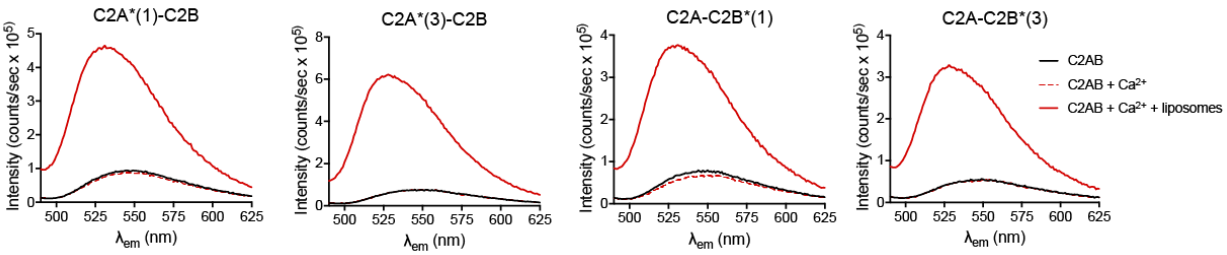

Figure S1. NBD emission intensity increases require the presence of lipids and Ca^{2+}. Labeled C2AB fragments of Doc2β were combined with Ca^{2+} (250 μM) prior to the addition of liposomes. No increase in NBD fluorescence was observed unless both Ca^{2+} and liposomes were present. Spectra are representative of results from two independent trials.

