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1 **Chromogranin A preferential interaction with Golgi phosphatidic acid induces**
2 **membrane deformation and contributes to secretory granule biogenesis**

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29
30 RUNNING TITLE: CgA/PA interaction and granulogenesis

31

32 NONSTANDARD ABBREVIATIONS

33

34 AFM: atomic force microscopy

35 CgA: chromogranin A

36 CgB: chromogranin B

37 DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine

38 FIPI: 5-Fluoro-2-indolyl des-chloroalopemide

39 gCW STED: time-gated continuous wave stimulated emission depletion

40 GUV: giant unilamellar vesicle

41 ITC: isothermal titration calorimetry

42 PA: phosphatidic acid

43 PABD: PA binding domain

44 PC: phosphatidylcholine

45 PE: phosphatidylethanolamine

46 PS: phosphatidylserine

47 PDE4A1: cAMP phosphodiesterase-4A1

48 PLD: phospholipase D

49 SgII: secretogranin II

50 Spo20p: Sporulation-specific protein 20

51 SUV: small unilamellar vesicle

52 TGN: *trans*-Golgi network

53 **ABSTRACT**

54 Chromogranin A (CgA) is a key luminal actor of secretory granule biogenesis at the *trans*-
55 Golgi network level but the molecular mechanisms involved remain obscure. Here, we
56 investigated the possibility that CgA acts synergistically with specific membrane lipids to
57 trigger secretory granule formation. We show that CgA preferentially interacts with the
58 anionic glycerophospholipid phosphatidic acid (PA). In accordance, bioinformatic analysis
59 predicted a PA-binding domain (PABD) in CgA sequence that effectively bound PA (36:1) or
60 PA (40:6) in membrane models. We identified PA (36:1) and PA (40:6) as predominant
61 species in Golgi and granule membranes of secretory cells and we found that CgA interaction
62 with these PA species promotes artificial membrane deformation and remodeling.
63 Furthermore, we demonstrated that disruption of either CgA PABD or phospholipase D
64 (PLD) activity significantly alters secretory granule formation in secretory cells. Our findings
65 show for the first time the ability of CgA to interact with PLD-generated PA, which allows
66 membrane remodeling and curvature, key processes necessary to initiate secretory granule
67 budding.

68

69 **KEY WORDS:** membrane dynamics / organelle biogenesis / phospholipase D / Golgi
70 apparatus

71

72

73 INTRODUCTION

74 Secretory granules are vesicular organelles which materialize the regulated secretory pathway,
75 allowing release of signal molecules such as hormones and neurotransmitters from endocrine,
76 neuroendocrine, and most neuronal cells. Secretory granules are formed by budding from the
77 trans-Golgi network (TGN) membrane in a very efficient manner and more than several
78 thousand can accumulate in neuroendocrine cells for active release upon cell stimulation,
79 most likely involving a specific budding process from the Golgi apparatus (1). It is now well
80 established that membrane remodeling at the TGN is an important process for secretory
81 granule budding, but the identity of the molecular actors, the sequence of events, and the
82 mechanisms involved are not elucidated to date.

83 The TGN membrane is composed of a broad spectrum of lipids with specific properties
84 that profoundly define its identity and function (2), but their contribution to secretory granule
85 biogenesis has only recently emerged. Membrane proteins are known to influence lipid
86 organization and conversely protein function and clustering are also under the control of
87 lipids, leading to the formation of TGN membrane microdomains that have been proposed to
88 regulate the budding of secretory granules (3). For instance, *in vitro* and *in vivo* studies
89 revealed that depletion of cholesterol alters secretory granule biogenesis (4, 5), and the
90 regulated production and organization of sphingomyelin, which assembles with cholesterol to
91 generate microdomains in the membranes, has been shown to be crucial for the biogenesis of
92 cargo carriers at the Golgi membranes (6). Moreover, the TGN membrane cytosolic leaflet is
93 highly enriched in phosphatidylinositol 4-phosphate (PI4P), which may directly induce
94 membrane curvature and/or recruit membrane-deforming cytosolic PI4P-binding proteins (7).
95 Thus, besides the TGN membrane components, cytosolic but also luminal molecular
96 components potentially influence membrane organization and shape. Among the luminal
97 components, several glycoproteins of the granin family were proposed to play a crucial role in
98 secretory granule biogenesis (8). In particular, chromogranin A (CgA) was shown to
99 contribute to hormone aggregation and secretory granule biogenesis (9–11). Indeed, deletion
100 of the CgA gene (*Chga*) led to decreased secretory granule number associated to metabolic
101 complications such as cardiovascular defects and obesity in knockout mice (12, 13), implying
102 a major contribution of CgA in catecholamine and insulin storage and release. In previous
103 studies, we have shown that ectopic CgA expression induced the formation of secretory
104 granule-like structures in COS7 cells, which are normally devoid of secretory granules and
105 granin expression (14, 15), confirming the important role of CgA in granulogenesis. These

106 observations suggested that CgA could act at the level of the TGN membrane to trigger the
107 formation of secretory granules; however, the mechanism involved is still unknown.

