Supplemental Materials

Molecular Biology of the Cell

Kabachinski et al.

SUPPLEMENTAL MATERIALS

CAPS and Munc13 utilize distinct PIP_2-linked mechanisms to promote vesicle exocytosis

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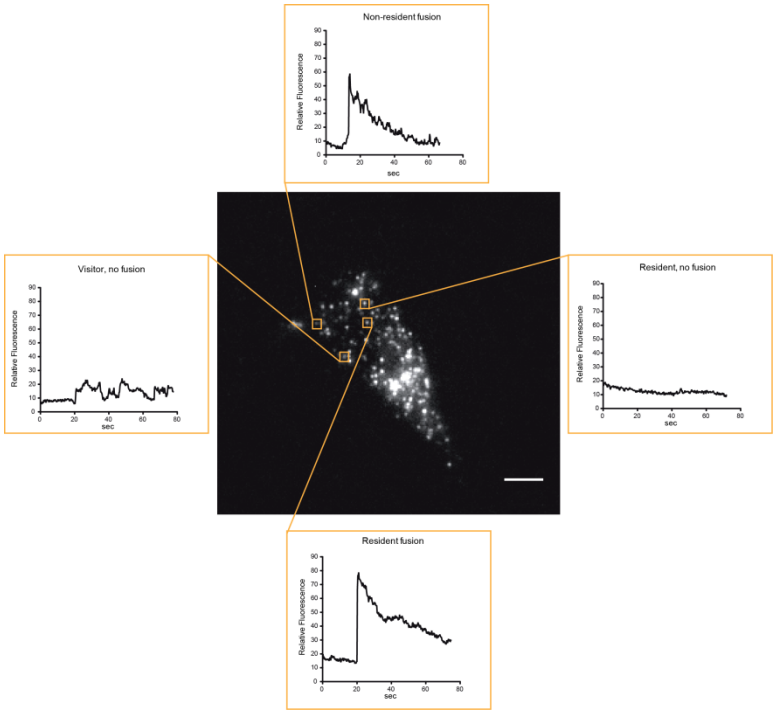
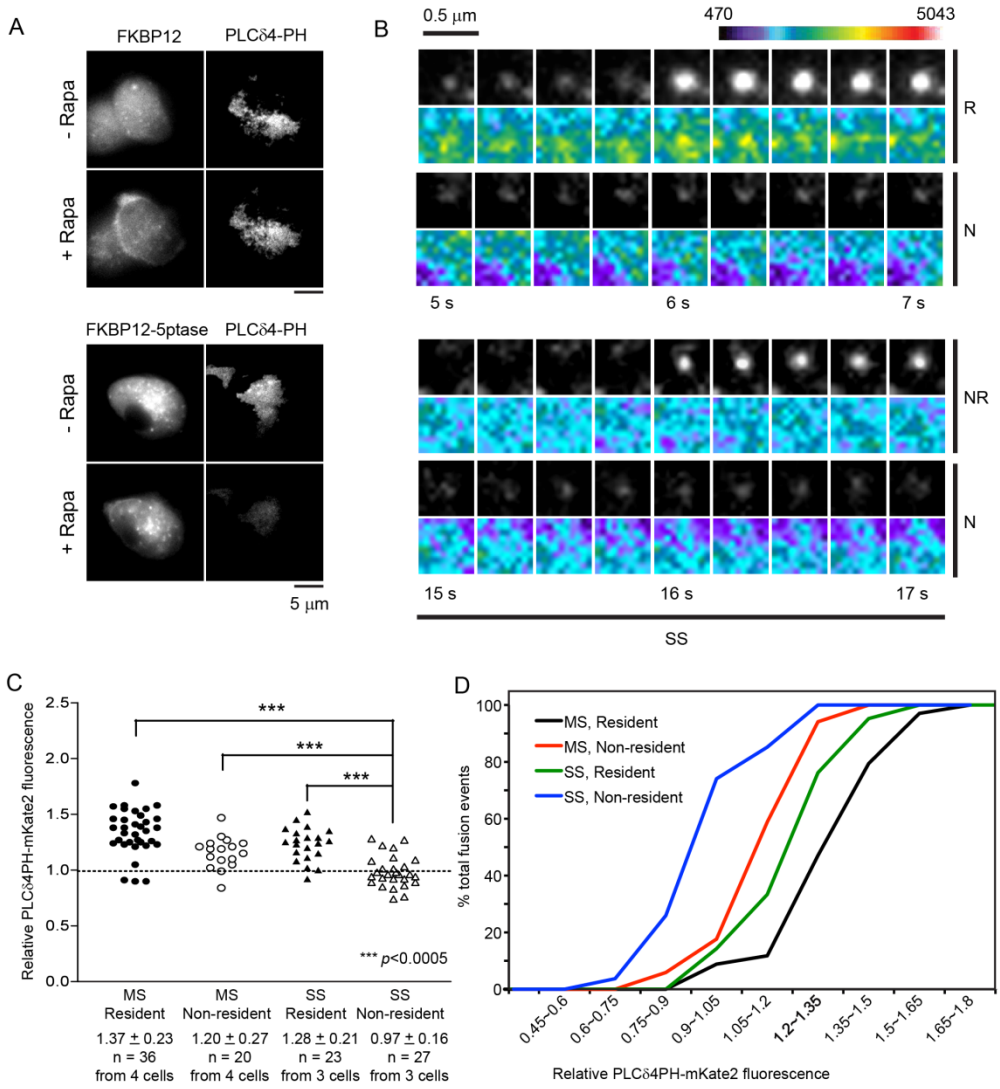
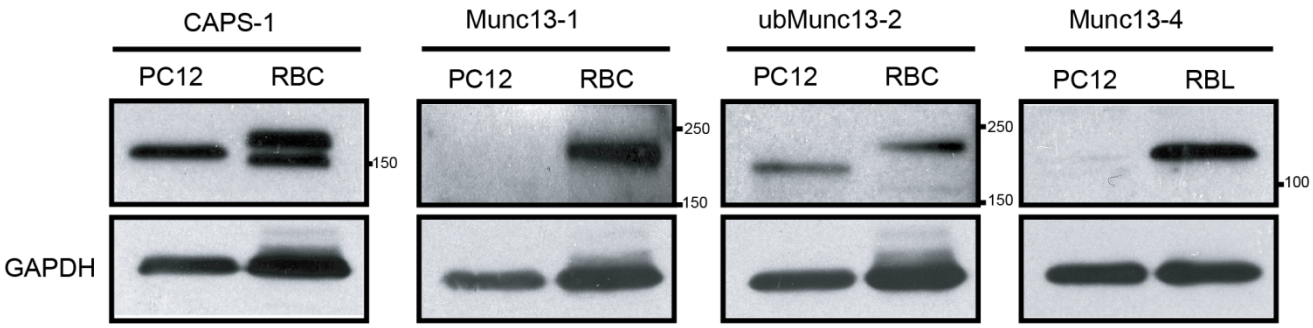