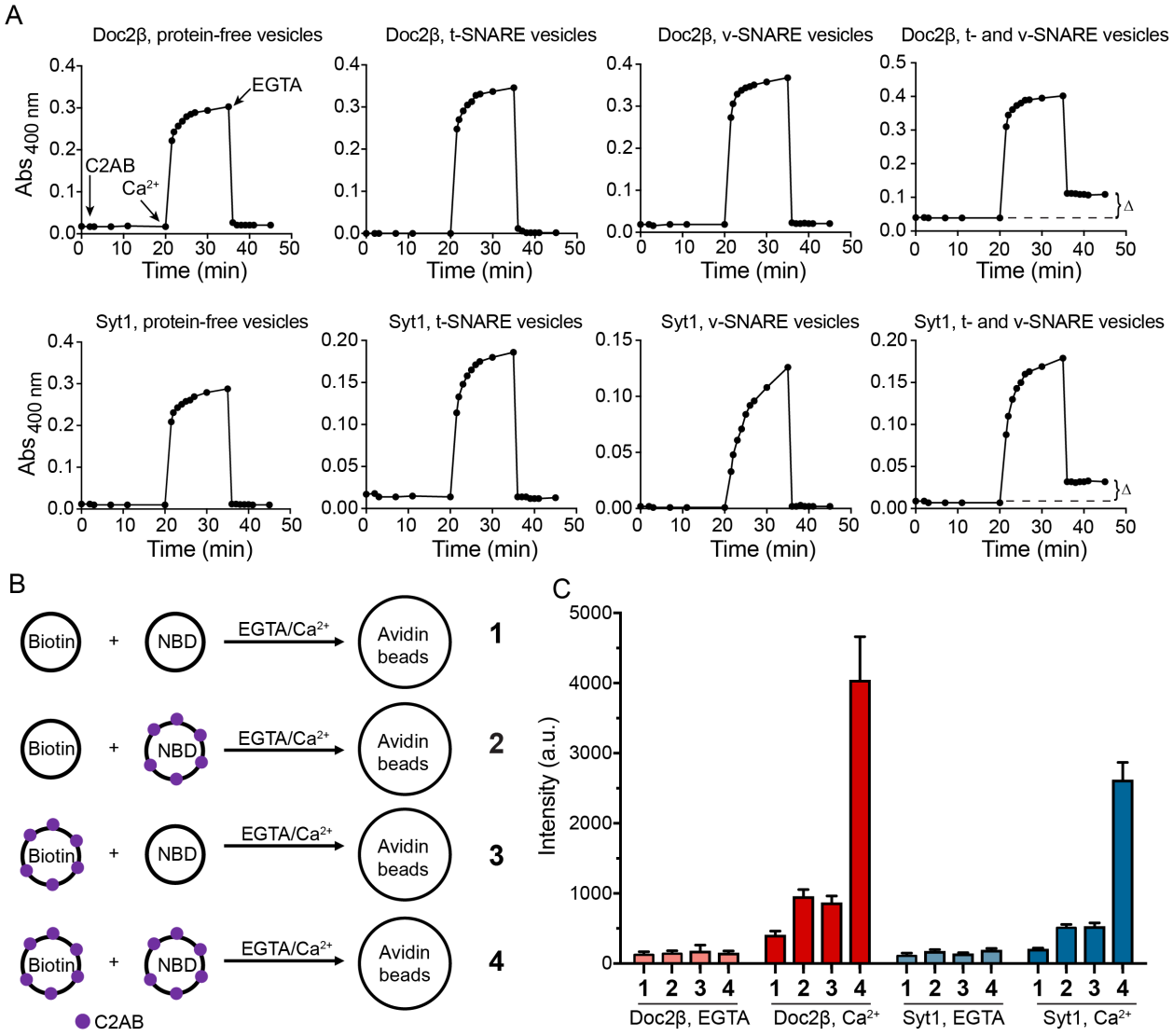

Figure S2. Doc2β, like syt1, aggregates membranes via a back-to-back *trans* mechanism. (A) Liposome aggregation mediated by syt1 and Doc2 was measured via absorbance at 400 nm after adding C2AB and Ca^{2+} to liposomes of the indicated composition. Ca^{2+} was added at 20 minutes, EGTA at 35 minutes. t-SNARE vesicles harbored reconstituted full-length SNAP-25:syntaxin-1 heterodimer and v-SNARE vesicles harbored reconstituted full-length synaptobrevin-2. Aggregation-induced absorbance increases were reversible for both proteins unless both t- and v-SNARE vesicles were included, in which case a residual absorbance increase persisted. (B) Schematic for pull-down assay to determine mechanism of vesicle aggregation. Liposomes bearing biotin-PE or NBD-PE were incubated with or without C2AB in EGTA or Ca^{2+}; in the latter case, C2AB is "pinned" to the vesicles due to its very slow off-rate in the presence of Ca^{2+}. NBD- and biotin-labeled liposomes were mixed, biotin liposomes were collected using avidin beads, and bead-bound NBD fluorescence was measured. (C) Quantification of bead-bound fluorescence. A small degree of liposome-liposome binding was observed when C2AB was pinned to one set of liposomes; pinning C2AB to both sets of liposomes dramatically increased liposome-liposome binding, suggesting that C2AB molecules associate in *trans* to mediate liposome aggregation. Error bars, standard error of the means (SEMs) of 3 independent trials.

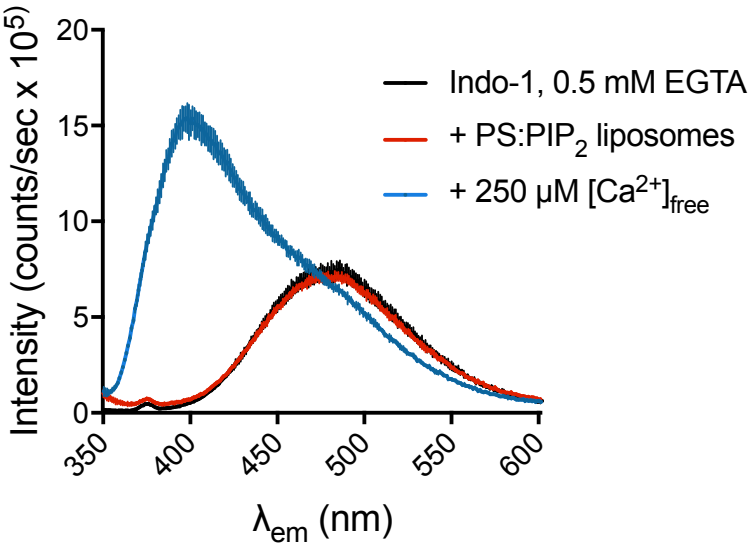

Fig. S3: PS:PIP_2 liposomes do not increase $[Ca^{2+}]_{free}$. Liposomes were added to 0.33 μM indo-1 in reconstitution buffer. A negligible increase at in the fluorescence at 400 nm, likely caused by scattering from liposomes, demonstrates that $[Ca^{2+}]_{free}$ remained < 10 nM after addition of PS:PIP_2 liposomes. Spectra are plotted as means +/- standard deviation for 3 separate batches of PS:PIP_2 liposomes.

