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Supplemental Information

TRIM67 regulates exocytic mode and neuronal morphogenesis via SNAP47

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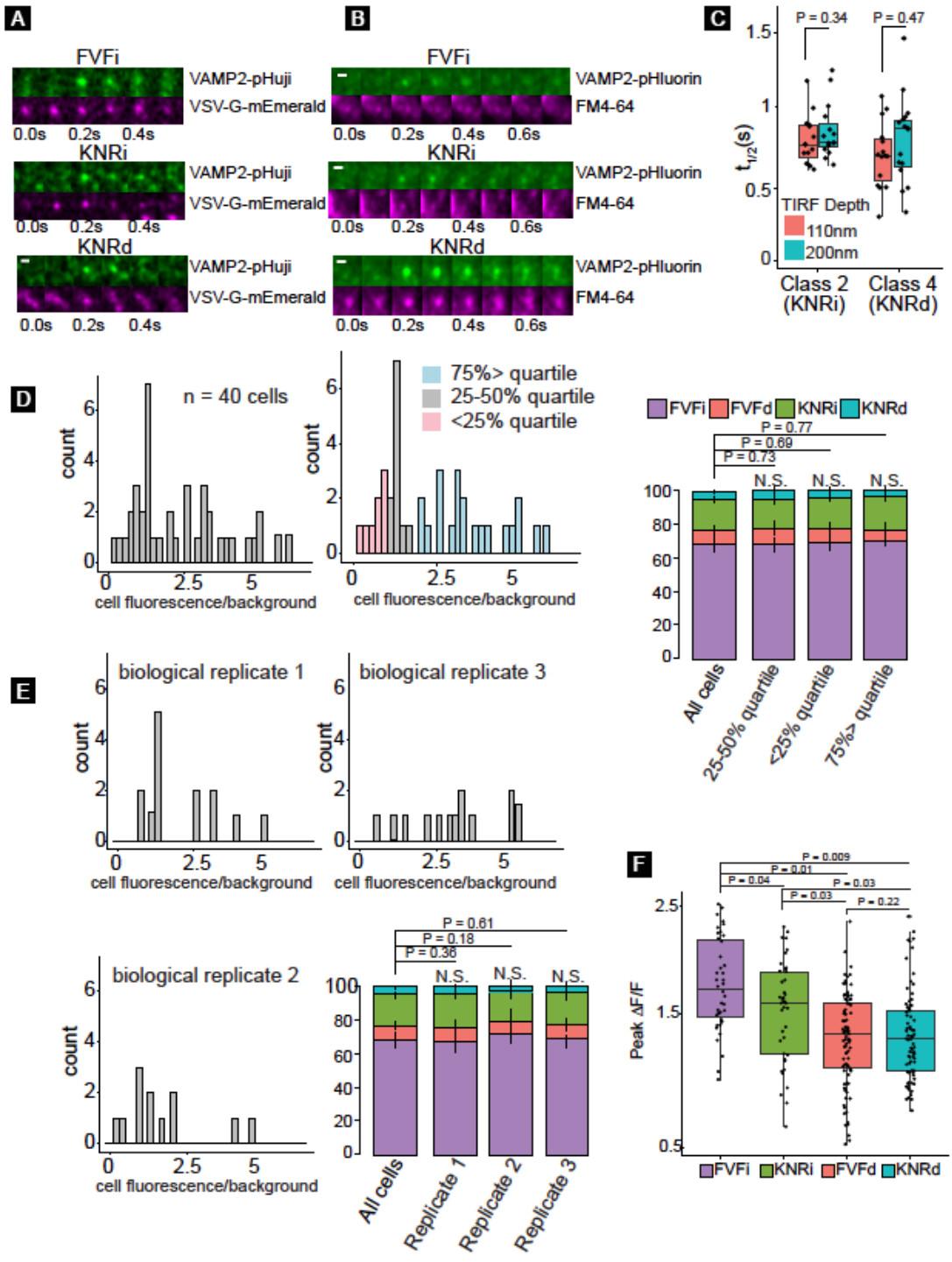

