3^N (p890) was produced from p526 and contains amino acids C₃ through S₅₇₆. pFSW Liprin- α 3^C (rat, p891) was produced from p526 and contains amino acids S₅₇₆ through C_{1,192}. Both p890 and p891 contain an HA tag at the N terminus (p890: M-YPYDVDPYA-GAPS-C₃...S₅₇₆; p891: M-YPYDVDPYA-GAP-S₅₇₆...C_{1,192}). pFSW HA-PTP σ (human, p846) was cloned from an open reading frame obtained from Jaewon Ko (pCMV HA-PTP σ in a modified pDisplay backbone⁷⁸), and sequence and numbering correspond to GenBank [AAI04813.1](#). pFSW HA-LAR (human, p1047) was produced from a plasmid obtained from the Harvard Plasmid Repository expressing human LAR, and sequence and numbering correspond to GenBank [AAH48768.1](#). The N-terminal signal peptide for each was replaced with a mouse IgK leader sequence followed by an HA tag and a short exogenous linker (sequence of p846: METDTLLLVWLLLVPGSTGD-YPYDVDPYA-GAQPARS-E₃₀...T_{1,501}; p1047: METDTLLLVWLLLVPGSTGD-YPYDVDPYA-GAQPARS-D₃₀...T_{1,898}).

Immunofluorescence staining for STED and confocal microscopy

Neurons grown on 12 mm, no. 1.5 glass coverslips were processed as previously established^{18,51}. They were fixed at DIV 15 to 16 in cold

phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA) for 10 min (for anti- $\text{Ca}_v2.1$ antibodies 2% PFA was used⁴⁸), followed by blocking and permeabilization in blocking solution (PBS, 3% BSA, 0.1% Triton X-100) for 1 h at room temperature (set point 23 °C). Incubations with primary and secondary antibodies were each done overnight at 4 °C in blocking solution. Samples were postfixed in 4% PFA for 10 min and mounted using ProLong diamond medium. Three 5 min washes with PBS were performed between steps. Primary antibodies used were rabbit anti-Liprin- $\alpha 3$ (lab antibody code A232, 1:500; home-made, knockout-verified for STED¹⁸), rabbit anti-RIM (A58, 1:500; RRID: AB_887774, knockout-verified for STED^{30,59}), rabbit anti- $\text{Ca}_v2.1$ (A46, 1:500; RRID: AB_2619841, knockout-verified for STED⁴⁸), rabbit anti-Munc13-1 (A72, 1:500; RRID: AB_887733, knockout-verified for STED⁶⁰), rabbit anti-RIM-BP2 (A126, 1:500; RRID: AB_2619739), mouse anti-HA (A12, 1:500; RRID: AB_2565006), mouse anti-Synaptophysin (A100, 1:500; RRID: AB_887824), mouse anti-PSD-95 (A152, 1:500; RRID: AB_10698024), mouse anti-Gephyrin (A8, 1:500; RRID: AB_2232546), mouse anti-Synapsin (A57, 1:500; RRID: AB_2617071) and guinea pig anti-Munc13-1 (A22, 1:500; RRID: AB_2619806). Secondary antibodies used were goat anti-rabbit Alexa Fluor 488 (lab antibody code S5, 1:250; RRID: AB_2576217), goat anti-mouse IgG1 Alexa Fluor 488 (S7, 1:250; RRID: AB_2535764), goat anti-mouse IgG1 Alexa Fluor 555 (S19, 1:250; RRID: AB_2535769), goat anti-mouse IgG2a Alexa Fluor 633 (S30, 1:250; RRID: AB_1500826), goat anti-guinea pig Alexa Fluor 405 (S51, 1:250; RRID: AB_2827755), goat anti-guinea pig Alexa Fluor 555 (S23, 1:250; RRID: AB_2535856) and goat anti-mouse IgG1 Alexa Fluor 633 (S29, 1:250; RRID: AB_2535768).

Image acquisition and analyses for confocal and STED microscopy

Images were acquired with previously established protocols^{18,51} using a Leica SP8 Confocal/STED 3X microscope equipped with an oil-immersion $\times 100$ objective (1.44 NA), a white laser, STED gated detectors and 592, 660 and 770 nm depletion lasers. To assess the localization of Munc13-1 relative to either $\text{Ca}_v2.1$ or Liprin- $\alpha 3$, triple confocal scans for Synapsin, Munc13-1 and either $\text{Ca}_v2.1$, Liprin- $\alpha 3$ or a different Munc13-1 antibody were followed by triple-color STED scans for the same proteins. For the remaining STED experiments, quadruple confocal scans for Synaptophysin, PSD-95, Gephyrin and a protein of interest were followed by triple-color STED scans for PSD-95, Gephyrin and the protein of interest (Synaptophysin was not imaged in STED mode because of depletion laser limitations). The two exceptions to these protocols were (1) when HA antibodies were used, Gephyrin antibodies could not be included because they are not compatible with our established HA antibodies and (2) for the assessment of Liprin- $\alpha 3$ fragments in Fig. 5b–d and Extended Data Fig. 7b–d, RIM-BP2 was used as a marker instead of PSD-95 and Gephyrin. The acquired images were 2,048 pixels $\times$ 2,048 pixels with a pixel size of 22.7 nm $\times$ 22.7 nm, and each image contained hundreds of synapses. Acquisition settings for a given staining and channel were identical for all images within a batch of culture in which all conditions from one experiment were compared. To quantify STED images, side-view synapses were selected by an experimenter who was blind to the protein of interest. Side-views were defined as synapses containing a synaptic vesicle cloud of >250 nm from the active zone to the inside of the presynaptic terminal and with a bar-like Munc13-1, Gephyrin, PSD-95 or RIM-BP2 structure along its edge. A 750 nm long, 250 nm wide line profile was then drawn perpendicular to the postsynaptic density marker and across its center. After applying a five-pixel rolled average to the protein of interest and PSD-95 or Gephyrin, line profiles of individual synapses were aligned to the peak of the postsynaptic marker and averaged. The maximum value of the resulting individual line profiles was used to calculate the peak intensity. In each culture, line profiles and peak intensities were normalized to the average signal in the condition that was used for comparison (which was control, conditional knockout or conditional knockout infected with a rescue lentivirus as noted in each figure legend). In line profile

plots, peaks appear below 100% because peak intensities for proteins of interest are not always at the same position. En-face synapses were visually selected by an experimenter as synapses for which the area of Munc13-1 staining was surrounded by Synapsin staining along more than three-quarters of its circumference, and the area that contained Munc13-1 covered approximately half or more of the Synapsin area. The channel of the protein of interest (either $\text{Ca}_v2.1$, Liprin- $\alpha 3$ or Munc13-1 labeled with another antibody) was not visible to the experimenter during en-face synapse selection. To assess colocalization between Munc13-1 and the proteins of interest, a binary mask for each individual channel was created using Otsu thresholding, and the pixel-to-pixel correlation between masks was measured using Pearson's correlation within a synaptic region of interest defined by Synapsin. In addition to the STED quantification, we quantified confocal signals for genotype comparisons because these methods complement one another. STED provides the spatial resolution required to resolve the narrow localization of active zone proteins, while confocal imaging does not involve signal depletion. To quantify synapse density in confocal images, whole images of individual confocal channels (excluding out-of-focus regions and somata) were analyzed with an automatic detection algorithm in MATLAB (https://github.com/kaeserlab/3DSIM_Analysis_CL and https://github.com/hmslcl/3D_SIM_analysis_HMS_Kaesler-lab_CL) using Otsu thresholding⁷⁹. To avoid detection artefacts, the area corresponding to the soma was removed. Out-of-focus regions were also removed. The synaptic mask was subsequently used to quantify the fluorescence intensity levels of the protein of interest. Confocal data were normalized to the average in the condition that was used for comparison (which was control, conditional knockout or conditional knockout infected with a rescue lentivirus as noted in each figure legend) per culture. For representative images, a smooth filter was added, brightness and contrast were linearly adjusted and images were interpolated. Identical adjustments were applied to representative images of the same channel within an experiment. Some example images may have saturated areas as a result of these adjustments, but quantifications were performed on original images without brightness and contrast adjustments and without background subtraction or resampling. Data were acquired and analyzed by an experimenter who was blind to genotype.

Immunofluorescence staining for DNA Exchange-PAINT

Neurons grown on MatTek dishes were fixed in 2% PFA and 4% glucose in PBS at DIV 15–17. They were next permeabilized in PBS supplemented with 100 mM glycine (PBS-G) containing 0.3% Triton X-100 and blocked in PBS-G containing 3% BSA and 0.1% Triton X-100. Five primary antibodies were used: rabbit anti-Munc13-1 (lab antibody code A72, 1:500; RRID: AB_887733), rabbit anti- $\text{Ca}_v2.1$ (A46, 1:500; RRID: AB_2619841), mouse anti-PSD-95 (A152, 1:500; RRID: AB_10698024), mouse anti-Bassoon (A85, 1:500; RRID: AB_11181058) and guinea pig anti-Bassoon (A67, 1:500; RRID: AB_2290619). The four primary antibodies for DNA-PAINT (A72, A46, A152 and A85) were pre-incubated with their respective DNA-conjugated secondary nanobodies (F2-anti-rabbit IgG (S65), F3-anti-rabbit IgG (S66), F1-anti-mouse kLC (S67) and F4-anti-mouse kLC (S68); custom-made by Massive Photonics using FAST-PAINT F-series DNA⁸⁰) in PBS at a 1:2.5 molar ratio for 20 min in tubes separated from one another. A rabbit Fc fragment was added to the pre-incubation mixes of $\text{Ca}_v2.1$ and Munc13-1 at two times higher concentration than the primary antibody. After pre-incubation, antibodies were mixed and neurons were incubated overnight at 4 °C. Next, goat anti-guinea pig Alexa Fluor 488 (S3, 1:500; RRID: AB_2534117) was added for 1 h at room temperature. Finally, the samples were postfixed in 4% PFA diluted in PBS with 4% glucose. Three 5 min washes with PBS-G were performed between steps.

Single-molecule imaging, processing and analysis for DNA Exchange-PAINT

Images were acquired on a Nikon Ti2 inverted microscope equipped with a $\times 100$ Apo TIRF oil-immersion objective (1.49 NA), 488, 561 and

640 nm excitation lasers, appropriate filters, an Ixon+ 897 EM-CCD camera and Nikon Elements and MicAO software. Before imaging, 90 nm gold nanoparticles at 1:10 dilution were added as fiducial markers. Imager strands (Massive Photonics; F1-Atto643, F3-Cy3B, F4-Atto643 and F2-Cy3B) were diluted in imaging buffer (containing PBS + 500 mM NaCl + PCA/PCD/Trolox, pH 7.4 (ref. 53)) at a final dilution of 0.25 nM (for Munc13-1 and Ca_v2.1) or 0.5 nM (for Bassoon and PSD-95). DNA-PAINT videos were acquired for 30,000 frames with 100 ms exposure in two exchange rounds to minimize chromatic aberration. First, PSD-95 (with F1-Atto643) and Munc13-1 (with F3-Cy3B) were imaged. Next, Bassoon (with F4-Atto643) and Ca_v2.1 (with F2-Cy3B) were imaged. One wash with PBS was applied between rounds. Finally, 100 nm Tetraspeck beads were immobilized on separate coverslips coated with poly-L-lysine and imaged to subsequently generate a transform.

Videos were processed with Picasso (<https://github.com/jungmannlab/picasso>)⁵³ and custom MATLAB scripts. Target protein DNA-PAINT videos were localized by minimizing a nearest neighbor search separately for each target (minimum net gradient 9,000). Resulting localization files were recombined to one file per exchange round using Picasso's *join* function, drift-corrected with Picasso's *undrift* function (segment 1,000) and corrected for chromatic aberrations using the MATLAB's *transformPointsInverse* function and a transform calculated from Tetraspeck bead images (which was calculated using MATLAB's *fitgeotrans* function with a second-degree polynomial, as before⁵⁵). Any residual linear offset between targets was corrected by cross-correlating each other target to the Munc13-1 localizations using a custom MATLAB function. Localizations with a standard deviation of the fit of <0.3 or >1.6 pixels, localization error of >20 nm or photon count larger than the mode bin of a histogram of photon counts were removed. Finally, localizations were temporally linked using Picasso's *link* function (0.3-pixel radius, five dark frames allowed). En-face synapses identified by Bassoon and PSD-95 were analyzed. Putative synaptic clusters of Bassoon and PSD-95 were first identified with Picasso's *dbscan* function (48 nm radius, 10 min points), and clusters with fewer than 75 localizations were removed. Clusters representing nonspecific imager binding were then removed if either their mean frame number was ± 2 times the standard deviation of a Gaussian fit of the mean frame number of all clusters or if the standard deviation of the frame number was <2,500 or >11,000, as nonspecific events are localized for only short time and thus their mean frame number deviates from $N \text{ frames} / 2$ (15,000) and the standard deviation of the frame number is small. Only en-face synapses in which the smoothed Bassoon alpha shape was ≤ 2 and the area overlap between Bassoon and PSD-95 alpha shapes was at least 70% were included.