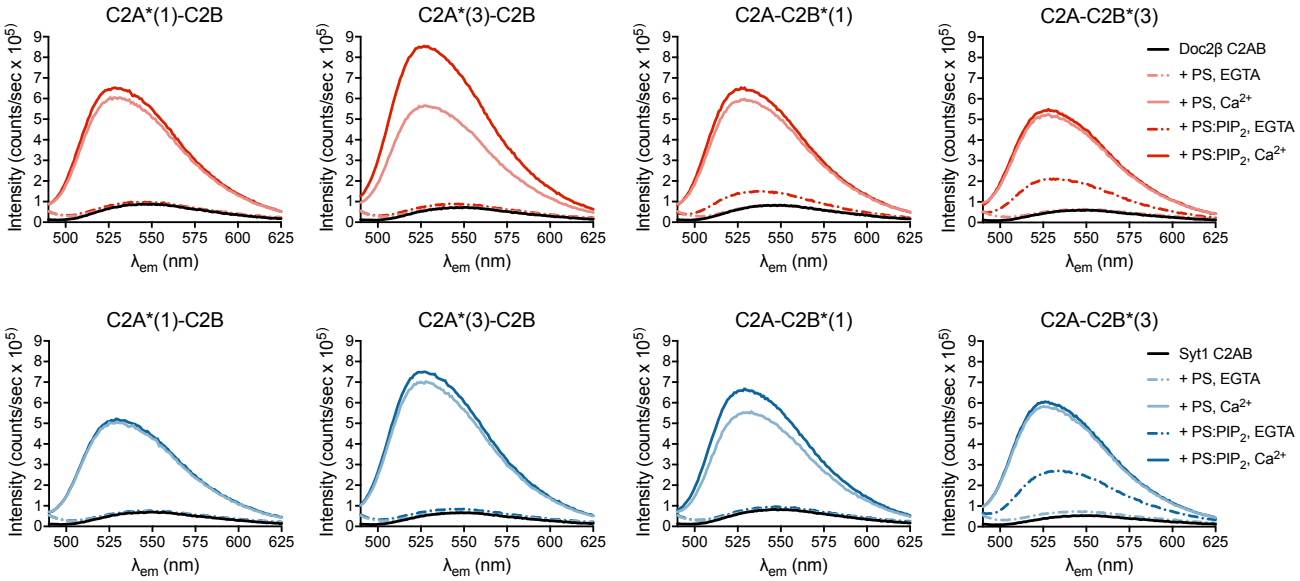

Fig. S4. Representative spectra from penetration assays demonstrating effect of 1% PIP_2 on penetration of liposomes containing PS in the presence and absence of 250 µM $[Ca^{2+}]_{free}$ (related to Figure 2, main text).

