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1 **Diacylglycerol-dependent hexamers of the SNARE-assembling chaperone**
2 **Munc13-1 cooperatively bind vesicles**

3

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16 **Keywords:** Munc13-1, cluster, SNARE, Syntaxin, synaptic vesicle, neurotransmission,
17 membrane fusion, TIRF, diacylglycerol, PI(4,5)P₂

18 **ABSTRACT**

19 Munc13-1 is essential for vesicle docking and fusion at the active zone of synapses. Here we
20 report that Munc13-1 self-assembles into molecular clusters within diacylglycerol (DAG)-rich
21 microdomains present in phospholipid bilayers. Although the copy number of Munc13-1
22 molecules in these clusters has a broad distribution, a systematic Poisson analysis shows that this
23 is most likely the result of two molecular species: monomers and mainly hexameric oligomers.
24 Each oligomer is able to capture one vesicle independently. Hexamers have also been observed
25 in crystals of Munc13-1 that form between opposed phospholipid bilayers (*1*). Mutations
26 targeting the contacts stabilizing the crystallographic hexagons also disrupt the isolated
27 hexamers, suggesting they are identical. Additionally, these mutations also convert vesicle
28 binding from a cooperative to progressive mode. Our study provides an independent approach
29 showing that Munc13-1 can form mainly hexamers on lipid bilayers each capable of vesicle
30 capture.
31

32 **SIGNIFICANCE STATEMENT**

33 Munc13-1 is a molecular chaperon that facilitates recruitment and docking of synaptic vesicles at
34 the active zone of the synapse. Using model membranes and statistical modeling, we show that
35 Munc13-1 forms mainly hexamers on diacyl-glycerol rich microdomains on lipid membranes.
36 These hexamers act cooperatively to capture vesicles from a solution. Statistics show that each
37 hexamer binds to one vesicle. Disruption of the interactions at Munc13-1 hexagonal interface by
38 point mutations based on crystallographic data alters its oligomerization state and hexamers are
39 no longer observed. Furthermore, the mutant oligomer loses its cooperativity in binding vesicles.
40 Our study suggests that the Munc13-1 hexamers observed on lipid bilayers resemble the
41 hexagons revealed by crystallography.

42

43

44

45 INTRODUCTION

46 Many cellular proteins form oligomeric structures to achieve their specific biological functions
47 (2-4). Numerous proteins required for neurotransmitter release are concentrated within the
48 crowded active zones of presynaptic terminals, potentially favoring the assembly of well-defined
49 oligomeric structures that would not be observed in dilute solution. Relatively small differences
50 in energy amongst a series of alternative oligomeric arrangements could then enable successive
51 rearrangements to occur in a defined order, thereby choreographing the process of vesicle
52 capture and activation for fusion. In this paper we will report novel properties of oligomers of the
53 synaptic protein Munc13-1 which assemble on lipid bilayers following concentration within
54 diacylglycerol (DAG)-rich microdomains likely mimicking portions of the pre-synaptic active
55 zone.

56 Munc13-1 is a key chaperone protein required for the assembly of the SNARE complex of
57 Syntaxin1, VAMP2, and SNAP25 which ultimately triggers membrane fusion for synaptic
58 transmission (5-15). Munc13-1 is a large molecule, the C-terminal half of which contains its
59 characteristic MUN domain, flanked at one end by a DAG-binding C₁ domain and a PIP₂ and
60 Ca⁺⁺ - binding C₂B domain, and at the other end by its phosphatidyl-serine (PS)-binding C₂C
61 domain. Munc13-1 is initially required to capture synaptic vesicles via its C₂C domain (16, 17),
62 it is later required to activate Syntaxin1 to initiate SNAREpin assembly (18) during which it
63 interacts with each of the three synaptic SNARE proteins (18-20), and approximately 5-10 copies
64 remain in close association with each primed, ready-release vesicle (21, 22) most likely
65 containing 6 clamped central SNAREpins arranged symmetrically on a ring (23, 24). Munc13-1
66 is therefore ideally positioned to choreograph this entire process via a series of oligomeric
67 assemblies.

68 We previously reported that purified Munc13-1 protein reconstituted onto supported lipid
69 bilayers containing DAG and PIP₂ is organized into clusters containing from 2 to ~20 copies, as
70 revealed by a combination of quantitative Total Internal Reflection Fluorescence (TIRF)
71 microscopy and stepwise photobleaching (17). We noted that only clusters containing a
72 minimum of 6 copies of Munc13-1 were capable of efficiently capturing and retaining PS-
73 containing small unilamellar vesicles (SUVs). This hinted that a hexamer of Munc13 could be a
74 basic functional unit for vesicle binding, a conclusion that had no structural precedent at the time.

75 Direct evidence for a hexameric organization then emerged when we solved the structure of two-
76 dimensional crystals of Munc13-1 that spontaneously assemble between bilayers containing
77 copious acidic phospholipids, revealing two novel oligomeric arrangements: a 21 nm high
78 ‘upright’ trimer that binds both bilayers, and a 14 nm high ‘lateral’ hexagon (*I*). The hexameric
79 structure of Munc13-1 is particularly of interest because it may represent a fusion intermediate,
80 with six Munc13-1 dependent protein complexes under each closely docked synaptic vesicle,
81 which is consistent with the electron tomography studies which showed that the protein density
82 between synaptic vesicles and the plasma membrane exhibited 6-fold symmetry (23, 24).

83 The straightforward interpretation of these results (*I*) is that upright trimers first capture synaptic
84 vesicles by PS-dependent binding to their plasma membrane-distal C₂C domains and then
85 transition closer to the plasma membrane as the trimers re-arrange to form hexamers. However,
86 this model would seem to require that the top surface of the hexamer retains the synaptic
87 vesicles, even though the known vesicle binding surface in C₂C is sterically unavailable in the
88 hexamer (*I*). The structural model (*I*) and *in vitro* & *in vivo* functional assays (25, 26) also
89 strongly predicted that DAG binding by the C₁ domain should trigger the rearrangement of
90 trimers into hexamers, which involves a simple intra-molecular rigid body rotation of C₁-MUN-
91 C₂C relative to C₂B, which remains fixed on the plasma membrane surface (*I*). This followed
92 because the DAG binding site is closely opposed to the bilayer surface in the hexamer but points
93 away from the bilayer in the trimer.

94 In this study we have built on the observation that clusters of approximately 6 copies of Munc13
95 bind a vesicle (*I7*) to test these predictions. Specifically, we now investigate whether isolated
96 hexamers of Munc13 can form on lipid bilayers, whether they require DAG to assemble, and
97 whether each hexamer can potentially retain synaptic vesicles following their initial capture by
98 upright trimers.

99

100 **RESULTS AND DISCUSSION**

101 **DAG forms microdomains within phospholipid bilayers**

102 Diacylglycerol (DAG) is a lipid in plasma membrane at relatively low concentration. However,
103 the membrane lipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) is known to form clustered

104 domain in lipid bilayers in the presence of Syntaxin-1 juxtamembrane domain (27).
105 Consequently, DAG may exist in the form of clustered microdomains when such enriched
106 PI(4,5)P₂ is hydrolyzed to DAG by the phospholipase C (28-30). DAG has been reported to
107 induce phase transition in phospholipid bilayers (31). Molecular dynamics simulations suggest
108 that DAG may form domains in the bilayers (32). Hence, such DAG domains may have been
109 present in our previous study on Munc13-dependent vesicle binding (17). To test this directly,
110 we examined the distribution of a fluorescently labelled analogue of DAG, TopFluor® DAG
111 (33), replacing ordinary DAG (2 mole%) in bilayers also comprised of 71 mole% PC, 25% PS,
112 and 2% PI(4,5)P₂, the same composition used in our previous study (see Materials and Methods
113 for acronyms). Fig. 1A (left panel) confirms that micro-domains of DAG were indeed present.
114 These DAG-rich domains ranged in area from less than 0.5 μm² up to about 2 μm², averaging 0.7
115 ± 0.5 μm² for 2 mole% total DAG (Fig. 1F, red bars). Higher concentrations of DAG, tested up
116 to a total of 10 mole% total (2 mole% TopFluor® DAG analogue with the balance of DAG being
117 underivatized DAG) resulted in somewhat larger microdomains, the largest being 2 – 3 μm² at
118 10% total DAG (Fig. 1F). The average DAG domain was 0.8 ± 0.7 μm² at 5 mole% total DAG
119 and 1.2 ± 0.8 μm² at 10 mole% total DAG.