108 The first indication for a role of CgA at the TGN level is related to its acidic nature
109 which confers to this granin the ability to aggregate with neuropeptides and hormones in the
110 luminal TGN condition (9). Besides these soluble aggregates, a fraction of CgA was found to
111 be tightly associated with secretory granule membrane (16), suggesting that CgA/hormone
112 aggregates might interact with the TGN membrane. Indeed, the conserved α -helical
113 organization of CgA terminal regions which confers to the protein the ability to trigger
114 secretory granule formation and to sort hormones to the regulated secretory pathway (11, 14),
115 the faculty of CgA to regulate fusion pore expansion (17) and the widespread expression of
116 this granin argue for a role of CgA in granulogenesis through direct interaction with
117 membranes. In fact, CgA-derived peptides with the conserved α -helical conformation have
118 been shown to interact with membrane lipids. For instance, the conserved N-terminal peptide
119 of CgA named vasostatin I (hCgA1-76) interacts with phosphatidylserine in phospholipid
120 monolayers (18) and cell surface (19), whereas the peptide catestatin (hCgA352-372) binds to
121 phosphatidylcholine micelles (20). As it is now well established that lipid composition and
122 properties define organelle identity and function, we explored the possibility that an
123 interaction between CgA and specific lipids of the TGN membrane could promote secretory
124 granule biogenesis. In this study, we observed that CgA interacts with phosphatidic acid (PA)
125 and phosphoinositides in membrane models and that ectopic expression of a cytosolic probe
126 which buffers Golgi PA in secretory cells impaired the formation of CgA-containing granules.
127 Moreover, we found that CgA preferentially interacts with few PA species enriched in
128 purified Golgi and secretory granule membranes *in vitro*, through a newly identified PA-
129 binding domain (PABD). Using giant unilamellar vesicles (GUVs) enriched with Golgi and
130 secretory granule PA species, we observed that CgA induces GUV membrane remodeling and
131 deformation in a dose-dependent manner. Interestingly, deletion of the identified PABD in
132 CgA significantly reduced secretory granule biogenesis. Finally, genetic and pharmacological
133 evidence revealed that inhibition of phospholipase D (PLD), a major source of PA production
134 in the Golgi, alters secretory granule formation in neuroendocrine cells.

135

136

137 MATERIALS AND METHODS

138 Cell culture and transfection

139 African green monkey kidney fibroblast-derived COS7 cells (American Type Culture
140 Collection; CRL 1651), wild-type or stably expressing CgA developed previously (15) and
141 mouse corticotrope-derived AtT20/DC16v-F2 cells (American Type Culture Collection; CRL
142 1795) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Thermo
143 Fisher Scientific) supplemented with 5% heat-inactivated and sterile-filtered fetal bovine
144 serum (FBS, Sigma - Aldrich), 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin (Gibco,
145 Thermo Fisher Scientific) and 300 µg ml⁻¹ geneticin (G-418 sulfate, Life Technologies, Inc,
146 UK) to maintain gene resistance selection, at 37°C in 5% CO₂. Rat pheochromocytoma PC12
147 cells (American Type Culture Collection; CRL 1721) were routinely grown in Dulbecco's
148 Modified Eagle's Medium (DMEM, Gibco, Thermo Fisher Scientific) supplemented with 5%
149 FBS, 10% sterile-filtered HyClone Donor Equine serum (GE Healthcare, Life Sciences), 100
150 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin (Gibco, Thermo Fisher Scientific) and 1% L-
151 glutamine 100X (Gibco, Thermo Fisher Scientific), at 37°C in 5% CO₂. For
152 immunofluorescence (IF) experiments, COS7-WT, PC12 and AtT20 cells were transfected
153 with 0.8 µg of DNA encoding GFP-tagged human CgA (CgA-GFP), GFP-tagged human
154 ΔPABD CgA (CgAΔPABD-GFP), GFP-tagged rat PDE4A1(PABD) (PDE4A1 WT) or GFP-
155 tagged rat PDE4A1(mutated PABD) (PDE4A1 Mut), and 2 µl Lipofectamine 2000
156 (Invitrogen) per well (24-well plate) according to the manufacturer's protocol. Four or five
157 hours after the beginning of transfection, the culture medium was replaced by supplemented
158 DMEM, and cells were additionally cultured for 24–48 h. For Western blot experiments,
159 COS7-WT and AtT20 cells were transfected with 4 µg of DNA encoding CgA-GFP or
160 CgAΔPABD-GFP, and 8 µl Lipofectamine 2000 (Invitrogen) per well (6-well plate)
161 according to the manufacturer's protocol. Four or five hours after the beginning of
162 transfection, the culture medium was replaced by supplemented DMEM, and cells were
163 additionally cultured for 48 h.

164

165 Plasmid constructs

166 Full-length CgA-GFP (pCgA-EGFP-N2) kindly provided by M. Courel (UPMC, Paris,
167 France) was used to amplify the sequence of CgA-encoding region by PCR using the sense
168 primer (5'-CTCGAGGCCACCATGCGCTCCGCCGCTGTCTCGGCTTCTT-3'), including
169 an XhoI restriction site (underlined bases), and the antisense primer (5'-

170 CCGCGGGCGCCCCGCCGTAGTGCCTGCA-3'), including a SacII restriction site before
171 cloning into a pGEM-T vector (Promega, Charbonnières, France). Site-directed mutagenesis
172 was conducted by using the QuickChange® II XL Site-Directed Mutagenesis Kit (Agilent
173 technologies, Les Ulis, France) and specific primers (sense: 5'-
174 CTCTCCTTCCGGGCCCCGGGCCTACGAGGACAGCCTTGAGGGCGGGCCTG-3';
175 antisense: (5'-CAGGCCCGCCTCAAGGCTGTCCTCGTAGGCCCGGGCCCCGGAAGGAG
176 AG-3') designed to remove the CgA PABD sequence (residues 364–381). The amplified
177 mutated CgA (with deleted PABD region) was digested with the appropriate enzymes (XhoI
178 and SacII) and subcloned into the eukaryotic expression vector pEGFP-N2. All the constructs
179 were verified by restriction enzyme digestion and DNA sequencing. PDE4A1 WT and
180 PDE4A1 Mut constructs were described previously (21).