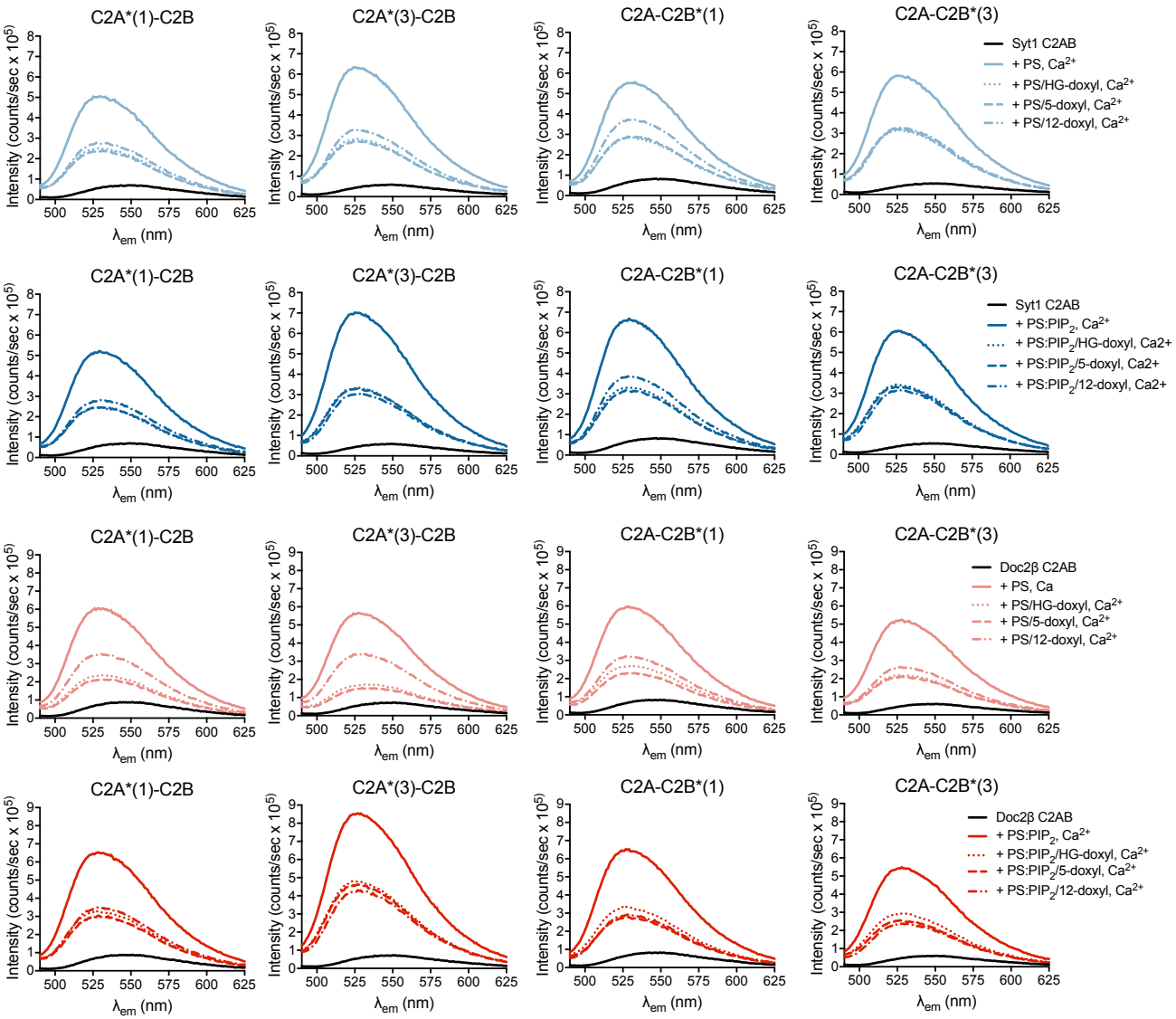

Fig. S5. Representative spectra from doxyl quenching experiments demonstrating effect of 1% PIP_2 on penetration of liposomes containing PS in the presence of 250 μM $[Ca^{2+}]_{free}$ (related to Figure 5, main text).

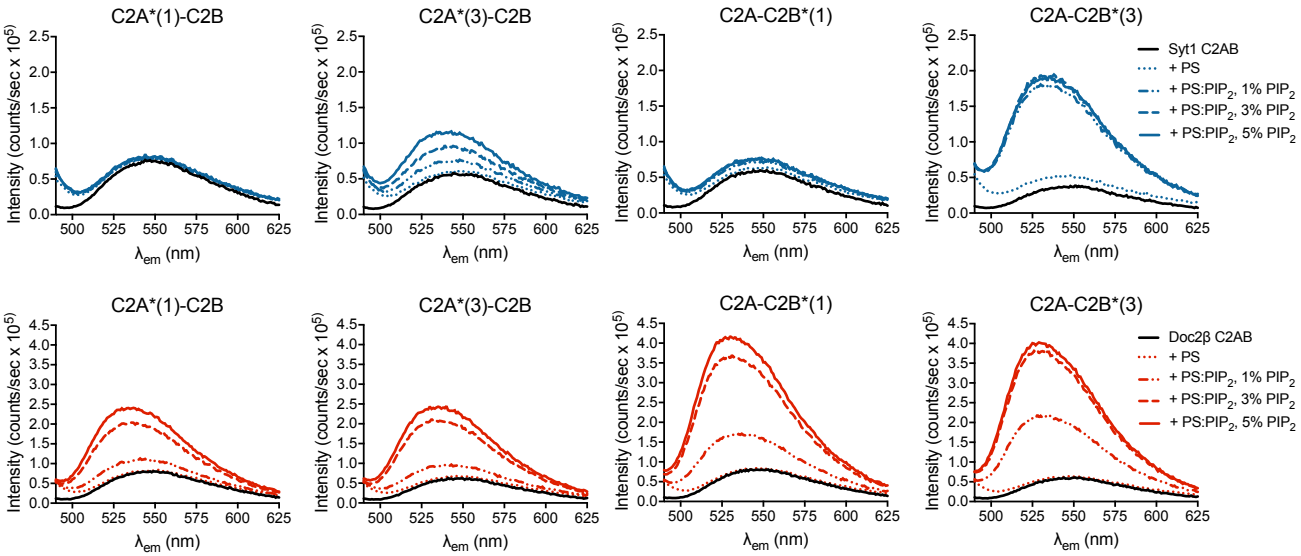

Fig. S6. Representative spectra demonstrating effect of increasing PIP_2 on penetration of liposomes containing PS in 500 µM EGTA (related to Figure 6, main text).