Suppl. Fig. S1. Examples of evoked exocytosis of BDNF-EGFP-containing vesicles. Boxes identify four different vesicles in the TIRF footprint of BDNF-EGFP-expressing PC12 cells. The vesicles (clockwise from left) either did not fuse, were non-resident vesicles that fused, were vesicles that did not fuse, or were resident vesicles that fused in response to depolarization in 56 mM K^+ buffer. Vesicles that fuse exhibit a brightening upon fusion pore formation that is followed by a gradual dimming as the fusion pore closes and the vesicle undergoes re-acidification. In non-resident fusion events, defined as lack of a vesicle in the TIRF field for 0.5s prior to fusion, the "fusion spike" is usually preceded by a small brief increase in fluorescence as the vesicle approaches the plasma membrane for fusion. Fluorescence traces were taken from a recording at 4Hz with a local background subtraction.

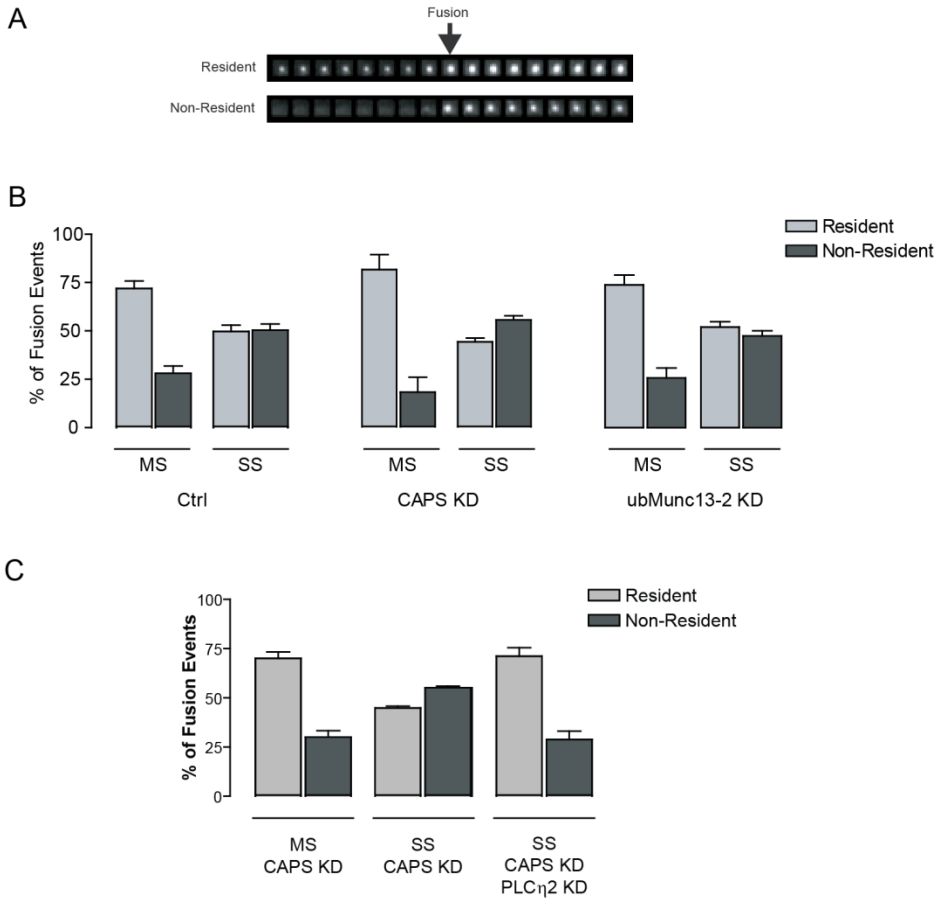


Suppl. Fig. S2. Evoked vesicle exocytosis occurs at PIP_2 domains. **A.** $PLC\delta_4$-PH binds to plasma membrane PIP_2. Left panels show cells expressing mRFP-FKBP12 or mRFP-FKBP12-5-phosphatase in epifluorescence; right panels show co-expressed $PLC\delta_4$-PH-EGFP in TIRF. Treatment with 100 nM rapamycin (Rapa) for 3 min reduced $PLC\delta_4$-PH-EGFP on the plasma membrane in FKBP12-5-phosphatase-expressing but not in FKBP12-expressing cells. Bar is 5 μm. **B.** Vesicle exocytosis was stimulated in 95 mM K^+ buffer (SS). Images of BDNF-EGFP-containing vesicles and $PLC\delta_4$-PH-mKate2 fluorescence were acquired at 4Hz. Resident (R) vesicles that fused were compared to non-fusing vesicles (N) in the same cells. Similarly, non-resident (NR) vesicles that fused were compared to non-fusing vesicles (N) in the same cells. **C.** Images similar to those of Fig. 1C,D and Fig. S2B were quantitated for fluorescence in the red channel ($PLC\delta_4$PH-mKate2) at sites of exocytosis and compared with sites in the same cells where vesicles did not exocytose. Relative fluorescence at sites of resident or non-resident vesicle exocytosis for cells in