181

182 **Animals**

183 *Pldl* knockout mice were described previously (22, 23). They were housed and raised at
184 Chronobiotron UMS 3415. All experiments were carried out in accordance with the European
185 Communities Council Directive of 24th November 1986 (86/609/EEC) and resulting French
186 regulations. Accordingly, the CREMEAS local ethical committee approved all experimental
187 protocols. Every effort was made to minimize the number of animals used and their suffering.

188

189 **Proteins and antibodies**

190 Recombinant CgA (human chromogranin A 19-457aa, His tag, *E. coli*, ATGP0323, Atgen
191 global) was used for lipid strips and GUVs experiments. For CgA-Alexa488 coupling, all
192 reactants and solvents were purchased from TCI-Chemicals and Alfa Aesar. CgA labeling
193 was performed according to a modified version of a protocol reported in Chevalier et al. (24).
194 First, 82 µl (1 eq.) of Alexa488 solution (2 mg ml⁻¹ in anhydrous N, N dimethylformamide)
195 were introduced into a 1 ml round-bottom flask. (O-(N-succinimidyl)-N,N,N',N'-
196 tetramethyluronium tetrafluoroborate (70 µg, 1.2 eq.) and N,N-diisopropylethyl-amine (0.04
197 ml, 1.2 eq.) were added and the reaction medium was placed under argon, with magnetic
198 stirring at RT during 1 h, to obtain the N-hydroxysuccinimidyl activated ester. A solution of
199 250 µg of CgA in PBS buffer (pH 7.2, 0.5 mg ml⁻¹, 1 eq.) was then slowly added dropwise in
200 the reaction medium, for a ratio Alexa488/CgA 10/1. Reaction was carried out under argon,
201 magnetic stirring and at RT overnight. Purification was performed using 10 kDa
202 centrifugation filters (Spin-X® UF 500, CORNING) previously conditioned with PBS to

203 remove glycerol and other biocidal compounds. Labeled protein was collected in PBS buffer
204 following 3 centrifugations of 10 min at 7,500 rpm. Grafting rate was evaluated by Western
205 blot and UV-Vis spectroscopy ($\lambda_{\text{abs}} = 494 \text{ nm}$), with a maximum labeling rate of 5.6
206 Alexa488 molecules per protein.

207 Primary antibodies used were goat polyclonal anti-CgA (sc-23556; Santa-Cruz
208 Biotechnology inc.) (1:200), rabbit polyclonal anti-CgA (WE-14 (25)) (1:1,000), rabbit
209 polyclonal anti-CgA (RV31.4 (14) (1:1,000), rabbit polyclonal anti-SgII (EM66 (26))
210 (1:1,000), goat polyclonal anti-CgB (sc-1489; Santa-Cruz Biotechnology inc.) (1:1,000),
211 mouse monoclonal anti-GM130 (BDBiosciences) (1:1,000), mouse monoclonal anti-CALR
212 (clone 1G11-1A9, Sigma-Aldrich) (1:1,000), mouse monoclonal anti-GLUD1 (clone 3C2,
213 Sigma-Aldrich) (1:500), mouse monoclonal anti-golgin 97 (A21270; Invitrogen) (1:500),
214 mouse monoclonal anti- α -tubulin (T5168, Sigma-Aldrich) (1:5,000). For IF, secondary
215 antibodies used were Alexa 488-conjugated donkey anti-rabbit IgG; Alexa 594-conjugated
216 donkey anti-mouse IgG (Invitrogen) (1:500). For Western blotting, anti-rabbit, anti-mouse
217 and anti-goat secondary antibodies conjugated to horseradish peroxidase (Thermo Fisher
218 Scientific) (1:5,000) were used.

219

220 **Protein-lipid binding assay**

221 Membrane lipid strips (P-6002, Echelon Biosciences) were saturated in PBS-T buffer (0.1%
222 v/v Tween 20 in PBS) supplemented with 3% fatty acid-free BSA (Sigma-Aldrich) for 1 h at
223 RT, and then incubated with 500 ng ml^{-1} recombinant human purified CgA diluted in this
224 buffer for an additional hour at RT. After several washes with PBS-T, the membranes were
225 incubated with anti-WE-14 antibody (1:1,000) 1 h at RT in the saturation buffer. After
226 washing with PBS-T, membranes were incubated with a secondary antibody coupled to
227 horseradish peroxidase (Thermo Fisher Scientific) and visualized by enhanced
228 chemiluminescence. Densitometric analysis was performed to determine the relative affinity
229 of CgA binding to various phospholipids.

230

231 **Subcellular fractionation**

232 Cells were collected in PBS and sedimented by centrifugation at $400 g$ for 5 min at 4°C . The
233 cell pellet was disrupted by 5 pulls/pushes through a 21- and then a 25-gauge needle attached

234 to a syringe, in ice-cold buffer (0.32 M sucrose, 20 mM Tris-HCl, pH 8; 1 ml g⁻¹ of cells). The
235 resulting lysate was centrifuged at 800 g for 30 min at 4°C.