120

121 **Munc13-1 binds preferentially to the DAG microdomains**

122 We next tested whether Munc13-1, previously shown (17) to bind to DAG-containing bilayers in
123 clustered regions that could not be resolved optically, might be selectively localized within DAG
124 domains. For this purpose, we employed a minimal fragment of Munc13-1 known to be
125 functional *in vivo* (10, 20), consisting of C₁-C₂B-MUN-C₂C (residues 529 to 1735; Munc13C)
126 with a Halo domain added at its C-terminus (Munc13C-Halo). This protein was rendered
127 fluorescent by reacting the Halo tag with Alexa Fluor 660 ligand. The theoretical molecular
128 weight of this protein is 167 kDa. It is soluble in buffer solution. In mass photometry
129 measurements, it displayed a large peak at 169 ± 15 kDa, and a barely noticeable second peak at
130 329 ± 21 kDa (Fig. 1G). This confirmed that the starting material in our experiments, 10 nM
131 Munc13-1 in solution, was primarily monomeric with an extremely small fraction of dimers.

132

133 We incubated the supported lipid bilayer with such 10 nM Munc13-1 solution. Then we
134 simultaneously imaged DAG (TopFluor®, 488 nm) and Munc13C (Alexa 660) on the lipid
135 bilayer by TIRF.

136 Fig. 1A-C (middle panels) reproduces our original finding that Munc13C forms clusters and goes
137 on to present new data that establishes that at 2 – 10 mole% DAG almost all of these Munc13-1
138 clusters are within the DAG microdomains (Fig. 1A-C, panels at right). This suggests that DAG
139 locally increases its own concentration through self-aggregation, resulting in DAG
140 microdomains which recruit and locally concentrate Munc13C molecules.

141 The number of copies of Munc13C in each DAG domain was then determined by a combination
142 of quantifying the TIRF intensity and stepwise photobleaching as before ((17), Material and
143 Methods). The frequency distributions of copy numbers were indistinguishable at 2 mole% (red)
144 and 5 mole% (blue) DAG, and the same whether the DAG was fluorescently tagged (red) or
145 natural DAG (dashed line), showing that Munc13-1 binding is not affected by the fluorescent
146 probe. Very little binding or clustering of Munc13-1 occurred when DAG was omitted from the
147 bilayers (Supporting Fig. S1).

148 Because Munc13-1 can potentially bind to the plasma membrane either to PI(4,5)P₂ (via C₂B) or
149 to DAG (via C₁) or to both lipids simultaneously, it was of special interest to determine whether
150 PI(4,5)P₂ is excluded from the DAG microdomains. For this purpose we prepared bilayers
151 containing 2 mole% non-fluorescent DAG and 2 mole% TopFluor® PI(4,5)P₂. We observed that
152 PI(4,5)P₂ was nearly uniformly distributed in the lipid bilayer and Munc13-1 clusters were
153 formed in PI(4,5)P₂ region (Fig. 1D), which suggested that PI(4,5)P₂ was neither concentrated
154 within nor excluded from the DAG microdomains. This implies that Munc13-1 bound to DAG
155 microdomains can potentially be bound to both DAG and PI(4,5)P₂ simultaneously.

156 Mass Photometry experiments showed Munc13-1 remained as monomer in solution, from 8 nM
157 to 170 nM, (Supporting Fig. S2). This result indicates that DAG, in addition to locally increasing
158 the Munc13-1 concentration, also provides a binding interface for correctly orientating Munc13-
159 1 for oligomer formation, as already suggested by the previous cryo-electron tomography study.

160

161 **Poisson distribution analysis reveals that Munc13-1 assembles into discrete oligomers**
162 **within DAG microdomains, most likely hexamers**

163 As reported previously (17), we are able to determine the number of Munc13-1 molecules, N , in
164 each cluster. For a given cluster, in principle such N Munc13-1 molecules may exist in the form
165 of monomers or oligomers, or a combination of these states. The likelihood of lateral diffusion of
166 the Munc13-1 molecules within the clusters makes it difficult to determine their oligomeric state
167 from optical microscopy alone, and this difficulty is compounded when the domains are close in
168 size to the wave length of the light. For example, a “cluster” with a copy number of 6 Munc13-1
169 molecules ($N = 6$) could be at one extreme 6 individual, non-interacting monomers diffusing
170 freely over the surface of a common lipid microdomain, or at the other extreme a rigidly
171 structured hexamer such as is observed bound to bilayers in the Munc13-1 crystal (1).

172 To rule out the trivial explanation (null hypothesis) that the observed Munc13-1 clusters are
173 merely a collection of individual proteins that happen to be bound to the same lipid microdomain
174 we can employ well-understood Poisson statistics to analyze the copy number distribution of the
175 apparent Munc13-1 clusters. In the null hypothesis the frequency distribution of copy numbers
176 will be given by:

$$177 P_M(m) = \frac{\langle m \rangle^m}{m!} e^{-\langle m \rangle} \quad (1)$$

178 Where m is the number of monomers and $\langle m \rangle$ the mean copy number of Munc13-1 molecules
179 per cluster. $\langle m \rangle$ cannot be directly measured experimentally since the domains without any
180 Munc13-1, *i.e.*, $P_M(0)$, is unknown because in most experiments we used unlabeled DAG.
181 Hence, the observed copy number probability needs to be corrected for it. This is a
182 straightforward process (see Supporting text). This model assumes that the DAG microdomains
183 are of uniform size which is reasonable considering the measured area distribution. With these
184 considerations, we determined that $\langle m \rangle \approx 4.8$ copies of Munc13-1 per cluster at 2 mole% total
185 DAG. This predicts a copy number distribution (Fig. 2A, bars) that clearly does not match with
186 the observed distribution (dashed line). We can conclude that the null hypothesis is incorrect.
187 That is, the observed copy number distribution can only be explained by molecular interaction
188 amongst the bound Munc13-1 into stable oligomers which distribute as such amongst the
189 individual cluster.

190 We can further refine the statistical model to provide deeper insight into the degree of
 191 oligomerization of Munc13-1 that is likely involved, which we term ‘ K ’. For example, if
 192 monomers were to assemble exclusively into hexamers, then $K = 6$, and a cluster containing 8
 193 copies of Munc13-1 ($N = 8$) would in fact most likely contain 1 copy of a hexamer and 2
 194 unassembled monomers or, less likely, 8 copies of individual Munc13-1. More generally, we will
 195 determine the best fit for K to the experimental data with the assumption that Munc13-1 is
 196 present in clusters only as combinations of monomers and K -mers. These two populations
 197 behave independently of each other and each randomly distributes amongst clusters following its
 198 own Poisson probability distribution, exactly as in the first model:

$$\begin{aligned}
 P_M(m) &= \frac{\langle m \rangle^m}{m!} e^{-\langle m \rangle} \\
 P_K(k) &= \frac{\langle k \rangle^k}{k!} e^{-\langle k \rangle}
 \end{aligned}
 \tag{2}$$

200 where P_M and P_K are the probability distributions for monomers and K -mers, respectively, and m
 201 and k are respectively the numbers of copies of monomers and K -mers in the cluster.

202 The observed copy number distributions of Munc13 cluster result from combining the monomers
 203 and K -mer distributions, as presented in Supporting Table S1. $\langle m \rangle$ and $\langle k \rangle$ can then be calculated
 204 (see Supporting text for explanations). The predicted probability distribution of copy numbers is
 205 fully described by these two parameters (Eq. S5 in the supporting information).

206 The resulting copy number distributions predicted from dimer to decamer, $K = 2$ to $K = 10$, are
 207 shown in Figure 2B-D and Supporting Figure S3. The predicted distribution for hexamers ($K =$
 208 6) closely matches the experimental results (dashed lines). In particular, this model correctly
 209 predicts the second peak at $N = 8$ and the third peak at $N = 14$. Flanking models ($K = 5$ and $K = 7$)
 210 do not predict these observed peaks. To objectively assess which model(s) seems to describe best
 211 the observed cluster size distribution, we use a parameter to test the oligomerization degree, O ,
 212 that quantitatively compares the predicted and observed histograms for each K -mer. A larger O
 213 value indicates a better prediction of the experimental distribution. The results confirm that the
 214 hexamer model has the highest O value (Fig. 2E and Supporting text).