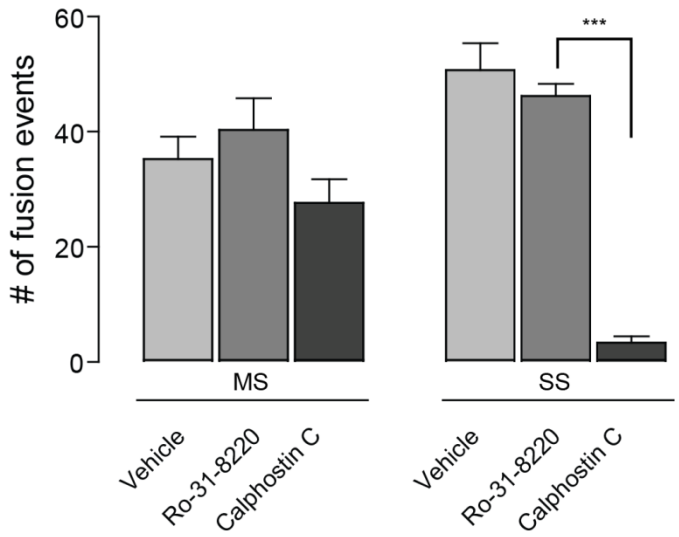
moderate stimulation buffer (MS = 56 mM K^+) or strong stimulation buffer (SS = 95 mM K^+) are shown with n corresponding to the number of exocytic events analyzed in the indicated number of cells. Except for non-resident vesicles fusing under SS conditions, fluorescence at exocytic sites was significantly greater (*p < 0.0005) than at sites where vesicles did not exocytose. **D.** A frequency distribution of the data for values of relative PLCδ_4PH-mKate2 fluorescence is shown for the indicated fraction of exocytic events.



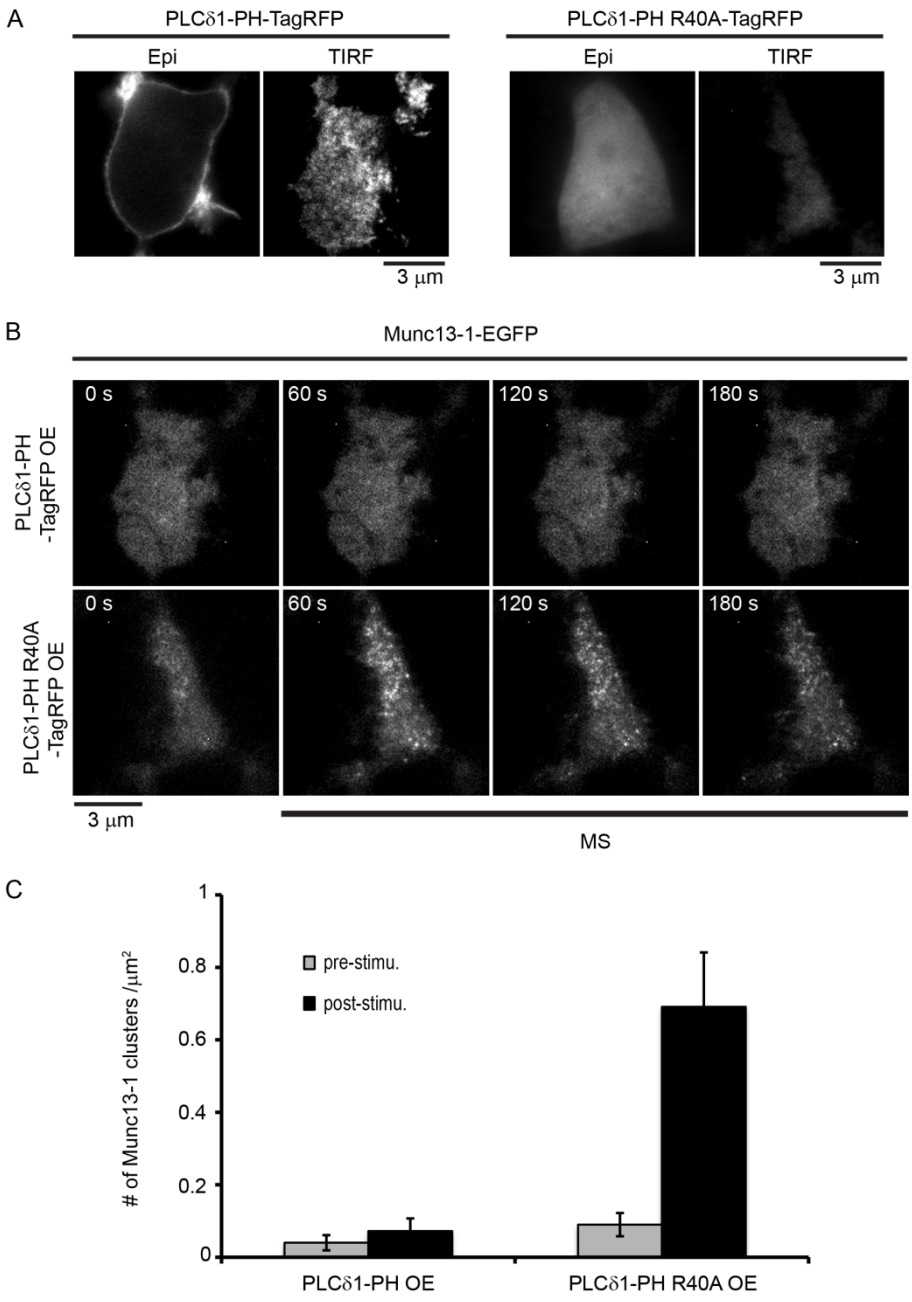
Suppl. Fig. S3. PC12 cells express CAPS-1 and ubMunc13-2. Western blotting was used to probe PC12 cell lysates for CAPS-1, Munc13-1, Munc13-2 and Munc13-4. Rat brain cytosol (RBC) was used as a positive control for CAPS-1, Munc13-1 and Munc13-2, and rat basophilic leukemic cell lysate (RBL) as a positive control for Munc13-4. GAPDH served as a loading control. PC12 cells express a ~160kDa form of CAPS-1 with intermediate mobility compared to two brain splicing isoforms, and a higher mobility ~185kDa form of Munc13-2 corresponding to ubMunc13-2. PC12 cells express small amounts of Munc13-4 but little detectable Munc13-1. Molecular weight markers (100, 150, 250kDa) corresponding to cut regions of blots are indicated.