236

237 *Golgi membrane purification*

238 The protocol used was adapted from that developed by Graham et al (27) for the isolation of
239 Golgi membranes from cultured cells by flotation through a discontinuous sucrose gradient.
240 Briefly, Golgi membranes are isolated from low-speed pellets traditionally referred to as the
241 nuclear pellet; hence the upper layer of the pellets (containing plasma, ER and Golgi
242 membranes) were suspended in 1.4 M sucrose. The homogenate was deposited on a
243 succession of 2 ml 1.4 and 2.0 M sucrose cushions and then covered with 2 ml 1.2 and 0.8 M
244 sucrose cushions and 10 ml 0.32 M sucrose cushion, all sucrose cushions containing 10 mM
245 Tris-HCl at pH 7.4. The extracts were then centrifuged at 10,000 g for 18 h at 4°C. The
246 different fractions were collected in hemolysis tubes and kept at -20°C. An aliquot of each
247 fraction was used for the localization of Golgi apparatus by Western blot using specific
248 antibodies to recognize plasma membrane, Golgi, nucleus, mitochondria and the endoplasmic
249 reticulum.

250

251 *Secretory granule purification*

252 Post-nuclear supernatants were centrifuged at 20,000 g for 20 min at 4°C. Pellets containing
253 dense core granules were centrifuged on a multi-step gradient of 1 to 2.2 M sucrose (1, 1.2,
254 1.4, 1.6, 1.8, 2 and 2.2 M sucrose; 5 ml steps), at 100,000 g for 12 h at 4°C. All gradient steps
255 were collected from the top of the tube in 5 ml fractions, and analyzed by western blotting to
256 identify the granule-containing fractions and to verify their degree of purification.

257

258 **Lipidomic analysis**

259 Total lipids from recovered Golgi and granule fractions were extracted by the method of
260 Bligh and Dyer (28). Lipid extracts were resuspended in 50 µl of eluent A. Liquid
261 chromatography coupled to tandem mass spectrometry (LC-MS/MS) analyses were
262 performed with a MS model QTRAP® 6500 (Sciex) coupled to an LC system (1290 Infinity
263 II, Agilent). Analyses were achieved in the negative (PA); nitrogen was used for the curtain
264 gas (set to 15), gas 1 (set to 20) and gas 2 (set to 0). Needle voltage was at -4,500 without
265 needle heating; the declustering potential was adjusted set at -180 V. The collision gas was
266 also nitrogen; collision energy is set to -50eV. Reversed phase separations were carried out
267 at 50°C on a Luna C8 150×1 mm column, with 100 Å pore size, 5 µm particles (Phenomenex,
268 Torrance, USA). Eluent A was isopropanol/CH₃OH/H₂O+0.2 % formic acid+0.028 % NH₃

269 and eluent B was isopropanol+0.2 % formic acid+0.028 % NH₃. The gradient elution program
270 was as follows: 0-5 min, 30-50 % B; 5 - 30 min, 50-80 % B; 31–41 min, 95 % B; 42–52 min,
271 30 % B. The flow rate was set at 40 µl min⁻¹; 3 µl sample volumes were injected. Quantitative
272 PA analyses were made based on MS/MS multiple reaction monitoring (MRM) as described
273 (29). Briefly, MRM transitions for individual PAs were determined using PA standards
274 (Avanti Polar Lipids, Alabaster, USA). The predominant daughter fragment ions were then
275 used for quantitative MRM analysis. MRM transitions and specific retention times were used
276 to selectively monitor PA using MultiQuant software (v3.0, Sciex).

277

278 **Liposome and giant unilamellar vesicle formation and use**

279 *Liposome flotation and binding assays*

280 Lipids solubilized in chloroform were purchased from Avanti Polar Lipids (Alabaster, USA)
281 and were used without further purification. Liposome mixtures were prepared in mass ratios
282 composed of 90% DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), 5% PE-NBD (1,2-
283 dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)), and 5%
284 PS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine), PI(4,5)P₂ (1,2-dioleoyl-*sn*-glycero-3-
285 phospho-(1'-myo-inositol-4',5'-bisphosphate)), egg PA mix or PA species (36:1 (1-stearoyl-2-
286 oleoyl-*sn*-glycero-3-phosphate), 36:2 (1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphate), 40:6
287 (1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphate) or 36:0 (1,2-distearoyl-*sn*-glycero-3-
288 phosphate). Lipids were dried in a stream of nitrogen and kept under vacuum for at least 2 h.
289 Dried lipids were then suspended in liposome-binding buffer (LBB: 20 mM HEPES, pH 7.4,
290 150 mM NaCl, 1 mM MgCl₂) by three freeze and thaw cycles and were extruded using a
291 Mini-Extruder (Avanti Polar Lipids, Alabaster, USA) through polycarbonate track-etched
292 membrane filters to produce liposomes of 200 nm in diameter. The liposomes were then
293 diluted in LBB (1:10) and incubated with 30 µg of COS7-CgA extract during 20 min. Then,
294 samples were centrifuged at 200,000 g (1 h at 4°C) on a multi-step gradient sucrose. To
295 determine the association of CgA with liposomes, the fractions were collected after
296 centrifugation, and the presence of CgA was revealed using western blot analysis of the
297 different fractions.

298 To quantify liposome binding to GST-CgA-PABD linked to GSH-Sepharose beads, GST and
299 GST-PABD constructs (330 pmol) bound to GSH beads were washed once with 1 ml of LBB
300 medium before incubation for 20 min in the dark at room temperature and under agitation
301 with liposomes containing a 10-fold molar excess of PA relative to the quantity of GST

302 proteins in a final volume of 200 μ l of LBB. Beads were washed three times with 1 ml of ice-
303 cold LBB and collected by centrifugation at 3,000 rpm for 5 min. Liposome binding to the
304 PABD was estimated by measuring the fluorescence at 535 nm with a Mithras fluorimeter
305 (Berthold). Triplicate measurements were performed for each condition. Fluorescence
306 measured with GST linked to GSH-Sepharose beads alone was between 3 and 4 A.U. and was
307 subtracted from sample measurements.

