**Supplementary Information**

**Supplementary Fig. 1. Representative proteins that contain multiple C2 domains.** The lengths (a.a.) of the predicted disordered linkers that join different protein domains or membranes are indicated.

**Supplementary Fig. 2. Two conserved membrane binding motifs in the C2 domains of synaptotagmins (Syts) and extended synaptotagmins (E-Syts)**. (**a**) Alignments of the C2 amino acid sequences showing two conserved membrane binding motifs highlighted in bold and color: the Ca2+-binding motif (blue) and the basic patch (red). (**b**) Comparison of the membrane binding motifs in the C2 domains of Syt1 and E-Syts.

**Supplementary Fig. 3. E-Syt1 C2AB only weakly binds to membranes enriched with negatively charged lipids.** (**a, b**) Diagrams showing no binding (a) and weak binding (b) of the E-Syt1 C2AB domain in the presence of 10% and 20% DOPS, respectively. (**c**) Force-extension curves showing no membrane binding of E-Syt1 C2AB domain in the absence of supported bilayer or in the presence of the supported bilayer containing 10% DOPS. Weak binding was detected in the presence of 20% DOPS, as indicated by the rip at low force (red arrow). (**d**) Extension-time trajectories at constant forces (black curves) and their idealized transitions derived from hidden-Markov modeling (red curves). (**e**) Unbinding probability and binding and unbinding rates (symbols) and their best model fits (lines). The fitting revealed an unbinding energy of 4.4 (±0.3) kBT for the E-Syt1 C2AB domain (Supplementary Table 1).

**Supplementary Fig. 4. E-Syt1 C2ABCDE binds to the membrane containing 30% DOPS and 0% PI(4,5)P2 via its C2CD domain, but not its C2E domain.** C2CD unbinding probability (top panel) and binding and unbinding rates (bottom panel) as a function of force. The experimental data (symbols) were fit by a nonlinear model to yield the best-fits (lines).

**Supplementary Fig. 5. E-Syt2 C2C undergoes a reversible force-dependent, but Ca2+-independent conformational change to inactivate its membrane binding.** (**a-c**) Extension-time trajectories at constant force in the absence (a) and presence (b) of Ca2+ for E-Syt2 C2ABC or in the presence of Ca2+ for E-Syt2 C2C (c). The long gaps in the unbound state highlighted blue represent the binding inactive state. (d) Diagram of the conformational transition of the C2 domain in the binding active and inactive states.

**Supplementary Fig. 6. Cytosolic E-Syt1 containing the SMP domain binds to membranes in a manner like its C2 repeat C2ABCDE.** (**a**) Schematic diagram showing different E-Syt1 binding states. (**b**) Force-extension curves obtained by pulling E-Syt1 under different conditions. (**c**) Extension-time trajectories of E-Syt1 at constant force in the presence or absence of Ca2+.

**Supplementary Fig. 7. Calculated state probabilities, forces, and energy as a function of membrane separation due to potential trans-membrane binding of E-Syt1 (left panel) and E-Syt2 (right panel) lacking a membrane-binding C-terminal C2 module.** (**a, c**) Schematics of different C2 binding and membrane tethering states in the absence and presence of Ca2+ for E-Syt1 (a) or E-Syt2 (c). The calculations were to simulate the results of membrane contact formation from in vivo imaging using E-Syts with mutant C-terminal C2 domains (C2E in E-Syt1 or C2C in E-Syt2 and E-Syt3) that did not bind to membranes or with the domains truncated. (**b, d**) Calculated probabilities (top panel), average stretching force (middle), and free energy (bottom) of different states for truncated E-Syt1 (b) or E-Syt2 (d). Calculations corresponding to the presence of Ca2+ or the absence of Ca2+ are indicated by solid and dashed lines, respectively, with their colors indicating different states as shown in a or b: red for the bound state I and black for the unbound state iii. Stable and unstable states are indicated by solid and hollow circles, respectively. The derived equilibrium distances and free energy are shown in Supplementary Table 2.

**Amino acid sequences of the E-Syt constructs**

Different sequence motifs are colored as follows: Avi-tags in red, the coding sequences of C2 domains in blue, the C-terminal cysteine or Snoop tag for DNA crosslinking in purple, and the linker sequences in black. The mutated amino acids are underlined.