Munc13-1 and Ca_v2.1 localizations were next restricted to active zone borders marked by Bassoon. Synapses with <25 localization for Munc13-1, Ca_v2.1 or PSD-95 within the Bassoon boundaries were excluded. Synapses were analyzed using a custom MATLAB script similar to those previously described, with modifications for 2d localizations^{55,81,82}. Autocorrelation was calculated as previously described^{55,82}, modified for 2d localizations. Nanoclusters of Munc13-1 and Ca_v2.1 were identified using MATLAB's *dbscan* function. This function requires two parameters, epsilon and minimum points that are sensitive to the density of localizations, which varied across synapses and between proteins. Hence, these two parameters were determined independently for each protein and each synapse. Localizations were randomized inside the Bassoon alpha shape, and epsilon was calculated as the mean minimal distance of the random points to each other plus $5 \times$ the standard deviation, and the minimum points as the mean number of points within that epsilon for each point plus $5 \times$ the standard deviation. For stability, the randomization was run 100 times for each protein or synapse, and the mean value was used. Nanoclusters were removed from the dataset if they had fewer than five localizations. After an initial round of detection, area outliers were identified with GraphPad

Prism's ROUT method using the strictest settings ($Q = 0.1\%$), and outlier nanoclusters with an area greater than the maximum non-outlier area were removed. The area of a nanocluster is defined by an alpha shape of the nanocluster localizations with an alpha radius of 150 nm. Cross-enrichment was calculated as previously described^{55,82}. Specifically, enrichments were normalized to a random distribution and smoothed by replacing high outliers (identified using Prism's ROUT with $Q = 0.1\%$) with the highest non-outlier value at each distance bin. To assess enrichment, the total enrichment curve from the center of each nanocluster of the opposite protein was calculated. Nanocluster enrichment was then categorized by comparing the average value of the cross-enrichment with the opposite protein within 60 nm of the nanocluster center with that following 50 randomizations of the opposite protein's distribution. Nanoclusters were considered enriched or de-enriched when the real average enrichment to the opposite distribution was greater than or less than the average ± 1.96 standard deviations of the enrichments to the random distributions, respectively. All other nanoclusters were designated as indistinguishable from randomized. The separation index was defined as $SI = d / (r_1 + r_2)$, where d is calculated as the Euclidean distance between the centroids of a nanocluster of interest to the nearest nanocluster of the opposite protein, r_1 is calculated as the distance along this line from the centroid to the border of the first nanocluster and r_2 is the same for the second nanocluster. The MATLAB code generated for image analysis is available at <https://github.com/blanpiedlab/EmperadorMelero-2024> and was used in parallel in another study⁸³. Example overview images were generated by rendering in Fiji using the ThunderSTORM plugin's *Averaged Shifted Histogram* method with a magnification of $\times 8$ (20 nm pixels).

Western blotting

To generate the homogenates, lysates from DIV 15 to 16 cultures were collected in standard $3 \times$ SDS sample buffer diluted with PBS. Brains of P21 to P28 mice were dissected and homogenized with a glass-Teflon homogenizer in a solution containing 150 mM NaCl, 25 mM HEPES, 4 mM EDTA and 1% Triton X-100 (pH 7.5), and then diluted with standard $3 \times$ SDS sample buffer. Samples were boiled at 100 °C for 10 min and run on standard SDS-PAGE gels. Proteins were electrophoretically transferred from gels to nitrocellulose membranes for 6.5 h at 4 °C in buffer containing (per l) 200 ml methanol, 14 g glycine and 6 g Tris. Membranes were blocked for 1 h at room temperature in TBST (Tris-buffered saline with 0.1% v/v Tween-20) containing 10% non-fat milk powder and 5% normal goat serum. Membranes were incubated with primary and secondary antibodies, and each step was done overnight at 4 °C. The antibody incubation solution contained TBST with 5% milk and 2.5% goat serum. Five 5 min washes were performed between steps. The primary antibodies used were rabbit anti-Liprin- $\alpha 1$ (A121, 1:500), rabbit anti-Liprin- $\alpha 2$ (A13, 1:500) and rabbit anti-Liprin- $\alpha 4$ (A2, 1:500) (gifts from S. Schoch)⁸⁴; mouse anti-HA (A12, 1:1,000; RRID: AB_2565006); mouse anti-Synaptophysin (A100, 1:5,000; RRID: AB_887824), mouse anti-Synapsin (A57, 1:5,000; RRID: AB_2617071), goat anti-PTP α (A114, 1:200; RRID: AB_2607944) and mouse anti-LAR (A156, 1:500; clone E9B9S from Cell Signaling). The secondary antibodies were peroxidase-conjugated goat anti-mouse IgG (S52, 1:10,000; RRID: AB_2334540), peroxidase-conjugated goat anti-rabbit IgG (S53, 1:10,000; RRID: AB_2334589) and peroxidase-conjugated anti-goat IgG (S60, 1:10,000).

Imaging and analyses of protein condensates in transfected HEK293T cells

HEK293T cells were plated on 12 mm, no. 1.0 or no. 1.5 glass coverslips for fixed samples or on MatTek dishes for fluorescence recovery after photobleaching (FRAP) experiments in HEK medium containing DMEM with 10% fetal bovine serum (Atlas Biologicals, F-0500-D) and 1% penicillin-streptomycin. They were transfected with the Ca²⁺ phosphate method with 150 ng of the plasmids expressing Liprin- $\alpha 3$ (or of RIM1 α

if Liprin- α was not included), and additional plasmids were added at 1:1 molar ratios (except for Ca $_v$ 2.1, which was added at 0.67:1). Phorbol 12-myristate 13-acetate and bisindolylmaleimide-I were added within 1 h after transfection at a concentration of 1 μ M and 0.1 μ M, respectively. At 16–20 h after transfection, cells were either fixed in 4% PFA in PBS and subsequently mounted for imaging or they were used for FRAP. For experiments in Fig. 6j,k, to assess the presence of Munc13-1 (not shown in the figure), cells were blocked and permeabilized in a blocking solution (PBS, 3% BSA, 0.1% Triton X-100) for 1 h at room temperature, followed by incubations with primary (rabbit anti-Munc13-1; A72, 1:500; RRID: AB_887733) and secondary (goat anti-rabbit Alexa Fluor 488; S5, 1:500, RRID: AB_2576217) antibodies overnight at 4 °C in blocking solution. Three 5 min washes with PBS were performed between steps. All images were acquired in confocal mode using a Leica SP8 Confocal/STED 3X microscope, using an oil-immersion $\times 63$ objective (1.44 NA). Images of 250 μ m $\times$ 250 μ m (pixel size, 240 nm $\times$ 240 nm), 185 μ m $\times$ 185 μ m (pixel size, 180 nm $\times$ 180 nm) or 125 μ m $\times$ 125 μ m (pixel size, 60 nm $\times$ 60 nm) containing multiple transfected cells were taken. Pearson's correlation was used to assess the colocalization of proteins in cells expressing all proteins of interest for any given experiment using the 'Coloc2' plugin of ImageJ/Fiji. Cells in which the fluorescent signal of at least one of the proteins was covering most of the cell area were not included. In experiments with mE-Ca $_v$ 2.1, cerulean was imaged last to prevent photoconversion of mEOS before other fluorophores were acquired. For FRAP, movies of variable dimensions containing transfected cells (one cell at a time) were taken at room temperature. Cells were maintained in HEK medium during image acquisition. Single condensates were photobleached using a 405 nm wavelength laser followed by image acquisition at 1 Hz in confocal mode at room temperature, as we previously described¹⁸. Regions of interest were drawn over pre-bleached structures and the percentage of intensity recovered was plotted as a function of time. Each individual trace was normalized to its maximum and minimum intensity values and plotted in Fig. 6f. The maximum value after photobleaching of the resulting normalized trace within the first 120 s of recovery was used to calculate the percentage of recovery (Fig. 6g). For representative images only, a smooth filter was added, brightness and contrast were linearly adjusted and images were interpolated. These adjustments were done either equally across conditions (Figs. 3 and 6b,k) or unequally to reach a similar level of signal intensity between condensates before bleaching (Fig. 6e). Quantifications were done on original images without adjustments. pCMV RIM1 α -tdTomato (rat, p1010) and pCMV RIM1 α -cerulean (rat, p1011) follow the same cDNA sequence variants and numbering as described for p592 above. The fluorescent tags were placed between E $_{1,379}$ and S $_{1,380}$ and flanked by exogenous linkers (p1010:...E $_{1,379}$ -AAAAA-V $_{2,\dots}$ K $_{476}$ -AAGGAP-S $_{1,380}$... with V $_{2,\dots}$ K $_{476}$ referring to tdTomato residues according to GenBank [LC311026.1](#); p1011:...E $_{1,379}$ -AAA-V $_{2,\dots}$ K $_{239}$ -GAP-S $_{1,380}$... with V $_{2,\dots}$ K $_{239}$ referring to cerulean residues according to GenBank [ACO48272.1](#)). pCMV cerulean-Liprin- α 3 (rat, p471) and pCMV mVenus-Liprin- α 3 (rat, p472) are the same sequence variant and numbering as outlined for p576 above and have been previously described¹⁸. The cerulean or mVenus tags were placed at the N terminus flanked by linker sequences (sequence M-AAA-V $_{2,\dots}$ K $_{239}$ -GAPS-C $_{3,\dots}$ C $_{1,192}$; V $_{2,\dots}$ K $_{239}$ refer to cerulean for p471 or mVenus for p472 according to GenBank [ACO48272.1](#) and GenBank [AAZ65844.1](#), respectively). pCMV HA-Liprin- α 3 (mouse, p470) corresponds to GenBank [NP_084017.2](#) with a T714A point mutation and the HA tag was positioned at the N terminus (sequence M-YPYDVPDYA-M $_{1,\dots}$ C $_{1,194}$). pCMV cerulean-RIM-BP2 (rat, p1009) and pCMV RIM-BP2 (rat, p205) correspond in sequence and numbering to [XP_038945062.1](#). The cerulean tag was placed at the N terminus between M $_1$ and R $_2$ and flanked by short linkers (sequence: M $_1$ -AAA-V $_{2,\dots}$ K $_{239}$ -GAP-R $_2$...P $_{1,068}$; V $_{2,\dots}$ K $_{239}$ refer to cerulean according to GenBank [ACO48272.1](#)). pCMV mEOS-Ca $_v$ 2.1 (mouse, p772) was produced based on a previous expression vector⁴⁸ and numbering and sequence correspond to GenBank [AAW56205.1](#).