308

309 *Giant unilamellar vesicle preparation*

310 Giant unilamellar vesicles (GUVs) were prepared by polyvinyl acetate (PVA, MW 145000,
311 purchased from VWR International, Fontenay-sous-Bois, France) – assisted swelling. Briefly,
312 a Teflon plate was used and was cleaned twice with 99% ethanol. A 5% (w/w) solution of
313 PVA was prepared by stirring PVA in water while heating at 90°C. PVA-coated substrates
314 were prepared by spreading 100–300 μ l of PVA solution on a clean Teflon plate, and dried
315 for 30 min at 80°C. 10–20 μ l of a lipid mixture (95% of DOPC, 4% of PA (36:1) or (40:6)
316 and 1% of PE-NBD) dissolved in chloroform (1 mg ml⁻¹) was spread on the dried PVA film
317 and placed under vacuum overnight at RT to evaporate the solvent. A chamber around the
318 drops of lipids was formed with Vitrex and filled with 100 mM sucrose and 150 mM NaCl
319 solution during 3 h at room temperature, allowing the formation of GUVs with a suitable size.
320 Next, the buffer containing GUVs is recovered using a rib with a wide tip to avoid destroying
321 the GUVs, and deposited in LabTek wells (LabTek I non-separable, on glass coverslip, 4
322 culture chambers, Nunc), which were previously coated with a 1 mg ml⁻¹ BSA (Bovine Serum
323 Albumin, Sigma – Aldrich) solution. To allow the sedimentation of GUVs, we added the
324 same volume (100 μ l) of a solution containing 90 mM glucose and 15 mM NaCl in LabTek
325 wells. The sedimentation of the GUVs takes place for about 1 h at RT.

326 To test the effect of CgA on the GUV membranes, we added CgA or CgA-Alexa488 in GUV-
327 containing Labtek wells at a final concentration of 2, 4 or 6 μ M just before the beginning of
328 confocal microscopy acquisitions.

329

330 *Small unilamellar vesicle preparation*

331 Small unilamellar vesicles (SUVs) were prepared using the standard extrusion method as
332 described previously for liposome formation. Liposome mixtures prepared in mass ratios
333 composed of 90% DOPC, 10% PA (36:1) were dissolved in a test tube with chloroform.
334 Lipids were dried under argon gaz. The lipid film thus obtained was hydrated with 500 μ l of

335 water and the solution was subjected to vortex mixing for 1 h at room temperature. The
336 multilamellar vesicles were extruded 27 times using the Mini-Extruder equipped with a
337 polycarbonate membrane filter of 100 nm pore diameter to obtain SUVs.

338

339 **Atomic force microscopy measurements**

340 *Membrane preparation*

341 Giant Unilamellar Vesicles (GUVs) were prepared by using 100% DOPC or a mix of 90%
342 DOPC and 10% PA (36:1). The vesicles were prepared in a 200 mM sucrose solution. GUV
343 solution was diluted 100 times in 250 mM glucose solution to promote their sedimentation
344 and to avoid multilayers. Final lipid concentration was 10 μM . The vesicle solution was
345 injected in the AFM liquid cell and put in contact with freshly cleaved mica. After 2 min, the
346 vesicle solution is replaced with 250 mM glucose solution in order to maintain the supported
347 lipid bilayers hydrated at all times during imaging. To study protein/lipid interactions, the
348 glucose solution was replaced with a CgA solution (0.6 or 1.2 μM), and the sample was
349 imaged again.

350

351 *Microscopy imaging*

352 AFM measurements were performed on a multimode atomic force microscope (Nanoscope
353 IIIA, Veeco, USA). Supported lipid bilayers images were taken in tapping mode in the fluid
354 cell. A soft cantilever with a typical spring constant of 0.06 N/m and equipped with a silicon
355 nitride tip was used. The tip velocity was set between 5-10 $\mu\text{m/s}$ by varying the scan rate
356 according to the scan size. The cantilever oscillation was tuned to a frequency between 20 and
357 30 kHz, and the amplitude was set between 0.8 V and 2 V. All the experiments were carried
358 out at a temperature of 21°C.

359

360 **High-sensitivity isothermal titration calorimetry**

361 Isothermal titration calorimetry (ITC) was performed on an Affinity instrument (TA
362 Instrument, New Castle, DE) equipped with a 185 μl reaction chamber. Solutions were
363 degassed under low pressure before use to avoid the formation of air bubbles while titrating.
364 The CgA solution (5 μM) was placed in the calorimeter chamber and SUV suspension (10
365 mM) was injected in aliquots of 2.5 μl . The period between two successive injections was
366 typically 200 sec to allow the system to reach equilibrium. The solution was stirred

367 continuously at 125 rpm and the experiments were carried out at a temperature of 10°C. The
368 resulting heat changes after SUV injection were recorded as a function of time. The heat of
369 reaction was obtained by integrating the area under each peak of heat flow tracings, using the
370 heat of SUV dilution in water as blank sample. The data were acquired by using the
371 Nanoanalyze software (v.3.10.0, TA Instrument).