215 Finally, another prediction from this model is that the fraction of DAG domains without any
 216 Munc13-1 is:

$$P_{Total}(0) = e^{-\langle k \rangle} e^{-\langle m \rangle}
 \tag{3}$$

218 In the case of hexamers, $\langle m \rangle = 2.4$ and $\langle k \rangle = 0.375$ which leads to $P_{Total}(0) = 0.06 = 6\%$.
219 Experimentally, we can estimate $P_{Total}(0)$ from experiments with bilayers containing
220 fluorescent DAG. We find $9 \pm 2\%$ of DAG domains lack Munc13C, which is reasonably close to
221 the predicted value considering the simplicity of our model.

222

223 **Targeted mutations predicted to destabilize the hexamers observed in crystals alter the** 224 **Munc13-1 copy number distribution and disrupts co-operative vesicle binding**

225 Are the predominantly hexameric units of Munc13-1 that assemble in clusters on the surface of
226 single lipid bilayers structurally equivalent to the Lateral Hexagons that assemble in protein
227 crystals between bilayers (*I*)? The Lateral Hexamer assembles when the Munc13-1 molecules
228 are in their ‘closed’ conformation and when the C₂C domain of one copy contacts the MUN
229 domain of its neighbor. This geometry results in a closed hexameric ring (Fig. 3A).

230 It was previously shown by cryo-electron tomography that Munc13-1 forms hexagonal lattice
231 between negatively charged lipid bilayers (*I*). We located possible interacting amino acid within
232 the lateral interfaces between C₂C domain and MUN domain of neighbor molecule in the
233 hexagonal formation of Munc13-1 (Fig. 3A). This interface was proposed to consist of two
234 regions – hydrophobic (a/a F1176 and I1215 on MUN domain and corresponding interaction
235 partners W1626 and W1684 on C₂C domain) and polar (a/a E1139 and D1039 on MUN domain
236 and R1678, R1673 on C₂C domain) (Fig. 3B). These two regions are well conserved in
237 vertebrates and the hydrophobic region is conserved in invertebrates as well (Supporting Figure
238 S4).

239 To test selected amino acid residues, we mutated them on MUN domain to N1176, N1215,
240 R1139 and R1039. We refrained from mutating the corresponding residues on C₂C domain due
241 to possible negative effect on membrane binding abilities. This quadruple mutation not only
242 disrupted the formation of the hexagonal crystal but also led to the assembly of a new rectangular
243 crystal lattice (Fig. 3D and Supporting Figure S5). Visually protein packing is much denser than
244 wild-type with visible rows of round-shaped protein densities and diagonally placed proteins
245 connections between them (Figs. 3C and 3D). When measured between the centers of round
246 elements, rows are spaced by 35 nm, and round elements within a row are spaced by 12 nm.
247 Distance between bilayers, as measured on the side views (Figs. 3C and 3D) is similar and ~21

248 nm suggesting the presence of upright open configuration of the protein in the new crystal. The
249 3D reconstruction of a new crystal is ongoing and will hopefully show the details of a new
250 crystal assembly.

251 If the oligomers assembling on Munc13-1 clusters on lipid bilayers are in fact Lateral Hexagons
252 in the cryo-electron tomography study, they too should be disrupted by these mutations. To test
253 this, we used the same mutant version of Munc13C as above, F1176N, I1215N, E1139R, and
254 D1039R, and measured its copy number distribution and vesicle capture property (Fig. 3E). The
255 mutant bound to DAG-containing lipid bilayers and formed cluster, similar to the wild-type
256 protein, but had a markedly reduced copy number distribution (Fig. 3F). In particular, the
257 predicted peaks for hexamers at $N = 8$ and $N = 14$ are abolished. Instead, a new peak is observed
258 at $N = 5$. Applying the same model and methodology as for the Poisson analysis of the wild-type
259 protein (Eq. 2 and Supporting Eq. S5) reveals that only a tetramer ($K = 4$) model predicts the new
260 peak at $N = 5$ (Supporting Fig. S6), and that a tetramer has by far the largest O value (Fig. 3G).
261 Further work involving direct structure determination will be required to independently confirm
262 this conclusion.

263 In any case, the new results imply that C_2C is required for the observed hexameric clustering of
264 Munc13C. Our previous report we suggested that C_2C could be deleted without affecting
265 clustering (Fig. 4B in ref. (17)). With the perspective of the current work, re-examination reveals
266 that clustering without C_2C results in smaller clusters and the distribution notably differs from
267 that of wild-type especially at copy numbers of 6 and 12, suggesting that there is a
268 C_2C -independent non-hexameric form of clustering that can occur when C_2C is artificially
269 removed.

270 We previously reported that Munc13-1 clusters formed on DAG-containing lipid bilayers bound
271 PS-containing SUVs depending on the number of copies of Munc13-1 present (17). In particular,
272 we found that clusters containing 6 or more copies of Munc13-1 always captured a vesicle, and
273 those with 4 or fewer copies almost never did, suggesting that 6 copies of Munc13-1 optimally
274 co-operate to capture a vesicle when Munc13-1 was bound to DAG via its C_1 domain, as
275 disrupting the interaction between DAG and C_1 reduced recruitment of Munc13-1 (Supporting
276 Fig. S7), while $PI(4,5)_2$ has a partial contribution to the recruitment but little effect on the size
277 distribution of the clusters (Supporting Fig. S8). We have confirmed and extended these data in

278 Fig. 4, which now tests the important prediction that vesicle binding, relying as it does, on
279 Munc13-1 binding should likewise be DAG-dependent (compare Figs 4A and B, with and
280 without DAG, respectively). Comparing the Munc13-1 copy number content of those clusters
281 which fail to capture a vesicle (Fig. 4C, blue bars) with those that do (orange bars) confirms that
282 it is only when the cluster contains 6 or more copies that it does not fail to capture a vesicle. This
283 is also true for Munc13-1 clusters on the bilayer in the absence of DAG, where there is barely
284 any co-localization with captured vesicles because the copy numbers of Munc13-1 molecules in
285 these clusters are usually less than 6 (Fig. 4D).

286 These experiments cannot in and of themselves distinguish whether the 6 co-operating copies of
287 Munc13-1 are pre-assembled into a hexamer as distinct from assembling around a vesicle during
288 the process of capturing it. However, the former model makes a unique prediction, that
289 disrupting the hexamers should eliminate the co-operativity of vesicle binding. To test this,
290 fluorescent vesicles containing 68 mole% PC, 30% PS and 2% PE-Atto647N were incubated
291 with supported lipid bilayers harboring the Munc13-1 quadruple mutant (F1176N I1215N
292 E1139R and D1039R) clusters. The bilayers were then washed to remove unbound vesicles and
293 imaged using TIRF microscopy (Fig. 3E, three panels at top). The results were quantified by
294 measuring the number of copies of the Munc13-1 mutant in each cluster and scoring that cluster
295 for the presence or absence of vesicles (Fig. 3H). As predicted for co-operative vesicle binding to
296 hexamers, the hexamer-destabilizing mutations (purple bars) reduced the probability of vesicle
297 capture at $N = 6$, the threshold observed for co-operative binding by the wild-type protein (Fig.
298 3H, green dashed line, reproduced from ref. (17)).

299 As strikingly, co-operativity was eliminated. Vesicle capture by the mutant increases
300 progressively (nearly linearly) with the number of copies present. This suggests that each copy
301 of the mutant, unable to form hexamers, now binds to vesicles independently.

302

303 **Each hexamer appears to capture a single vesicle**

304 So far, we have not attempted to distinguish the number of vesicles bound to each Munc13-1
305 cluster. Two vesicles bound to the same cluster would be difficult or impossible to resolve
306 optically. However, the fluorescent intensities of these vesicles would be additive. With this in
307 mind, we measured the total vesicle fluorescence intensity colocalizing with each Munc13-1

308 cluster and related it to the number of copies of Munc13-1 bound to the same cluster (Fig. 5A
309 and B). There is a clear increase in vesicle intensity with the copy number of Munc13-1
310 molecules in the cluster (Fig. 5C).

311 The simplest model would be that each hexamer can bind one vesicle. If this were the case, we
312 would observe a quantal rather than a progressive increase in the number of vesicles bound as a
313 function of copy number. In particular, these steps should occur around $N > 8$ and $N > 14$, where
314 one and two hexamers predominate (Fig. 2C). In fact, this is exactly what we observe (Fig. 5C).