It has an mEOS3.2 tag between V $_{27}$ and G $_{28}$, that is flanked by short linkers (sequence M $_1$...V $_{27}$ -AS-S $_{2,\dots}$ R $_{226}$ -ACR-G $_{28}$...C $_{2,369}$). S $_{2,\dots}$ R $_{226}$ refer to mEOS3.2 as previously described⁸⁵ (<https://www.fpbase.org/protein/meos32>). mEOS3.2 was obtained as a gift from M. Davidson and T. Xu (Addgene plasmid no. 54550; <http://n2t.net/addgene:54550>; RRID: Addgene_54550). pcDNA Munc13-1 (rat, p202) corresponds in sequence and numbering to NCBI Reference Sequence [NP_074052.2](#) without G $_{1,415}$ TLLRKHGKGLKGRVKLPESHSD $_{1,437}$ and V $_{1,533}$ HGGKGRFTLSE DVCPEM $_{1,551}$; see NCBI Reference Sequence [XP_017168369.1](#) for the same splice variant). pcDNA3.1 Dynamin 1 (human, p880) was a gift from S. Schmid and was obtained from Addgene (Addgene plasmid no. 34682; <http://n2t.net/addgene:34682>; RRID: Addgene_34682). It contains a partial N-terminal HA tag and a sequence that corresponds to PDB 7AX3_A (sequence: ME-YDVPDYA-H-M $_{1,\dots}$ L $_{864}$). pCMV HA-PTP δ (human, p844, obtained as a gift from J. Ko with a modified pDisplay backbone) and pCMV tdTomato-HA-PTP δ (human, p889) follow the same sequence variant and numbering as outlined for p846 above. The N-terminal signal peptide was substituted by a mouse IgK leader sequence and followed by the corresponding tag and a short exogenous linker (p844, METDTLLWVLLWVPGSTGD-YPYDVPDYA-GAQPARS-E $_{30,\dots}$ T $_{1,501}$; p889, METDTLLWVLLWVPGSTGD-YPYDVPDYA-GAQPARS-V $_{2,\dots}$ K $_{476}$ -RS-E $_{30,\dots}$ T $_{1,501}$; V $_{2,\dots}$ K $_{476}$ refer to tdTomato residues according to GenBank [LC311026.1](#)). pCMV PTP δ (mouse, p848, in a modified pDisplay vector) was produced from a plasmid containing a PTP δ open reading frame obtained as a gift from J. Ko (pcDNA myc-PTP δ ⁷⁸). The numbering and the sequence of p848 correspond to GenBank [XP_036019750.1](#) but with two variants (without amino acids V $_{51}$ and E $_{189}$ -I $_{191}$; see NCBI Reference Sequence [XP_036019737.1](#)). The signal peptide was replaced with a mouse IgK leader sequence followed by a short exogenous linker (sequence METDTLLWVLLWVPGSTGD-GAPGPV-E $_{28}$ -T $_{1,921}$). pCMV LAR (human, p849) was cloned from a plasmid obtained from the Harvard Plasmid Repository expressing human LAR identical to the sequence in GenBank [AAH48768.1](#).

Correlative light-electron microscopy

HEK293T cells were plated on 25 mm, no. 2 photo-etched, gridded coverslip in HEK medium, transfected as described for protein condensate experiments above and fixed in 2.5% glutaraldehyde in PBS buffer (pH 7.4) for 30 min at room temperature 16–20 h after transfection. Fluorescent and wide-field images of transfected cells expressing the proteins of interest were acquired using either a Nikon Ti inverted microscope equipped with a Yokagawa CSU-W1 spinning disc confocal system and a $\times 40$ objective (0.75 NA) or a Leica SP8 Confocal/STED 3X microscope, using an oil-immersion $\times 63$ objective (1.44 NA). After acquiring images, samples were washed three times for 5 min each with ice-cold 100 mM PIPES pH 7.4 and then incubated in staining solution I (consisting of 1% OsO $_4$, 1.25% potassium hexacyanoferrate in 100 mM PIPES pH 7.4) for 2 h followed by staining solution II (prepared by diluting staining solution I 100 $\times$ in a solution of 1% tannic acid) for 30 min and, finally, in 1% uranyl acetate overnight. The samples were kept at 4 °C and protected from light during all the staining steps. Three 5 min washes with ice-cold milli-Q water were performed between steps. Samples were dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 90%, 100%; ice-cold), followed by two washes in 100% ice-cold acetone, embedded in epoxy resin and baked at 60 °C for 36 h. The coverslip was removed from the resin by sequential immersions into liquid nitrogen and boiling water. Then, 50 nm-thick sections from blocks containing grid areas with the cells of interest were cut with a Leica EM UC7 ultramicrotome. A JEOL 1200EX transmission electron microscope equipped with an AMT 2k CCD camera was used for image acquisition of the target cells (identified by their position on the gridded coverslips and by their shape matching that of the wide-field image). Fluorescent and electron microscopy images were aligned using the BigWarp plugin (ImageJ/Fiji). The electron micrograph was used as a fixed image, and arbitrary

references (such as the nucleus or the plasma membrane) were used for alignment. The alignment for each cell was conducted multiple times, using different features as landmarks each time to independently confirm the alignments. For representative images, a smooth filter was added to fluorescent images, and brightness and contrast in all images were linearly adjusted and interpolated.

Electrophysiology of cultured neurons

Whole-cell patch clamp recordings of cultured hippocampal neurons plated on 12 mm, no. 1.0 or no. 1.5 glass coverslips were done as established before at DIV 15 to 16 (refs. [18,19,24,30,48,51](#)). Glass pipettes (resistance of 1.5–4 M Ω) were filled with an intracellular solution containing 120 mM Cs-methanesulfonate, 10 mM EGTA, 2 mM MgCl₂, 10 mM HEPES-CsOH (pH 7.4), 4 mM Na₂-ATP and 1 mM Na-GTP for excitatory transmission; and 40 mM CsCl, 90 mM K-gluconate, 1.8 mM NaCl, 1.7 mM MgCl₂, 3.5 mM KCl, 0.05 mM EGTA, 10 mM HEPES, 2 mM MgATP, 0.4 mM Na₂-GTP and 10 mM phosphocreatine, CsOH (pH 7.4) for inhibitory transmission. Neurons were clamped at –70 mV (or at +40 mV for NMDAR-EPSCs) and series resistance was compensated to <5 M Ω . Any recording with a series resistance of >15 M Ω before compensation during acquisition was discarded. Recordings were done at room temperature in extracellular solution containing 140 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES (pH 7.4) and 10 mM glucose. For mEPSCs, mIPSCs and sucrose-evoked release, the extracellular solution was supplemented with 1 μ M TTX, 50 μ M D-AP5 and either 50 μ M picrotoxin (for EPSCs) or 20 μ M CNQX (for IPSCs). For electrically evoked currents, the extracellular solution was supplemented with 20 μ M CNQX and either 50 μ M D-AP5 (for IPSCs) or 20 mM PTX (for NMDAR-EPSCs). Electrical stimulation was applied using a bipolar electrode custom-made from Nichrome wire. Hyper-tonic sucrose (500 mM) was locally applied with a pump for 10 s at a flow rate of 10 μ l min^{–1}, and the integral of the first 10 s of the response was used to estimate the readily releasable pool. A Multiclamp 700B amplifier and a Digidata 1550 digitizer were used, sampling at 10 kHz and filtering at 2 kHz. Data were analyzed using pClamp. Data were acquired and analyzed by an experimenter who was blind to genotype.

Electron microscopy of cultured neurons

Electron microscopy was performed as previously established^{[18,19,24,30,48,51,60](#)}. In brief, DIV 15 neurons grown on 0.12 mm thick, 6 mm diameter Matrigel-coated sapphire coverslips were transferred to an extracellular solution containing 140 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, 20 mM CNQX, 50 mM D-AP5, 50 mM PTX and 10 mM HEPES (pH 7.4, ~310 mOsm) and high-pressure frozen with a Leica EM ICE freezer. The samples were then freeze-substituted using an acetone solution supplemented with 1% osmium tetroxide, 1% glutaraldehyde and 1% H₂O following this protocol: –90 °C for 24 h, 5 °C h^{–1} to –20 °C, –20 °C for 12 h and 10 °C h^{–1} to 20 °C. Next, samples were embedded in epoxy resin and baked for 48 h at 60 °C. The sapphire coverslip was then removed from the resin block by sequential immersions into liquid nitrogen and boiling water. The process was repeated until the coverslip detached. The resin block containing the neurons was divided into four pieces and each piece was mounted on a stub. A Leica EM UC7 ultramicrotome was used for sectioning at 50 nm, and sections were collected on a nickel slot grid (2 mm $\times$ 1 mm) with a carbon-coated formvar support film. The sections were counter-stained with 2% lead acetate solution for 10 s followed by rinsing with distilled water. Images were taken with a JEOL 1200EX transmission electron microscope equipped with an AMT 2k CCD camera. Single cross-sections of synapses containing a cloud of vesicles and with an evident, well-preserved synaptic cleft were selected for quantification. A MATLAB macro provided by M. Verhage and J. Broeke was used for analyses. Docked vesicles were defined as those in contact with the presynaptic plasma membrane apposed to the PSD and with no white space between the electron-dense vesicular and target membranes.

Bouton size is expressed as the area within the perimeter of a bouton in a cross-section. Brightness and contrast were adjusted for representative examples to match appearance. Data were acquired and analyzed by an experimenter who was blind to the conditions.

Statistics

Data are shown as mean $\pm$ s.e.m. unless noted otherwise. Violin plots are shown instead of scatter plots when the number of data points for at least one of the groups was >100. Significance was assessed using parametric (*t*-test or one-way ANOVA) or non-parametric (Mann–Whitney *U* or Kruskal–Wallis) tests depending on whether assumptions of normality and homogeneity of variances were met (assessed using Shapiro or Levene’s tests, respectively). Tukey–Kramer or Holm corrections for multiple testing were applied. Post hoc comparisons between all groups were performed, and only significance relative to the respective conditional knockout group (or to the control group in Fig. [3h](#), the ‘no PTP’ group in Fig. [6c,d](#) and LAR in Fig. [6g](#)) is reported in figures. For STED images, statistical analyses were performed on the peak values of the line profiles. For paired pulse ratios, a two-way ANOVA was used followed by Dunnett’s post hoc test. A Pearson’s chi-squared test was used to assess mouse survival ratios. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications^{[16,18,30,48,49,52,55,64](#)}. All data that met quality standards described throughout the methods were included and there was no exclusion of outliers for genotype comparisons. Experimental groups and data were not randomized but were sorted by genotype or condition after they were analyzed by an experimenter who was blind to genotype and condition. Statistical analyses were performed in R or in GraphPad Prism. Exact *P* values for statistical tests are provided in Supplementary Table 1.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Data points generated for this study are included in the figures whenever possible. Numerical data for all figures are available at <https://doi.org/10.5281/zenodo.12205516> (ref. [86](#)). Additional data are available from the corresponding author upon request. Source data are provided with this paper.

Code availability

Custom MATLAB codes for automatic two-dimensional segmentation (https://github.com/kaeserlab/3DSIM_Analysis_CL)^{[79](#)} and DNA Exchange-PAINT image analyses (<https://github.com/blampiedlab/EmperadorMelero-2024>) have been deposited to Github.

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Author contributions

J.E.-M. and P.S.K. conceived the study. J.E.-M., J.W.A., S.R.M., A.D.L., P.A.D. and G.dN. devised the methodology. J.E.-M., J.W.A., S.R.M. and G.dN. performed the investigation. J.E.-M., S.R.M., A.D.L., P.A.D., G.dN., T.A.B. and P.S.K. conducted the formal analysis. T.A.B. and P.S.K. supervised the project. J.E.-M., T.A.B. and P.S.K. acquired funding. J.E.-M. and P.S.K. wrote the original draft of the paper; J.E.-M., J.W.A., S.R.M., A.D.L., P.A.D., G.dN., T.A.B. and P.S.K. reviewed and edited the final version.

Competing interests

The authors declare no competing interests.

Additional information

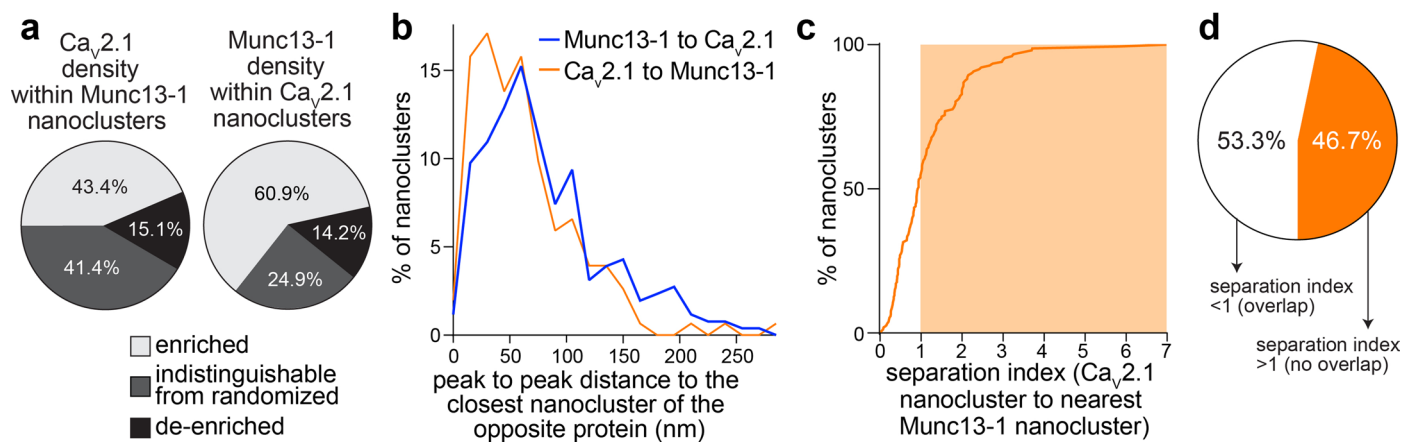
Extended data is available for this paper at <https://doi.org/10.1038/s41593-024-01720-5>.