E-Syt1 C2ABCDE

MHHHHHHAIAGLNDIFEAQKIEWHELEGGSDEGSQGDNGSGDGSKGSGNPWQLRSPLPRGIIRIHLLAARGLSSKDKYVKGLIEGKSDPYALVRLGTQTFCSRVIDEELNPQWGETYEVMVHEVPGQEIEVEVFDKDPDKDDFLGRMKLDVGKVLQASVLDDWFPLQGGQGQVHLRLEWLSLLSDAEKLEQVLQWNWGVSSRPDPPSAAILVVYLDRAQDLPLKKGNKEPNPMVQLSIQDVTQESKAVYSTNCPVWEEAFRFFLQDPQSQELDVQVKDDSRALTLGALTLPLARLLTAPELILDQWFQLSSSGPNSRLYMKLVMRILYLDSSEICFPTVPGCPGAWDVDSENPQRGSSVDAPPRPCHTTPDSQFGTEHVLRIHVLEAQDLIAKDRFLGGLVKGKSDPYVKLKLAGRSFRSHVVREDLNPRWNEVFEVIVTSVPGQELEVEVFDKDLDKDDFLGRCKVRLTTVLNSGFLDEWLTLEDVPSGRLHLRLERLTPRPTAAELEEVLQVNSLIQTQKSAELAAALLSIYMERAEDLPLRKGTKHLSPYATLTVGDSSHKTKTISQTSAPVWDESASFLIRKPHTESLELQVRGEGTGVLGSLSLPLSELLVADQLCLDRWFTLSSGQGQVLLRAQLGILVSQHSGVEAHSHSYSHSSSSLSEEPEL SGGPPHITSSAPELRQRLTHVDSPLEAPAGPLGQVKLTLWYYSEERKLVSIVHGCRSLRQNGRDPPDPYVSLLLLPDKNRGTKRRTSQKKRTLSPEFNERFEWELPLDEAQRRKLDVSVKSNSSFMSRERELLGKVQLDLAETDLSQGVARWYDLMDNKDKGSS GAPGSGESGKLGDIEFIKVNK

E-Syt1 C2AB

GLNDIFEAQKIEWHELEGGSDEGSQGDNGSGDGSKGSGNESGQGTGEGSNGSGDGSGELPWQLRSPLPRGIIRIHLLAARGLSSKDKYVKGLIEGKSDPYALVRLGTQTFSSRVIDEELNPQWGETYEVMVHEVPGQEIEVEVFDKDPDKDDFLGRMKLDVGKVLQASVLDDWFPLQGGQGQVHLRLEWLSLLSDAEKLEQVLQWNWGVSSRPDPPSAAILVVYLDRAQDLPLKKGNKEPNPMVQLSIQDVTQESKAVYSTNCPVWEEAFRFFLQDPQSQELDVQVKDDSRALTLGALTLPLARLLTAPELILDQWFQLSSSGPNSRLYMKLVMRILYLDSSEIC

E-Syt2 C2ABC

GLNDIFEAQKIEWHELEGGSDEGSQGDNGSGDGSKGSGNPWSEVQIAQLRFPVPKGVLRIHFIEAQDLQGKDTYLKGLVKGKSDPYGIIRVGNQIFQSRVIKENLSPKWNEVYEALVYEHPGQELEIELFDEDPDKDDFLGSLMIDLIEVEKERLLDEWFTLDEVPKGKLHLRLEWLTLMPNASNLDKVLTDIKADKDQANDGLSSALLILYLDSARNLPSGKKISSNPNPVVQMSVGHKAQESKIRYKTNEPVWEENFTFFIHNPKRQDLEVEVRDEQHQCSLGNLKVPLSQLLTSEDMTVSQRFQLSNSGPNSTIKMKIALRVLHLEKRERPPDHQHSAQVKRPSVSKEGRKTSIKSHMSGSPGPGGSNTAPSTPVIGGSDKPGMEEKAQPPEAGPQGLHDLGRSSSSLLASPGHISVKEPTPSIASDISLPIATQELRQRLRQLENGTTLGQSPLGQIQLTIRHSSQRNKLIVVVHACRNLIAFSEDGSDPYVRMYLLPDKRRSGRRKTHVSKKTLNPVFDQSFDFSVSLPEVQRRTLDVAVKNSGGFLSKDKGLLGKVLVALASEELAKGWTQWYDLTEDGTRPQAMTGAPGSGESGKLGDIEFIKVNK