315 Because the size of each vesicle (and thus its intensity) follows a probability distribution, it is not
316 necessarily straightforward to relate the intensities in each step to the number of vesicles, even
317 though the vesicles in the capture experiments have a narrow size distribution with a mean radius
318 of $\sim 31 \pm 9$ nm (Supporting Fig. S9). However, this can be done with a normalization process that
319 takes into account the various combinations of monomers and hexamers that can combinatorially
320 result in a given copy number for a cluster (see Table S1) and their associated probabilities for
321 vesicle binding. As a result, we can calculate the mean number of vesicles bound to a cluster as
322 a function of its overall copy number as predicted by the quantal binding model (Fig. 5D). The
323 good agreement between the predictions and observations suggests that our assumptions are
324 correct and that each DAG-dependent hexamer captures a single vesicle as an independent co-
325 operative unit.

326

327 **CONCLUDING REMARKS**

328 In summary, a combination of biochemical and structure-based mutation data imply that
329 hexamers corresponding to the lateral hexamers in protein-bilayer crystals (*I*) form as stand-
330 alone structures on DAG-rich microdomains (Figs. 1-3); that hexamer assembly depends on
331 binding to DAG microdomains (Fig. S1C); and that each hexamer co-operatively captures a
332 single vesicle (Fig. 5). Therefore, hexamers are predicted to bind two bilayers simultaneously. It
333 will be important to directly visualize such structures by cryo-electron microscopy in future
334 studies.

335 Our conclusion that hexamers can still bind vesicles is satisfying biologically because it would
336 explain how a synaptic vesicle captured initially by the C₂C domains of upright trimers of

337 Munc13-1 (21 nm high) could be retained at the plasma membrane after these trimers have
338 putatively transitioned into hexamers (now 14 nm high) as Munc13-1 transitions from its open to
339 closed conformation, putatively driven by DAG binding. Yet, this conclusion is also surprising
340 from a structural perspective. The upright trimers bind bilayers representing the vesicle by their
341 C₂C domains, and our cryo-EM structure (*I*) directly visualized the surface of C₂C that would be
342 in contact with a synaptic vesicle. But as we have detailed (*I*) in the closed conformation of the
343 subunits of a hexamer, this very surface of C₂C is engaged in binding to the neighboring MUN
344 domain and thus sterically unavailable.

345 Presumably a new surface is created on the top of the hexamer that contains a combination of
346 hydrophobic and basic residues which can multivalently bind negatively charged PS-containing
347 phospholipid vesicles (*17*). It remains for future work to identify the constituents of this
348 unanticipated vesicle binding surface.

349 Moreover, our work suggest that oligomerization of synaptic proteins is a critical form of
350 organization at the presynaptic membrane. Many important tethers in synaptic fusion, such as the
351 RIM protein, RIM-BP, and Synapsin, are known to undergo phase separation and form
352 condensed liquid droplets in the active zone (*34, 35*). These droplets are a form of
353 oligomerization. These findings show that oligomerization occurs quite often, and is probably a
354 general mechanism for locally obtaining high concentration.

355

356 **MATERIALS AND METHODS**

357 **Chemicals.** 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Potassium hydroxide
358 (KOH), Potassium chloride (KCl), Magnesium chloride (MgCl₂), Glycerol, DNase I, RNase A,
359 Benzonase, Roche complete protease inhibitor cocktail tablets, Phorbol 12-myristate 13-acetate
360 (PMA), and DL-Dithiothreitol (DTT) were purchased from Sigma-Aldrich. Nickel-NTA agarose,
361 TCEP-HCl, and Expi293™ Expression System Kit were supplied by Thermo Fisher Scientific.
362 The lipids used in this study, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-
363 oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine)
364 (sodium salt) (DOPS), L- α -phosphatidylinositol-4,5-bisphosphate (Brain, Porcine) (ammonium
365 salt) (brain PI(4,5)P₂), 1-2-dioleoyl-sn-glycerol (DAG), 1,2-dioleoyl-sn-glycero-3-
366 phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (DOPE-NBD), 1-

367 palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycerol (TopFluor® DAG), 1-
368 oleoyl-2-{6-[4-(dipyrrometheneboron difluoride)butanoyl]amino}hexanoyl-sn-glycero-3-
369 phosphoinositol-4,5-bisphosphate (ammonium salt) (TopFluor® PI(4,5)P₂), and 1-oleoyl-2-(6-
370 ((4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-2-
371 propionyl)amino)hexanoyl)-sn-glycero-3-phosphoinositol-4.5-bisphosphate (ammonium salt)
372 (TopFluor® TMR PI(4,5)P₂) were purchased from Avanti Polar Lipids. 1,2-Dioleoyl-sn-glycero-
373 3-phosphoethanolamine ATTO 647N (DOPE-Atto647N) was from ATTO-Tec. The plasmid
374 maxi prep kit was from QIAGEN. HaloTag® Alexa Fluor® 488 Ligand and HaloTag® Alexa
375 Fluor® 660 Ligand were purchased from Promega. All aqueous solutions were prepared using
376 18.2 MΩ ultra-pure water (purified with the Millipore MilliQ system).

377 ***Protein constructs, expression and purification.***

378 The original vector expressing rat Munc13 was a kind gift from Dr. Claudio Giraudo. Similar to
379 our previous report (17), the expression plasmids His₁₂_PreScission
380 _C₁_C₂B_MUN_C₂C_tev_Halo and His₁₂_PreScission _C₁_C₂B_MUN_C₂C_tev_Halo
381 quadruple mutant (F1176N I1215N E1139R D1039R), were produced by cloning rat Munc13-1
382 residues 529 to 1735, respectively, to a pCMV-AN6 plasmid. Munc13-1 residues 1408-1452
383 were deleted and residues EF were added in their place (36). A short linker sequence containing
384 a TEV cut site was subcloned in followed by the Halo tag. For CryoET studies, the cDNA
385 construct with His₁₂-Munc13C (Munc13-1 residues 529 to 1735 with residues 1408 to 1452
386 replaced by the sequence EF) was expressed in a modified pCMV-AN6 vector, which includes a
387 PreScission cut site following the His₁₂ tag. The quadruple mutant
388 (F1176N/I1215N/E1139R/D1039R) was generated using a QuickChange mutagenesis kit
389 (Agilent Technologies).

390 The resulting plasmids were amplified with maxi prep using QIAGEN Plasmid Maxi kit and
391 were used to transfect Expi293F™ human cells. Proteins were expressed with Expi293™
392 expression system following the manufacturer's protocol.

393 The Munc13-1 proteins were then purified using Ni-NTA affinity beads as described before (37-
394 39). To summarize, the cell pellet was thawed on ice and disrupted with a homogenizer, and then
395 spun in an ultracentrifuge for 30 minutes at ~142,400xg at 4°C. The supernatant was removed
396 and 2 mL Qiagen Ni-NTA slurry along with 10 uL Benzonase were added, and subsequently

397 rotated using an orbiting wheel overnight at 4°C. The beads were washed at 4°C with 30mL
398 buffer containing 50 mM HEPES at pH 7.4, 400 mM KCl, 10% glycerol, 1 mM TCEP, and 10
399 mM Imidazole, then with another 30mL buffer containing 50 mM HEPES at pH 7.4, 400 mM
400 KCl, 10% glycerol, 1 mM TCEP, and 25 mM Imidazole, followed the third 30 mL buffer
401 containing 50 mM HEPES at pH 7.4, 270 mM KCl, 10% glycerol, 1 mM TCEP, and 25 mM
402 Imidazole. 100 µL of PreScission protease (~2 mg.mL⁻¹) in 1 mL buffer was added to the beads
403 and incubated for 3 hours at room temperature with shaking to remove the 12xHis tag. After the
404 cleavage reaction, elusions were collected and gel filtrated using a Superdex 200 column. The
405 protein concentration was typically 1 to 2 mg.mL⁻¹ as determined by using a Bradford protein
406 assay with Bovine Serum Albumin (BSA) as the standard.

407 ***Protein labeling***

408 The Munc13-1-Halo proteins were labeled by incubating the proteins with Alexa488 or
409 Alexa660 conjugated with Halo ligand from Promega, as described before (13). The protein was
410 first centrifuged at 14,000 rpm for 20 minutes at 4°C to remove any precipitation. Fluorescence
411 dye was added into the protein solution at dye:protein = 5:1 molar ratio and the mixture was
412 incubated for 30 min at room temperature with gentle rotation. Unreacted dye was removed by
413 passing through the PD MidiTrap G-25 column (GE Healthcare) three times at room
414 temperature. The labeling efficiencies were about 97%.