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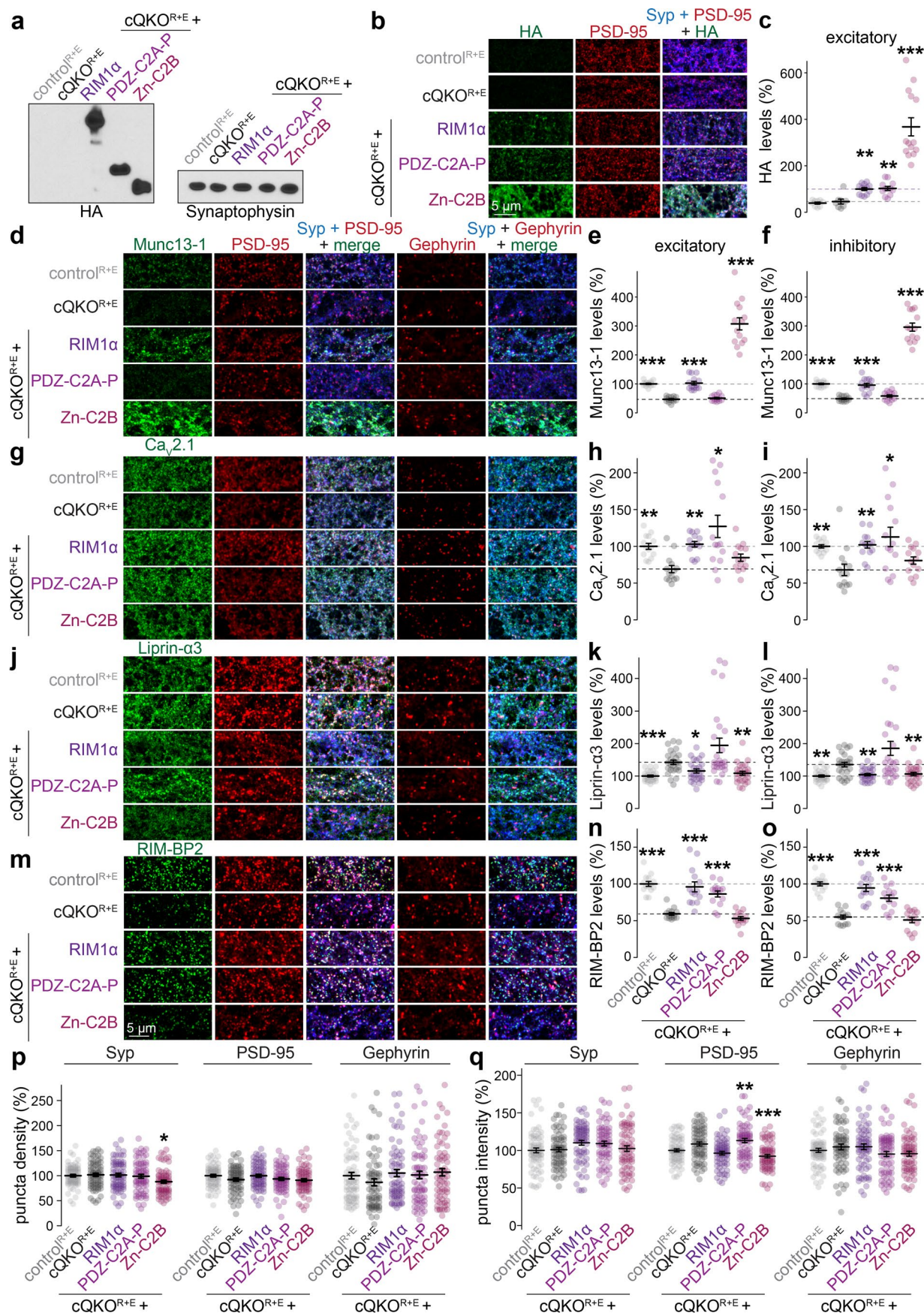
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Extended Data Fig. 1 | Additional assessment of Munc13-1 and $\text{Ca}_v2.1$ with DNA Exchange-PAINT. (a) Percentage of Munc13-1 nanoclusters (left) enriched with $\text{Ca}_v2.1$, containing a $\text{Ca}_v2.1$ density indistinguishable from its randomized distribution, or de-enriched of $\text{Ca}_v2.1$, and vice versa for $\text{Ca}_v2.1$ nanoclusters (right); Munc13-1 350 nanoclusters/3 cultures, $\text{Ca}_v2.1$ 169/3. (b) Histogram showing the binned peak-to-peak distance from Munc13-1 nanoclusters to the

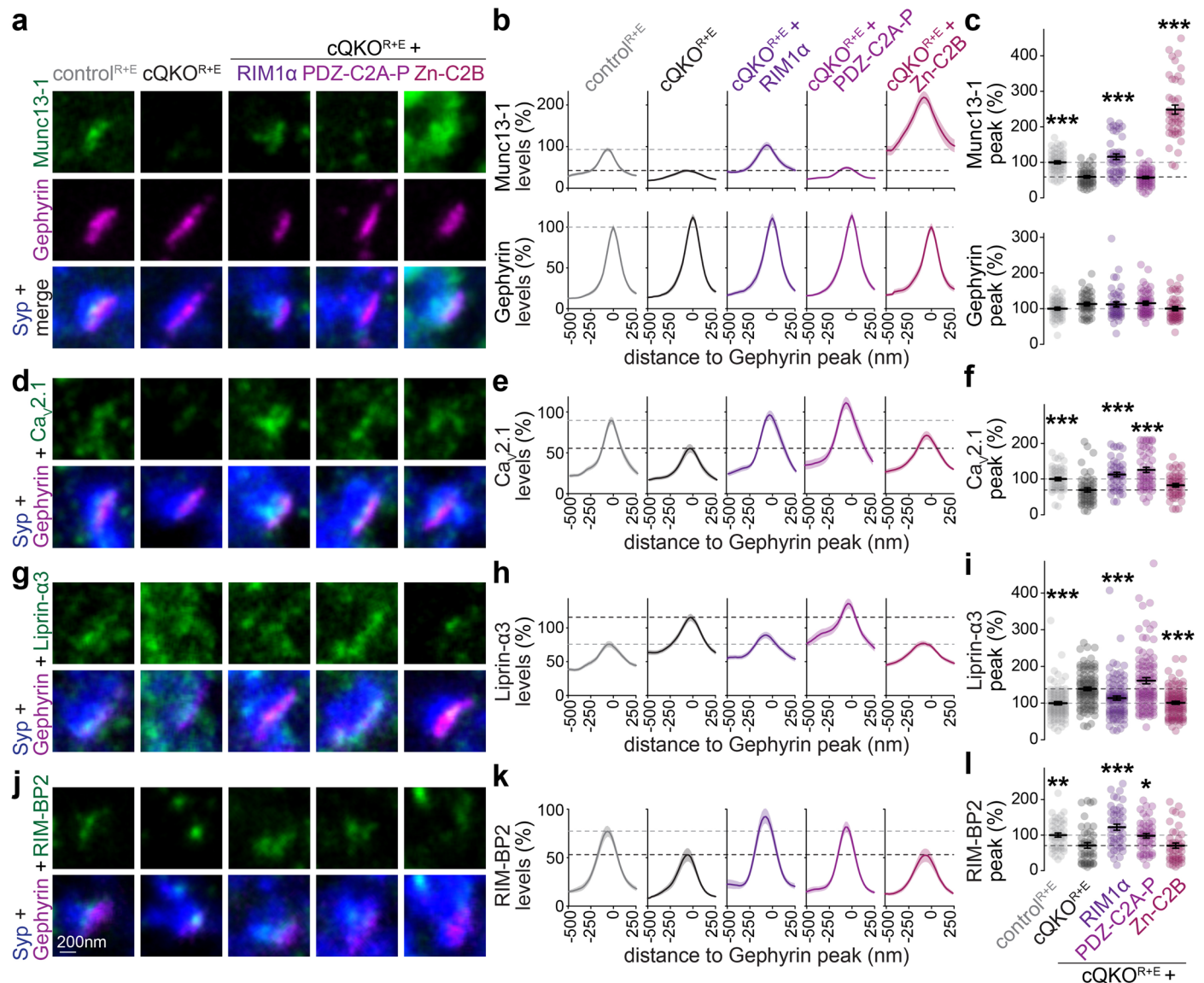
closest $\text{Ca}_v2.1$ nanocluster, and vice versa. The estimated nanocluster radii are ~23 nm for Munc13-1 and ~30 nm for $\text{Ca}_v2.1$; N as in a. (c, d) Histogram showing the index of separation from $\text{Ca}_v2.1$ nanoclusters to the nearest Munc13-1 nanocluster (c) and pie chart (d) showing the percentage of clusters that show any overlap (separation index < 1) or no overlap (separation index > 1); N as in a.



Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Assessment of RIM1 constructs with Western blot and confocal microscopy. (a) Western blot to assess expression of RIM1 α , PDZ-C2A-P and Zn-C2B in cultured neurons. (b, c) Example confocal images (b) and quantification (c) of the average intensity of HA (to detect rescue proteins) at excitatory synapses identified as PSD-95 regions of interest (ROIs). Neurons were stained for HA, PSD-95 and Synaptophysin (Syp). Intensity is normalized to the average signal in cQKO^{R+E} + RIM1 α per culture. Levels at inhibitory synapses were not assessed due to incompatibility of HA and Gephyrin antibodies; control^{R+E} 9 images/3 cultures, cQKO^{R+E} 8/3, cQKO^{R+E} + RIM1 α 13/3, cQKO^{R+E} + PDZ-C2A-P 12/3, cQKO^{R+E} + Zn-C2B 14/3. (d–o) As in b and c, but for excitatory and inhibitory synapses and for Munc13-1 (d–f), Ca_v2.1 (g–i), Liprin- α 3 (j–l) and RIM-BP2 (m–o). Neurons were stained for a protein of interest (Munc13-1, Ca_v2.1, Liprin- α 3 or RIM-BP2), postsynaptic markers PSD-95 and Gephyrin), and Synaptophysin. Excitatory synapses were defined as PSD-95 ROIs and inhibitory synapses as Gephyrin ROIs. Data are normalized to the average signal in control^{R+E} per