Figure S1: Distinguishing features of four modes of exocytosis, Related to Figures 1 and 3**. A)** Example images of different exocytic modes demonstrating VAMP2-pHuji co-localized with VSV-G-mEmerald. White bar = 1 μm. **B)** Example images of different exocytic modes demonstrating VAMP2-pHuji co-localized with FM4-64 dye. White bar = 1 μm. **C)** $t_{1/2}$ of VAMP2-phluorin fluorescent decay in KNRi and KNRd events is the same at 110 nm and 200 nm evanescent wave TIRF penetration depth, suggesting fluorescence decay is not caused by vesicle retreat from the PM. **D)** Cellular VAMP2-pHluorin fluorescence or **E)** biological replicate did not affect exocytic mode distribution. No significant difference in mode of exocytosis was found when partitioning cells based on fluorescent signal or grouping by biological replicate. **F)** Peak ΔF/F of fusion events of each exocytic mode (t-test with Benjamini-Hochberg correction).

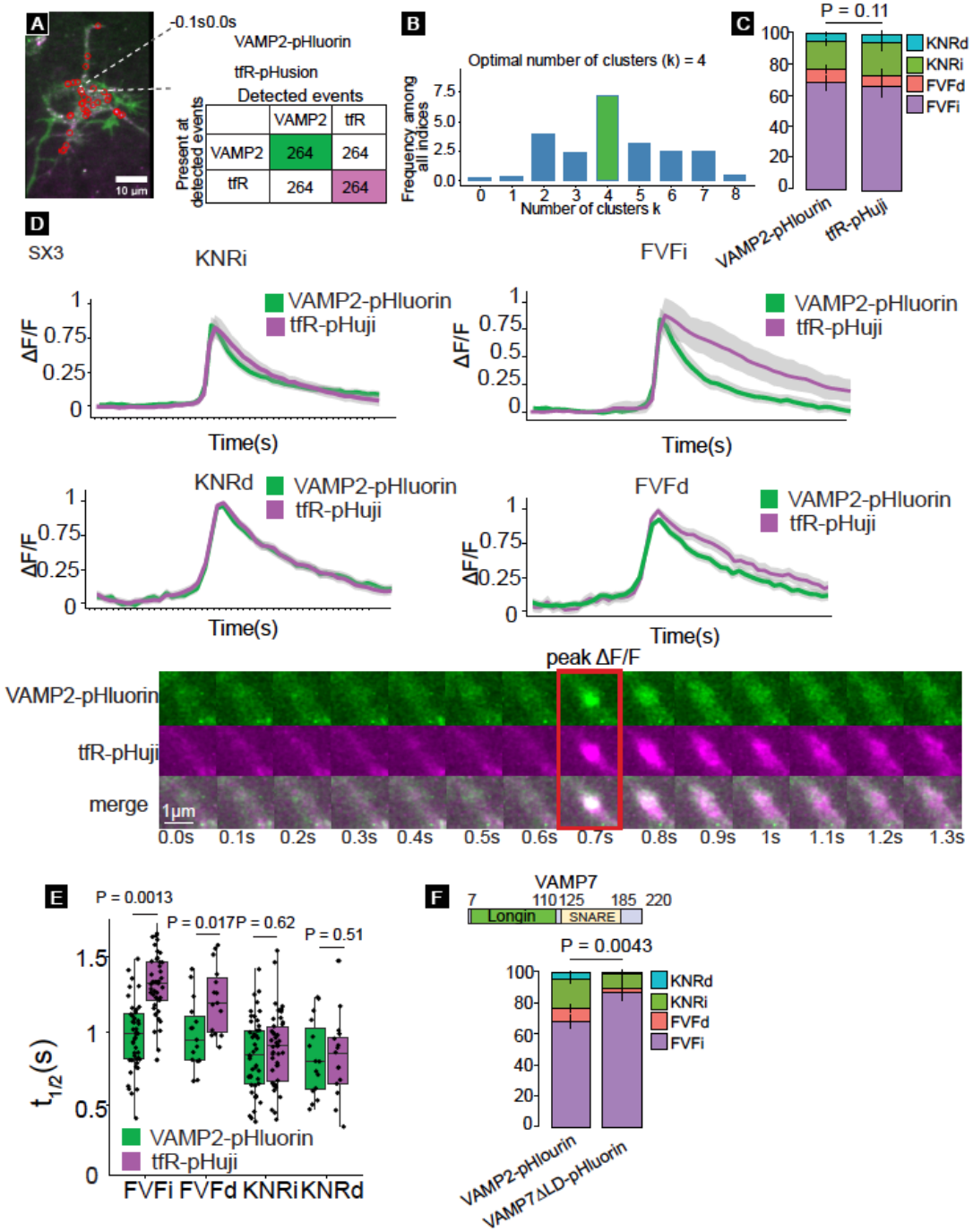