Suppl. Fig. S4. Resident and newly-arrived vesicles exhibit the same requirements for exocytosis. A. Images of single BDNF-EGFP-containing vesicles acquired at 4Hz. Resident vesicles are present in the evanescent field >0.5s prior to fusion whereas non-resident vesicles suddenly (<0.5s) appear and undergo fusion. **B.** Strength of stimulation modifies the ratio of resident to non-resident vesicle exocytic events. Left panel: Under moderate stimulation (MS) conditions, ~75% of exocytic events are from resident vesicles and ~25% from non-resident vesicles. Under strong stimulation (SS) conditions, ~50% of exocytic events are from resident vesicles and ~50% from non-resident vesicles. Even though the number of exocytic fusion events is strongly decreased, similar ratios were obtained for the residual exocytic events in CAPS knockdown cells (middle panel) and in ubMunc13-2 knockdown cells (right panel). Bars represent SE (n = 15 cells). **C.** As in panel B for CAPS knockdown cells, strong stimulation (SS) increased fusion events from non-resident vesicles and decreased those from resident vesicles compared to moderate stimulation (MS). The additional knockdown of PLCη2 prevented the shift observed under SS conditions and restored the ratio to what was observed in MS conditions. Bars represent SE (n = 5-6 cells).

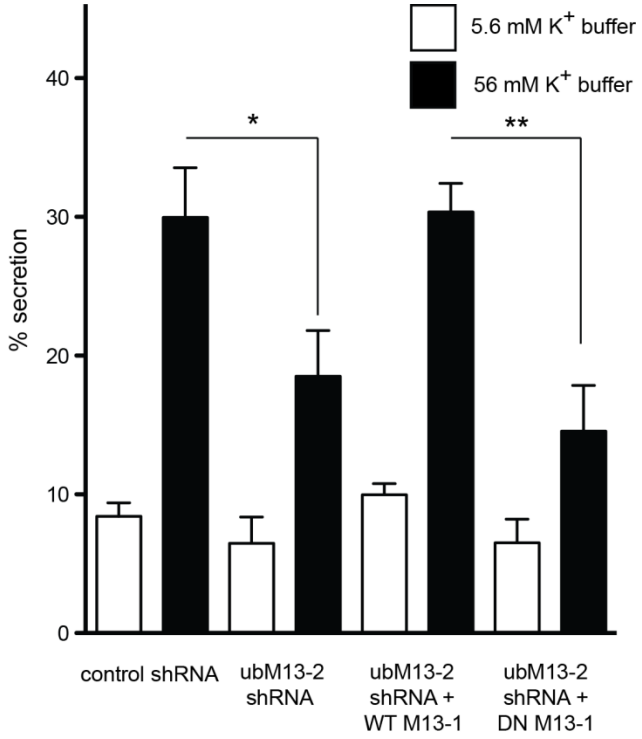


Suppl. Fig. S5. Munc13 employs its C1 domain under strong stimulation conditions. Because strong stimulation activated PLCη2 and elicited exocytosis efficiently without CAPS, we probed for a role of DAG in exocytosis. DAG stimulation of exocytosis is mediated by the activation of either PKC or Munc13 through C1 domain interactions (Brose and Neher, 2002; Brose and Rosenmund, 2002; Wierda et al., 2007). Ro-31-8220 (bisindolylmaleimide), a PKC inhibitor, had no effect on evoked exocytosis regardless of stimulation conditions suggesting that PKC activity is not essential. By contrast, treatment with calphostin C, a C1 domain antagonist (Betz et al., 1998), selectively inhibited exocytosis but only under the strong stimulation conditions. While ubMunc13-2 is essential for exocytosis in both stimulation conditions (see Fig. 4D), the result with calphostin C indicated that ubMunc13-2 employs its C1 domain for activity only under strong stimulation conditions. Cells were treated for 15 min with vehicle (0.1% DMSO), 1μM calphostin C, or 10μM Ro-31-8220. Exocytosis was evoked with either MS or SS buffer and the total number of exocytic events in 300s was plotted. Exocytosis with Ro-31-8220 and calphostin C under SS conditions differed (*** = $p < 0.0005$). Bars represent SE (n = 8-10 cells).



Suppl. Fig. S6. PLCδ_1-PH domain blocks stimulation-dependent Munc13-1 translocation. A. Epifluorescent and TIRF views are shown for the overexpression of tagRFP versions of PLCδ_1-PH or its corresponding R40A mutant. The wild-type protein localized to the plasma membrane whereas the mutant protein remained cytosolic. **B.** Similar overexpression studies with PLCδ_1-PH (upper panels) and the cognate mutant (lower panels) were conducted in cells co-expressing Munc13-1-EGFP. Cells were shifted into 95 mM K^+ buffer at time zero. The wild-type but not mutant PLCδ_1-PH protein blocked the stimulus-dependent translocation of Munc13-1-EGFP. **C.** Data in panel B were

quantitated to obtain the number of Munc13-1-EGFP clusters per surface area. Mean values ± SD are shown (n = 6-7).



Suppl. Fig. S7. DN C2B mutant Munc13-1 fails to rescue evoked vesicle exocytosis in ubMunc13-2 knockdown cells. Stable NPY-EGFP- expressing cells were transfected with a ubMunc13-2 shRNA knockdown construct or sham vector with or without co-expression of Munc13-1-ECFP or C2B D705N/D711N Munc13-1-ECFP. Four days post transfection cells were incubated with 5.6 mM K^+ basal buffer or 56 mM K^+ stimulation buffer for 10 min and the secretion of NPY-EGFP was measured. Percent secretion was calculated as 100 x $GFP_{supernatant}$/($GFP_{supernatant}$ + GFP_{cells}). Mean values ± SD are shown for 4 independent experiments with triplicates in each. Student's t-test was used to assess statistical significance (* $p \leq 0.05$ ** $p \leq 0.01$).

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