415 ***Liposome formation***

416 Protein-free liposomes were prepared by extrusion using an Avestin mini-extruder (7, 17). To
417 make the liposomes for preparing bilayers, DOPC, DOPS, DAG, TopFluor® DAG, PI(4,5)P₂,
418 TopFluor® PI(4,5)P₂, and/or TopFluor® TMR PI(4,5)P₂ were mixed at proper mole ratio. Total
419 amount was 3 µmoles. Nitrogen flow was use to remove the liquid solvents, and lipids were then
420 dried in vacuum for 2 hours, followed by re-suspension with 500 µL buffer containing 50 mM
421 HEPES, 140 mM KCl, and 10% glycerol. The resuspended lipids were treated with freezing
422 (using liquid nitrogen) and thawing (in 37°C water bath) cycles for 8 times, followed by
423 extrusion using 100 nm or 50 nm membrane for 21 times.

424 We usually had 2% DAG, a reasonable estimate of the physiological DAG level, because it was
425 previously reported that DAG level is around 0.4% to 2% in hepatocytes (40). At the plasma

426 membrane, PI(4,5)P₂ is about 0.4 mol% to 4 mol% (41), and can be converted to DAG through
427 hydrolysis (28). Both PI(4,5)P₂ and DAG are able to be enriched to high local concentration in
428 the presence of Syntaxin-1 juxtamembrane domain (27).

429 To make the liposomes for preparing vesicles, 68 mol% DOPC, 30 mol% DOPS, and 2 mol%
430 DOPE-Atto647N were mixed. Total amount was 3 μmoles, and liposomes were produced
431 similarly as above.

432 ***Bilayer Preparation and TIRF microscopy***

433 Bilayers were prepared by bursting liposomes on the glass surface using a glass-bottomed μ-
434 Slide V1^{0.5} chip from Ibidi. 2.5 μL MgCl₂ at 500 mM were added into 122.5 μL buffer
435 containing 50 mM HEPES (pH 7.4), 140 mM KCl, and 10% glycerol. Then 125 μL extruded
436 bilayer liposomes were added. 60 μL MgCl₂-liposome solution were loaded into the channel of
437 the ibidi chip and incubate for 40 min at room temperature. The channel was washed with the
438 same buffer supplemented with 6 mM EDTA, and then with buffer supplemented with 1 mM
439 DTT. Depending on the purpose of experiments, MgCl₂ or CaCl₂ may be added in the buffer to
440 the desired concentration. 60 μL of 10 nM Munc13-1-Halo-Alexa488 were loaded into the
441 channel and incubate with the bilayer for 60 min at room temperature. The channel was washed
442 with the buffer supplemented with 1 mM DTT. The vesicle liposomes were diluted 30 times. 60
443 μL diluted vesicle liposomes were loaded into the channel and incubate for 5 min at room
444 temperature. The channel was washed with buffer supplemented with 1 mM DTT.

445 The Ibidi chip was then mounted to the stage of a Nikon TIRF microscope. Bilayers, Munc13
446 particles on bilayers, and vesicles attached to bilayers were respectively imaged at room
447 temperature with the TIRF microscope using the corresponding laser.

448 ***Counting Munc13 copy numbers***

449 To determine the number of copies of Munc13C in each DAG domain, we gradually bleached
450 the image frames using suitable laser power at different positions. The bleaching profiles
451 (particle fluorescence intensity versus time) were plotted and a variety of bleaching patterns were
452 found. When the Munc13C copy number was small, the bleaching profile displayed apparent
453 discrete steps, and the actual number of proteins can be determined from counting the number

454 and intensity of steps. This method only works for relatively small copy numbers (generally, 5–6
455 or fewer) because the bleaching profile becomes smooth when the copy number is large. As an
456 alternative for larger copy numbers, we fitted the intensity profile using:

$$457 \quad I(t) = I_0 e^{-\frac{t}{\tau}} + B \quad (4)$$

458 where $I(t)$ is the intensity at time t , I_0 is the initial intensity before bleaching, τ is the decay time
459 constant, and B is the background. Hence, the copy number N can be obtained through

$$460 \quad N = \frac{I_0}{i} \quad (5)$$

461 where i is the unit intensity of a single fluorophore, *i.e.* the average intensity of a single
462 bleaching step determined using small clusters.

463 ***Mass photometry***

464 Mass photometry experiments were performed on a OneMP instrument (Refeyn, Oxford, UK) at
465 room temperature (42, 43). The microscope coverslips (24 × 50 mm, Fisher Scientific) and
466 pre-cut 2 × 2 silicon gasket wells (Sigma) were cleaned with MilliQ water, isopropanol, and dried
467 with clean nitrogen flow, and assembled with an adjustable cover-slip rack. The instrument was
468 calibrated using β -Amylase and Thyroglobulin from Refeyn as protein standards: 18 μ L of the
469 buffer was added in an empty well on the coverslip and the laser was focused; then 2 μ L of β -
470 Amylase or Thyroglobulin was added and mixed by pipetting; data acquisition was started
471 immediately and the MP video was recorded using the AcquireMP software, and then the
472 software DiscoverMP was used to analyze the data and generate the calibration function. To
473 measure the mass of the protein of interest, 10 μ L buffer was added to an empty well on the
474 coverslip, then 10 μ L of Munc13-1 solution at various concentrations (from 8 nM to 170 nM)
475 was added and well mixed. Data collection was then started and the MP video was recorded.
476 DiscoverMP was used to process the data (44). The distribution of molecular mass was plotted
477 as histograms and fit with Gaussian peaks to obtain the average mass of different species and
478 determine the molecular weight of Munc13-1.

479 ***Lipid Membrane Preparation for CryoET***

480 Vesicles (DOPC/DOPS/PI(4,5)P₂ in a molar ratio of 14/80/6) were prepared as described
481 previously (1). Briefly, the lipid stocks were mixed in a chloroform with addition of 20 μ L

482 methanol to dissolve PI(4,5)P₂ and the solvent was evaporated under N₂ gas followed by vacuum
483 drying for 1 h. The dried lipid film was rehydrated for 1 h at room temperature with constant
484 vortexing in buffer containing 20 mM MOPS pH 7.4, 150 mM KCl, 1 mM EDTA, and 0.5 mM
485 TCEP at a final lipid concentration of 1 mM. Next, the mixture was sonicated for 5 min using a
486 bath sonicator (Branson Ultrasonics). Vesicles were used for crystallization next day after
487 storage at 4°C to sediment large lipid aggregates.

488

489 ***Protein Crystallization for CryoET***

490 Vesicles were diluted down to lipid concentration of 100 μM and mixed with 1 μM Munc13C
491 protein in 1:1 (vol/vol) ratio. Once mixed, the samples were incubated at room temperature for 5
492 min then kept on ice until freezing.

493 ***Electron Microscopy Sample Preparation and Data Acquisition***

494 Samples (2.5 μL) were vitrified using a Vitrobot Mark IV (Thermo Fisher Scientific) held at 8°C
495 with 100% humidity. They were applied to freshly glow-discharged 200 mesh Lacey
496 Formvar/carbon grids and grids were blotted for 5 s with blot force -1 and then plunged frozen in
497 liquid ethane cooled by liquid nitrogen. Samples with wild-type protein were imaged using
498 Glacios Cryo TEM 200 kV (Thermo Fisher Scientific) equipped with a K2 Summit direct
499 electron detector (Gatan). The K2 camera was used in counting mode, and detector dark and gain
500 references were collected prior to each data acquisition session. The tilt-series movies were
501 acquired using SerialEM with a bidirectional scheme starting from +20°; -43 to +44° tilt range
502 with 3° increment, 80 e/Å² total dose. The nominal magnification was 13,500× resulting in a
503 pixel size of 3.02 Å. Nominal defocus was set to -4.5 μm. Quadruple mutant tilt-series were
504 collected using a 300 kV Titan Krios G2 transmission electron microscope (Thermo Fisher
505 Scientific) equipped with GIF Quantum LS energy filter and K3 Summit direct electron detector
506 (Gatan). The slit width of the filter was set to 20 eV. Filter tuning was done using Digital
507 Micrograph software (Gatan). The tilt-series movies were acquired using SerialEM at a nominal
508 magnification of 42,000× (corresponding to a calibrated physical pixel size of 2.1 Å) using dose
509 symmetric scheme (45) with tilt range ± 51° and 3° increment. The tilt images were acquired as
510 11,520 × 8,184 super-resolution movies of 10 frames per each tilt corresponding to a tilt series
511 total dose ~120 e/Å², respectively. Nominal defocus was set to -5 μm. Movies were aligned and

512 saved as mrc stacks using alignframes from IMOD package (46). The resulting tilt series were
513 aligned and reconstructed with 4x binning for and 8x binning by AreTomo (47) so the final pixel
514 size of reconstructed tomograms was 12.08 Å and 16.8 Å. Topaz denoising was used to denoise
515 selected tomograms using unet-3d-20a pretrained model (48).