Figure S2: Four exocytic modes are also detected with TfR-pHuji and VAMP7ΔLD-pHluorin, Related to Figure 3. **A)** Colocalization of VAMP2-pHluorin and TfR-pHuji. 100% of VAMP2-pHluorin detected events colocalized with TfR-pHuji events, and 100% of TfR-pHuji detected events colocalized with VAMP2-pHluorin (n = 6 cells, n = 264 events, white scale bar in inset = 1µm). **B)** Decision histogram of the committee of indices for TfR-pHuji, with the plurality of indices choosing four classes. **C)** Proportion of FVFi, FVFd, KNRi, and KNRd in wildtype neurons expressing VAMP2-pHluorin or tfR-pHuji (n = 11 neurons, 4 biological replicates, multivariate linear regression). **D)** Mean fluorescent curves +/- SEM (grey) of each class of VAMP2-pHluorin and TfR-pHuji. (lower) representative images of ad VAMP2-pHluorin exocytic event with TfR-pHuji. **E)** Half-life of fluorescence decay ($t_{1/2}$) of each class of VAMP2-pHluorin (green) and TfR-pHuji (n = 11 cell, n = 4 biological replicates, paired t-tests with Benjamini-Hochberg correction) **F)** Proportion of FVFi, FVFd, KNRi, and KNRd in wildtype neurons expressing VAMP2-pHluorin or VAMP7ΔLD-pHluorin (n = 8 cells per condition, n = 3 biological replicates, multivariate linear regression)