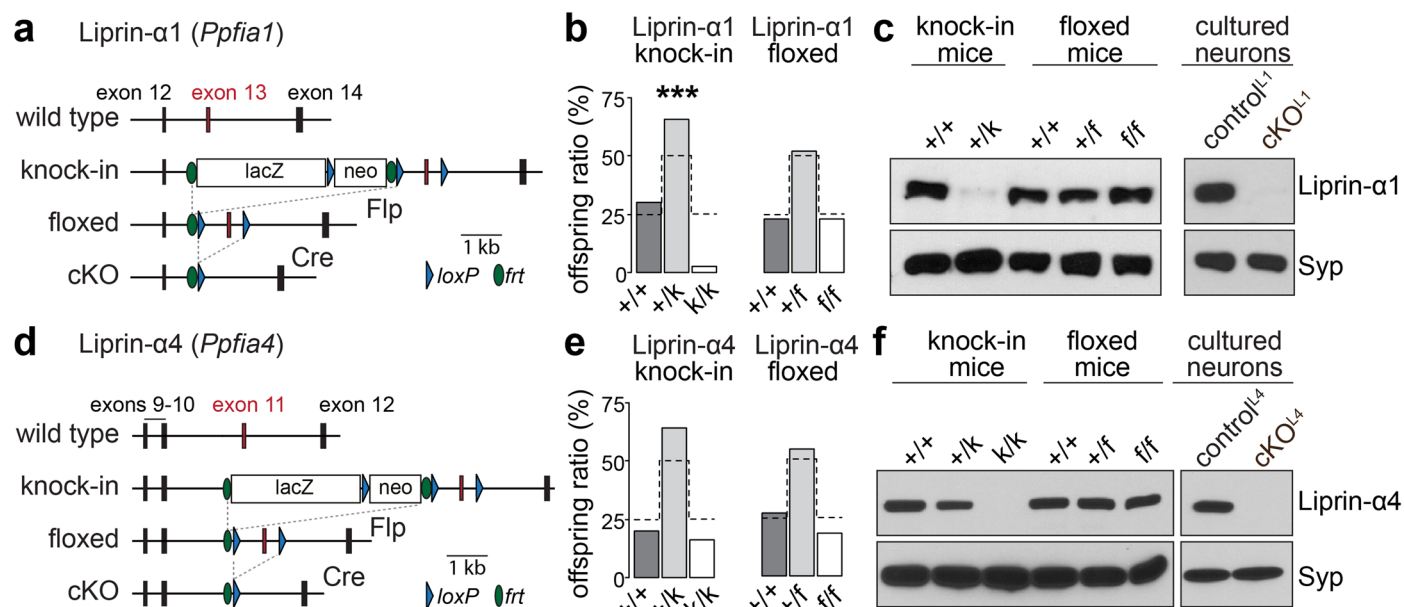
culture, dotted lines mark the levels of cQKO^{R+E} (black) or control^{R+E} (gray); d–f, control 14/3, cQKO^{R+E} 14/3, cQKO^{R+E} + RIM1 α 14/3, cQKO^{R+E} + PDZ-C2A-P 13/3, cQKO^{R+E} + Zn-C2B 14/3; g–i, 14/3 each; j–l, 26/6 each; m–o, 14/3 each. The scale bar in m applies to all confocal images in d, g, j and m. (p, q) Quantification of Synaptophysin, PSD-95 and Gephyrin puncta densities (p) and of their fluorescence intensities (q) normalized to the average signal in control^{R+E} per culture. Small changes in Synaptophysin and PSD-95 in some conditions do not confound the conclusion that independent assembly pathways recruit Munc13-1 and Ca_v2.1; control^{R+E} 68/6, cQKO^{R+E} 68/6, cQKO^{R+E} + RIM1 α 68/6, cQKO^{R+E} + PDZ-C2A-P 67/6, cQKO^{R+E} + Zn-C2B 68/6. Data are mean $\pm$ SEM; * p < 0.05, ** p < 0.01, *** p < 0.001 compared to cQKO^{R+E} determined by Kruskal-Wallis and Holm multiple comparisons post hoc tests in c, e, f, h, i, k, l, n, p (Synaptophysin and Gephyrin) and q, or one-way ANOVA and Tukey-Kramer multiple comparisons post hoc tests in o and p (PSD-95).



Extended Data Fig. 3 | Assessment of inhibitory synapses by STED

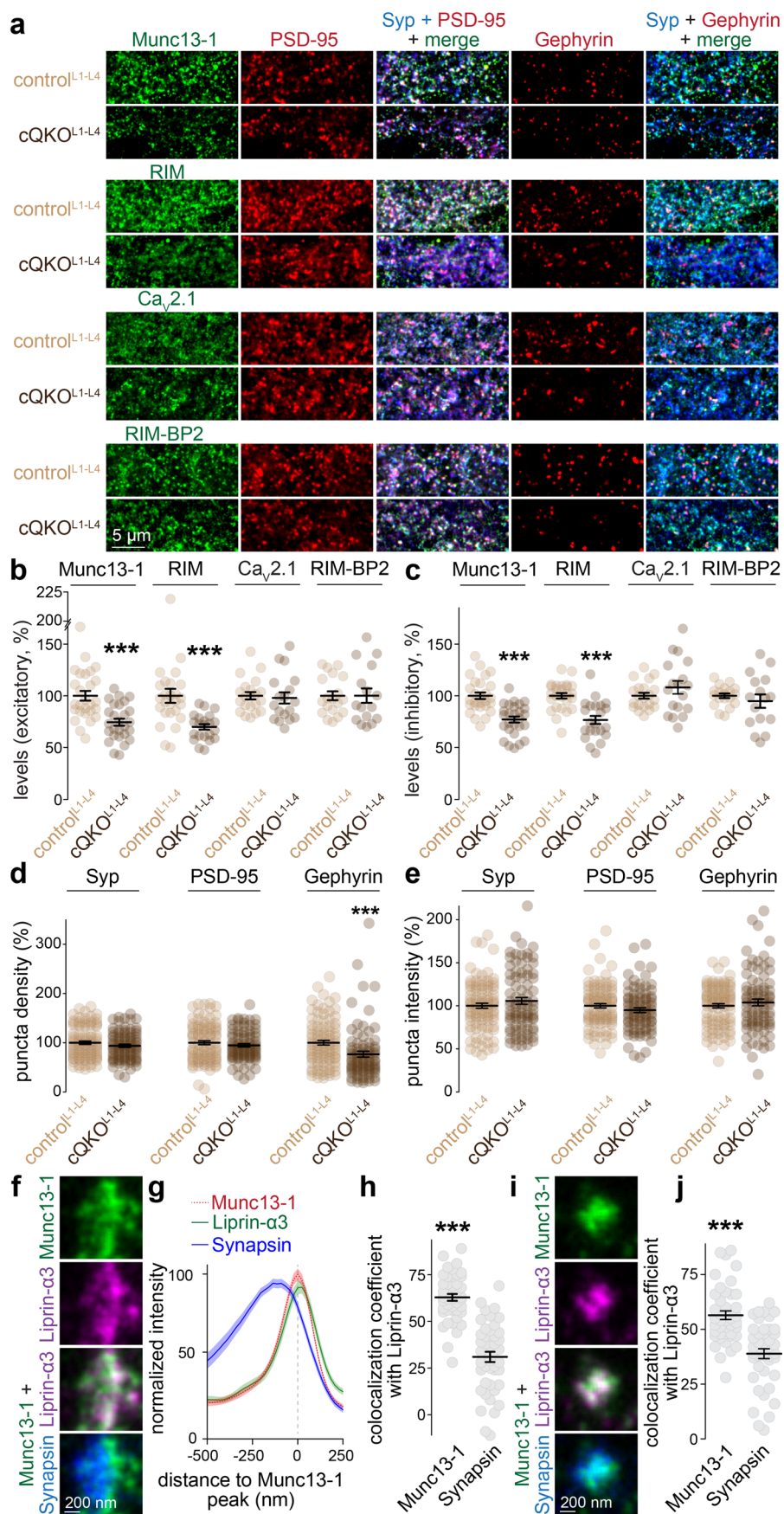
microscopy after RIM rescue. (a-l) Example STED images, average line profiles and quantification of the peak intensity of Munc13-1 and Gephyrin (a-c), Ca_v2.1 (d-f), Liprin-α3 (g-i) and RIM-BP2 (j-l) at inhibitory side-view synapses identified by Synaptophysin (Syp) and Gephyrin. Analyses were performed on the experiment shown in Fig. 2f–q as the neurons were co-stained for Gephyrin. A line profile (750 nm x 250 nm) was positioned perpendicular to the center of the elongated Gephyrin object and profiles of all synapses were aligned to the Gephyrin peak. The maximum value of each individual profile was used to calculate the peak. Dotted lines mark the levels of cQKO^{R+E} (black) or control^{R+E} (gray), line profiles

and peak intensities are normalized to the signal in control^{R+E} per culture; a–c, control^{R+E} 52 synapses/3 cultures, cQKO^{R+E} 45/3, cQKO^{R+E} + RIM1α 44/3, cQKO^{R+E} + PDZ-C2A-P 49/3, cQKO^{R+E} + Zn-C2B 45/3; d–f, control^{R+E} 49/3, cQKO^{R+E} 52/3, cQKO^{R+E} + RIM1α 44/3, cQKO^{R+E} + PDZ-C2A-P 51/3, cQKO^{R+E} + Zn-C2B 47/3; g–i, control^{R+E} 94/6, cQKO^{R+E} 97/6, cQKO^{R+E} + RIM1α 94/6, cQKO^{R+E} + PDZ-C2A-P 95/6, cQKO^{R+E} + Zn-C2B 91/6; j–l, control^{R+E} 46/3, cQKO^{R+E} 46/3, cQKO^{R+E} + RIM1α 45/3, cQKO^{R+E} + PDZ-C2A-P 47/3, cQKO^{R+E} + Zn-C2B 44/3. The scale bar in j applies to all STED images. Data are mean ± SEM; **p < 0.01, ***p < 0.001 compared to cQKO^{R+E} as determined by Kruskal-Wallis followed by Holm multiple comparisons post hoc tests.



Extended Data Fig. 4 | Liprin- $\alpha 1$ and - $\alpha 4$ mutant alleles. (a) Diagram outlining the gene targeting strategy of *Ppfia1* to remove Liprin- $\alpha 1$. The knock-in allele containing loxP sites flanking exon 13 (numbering follows Ensembl ENSMUST00000182226.8) was generated by homologous recombination, chimeric founders were used to establish the knock-in line and subsequently crossed to Flp-transgenic mice⁷⁷ to generate the floxed allele. (b) Offspring ratios from Liprin- $\alpha 1$ knock-in (k) or floxed (f) heterozygous breeding pairs. Dotted lines show expected Mendelian ratios; Liprin- $\alpha 1$ knock-in 26 mice/5 litters; Liprin- $\alpha 1$ floxed 35/5. (c) Western blots of whole brain homogenates of wild type,

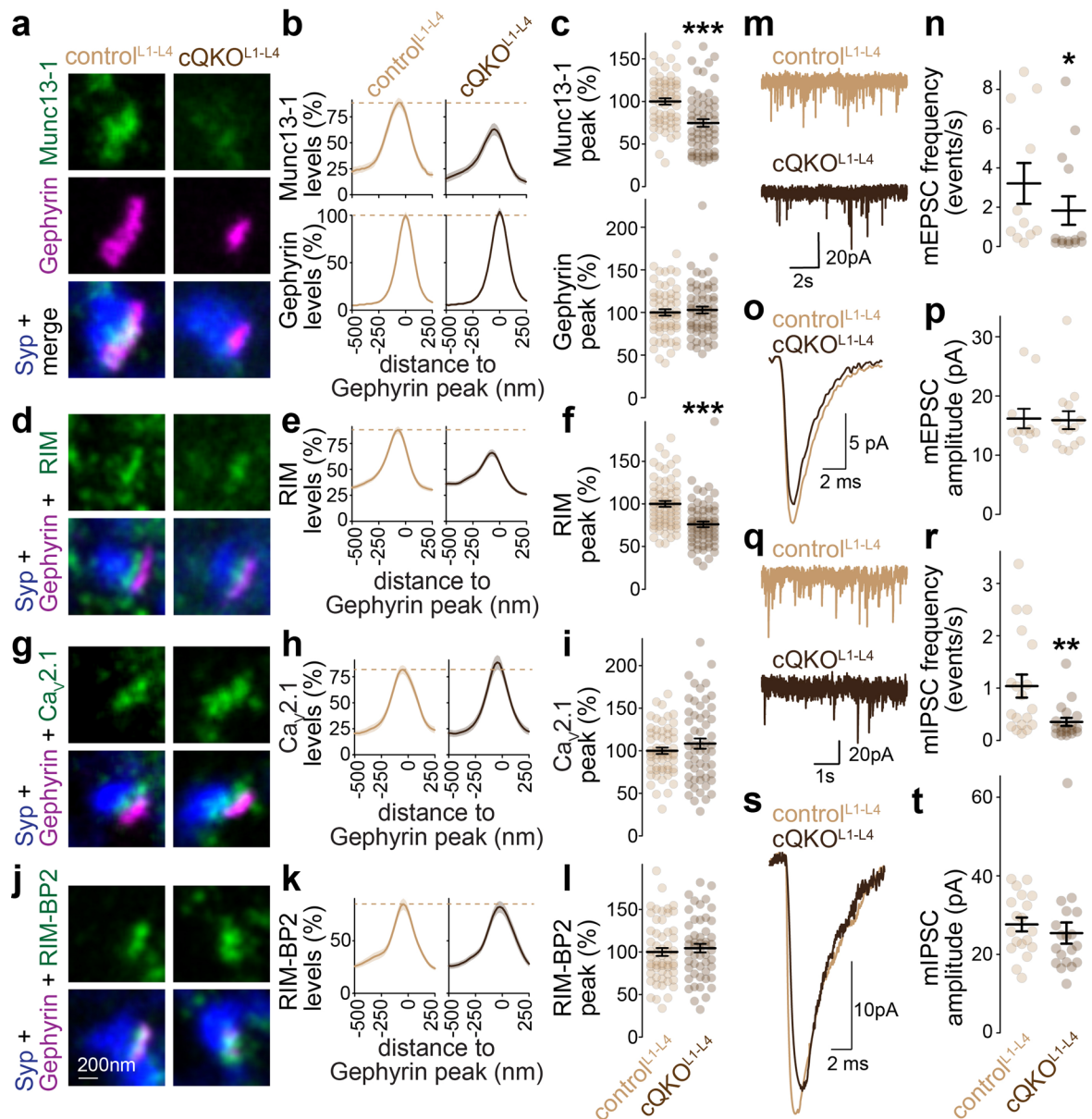
heterozygous and homozygous littermate Liprin- $\alpha 1$ mice before (knock-in) or after Flp recombination (floxed), or of lysates from hippocampal cultures of Liprin- $\alpha 1$ floxed mice infected with a lentivirus expressing Cre (cKO^{L1}) or a recombination deficient truncation of Cre (control^{L1}). (d-f) Same as a-c but for *Ppfia4* to remove Liprin- $\alpha 4$, exon 11 is flanked by loxP sites (numbering follows Ensembl ENSMUST00000168515.8); e, Liprin- $\alpha 4$ knock-in 25 mice/4 litters; Liprin- $\alpha 4$ floxed 44/5. Data in b and e are shown as observed offspring ratios. *** $p < 0.001$ determined by two-sided Chi-square tests comparing obtained ratios with expected Mendelian ratios (dotted line).



Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | Additional microscopic assessment of synaptic proteins after ablation of Liprin- α 1 to - α 4. (a-c) Example confocal images (a) and quantification of fluorescence intensity levels at excitatory and inhibitory synapses (b, c). Neurons were stained for a protein of interest (Munc13-1, RIM, Ca_v2.1 or RIM-BP2), two postsynaptic markers (PSD-95, excitatory, and Gephyrin, inhibitory), and Synaptophysin (Syp). Data are normalized to the average signal in control^{L1-L4} per culture; Munc13-1, control^{L1-L4} 27 images/3 cultures, cQKO^{L1-L4} 27/3; RIM, control^{L1-L4} 26/3, cQKO^{L1-L4} 22/3; Ca_v2.1, control^{L1-L4} 21/3, cQKO^{L1-L4} 18/3; RIM-BP2, control^{L1-L4} 19/3, cQKO^{L1-L4} 16/3. (d, e) Quantification of Synaptophysin, PSD-95 and Gephyrin puncta densities (d) and of their fluorescence intensities (e) normalized to the average signal in control^{L1-L4} per culture. The ~20% reduction

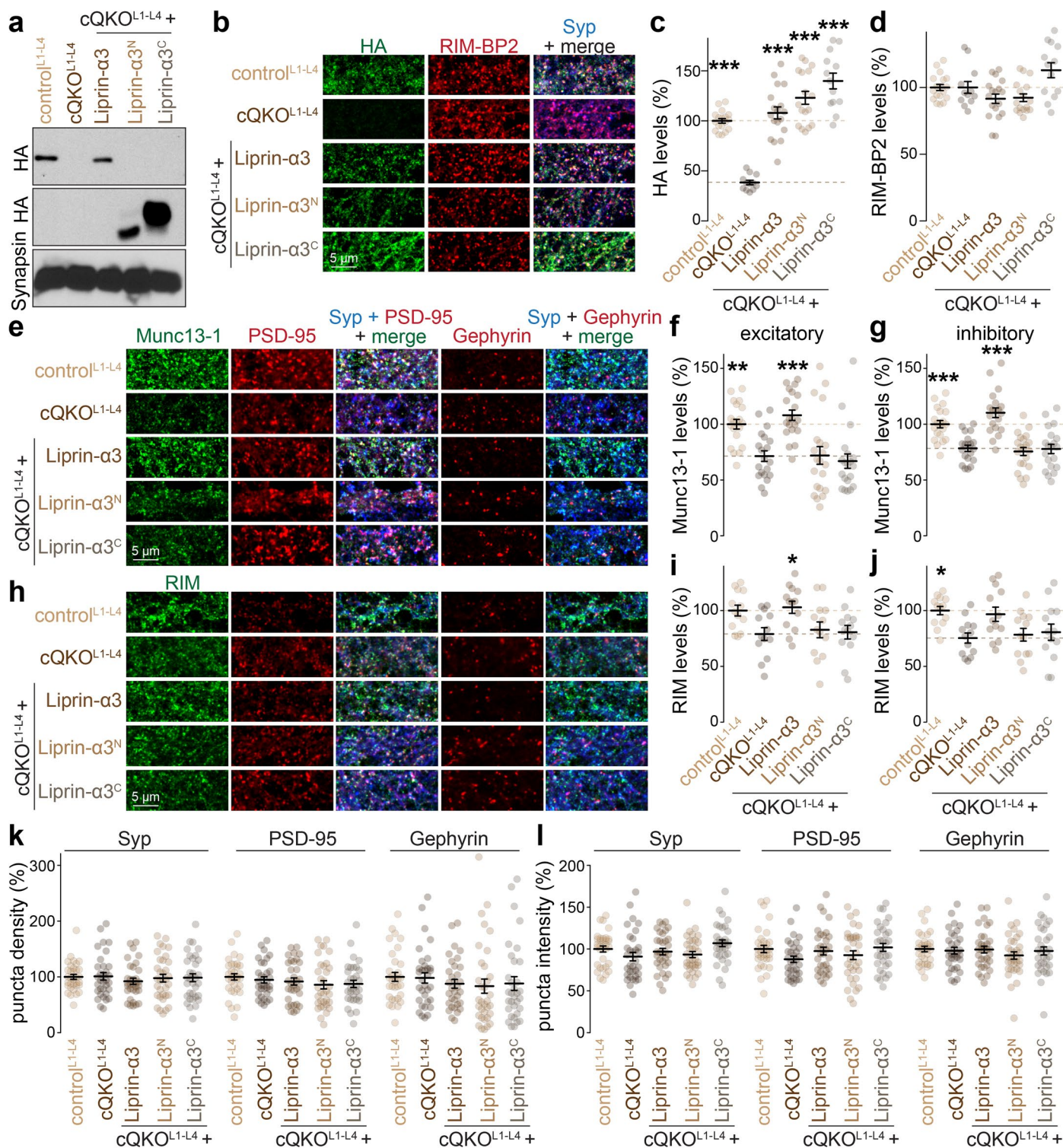
in inhibitory synapses is unlikely to fully account for the ~60% decrease in mIPSC frequency and the ~45% decrease in sucrose-evoked responses (Fig. 4w, x and Extended Data Fig. 6q, r); control^{L1-L4} 93/3, cQKO^{L1-L4} 83/3. (f-h) Example STED images of side-view synapses (f) and quantification of line profiles (g) and Pearson's colocalization coefficients (h). Neurons were stained for Synapsin, Munc13-1 and Liprin- α 3; 45 synapses/3 cultures. (i, j) Same as f-h, but for en-face synapses and without line profiles; 45 synapses/3 cultures. Data are mean $\pm$ SEM; *** $p < 0.001$ compared to cQKO^{L1-L4} determined by two-sided Mann-Whitney U tests (b for RIM and RIM-BP2, c for RIM-BP2 and Ca_v2.1, d, e, h and j), or two-sided Student's t-tests (b for Munc13-1 and Ca_v2.1, and c for Munc13-1 and RIM).



Extended Data Fig. 6 | Assessment of inhibitory synapses by STED microscopy and of spontaneous transmission after Liprin- α ablation.

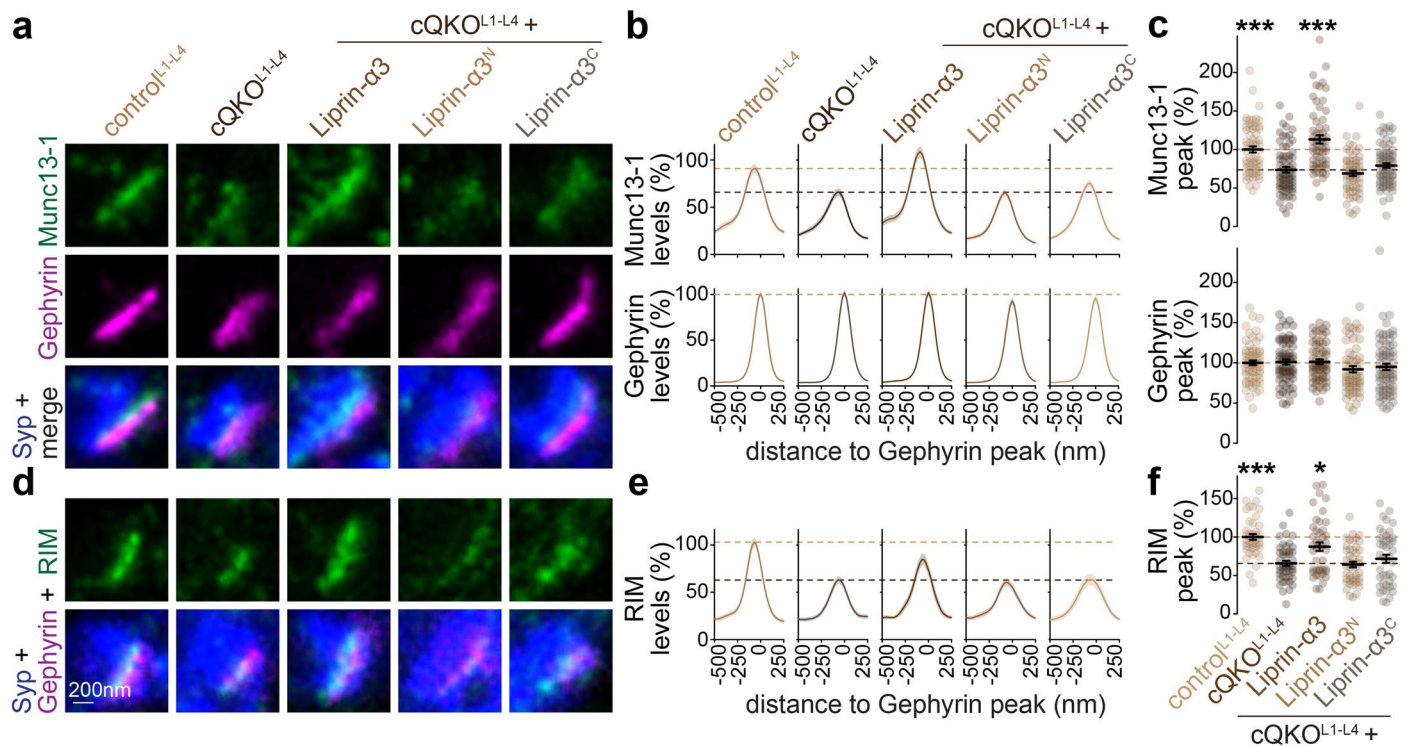
(a-l) Example STED images, average line profiles and quantification of the peak intensity of Munc13-1 and Gephyrin (a-c), RIM (d-f), Ca_v2.1 (g-i) and RIM-BP2 (j-l) at inhibitory side-view synapses identified by Synaptophysin (Syp) and Gephyrin. Analyses were performed on the experiment shown in Fig. 4c-n as the neurons were co-stained for Gephyrin. Dotted lines in line profile plots mark the levels of control^{L1-L4}, line profiles and peak intensities are normalized to the average signal in control^{L1-L4} per culture; a-c, control^{L1-L4} 60 synapses/3 cultures, cQKO^{L1-L4}

61/3; d-f, control^{L1-L4} 68/3, cQKO^{L1-L4} 65/3; g-i, control^{L1-L4} 58/3, cQKO^{L1-L4} 56/3; j-l, control^{L1-L4} 57/3, cQKO^{L1-L4} 51/3. The scale bar in j applies to all STED images. (m, n) Example traces (m) of spontaneous miniature excitatory postsynaptic current (mEPSC) recordings and quantification (n) of mEPSC frequency; control^{L1-L4} 11 cells/4 cultures, cQKO^{L1-L4} 14/4. (o, p) Example traces (o) of an averaged mEPSC from a single cell and quantification of the mEPSC amplitude (p), N as in m + n. (q-t) As in m-p but for mIPSCs; 19/3 each. Data are mean $\pm$ SEM; *p < 0.05, **p < 0.01, ***p < 0.001 compared to cQKO^{L1-L4} determined by two-sided Mann-Whitney U tests (c, f, i, n, p, r and t) or two-sided Student's t-tests (l).