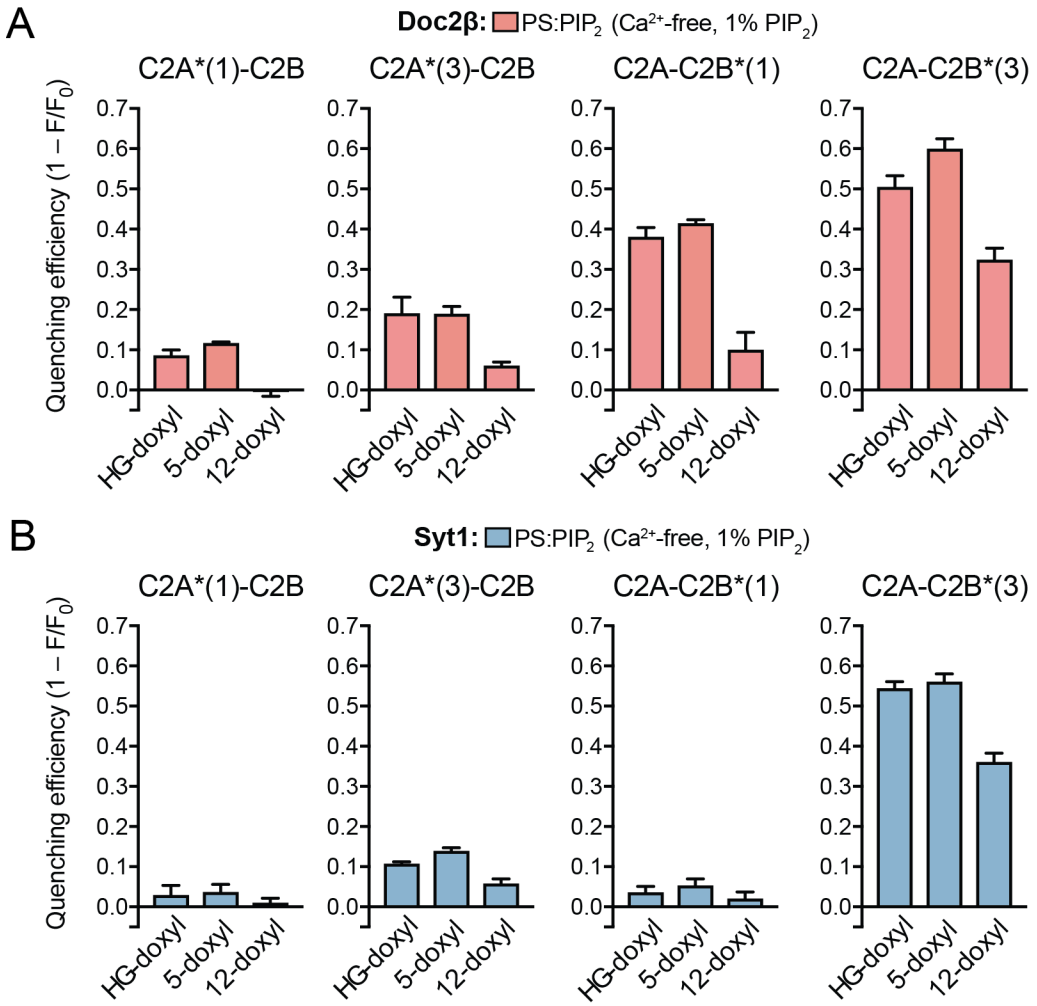

Figure S7. Doxyl quenching of NBD-labeled Doc2β and syt1 in EGTA. (A) NBD-labeled Doc2β C2AB was combined with PS:PIP_2 liposomes (1 mol % PIP_2) in 500 μM EGTA, and quenching efficiencies of doxyl-PC liposomes were quantified. Quenching of loops in C2A was far weaker than in C2B, consistent with localization of Ca^{2+}-independent penetration to the C2B domain. (B) as in (A) but for syt1 C2AB. In contrast to Doc2β minimal quenching was observed for C2B, loop 1. Both proteins showed weak quenching of C2A, loop 3, along with the expected robust quenching of C2B, loop 3. Error bars, SEMs of 4 independent trials.