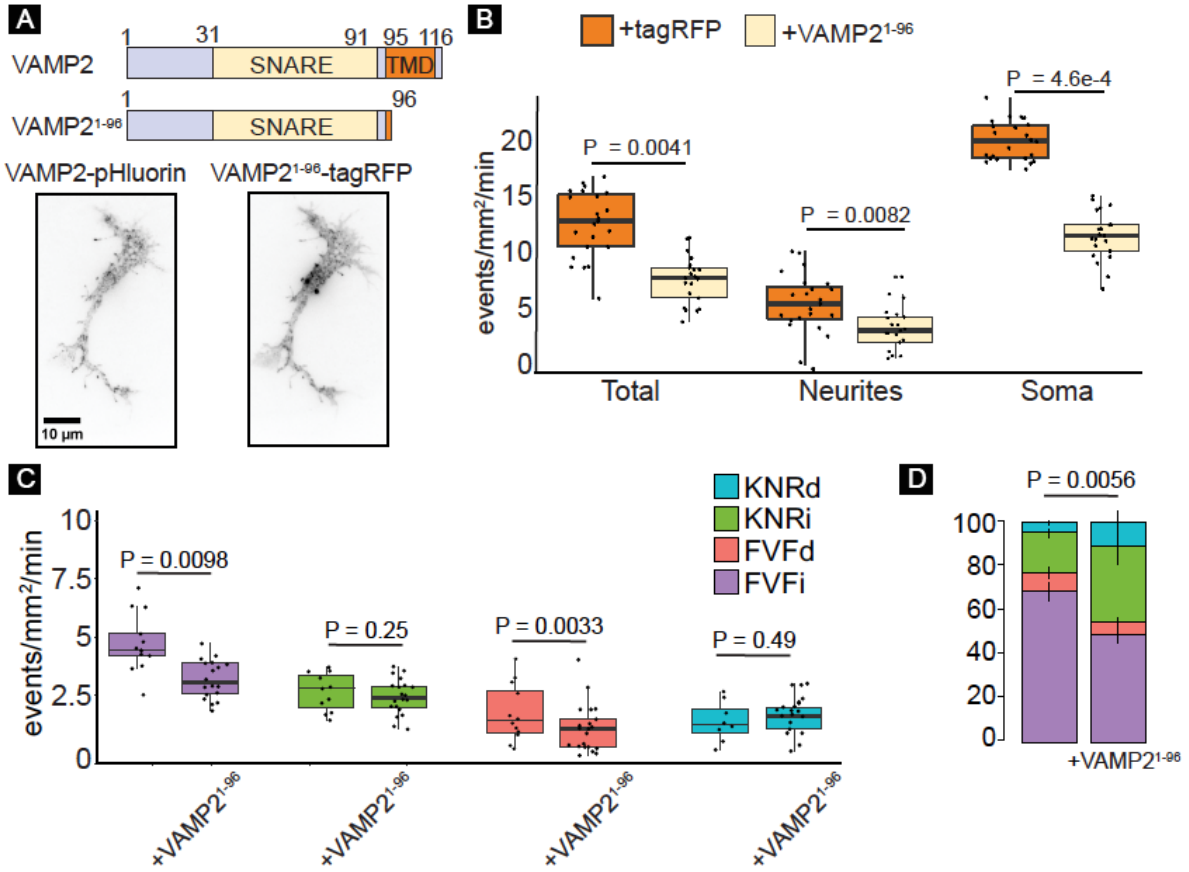

Figure S3: Expression of truncated VAMP2 alters exocytic mode, Related to Figure 3**. A)** VAMP2 and VAMP2$^{1-96}$ constructs and representative images of a neuron expressing both of VAMP2-pHluorin and VAMP2$^{1-96}$-tagRFP. **B)** Frequency of exocytic events in the whole cell, soma, and neurites of neurons expressing VAMP2-pHluorin +/- VAMP2$^{1-96}$-tagRFP (n = 23 cells per condition; n = 3 biological replicates; Welch's t-test followed by Benjamini-Hochberg correction). **C)** Frequency of exocytic events of each class of in whole neurons expressing VAMP2-pHluorin +/- VAMP2$^{1-96}$-tagRFP (n = 17 cells per condition; Welch's t-test followed by Benjamini Hochberg correction). **D)** Relative proportions of each class of exocytosis in whole neurons expressing VAMP2-pHluorin +/- VAMP2$^{1-96}$-tagRFP (n = 17 cells per condition; multivariate linear regression).