372

373 **Immunofluorescence labelling**

374 COS7-CgA cells were cultured in 24-well plates (Costar® 24 Well Clear TC-Treated
375 Multiple Well Plates, Corning), onto glass coverslips and fixed with 4% paraformaldehyde
376 (Sigma – Aldrich) in PBS at RT for 15 min. Cells were permeabilized and blocked for 30 min
377 with 0.3% Triton X-100 (Thermo Fisher Scientific), in PBS containing 5% normal donkey
378 serum (Sigma – Aldrich) (1:50) and 1% BSA. Cells were then incubated for 2 h at RT with
379 primary antibodies, and, after washing with PBS, for 1 h with secondary antibodies. Nuclei
380 were stained with DAPI (4',6-diamidino-2-phenylindole, Molecular probes) ($1 \mu\text{g ml}^{-1}$). To
381 verify the specificity of the immunoreactions, the primary or secondary antibodies were
382 substituted with PBS.

383

384 **Protein electrophoresis and Western blotting**

385 Cells were harvested by scraping in NP40 cell lysate buffer (Thermo Fisher Scientific)
386 supplemented with a cocktail of protease inhibitors (Thermo Fisher Scientific), homogenized,
387 and proteins were separated by SDS-PAGE followed by Western blotting. Membranes were
388 incubated in a blocking buffer containing 5% non-fat dry milk in PBS containing 0.5% Tween
389 20 (Sigma - Aldrich) (PBS-T) for 1 h at RT, and overnight with primary antibodies at 4°C.
390 Then, membranes were washed for 45 min with PBS-T. Blots were subsequently incubated
391 for 1 h with appropriate HRP (Horse Radish Peroxidase)-conjugated secondary antibody
392 (Thermo Fisher Scientific) in blocking buffer. Membranes were washed for 45 min with PBS-
393 T and immunoreactive proteins were detected by chemiluminescence (BioRad
394 Biotechnology). Quantification was performed using Image Lab software (5.0 build 18, 2013
395 version, Bio-Rad Laboratories). The mean intensity of each individual band of interest was
396 calculated after background value subtraction.

397

398 **Image acquisition**

399 For GUVs experiments, real-time videomicroscopy was carried out with an inverse confocal
400 microscope TCS-SP5 AOBS (*Acousto-Optical Beam Splitter*), equipped with pulsed white
401 light laser (WLL) and with a 63X oil immersion objective (Leica Microsystems). PE-NBD
402 was excited at 463 nm and observed in a 510-550 nm window. Images were acquired at a
403 speed of one frame per 2 s during 1 min.

404 For immunocytochemistry experiments, confocal microscopy was carried out with a TCS-SP8
405 upright confocal laser-scanning microscope equipped with 63X oil immersion objective
406 (NA=1.4; Leica, Microsystems). Alexa 488 and GFP were excited at 488 nm and observed in
407 a 505–540 nm window. Alexa 594 was excited at 594 nm and observed in a 600–630nm
408 window. For dual color acquisition, images were sequentially acquired in line scan mode
409 (average line = 2). Overlays were performed with post acquisition Leica Confocal Software
410 functions to obtain the presented snapshots.

411 For super-resolution experiments, image acquisitions were performed with a 100X oil
412 immersion objective (NA 1.4) through time-gated continuous wave stimulated emission
413 depletion (gCW STED) imaging (TCS SP5-X; Leica Microsystems) (30) with optimized
414 parameters for GFP detection (Supplemental Fig. 2). Samples (zoom 8, pixel size = 18.95 nm)
415 were excited with a 488 nm wavelength of a supercontinuum laser (20–30% AOTF). A
416 conventional scanner (400 Hz, Line Average 1, Frame Average 1, Frame Accumulation 3,
417 1024 × 1024) was used. Depletion was obtained with a 592 nm laser (70% AOTF).
418 Fluorescence (500–550 nm) was collected with a hybrid detector (Gain 100) in the gated
419 mode and a pinhole for Airy 1.

420

421 **Transmission electron microscopy of WT and *Pld1*^{-/-} chromaffin cells in situ**

422 WT and *Pld1*^{-/-} mice were anesthetized with a mixture of ketamine (100 mg kg⁻¹) and xylazine
423 (5 mg kg⁻¹) and transcardiacally perfused with 0.1 M phosphate buffer, pH 7.3, containing 2%
424 paraformaldehyde and 2.5% glutaraldehyde. The 2-mm-thick slices were cut from the adrenal
425 glands and postfixed in 1% glutaraldehyde in phosphate buffer overnight at 4°C. The slices
426 were then immersed for 1 h in OsO₄ 0.5% in phosphate buffer. 1-mm³ blocks were cut in the
427 adrenal medulla, dehydrated, and processed classically for embedding in Araldite and
428 ultramicrotomy. Ultrathin sections were counterstained with uranyl acetate and examined with
429 a Hitachi 7500 transmission electron microscope.

430

431 **Post-acquisition analysis**

432 The following procedure was used to measure the number of CgA granules. The confocal
433 section generated by the Leica TCS-SP8 confocal microscope was analyzed with Imaris and
434 converted into an Imaris file. A broad region of interest (ROI) was defined around a cell.
435 Then with the tool 'spots detection' on Imaris, the number of spots with a diameter > 200 nm
436 was quantified. Spots statistics are automatically computed for each spot object. It provides a
437 procedure to automatically detect point-like structures, an editor to manually correct detection
438 errors, a viewer to visualize the point-like structures as spheres, and statistics output.

439 To analyze secretory granule density in wild-type (WT) and *Pld1* knock-out chromaffin cells,
440 secretory granules were counted in 50 chromaffin cells from WT and *Pld1* KO mice with a
441 visible nucleus randomly selected in ultrathin sections from several blocks (1 section/block)
442 from each mouse (n=3 mice per genotype). Dense core diameter was measured from 950
443 randomly selected granules for each genotype using the line segment tool of Image J. To
444 minimize the bias measurements, samples were blinded.