516

517 **SUPPORTING INFORMATION**

518 Supporting Information includes: Supporting Text, Supporting Table, and Supporting Figures.

519

520 **AUTHOR CONTRIBUTIONS**

521 F.L., F.P. and J.E.R designed research; F.L., J.C. and K.G. performed research; F.L., F.P. and
522 J.E.R. analyzed data; and F.L., F.P. and J.E.R wrote the paper.

523

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526

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631

632 **FIGURE LEGENDS**

633

634 **Figure 1. DAG forms microdomains on lipid bilayer, and wildtype Munc13-1 clusters are**
635 **located at these microdomains.** (A) to (C) Representative TIRF images of fluorescent DAG in
636 bilayers and Munc13-1 bound to bilayers: (A) bilayer with 2% TopFluor® DAG, 0% non-
637 fluorescent DAG, and 2% non-fluorescent PI(4,5)P₂; (B) bilayer with 2% TopFluor® DAG, 3%
638 non-fluorescent DAG, and 2% non-fluorescent PI(4,5)P₂; and (C) bilayer with 2% TopFluor®
639 DAG, 8% non-fluorescent DAG, and 2% non-fluorescent PI(4,5)P₂. Left panels are fluorescent
640 DAG, the middle panels are Munc13-1 labeled with Alexa 660, and right panels are the merge
641 the previous two panels. (D) Representative TIRF images of fluorescent PI(4,5)P₂ in bilayers
642 which contains 2% non-fluorescent DAG and 2% TopFluor® PI(4,5)P₂. Left panel is fluorescent
643 PI(4,5)P₂, the middle panel is Munc13-1 labeled with Alexa 660, and right panels are the merge
644 the previous two panels. (E) Distributions of the copy number of Munc13-1 molecules in the
645 clusters with various DAG amounts (sample size: $n = 4$ for 2% Top-Fluor DAG; $n = 3$ for 2%
646 Top-Fluor DAG + 3% DAG; and $n = 5$ for 2% DAG,); and (F) Distribution of the area of DAG
647 microdomains (sample size: $n = 4$ for 2% Top-Fluor DAG; $n = 3$ for 2% Top-Fluor DAG + 3%
648 DAG; and $n = 3$ for 2% Top-Fluor DAG + 8% DAG). (G) The molecular weight of Munc13C-
649 Halo-Alexa 660 in solution was measured with Mass Photometer. Final concentration of
650 Munc13C-Halo-Alexa 660 in this measurement was around 10 nM. The experimental molecular
651 mass nearly equals to its theoretical molecular weight.

652

653 **Figure 2. Poisson distribution modeling of oligomers in wildtype Munc13-1 clusters.** (A)
654 Single Poisson distribution, assuming all wildtype Munc13-1 in monomer state; (B) through (D)
655 Dual Poisson distribution (monomer + uniform size oligomer), 2-step process: monomer with
656 pentamer (B), monomer with hexamer (C), and monomer with heptamer (D). The orange bars
657 represent predicted distribution of the copy number of Munc13-1 molecules in the clusters, and
658 the green dashed lines are experimental data (sample size $n = 5$). (E) The O value of the Poisson
659 modelling as function of the assumed oligomeric state of wildtype Munc13-1. Larger O value
660 suggests the predicted oligomeric state fits better with observed data.

661

662 **Figure 3. Mutations in interaction interface between C₂C and neighboring MUN domain in**
663 **the Munc13-1 hexagon affect the protein organization, cluster size distribution, and vesicle**
664 **capture.** (A) Top and side views of the hexagonal cage of Munc13-1 oligomer that binds a
665 vesicle (based on PDB 7T7C). C₂C domains are colored in purple, MUN domains in grey and
666 C₂B in blue. The red rectangular area shows the position of interaction interface between C₂C
667 and MUN domain in Munc13-1 hexagon. (B) Close-up view of interface in ribbon
668 representation. Possibly involved residues in interaction are labeled. The interface contains two

669 separate regions: hydrophobic (green colored amino acid residues) and polar (red and blue
670 colored amino acid residues). (C) and (D) Slices through the reconstructed tomograms. (C) Wild
671 type Munc13C hexagonal crystal between negatively charged lipid bilayers. Top panel is the
672 Topaz-denoised (48) top view of the crystal. Bottom is the side view of the crystal with visible
673 protein density lane in the middle between lipid bilayers corresponding to the top of hexagons
674 (A) formed by lateral conformation of Munc13-1. (D) Top panel is the Topaz-denoised top view
675 of the crystal formed by F1176N/I1215N/E1139R/D1039R Munc13-1 mutant. This crystal is
676 characterized as a rectangular lattice with denser protein packing in form of rows of
677 round-shaped densities and protein-filled space between them. Lower panel is the slice through
678 the side of the crystal. The middle protein density lane is no longer visible. Scale bars 50 nm.
679 (E) Overlay of TIRF image of Munc13-1 interface mutant labeled with Alexa 488 on lipid
680 bilayer membrane containing PC, PS, DAG, and PI(4,5)P₂, with TIRF image of captured vesicles
681 by the same Munc13-1 interface mutant (vesicles labeled with Atto647N, pink). (F) Distribution
682 of the copy number of Munc13-1 interface mutant molecules in the clusters (purple columns)
683 (sample size $n = 4$). The green dots represent the distribution of the copy number in the clusters
684 formed by wildtype Munc13-1, which serves as a reference. (G) The O value of the Poisson
685 modelling as function of the assumed oligomeric state of the Munc13-1 interface mutant (purple
686 solid line). The green dashed line represent the O value of wildtype Munc13-1 clusters, which
687 serves as a reference (H) Probability of vesicle capture by the clusters of Munc13-1 interface
688 mutant (purple columns) as a function of their cluster size which is the copy number of the
689 protein molecules in the cluster (sample size $n = 4$). The green dashed line represent the
690 probability of wildtype Munc13-1 clusters, which serves as a reference.

691

692 **Figure 4. DAG plays a critical role in wildtype Munc13-1's function of vesicle capture.**