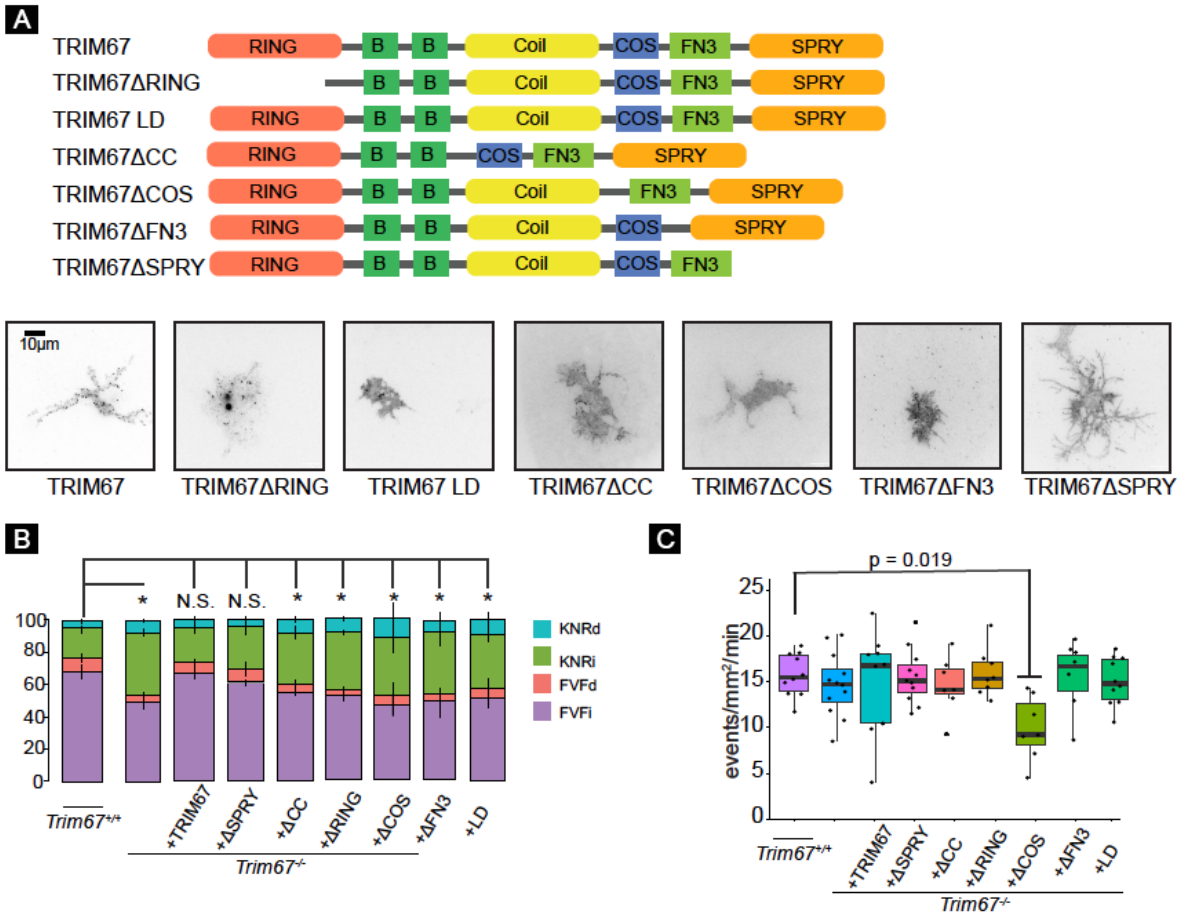

Figure S4: Multiple domains of TRIM67 and ligase function are required for exocytic mode regulation, Related to Figure 5**. A)** Domain architecture of TRIM67 constructs tagged with TagRFP for structure:function assays. TRIM67 is a TRIpartite Motif (TRIM) E3 ubiquitin ligase, characterized by a ubiquitin ligase RING domain, two BBox domains, a coiled-coil motif (CC) that mediates multimerization, a COS domain, FN3 domain, and SPRY domain Example fluorescence images of each mutant (below). **B)** Relative proportion of each exocytic class (n = 16 cells per condition; n = 4 biological experiments; multivariate linear regression. P-values from left to right: p = 0.0004; p = 0.49; p = 0.20; p = 0.024; p = 0.009; p = 0.029; p = 0.0073; p = 0.014) and **C)** exocytic frequency in *Trim67*$^{+/+}$ neurons, *Trim67*$^{-/-}$ neurons, or *Trim67*$^{-/-}$ neurons expressing VAMP2-pHluorin and TRIM67-tagRFP constructs (n = 14 cells per condition; n = 3 biological replicates; l Welch's t-test with Benjamini-Hochberg correction).