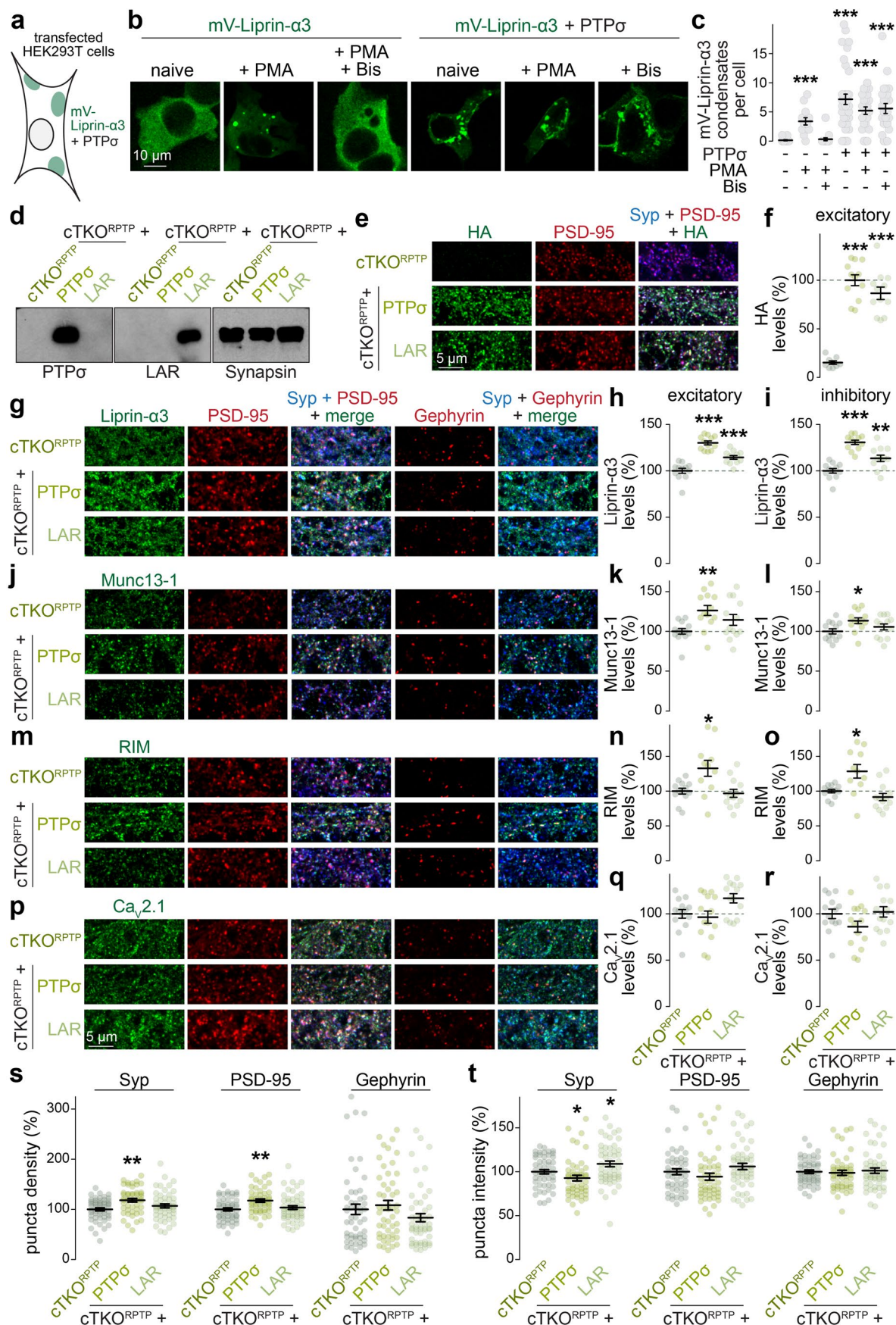
Extended Data Fig. 7 | Assessment of Liprin-α rescue constructs with Western blot and confocal microscopy. (a) Western blot to assess expression of Liprin-α3, Liprin-α3^N and Liprin-α3^C; note that control^{L1-L4} has an HA signal because of the lentiviral expression of HA-tagged Liprin-α3. (b–d) Example confocal images (b) and quantification of fluorescence intensity levels (c + d) of HA to detect Liprin-α3 and of RIM-BP2 at synapses defined as Synaptophysin (Syp) ROIs. Intensity is normalized to the average signal in control^{L1-L4} per culture, dotted lines mark the levels of cQKO^{L1-L4} (dark brown) or control^{L1-L4} (light brown); control^{L1-L4} 18 images/5 cultures, cQKO^{L1-L4} 12/5, cQKO^{L1-L4} + Liprin-α3 18/5, cQKO^{L1-L4} + Liprin-α3^N 17/5, cQKO^{L1-L4} + Liprin-α3^C 13/5. (e–j) Example confocal images and quantification of fluorescence intensity levels at excitatory and inhibitory synapses of Munc13-1 or RIM (h–j). Neurons were stained for a protein of interest (Munc13-1 or RIM), postsynaptic markers (PSD-95 and

Gephyrin), and Synaptophysin. Data are normalized to the average signal in control^{L1-L4} per culture, dotted lines mark the levels of cQKO^{L1-L4} (dark brown) or control^{L1-L4} (light brown); e–g, control^{L1-L4} 20/4, cQKO^{L1-L4} 20/4, cQKO^{L1-L4} + Liprin-α3 20/4, cQKO^{L1-L4} + Liprin-α3^N 21/4, cQKO^{L1-L4} + Liprin-α3^C 20/4; h–j, 13/3 each. (k, l) Quantification of Synaptophysin, PSD-95 and Gephyrin puncta densities (k) and of their fluorescence intensities (l) normalized to the average signal in control^{L1-L4} per culture; control^{L1-L4} 33/4, cQKO^{L1-L4} 33/4, cQKO^{L1-L4} + Liprin-α3 33/4, cQKO^{L1-L4} + Liprin-α3^N 34/4, cQKO^{L1-L4} + Liprin-α3^C 33/4. Data are mean ± SEM; *p < 0.05 **p < 0.01, ***p < 0.001 compared to cQKO^{L1-L4} determined by Kruskal-Wallis and Holm multiple comparisons post hoc tests in c, d, f, k (Gephyrin and Synaptophysin) and l (Synaptophysin), or one-way ANOVA and multiple comparisons Tukey-Kramer post hoc tests in g, i, j, k (PSD-95) and l (PSD-95 and Gephyrin).



Extended Data Fig. 8 | Assessment of inhibitory synapses by STED microscopy after Liprin-α3 rescue. (a-f) Example STED images, average line profiles and quantification of the peak intensity of Munc13-1 and Gephyrin (a-c), and RIM (d-f) at inhibitory side-view synapses identified by Synaptophysin (Syp) and Gephyrin. Analyses were performed on the experiment shown in Fig. 5e-j as the neurons were co-stained for Gephyrin. Dotted lines mark the levels of cQKO^{L1-L4} (dark brown) or control^{L1-L4} (light brown), line profiles and

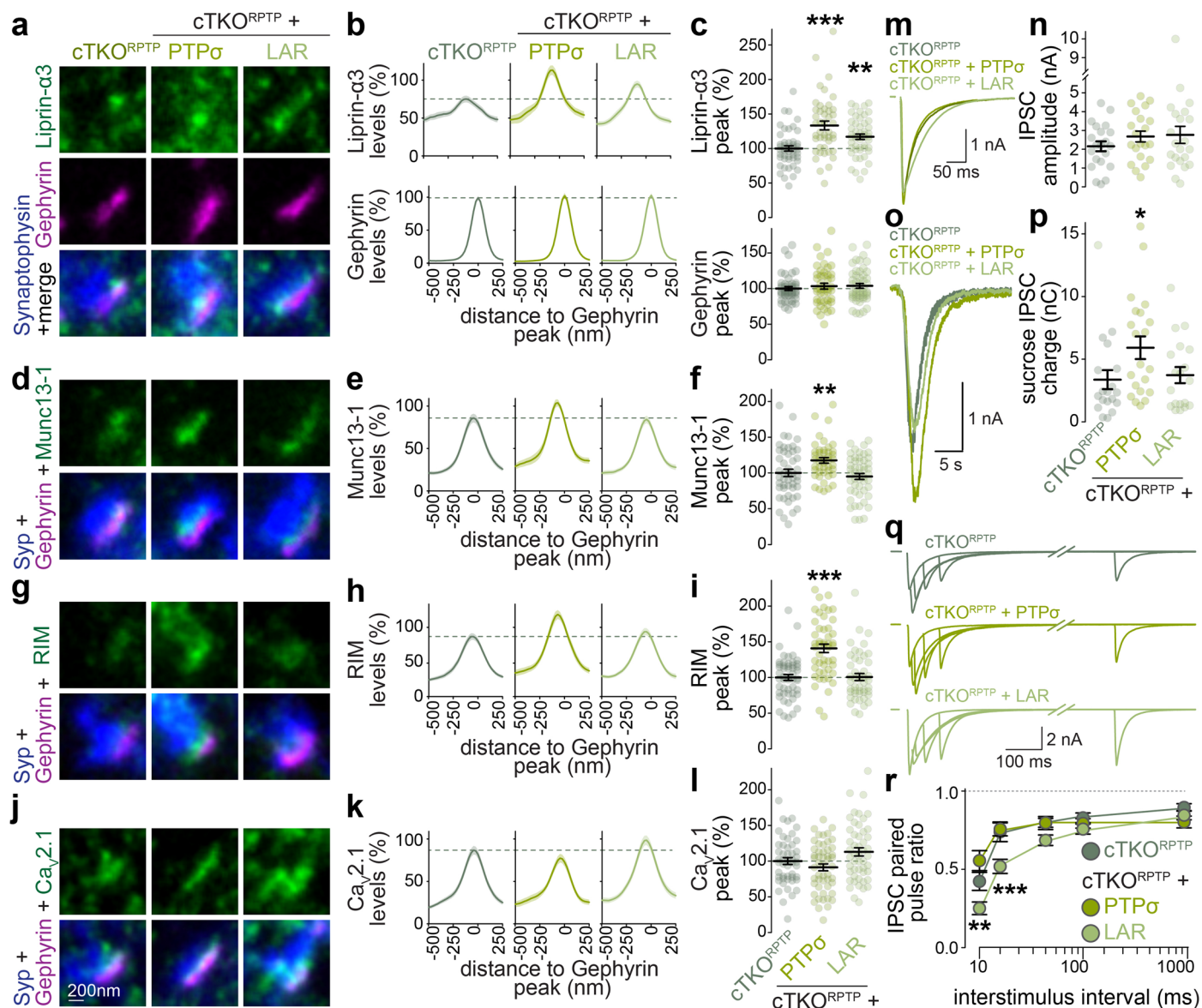
peak intensities are normalized to the average signal in control^{L1-L4} per culture; a-c, control^{L1-L4} 67 synapses/4 cultures, cQKO^{L1-L4} 69/4, cQKO^{L1-L4} + Liprin-α3 61/4, cQKO^{L1-L4} + Liprin-α3^N 60/4, cQKO^{L1-L4} + Liprin-α3^C 74/4; d-f, control^{L1-L4} 45/3, cQKO^{L1-L4} 48/3, cQKO^{L1-L4} + Liprin-α3 43/3, cQKO^{L1-L4} + Liprin-α3^N 47/3, cQKO^{L1-L4} + Liprin-α3^C 44/4. The scale bar in d applies to all STED images. Data are mean ± SEM; *p < 0.05, ***p < 0.001 compared to cQKO^{L1-L4} as determined by Kruskal-Wallis followed by Holm multiple comparisons post hoc tests.



Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | Assessment of LAR-RPTP proteins with Western blot and confocal microscopy. (a) Overview of the HEK293T cell experiment. (b, c) Example confocal images (b) and quantification of the number of mV-Liprin- α 3 condensates per transfected cell (c). PMA, which activates PKC, promotes mV-Liprin- α 3 condensate formation and co-addition of the PKC blocker BIS inhibits this effect. Neither drug influences mV-Liprin- α 3 condensate formation when PTP σ is present; mV-Liprin- α 3, naive 14 transfected cells/3 transfections, + PMA 13/3, + PMA + BIS 16/3, mV-Liprin- α 3 + PTP σ , naive 37/3, + PMA 23/3, + BIS 24/3. Images are 185 $\times$ 185 μ m and contain multiple cells. (d) Western blot of neurons. (e, f) Example confocal images and quantification of HA intensity to detect PTP σ or LAR at excitatory synapses. Neurons were stained for HA, PSD-95 and Synaptophysin (Syp). Intensity is normalized to the cTKO^{RPTP} average per culture, dotted lines mark the cTKO^{RPTP} + PTP σ levels; cTKO^{RPTP} 9 images/3 cultures, cTKO^{RPTP} + PTP σ 12/3, cTKO^{RPTP} + LAR 12/3. (g-r) Example confocal images and quantification of fluorescence intensity levels at excitatory

and inhibitory synapses of Liprin- α 3 (g-i), Munc13-1 (j-l), RIM (m-o) and Ca_v2.1 (p-r). Neurons were stained for a protein of interest (Liprin- α 3, Munc13-1, RIM or Ca_v2.1), postsynaptic markers (PSD-95 and Gephyrin), and Synaptophysin. Data are normalized to the cTKO^{RPTP} average per culture, dotted lines mark cTKO^{RPTP} levels; g-i, 12/3 each; j-l, 13/3 each; m-o, cTKO^{RPTP} 13/3, cTKO^{RPTP} + PTP σ 11/3, cTKO^{RPTP} + LAR 13/3; p-r, 14/3 each. The scale bar in p applies to g, j and m. (s, t) Quantification of Synaptophysin, PSD-95 and Gephyrin puncta densities (s) and of their fluorescence intensities (t) normalized to the cTKO^{RPTP} average per culture. Expression of PTP σ mildly increased the number of Synaptophysin and PSD-95 puncta, possibly reflecting synaptogenic effects, and mildly decrease Synaptophysin intensity; cTKO^{RPTP} 52/3, cTKO^{RPTP} + PTP σ 50/3, cTKO^{RPTP} + LAR 52/3. Data are mean $\pm$ SEM; * p < 0.05, ** p < 0.01, *** p < 0.001 compared to naive (c) or cTKO^{RPTP} by Kruskal-Wallis and Holm post hoc tests (c, f, h, n, o, s for Gephyrin, t) or one-way ANOVA and Tukey-Kramer post hoc tests (i, k, l, q, r, s for Synaptophysin and PSD-95).



Extended Data Fig. 10 | Assessment of inhibitory synapses by STED microscopy and of synaptic transmission after expression of LAR-PTPs in cTKO^{RTP} neurons. (a–l) Example STED images, average line profiles and quantification of the peak intensity of Liprin-α3 and Gephyrin (a–c), Munc13-1 (d–f), RIM (g–i) and Ca_v2.1 (j–l) at inhibitory side-view synapses identified by Synaptophysin (Syp) and Gephyrin. Analyses were performed on the experiment shown in Fig. 7f–q as the neurons were co-stained for Gephyrin. Dotted lines mark the levels of cTKO^{RTP}, line profiles and peak intensities are normalized to the average signal in cTKO^{RTP} per culture; a–c, cTKO^{RTP} 48 synapses/3 cultures cTKO^{RTP} + PTPσ 45/3, cTKO^{RTP} + LAR 50/3; d–f, cTKO^{RTP} 49/3, cTKO^{RTP} + PTPσ 45/3, cTKO^{RTP} + LAR 50/3; g–i, cTKO^{RTP} 51/3, cTKO^{RTP} + PTPσ 49/3, cTKO^{RTP} + LAR 47/3; j–l, cTKO^{RTP} 49/3, cTKO^{RTP} + PTPσ 51/3, cTKO^{RTP} + LAR 50/3. The scale

bar in j applies to all STED images. (m, n) Example traces (m) and average amplitudes (n) of single action potential-evoked IPSCs; cTKO^{RTP} 21 cells/3 cultures cTKO^{RTP} + PTPσ 21/3, cTKO^{RTP} + LAR 22/3. (o, p) Example traces (o) and average GABAR-mediated charge transferred in response to hypertonic sucrose superfusion (p); cTKO^{RTP} 19/3, cTKO^{RTP} + PTPσ 21/3, cTKO^{RTP} + LAR 19/3. (q, r) Example traces (q) and average IPSC paired pulse ratios (r) at various interstimulus intervals to estimate P; N as in m + n. Data are mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 compared to cTKO^{RTP} determined by Kruskal–Wallis and Holm multiple comparisons post hoc tests (c, f, i, n, and p), by one-way ANOVA and Tukey–Kramer post hoc tests (l), or by two-way ANOVA and Dunnett post hoc tests (r).

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- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☐ ☒ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection Commercially available software was used for data collection.

Data analysis Commercially available software and open source software were used for data analyses. A custom MATLAB code (run on version 2019b) for automatic two-dimensional segmentation was used for detection of synaptic objects, was previously published (PMID: 29398114), and is available on Github (https://github.com/kaeserlab/3DSIM_Analysis_CL and https://github.com/hmslcl/3D_SIM_analysis_HMS_Kaeser-lab_CL). A custom MATLAB code (run on version R2021a) for analyses of DNA Exchange-PAINT images has been deposited to Github (<https://github.com/blanpiedlab/EmperadorMelero-2024>). Fiji/ImageJ (RRID: SCR_002285, most recent version at any given time) was used for image analysis. GraphPad Prism8 (RRID:SCR_002798, <https://www.graphpad.com/scientific-software/prism>) and R (most recent version of RStudio at any given time, RStudio is an integrated development environment for R; RRID:SCR_000432) were used for statistical analysis and generation of plots.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

Data points generated for this study are included in the figures whenever possible. Numerical data for all figures are available at <https://doi.org/10.5281/zenodo.12205516>. Additional data are available from the corresponding author upon request. Source data are provided with this paper.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

n/a

Reporting on race, ethnicity, or other socially relevant groupings

n/a

Population characteristics

n/a

Recruitment

n/a

Ethics oversight

n/a

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences

☐ Behavioural & social sciences

☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Sample size was determined based on the number of observations that has been used in similar studies published in the field (PMIDs: 34031393, 35176221, 32616470, 15294146, 20332206, 31985401, 27462810, 38625946).

Data exclusions

All data that met quality standards described throughout the methods were included and there was no exclusion of outliers for genotype comparisons.

Replication

The number of observations is reported in each figure. For cultured neurons, data were acquired from at least three independent cultures, with the exception of electron microscopy (two independent cultures). Correlative light and electron microscopy was performed in multiple cells from one or two transfections.

Randomization

Data derived from animals were not randomized, but pooled by genotype and/or condition after analyses by a blinded experimenter were completed.

Blinding

The experimenter was blind throughout data collection and analyses.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

The following primary antibodies were used: rabbit anti-Liprin-α3 (lab antibody code A232, 1:500; homemade, knockout-verified for STED in PMID 34031393), rabbit anti-RIM (A58, 1:500; RRID: AB_887774, knockout-verified for STED in PMID 29606581; SySy catalogue # 140 003), rabbit anti-CaV2.1 (A46, 1:500; RRID: AB_2619841, knockout verified for STED in PMID 32616470; SySy catalogue # 152 203), rabbit anti-Munc13-1 (A72, 1:500; RRID: AB_887733, knockout-verified for STED in PMID 36398873; SySy catalogue # 126 103), rabbit anti-RIM-BP2 (A126, 1:500; RRID: AB_2619739; SySy catalogue # 316 103), mouse anti-HA (A12, 1:500; RRID: AB_2565006; Biolegend catalogue # 901501), mouse anti-Synaptophysin (A100, 1:500; RRID: AB_887824; SySy catalogue # 101 011), mouse anti-PSD-95 (A152, 1:500; RRID: AB_10698024; NeuroMab catalogue # 73-028), mouse anti-Gephyrin (A8, 1:500; RRID: AB_2232546; SySy catalogue # 147 021), mouse anti-Synapsin (A57, 1:500; RRID: AB_2617071; SySy catalogue # 106 001), guinea pig anti-Munc13-1 (A22, 1:500; RRID: AB_2619806; SySy catalogue # 126 104), rabbit anti-Liprin-α1 (A121, 1:500; gift from Dr. Schoch), rabbit anti-Liprin-α2 (A13, 1:500; gift from Dr. Schoch), rabbit anti-Liprin-α4 (A2, 1:500; gift from Dr. Schoch), goat anti-PTPσ (A114, 1:200, RRID:AB_2607944; Thermo Fisher catalogue # PA5-47487), mouse anti-LAR (A156, 1:500, clone E9B9S; Cell signaling; catalogue # 48701).

The following secondary antibodies were used: goat anti-rabbit Alexa Fluor 488 (lab antibody code S5; 1:250, RRID: AB_2576217; Thermo Fisher catalogue # A-11034), goat anti-mouse IgG1 Alexa Fluor 488 (S7; 1:250, RRID: AB_2535764; Thermo Fisher catalogue # A-21121), goat anti-mouse IgG1 Alexa Fluor 555 (S19, 1:250, RRID: AB_2535769; Thermo Fisher catalogue # A-21127), goat anti-mouse IgG2a Alexa Fluor 633 (S30, 1:250, RRID: AB_1500826; Thermo Fisher catalogue # A-21136), goat anti-guinea pig Alexa Fluor 405 (S51, 1:250, RRID: AB_2827755 Abcam catalogue # ab-175678), goat anti-guinea pig Alexa Fluor 555 (S23, 1:250, RRID: AB_2535856 Thermo Fisher catalogue # A-21435), goat anti-mouse IgG1 Alexa Fluor 633 (S29, 1:250, RRID: AB_2535768 Thermo Fisher catalogue # A-21126), peroxidase-conjugated goat anti-mouse IgG (S52, 1:10000, RRID:AB_2334540; MP Bio AB_2334540), peroxidase-conjugated goat anti-rabbit IgG (S53, 1:10000, RRID:AB_2334589; MP Bio AB_2334589), peroxidase-conjugated anti-goat IgG (S60, 1:10000).

DNA-conjugated secondary nanobodies were custom made by Massive Photonics using FAST-PAINT F-series DNA.

Validation

Primary antibodies that are central to the conclusions of the manuscript have been knockout-validated in our laboratory for confocal and STED experiments. This includes Liprin-alpha3 in PMID 34031393, CaV2.1 in PMID 32616470, RIM in PMID 29606581 and Munc13-1 (rabbit) in PMID 36398873.

Primary antibodies against Liprin-alpha1, -alpha2 and -alpha4, PTPsigma and LAR have also been knockout-validated in our laboratory for Western blots in this study and in PMID 33656439.

This study validates HA antibodies for confocal, STED and Western blot experiments by using negative controls that do not contain an HA-tagged protein.

Munc13-1 (guinea pig), PSD-95, Gephyrin, Synaptophysin and Synapsin have been all knockout-validated according to the manufacturer's website; the specific information for each antibody can be found via catalogue numbers and additional information provided above.

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)

HEK293T cells: purchased from ATCC; Cat# CRL-3216; RRID: CVCL_0063.

Authentication

HEK293T cells were purchased from ATCC as a validated cell line. We did not further authenticate them in the laboratory.

Mycoplasma contamination

Purchased as mycoplasma free cell line from ATCC, Cat#: CRL-3216, RRID:CVCL_0063.

Commonly misidentified lines
(See [ICLAC](#) register)

None

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	Liprin-alpha quadruple mutant mice, PTP triple conditional knockout mice and RIM+ELKS quadruple conditional knockout mice were maintained on a mixed background (containing at least C57BL/6 and 129/Sv). Neuronal cultures from these mouse lines were generated from postnatal day 0-1 pups. Mice were maintained at a standard 12 h dark/light cycle in a room set to 20-24 °C and 35-70 % humidity (set point 50 %).
Wild animals	No wild animals were used in this study.
Reporting on sex	Cells from a mix of male and female mouse pups were pooled together to generate primary neuronal cultures.
Field-collected samples	No field-collected samples were used in this study.
Ethics oversight	All animal experiments were approved by the Harvard University Animal Care and Use Committee.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	n/a
Novel plant genotypes	n/a
Authentication	n/a