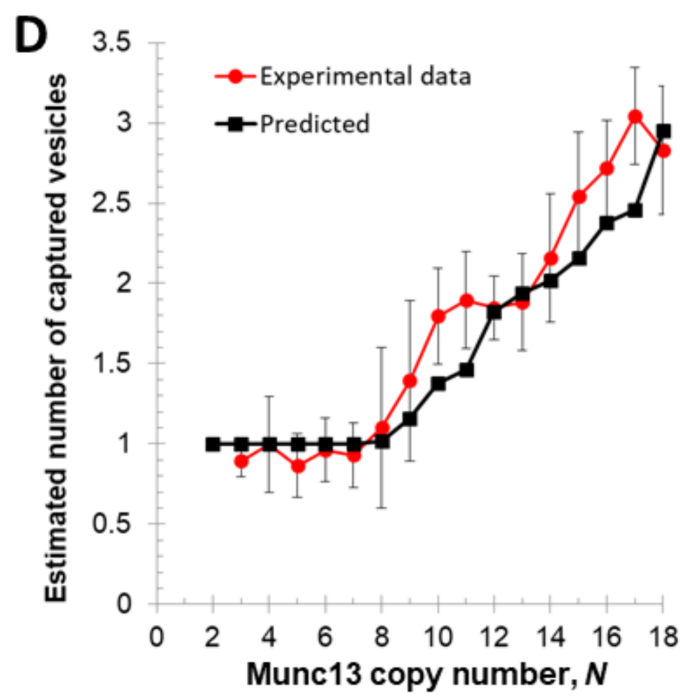
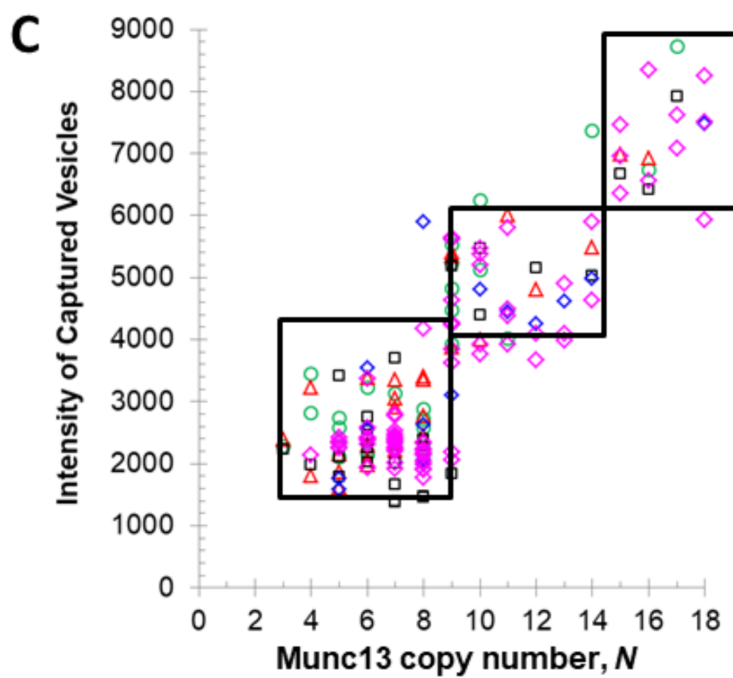
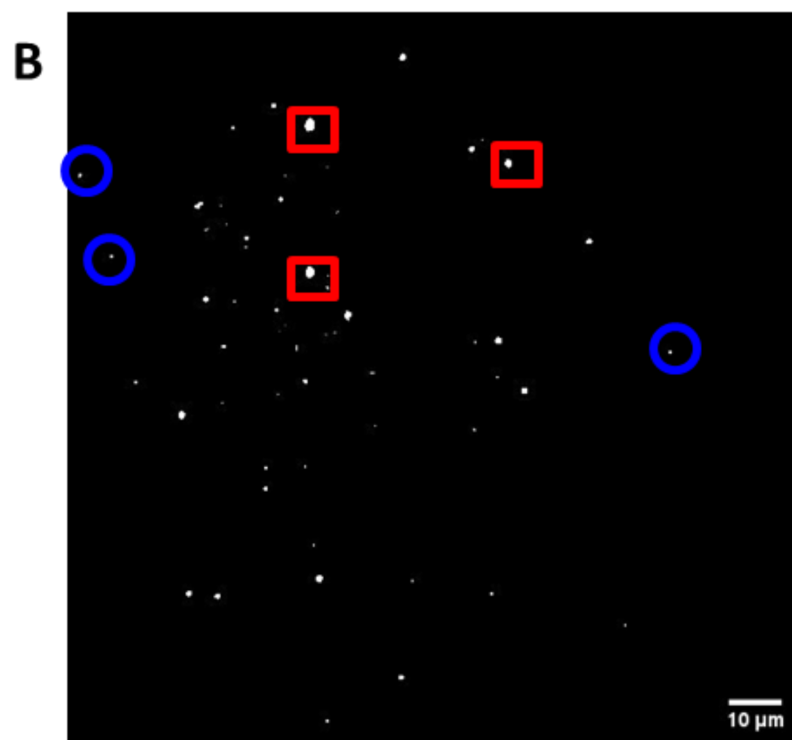
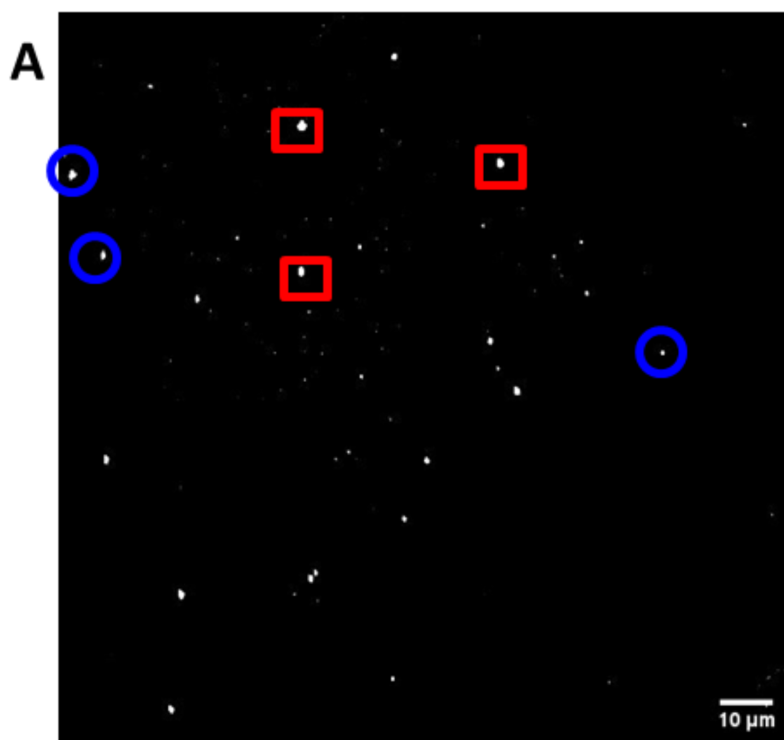
693 (A) Overlay of TIRF image of wildtype Munc13-1 clusters formed on a bilayer with 2% DAG
694 (wildtype Munc13-1 labeled with Alexa488, green) with TIRF image of captured vesicles
695 (vesicles labeled with Atto647N, pink). (B) Overlay of TIRF image of wildtype Munc13-1
696 clusters that were formed on bilayer in the absence of any DAG (Munc13-1, green) with vesicles
697 bound to the same bilayer (vesicles, pink). (C) Distribution of the copy number of wildtype
698 Munc13-1 molecules in the clusters on lipid bilayer in the presence of DAG that are capable
699 (orange) and incapable (blue) of capturing vesicle, respectively (sample size $n = 5$). (D)
700 Distribution of the copy number of wild-type Munc13-1 molecules in the clusters on lipid bilayer
701 in the absence of DAG that are capable (orange) and incapable (blue) of capturing vesicle,
702 respectively (sample size $n = 4$).