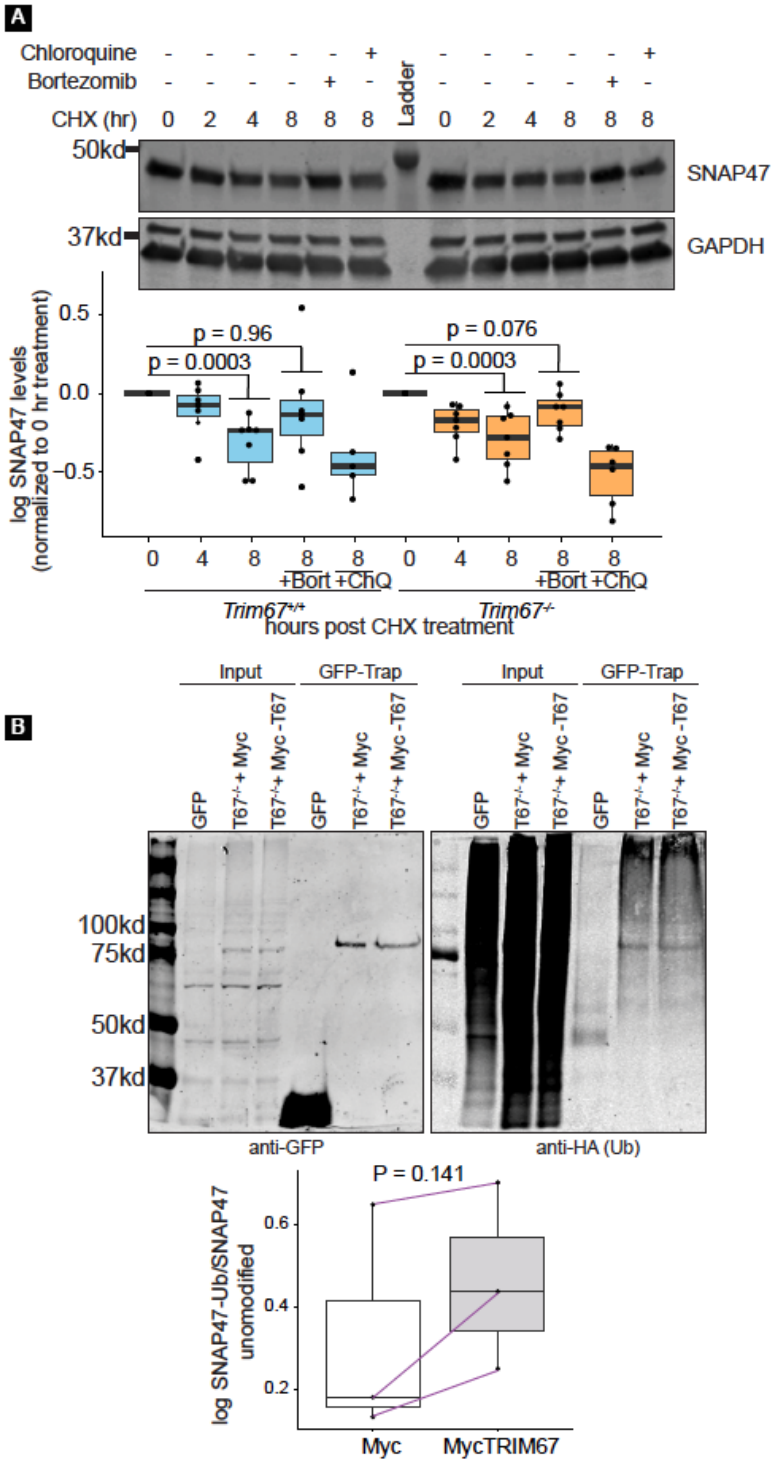

Figure S5: Proteasomal degradation and ubiquitination of SNAP47 are TRIM67 independent, Related to Figure 6**. A)** Immunoblot of neuronal lysates at 2 DIV after treatment with cycloheximide (CHX,50 µg/µl) for 0, 2, 4, 8 hrs or cycloheximide (50 µg/µl) +/- bortezomib (Bort) (200 nM) for 8 hrs or cycloheximide (50 µg/µl) +/- chloroquine (ChQ) for 8 hours (log-ratio paired t-test (methods) performed between genotypes at each timepoint followed by Benjamini-Hochberg correction; n = 7 blots, n =5 for CHX+ChQ in *Trim67*$^{+/+}$, n = 6 for CHX+ChQ in *Trim67*$^{-/-}$). **B)** Immunoblot of lysates and immunoprecipitation in *TRIM67*$^{-/-}$ HEK cells expressing HA- tagged ubiquitin (HA-Ub) along with empty GFP or GFP-tagged SNAP47 (GFP-SNAP47) with empty-Myc or Myc-tagged TRIM67 (Myc-TRIM67). Cells overexpressing these constructs were treated with MG132 for 4 hrs prior to lysis to prevent proteasomal degradation (log-ratio paired t-test (methods), n = 3).