E-Syt2 C2C

GLNDIFEAQKIEWHEGSSHHHHHHSGLVPRGSRLRQLENGTTLGQSPLGQIQLTIRHSSQRNKLIVVVHACRNLIAFSEDGSDPYVRMYLLPDKRRSGRRKTHVSKKTLNPVFDQSFDFSVSLPEVQRRTLDVAVKNSGGFLSKDKGLLGKVLVALASEELAKGWTQWYDLTEDGTRPQAMTC

**Supplementary Table 1**. Binding energies, binding rates, and unbinding rates of C2 domains at zero force. The bimolecular binding energies (Eon) and rates (kon) in the absence of membrane tethers were derived from the corresponding energies (Eb) and rates (kb) measured by our assay in the presence of membrane tethers, whereas the unbinding rates (kub) are independent of membrane tethers. “NA” means the C2-membrane binding is too weak to be detected by our assay.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Protein** | **C2 domain** | **[Ca2+]**  **(µM)** | **PI(4,5)P2%** | **DOPS%** | **Binding energy with tether (Eb) (kBT)** | **Binding energy without tether (Eon) (kBT)** | **Log10[kb (s-1)]** | **Log10[kon (M-1s-1)]** | **Log10[kub**  **(s-1)]** |
| E-Syt1 C2ABCDE | C2AB | **0** | 5 | 10 | NA |  |  |  |  |
| C2CD | NA |  |  |  |  |
| C2E | 5 (1) | 7 (1) | 4.5 (0.6) | 5.4 (0.6) | 2.3 (0.8) |
| C2AB | 100 | 5 | 10 | NA |  |  |  |  |
| C2CD | 8.5 (0.9) | 10.4 (0.9) | 4.0 (0.3) | 4.8 (0.3) | 0.3 (0.2) |
| C2E | 5 (1) | 6 (1) | 4.1 (0.1) | 4.6 (0.1) | 1.8 (0.1) |
| C2AB | 100 | **10** | 10 | NA |  |  |  |  |
| C2CD | 13.3 (0.9) | 14.5 (0.9) | 4.9 (0.3) | 5.4 (0.3) | -0.9 (0.5) |
| C2E | 7.2 (0.3) | 8.4 (0.3) | 4.2 (0.1) | 4.7 (0.1) | 1.1 (0.1) |
| C2AB | 100 | **0** | **30** | NA |  |  |  |  |
| C2CD | 7.6 (0.7) | 9.5 (0.7) | 3.9 (0.1) | 4.8 (0.1) | 0.6 (0.2) |
| C2E | NA |  |  |  |  |
| E-Syt1 C2AB | C2AB | 100 | 5 | **20** | 4.4 (0.3) | 6.5 (0.3) | 3.2 (0.1) | 4.1 (0.1) | 1.33 (0.05) |
| E-Syt2 C2ABC | C2AB | 100 | 5 | 10 | 4.9 (0.4) | 7.1 (0.4) | 4.8 (0.3) | 5.8 (0.3) | 2.6 (0.3) |
| C2C | 11.5 (0.7) | 13.0 (0.7) | 6.5 (0.5) | 7.2 (0.5) | 1.5 (0.5) |

**Supplementary Table 2**. Comparisons of predicted and measured equilibrium membrane distance, energy or population of the membrane contacts mediated by wide-type E-Syts or E-Syts lacking the C-terminal C2 domains (C2E in E-Syt1 or C2C in E-Syt2)18. See also Fig. 6 and Supplementary Fig. 7. ‘NA’ indicates that the corresponding data is ‘not available’.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Protein** | **Ca2+** | **C-terminal C2 domain** | **Predicted Equilibrium membrane separation (nm)** | **Predict equilibrium energy (kBT)** | **Observed equilibrium membrane separation (nm)** | **Observed population of membrane contacts** |
| E-Syt1 | - | + | 19.5 | 4.3 | 22-25 | + |
| + | + | 15.8 | -1.5 | ~15 | +++ |
| + | - | 15.7 | 3.5 | NA | - |
| E-Syt2 | - | + | 19.2 | -2.2 | ~19 | +++ |
| + | + | 11.3 | -0.75 | NA | +++ |
| + | - | 11.4 | 10 | NA | - |