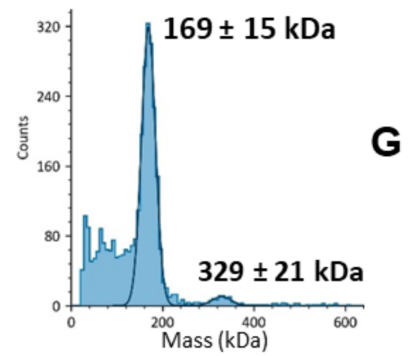
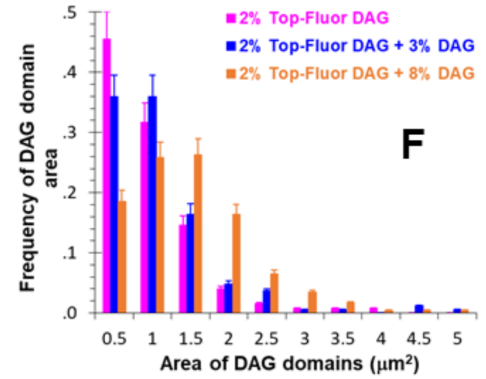
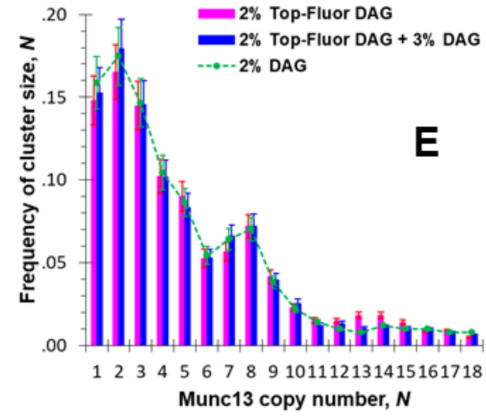
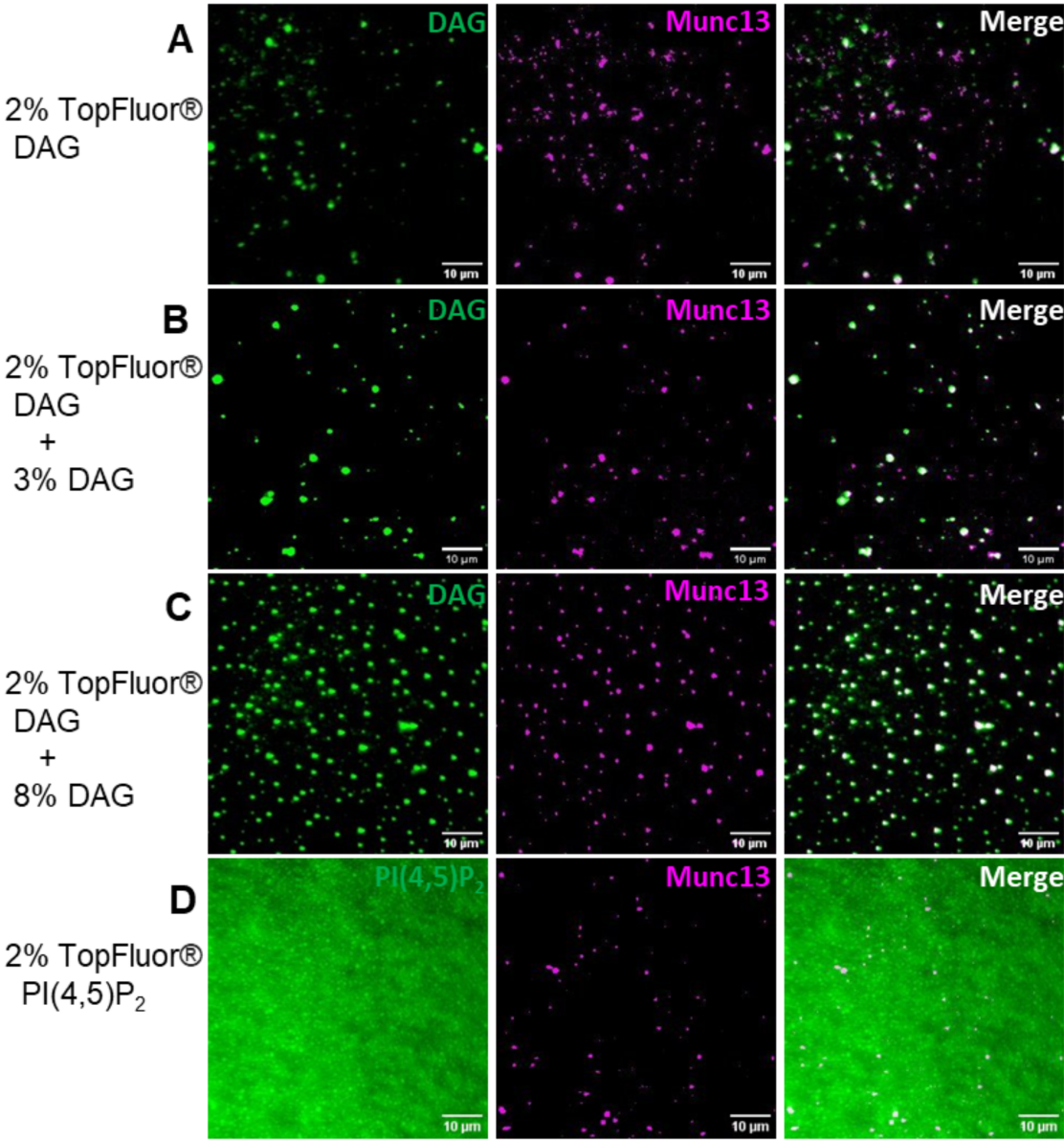
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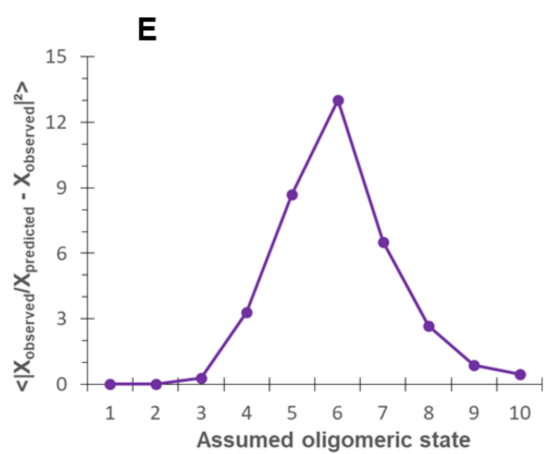
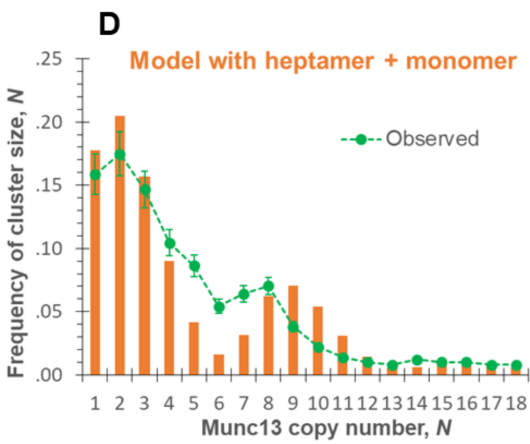
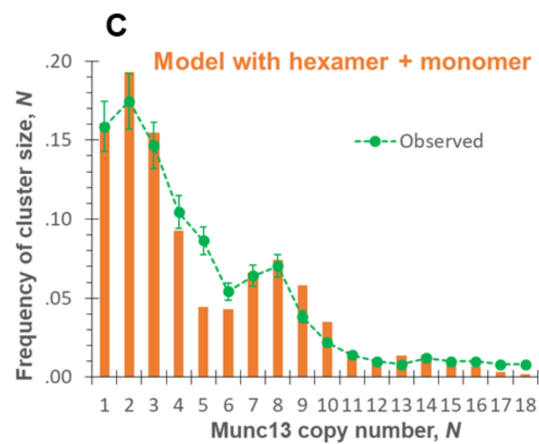
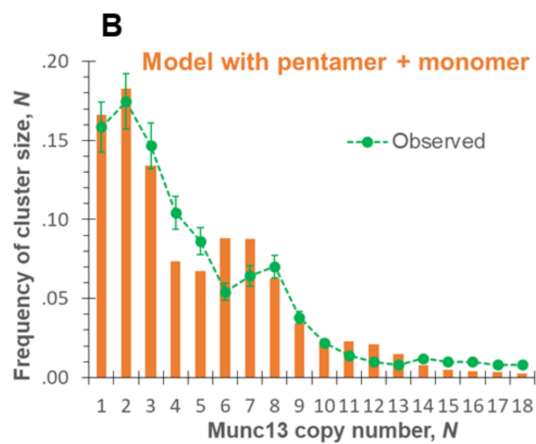
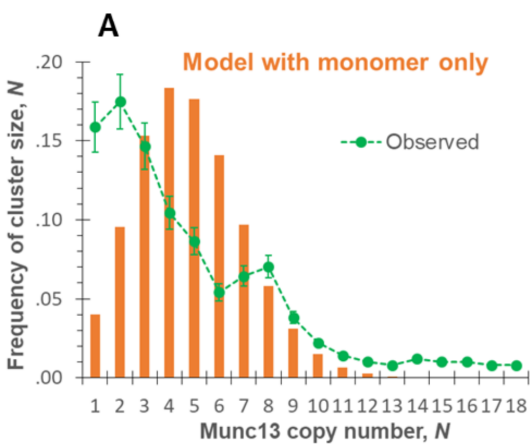
704 **Figure 5. Quantal relationship of the number of captured vesicles by a cluster and the**
705 **number of copies of Munc13-1 molecules in the same cluster. (A) & (B) Qualitative**

706 correlation between optical cluster sizes and the optical sizes of captured vesicles. When the
707 intensities of Munc13-1 clusters (**A**) are bigger, the corresponding intensities of captured vesicles
708 (**B**) are larger. (**C**) Intensity distribution of captured vesicles as a function of the copy numbers of
709 Munc13-1 molecules in the corresponding clusters shows three distinct regimes. Different
710 symbols and colors represent the data points were from different independent experiments,
711 sample size $n = 5$. (**D**) Comparison of predicted number of captured vesicle as a function of the
712 copy number of Munc13-1 molecules in the cluster (black squares), and experimental average
713 number of captured vesicles that was determined from vesicle intensities, versus the copy
714 number of Munc13-1 molecules in the cluster (red spheres).

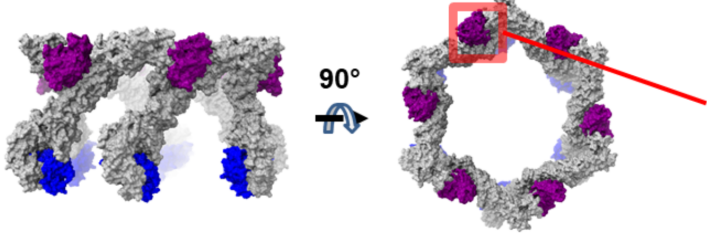
715







A Binding surface of hexagonal cage



B