445 To improve signal-to-noise ratio in STED images, deconvolution of raw data was obtained
446 through image processing with Huygens professional 4.5.1 software (SVI, The Netherlands)
447 (31).

448

449 **Statistics**

450 Data were analyzed with the Prism program (GraphPad 6.04 Software). For the quantification
451 of the number of CgA granules in IF studies, statistical significance was determined by Mann-
452 Whitney U test. Values are expressed as means \pm s.e.m. or S.D., and the level of significance
453 is designated in the figure legend as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For the
454 quantification of CgA signal in lipid strip experiments, statistical significance was determined
455 by an Anova one-way analysis of variance test with Bonferroni's comparison test. Values are
456 expressed as means \pm s.e.m., and the level of significance is designated in the figure legend as
457 follows: *** $P < 0.001$. For the quantification of membrane deformation after CgA addition on
458 GUVs, statistical significance was determined by Mann-Whitney U test. Values are expressed
459 as means \pm s.e.m., and the level of significance is designated in the figure legend as follows:
460 *** $P < 0.001$. For the quantification of the number of secretory granules and their dense core
461 diameter in MET study, statistical significance was determined by Mann-Whitney U test.
462 Values are expressed as means \pm s.e.m., and the level of significance is designated in the
463 figure legend as follows: * $P < 0.05$.

464

466 **RESULTS**

467 **Chromogranin A interacts preferentially with PA**

468 Due to the presence of two helicoidal motives in its N- and C-terminal conserved regions
469 which bear the granulogenic activity of CgA and which can promote binding to lipids (11),
470 we analyzed the capacity of the granin to interact with membrane lipids. A lipid overlay assay
471 was performed by incubating recombinant CgA with a nitrocellulose sheet where different
472 membrane lipids were adsorbed. Using an antibody raised against CgA (25), this overlay
473 assay revealed that CgA interacts with PA and phosphoinositides (PIP, PIP₂ and PIP₃) (Fig. 1
474 A). Quantification of the CgA-lipid interaction signals revealed that CgA preferentially
475 interacts with PA (Fig. 1 A).

476

477 **Golgi PA is involved in the biogenesis of CgA-containing secretory granules**

478 To examine the importance of PA in the formation of CgA-induced secretory granules, we
479 overexpressed the PABD of the cytosolic phosphodiesterase PDE4A1 in neuroendocrine
480 PC12 cells in order to reduce the ability of CgA to interact with PA in Golgi membranes.
481 Indeed, PDE4A1 PABD was previously shown to interact with PA specifically at the TGN in
482 COS1 cells (32) and RAW264.7 macrophages (21), and its overexpression should reduce the
483 amount of available PA at this level. We found that PDE4A1(PABD) not only exhibited the
484 expected Golgi-like confined localization, but also significantly reduced the number of CgA
485 secretory granules, without affecting CgA expression (Fig. 1 B). Moreover, expression of
486 PDE4A1(PABD Mut) which does not bind to PA and which was not detected in the Golgi
487 area did not impair the biogenesis of CgA-induced granules (Fig. 1 B), strongly supporting
488 the crucial role of Golgi PA in the formation of CgA secretory granules.

489

490 **Chromogranin A interacts with PA through a putative PA-binding domain**

491 To characterize the CgA region involved in PA binding, we compared the CgA helicoidal
492 motifs with those of proteins with known PABD, notably the Golgi PA-binding protein
493 PDE4A1. This analysis revealed a potential PABD within residues 352-381 of CgA, a
494 sequence containing positively charged and hydrophobic amino acids (Fig. 1 C). Indeed, a
495 bioinformatics analysis using Heliquest software (33) further refined this PABD to residues
496 364 - 381 (Fig. 1 C), which shares the characteristics of typical PABDs (34). It is predicted
497 that this PABD adopts an amphipathic α -helical conformation with five positively charged

498 residues (arginines) sequestered in one side of the helix, which could interact with the
499 negatively charged polar head of PA, and three surface exposed hydrophobic residues
500 (tryptophane, phenylalanine, leucine) on the other side of the helix which could insert within
501 the hydrophobic part of membranes and interact with the fatty acid chains of PA (Fig. 1 D).
502 Interestingly, a 3D structural model of CgA PABD confirmed that this domain displays an α -
503 helix when its structure was examined using MacPyMol software (shown as a ribbon, Fig. 1
504 E). We then used a semi-quantitative *in vitro* assay with NBD-labeled liposomes (21) to
505 investigate the PA binding capacity of three different GST fusion proteins containing the C-
506 terminal helicoidal motif of CgA or parts of it: CgA(352-381) (CgA PABD), CgA(352-372)
507 (catestatin) and CgA(373-381). We found that GST-CgA(352-381) very efficiently bound
508 PA-containing liposomes, while the binding of GST-CgA(352-372) was greatly reduced and
509 that of GST-CgA(373-381) was even lower (Fig. 1 F). In contrast, none of the proteins tested
510 efficiently bound PC NBD-liposomes (Fig. 1F), highlighting the preferential affinity of the
511 CgA α -helix for PA rather than for curved membranes. These data not only indicate that the
512 entire sequence corresponding to CgA PABD is important for PA binding but also that CgA
513 proteolysis, as it occurs in secretory granules and which generates the peptide catestatin
514 (CgA(352-372)) (Fig. 1 C), should disrupt this binding.

515

516 **Specific PA species are predominant and common to Golgi and secretory granule** 517 **membranes from CgA-expressing cells**

518 We analyzed and compared the PA content of Golgi and secretory granule membrane
519 fractions purified from COS7 cells stably expressing CgA (COS7-CgA). These cells display
520 CgA-containing secretory granule-like structures in contrast to their WT counterparts with no
521 CgA (14, 15). The enrichment of secretory granule and Golgi membrane fractions was
522 evaluated by western blotting (Supplemental Fig. 1). Detailed lipidomics analysis of these
523 fractions revealed that over the 44 distinct species detected, only few specific PA species are
524 predominantly identified in secretory granule and Golgi membranes of COS7-CgA cells, *i.e.*
525 PA (36:1), PA (38:2), PA (38:6) and PA (40:6) (Fig. 2 A). Furthermore, we compared the
526 Golgi membrane of COS7-WT and COS7-CgA cells to examine the potential impact of CgA
527 expression on the PA content. We found that the Golgi membrane of COS7-WT and COS7-
528 CgA cells exhibit the same predominant PA species, *i.e.* PA (36:1), PA (38:2) and PA (40:6),
529 but we also noticed a significant enrichment in PA (36:1) in Golgi membranes after CgA
530 expression, mainly at the expense of PA (40:6) (Fig. 2 B). The presence of the same major PA

531 species, namely PA (36:1), PA (38:2) and PA (40:6), was also found in secretory granule
532 membranes isolated from the neuroendocrine PC12 cells (Fig. 2 C). These data suggest that
533 PA (36:1), PA (38:2) and PA (40:6) could represent crucial species for secretory granule
534 formation.

535

536 **Chromogranin A interacts with major Golgi/secretory granule PA species included in** 537 **giant liposomes and induces membrane deformation and vesicle budding**

538 To test the interaction between CgA from COS7-CgA cell extracts and the predominant PA
539 species identified in Golgi and secretory granule membranes, we used a liposome flotation
540 assay (Fig. 3 A). Following the flotation assay, the different fractions were analyzed by
541 western blotting using a CgA specific antibody (25). This analysis revealed a strong
542 interaction between CgA and liposomes enriched with PA species identified previously by
543 lipidomics analysis, with an apparent preference for PA (36:1), and far less interaction with
544 PIP₂-enriched liposomes (Fig. 3 B). In contrast, no interaction was found with liposomes
545 enriched with phosphatidylcholine (PC), phosphatidylserine (PS) or PA (36:0), in accordance
546 with the results obtained using lipid strips (Fig. 1 B; Fig. 3 B). These results demonstrate that
547 endogenous CgA interacts specifically with predominant PA species identified in Golgi and
548 secretory granule membranes from CgA-expressing cells.

549 To examine the membrane dynamics following CgA and PA interaction, we developed
550 a model of GUVs enriched in PA (36:1) or PA (40:6), whose formation is assisted by a
551 polyvinyl alcohol gel. To analyze their morphology using confocal microscopy, we generated
552 PA-enriched GUVs containing a fluorescent lipid, phosphatidylethanolamine (PE)-NBD.
553 After image acquisitions, we observed that the formed GUVs exhibit a diameter ranging
554 between 5 and 50 μm , that the fluorescence is homogeneously distributed around the GUVs
555 and that there are no budding events (Fig. 3 C; supplemental video 1). Addition of 2 μM CgA
556 to PA-enriched GUVs provoked a marked fluorescence concentration in confined membrane
557 domains of several GUVs just before budding events which resulted in the formation of small
558 vesicles (Fig. 3 D; supplemental video 2). Quantification of membrane deformations
559 generating vesicles revealed that the number of budding events depends on CgA
560 concentration (Fig. 3 E). Finally, to analyze the direct involvement of CgA in GUV
561 membrane remodeling, we generated a CgA fluorescent probe, CgA-Alexa488, and we
562 observed that addition of 2 μM CgA-Alexa488 on non-fluorescent PA-enriched GUVs results
563 first in a rapid and homogenous fluorescence distribution in GUV membranes, and then

564 within a minute budding events were detected in fluorescent CgA-enriched membrane
565 domains (Fig. 3 F). Altogether, these results indicate that CgA interacts with membrane PA
566 and that this interaction could induce a membrane remodeling resulting in vesicle budding.

567

568 **Thermodynamic characteristics of CgA/PA interaction indicate that PA favors CgA** 569 **incorporation in liposome membranes**

570 To investigate the CgA/PA interaction in more details, we conducted an ITC analysis, which
571 quantifies the binding equilibrium directly by measuring the heat change resulting from the
572 association of a ligand with its binding partner (35, 36). ITC thermograms showed that
573 injection of a water suspension of small unilamellar vesicles (SUVs) composed of 1,2-
574 dioleoyl-sn-glycero-3-phosphocholine (DOPC) or DOPC/PA (36:1) induced an exothermic
575 process resulting from the dilution process of SUVs in water (Fig. 4, A and B, upper panels).
576 When we injected DOPC or DOPC/PA (36:1) SUV suspension into a CgA solution, the ITC
577 results showed sequential exothermic and endothermic processes (Fig. 4, A and B, lower
578 panels). Enthalpy curves (Fig. 4 C) were obtained from the integrated energy values obtained
579 from A and B traces in lower panels, using A and B traces in upper panels as blank,
580 respectively. These curves were fitted using two simple one-site binding model to yield
581 thermodynamic parameters (Table 1). Comparing the enthalpy curves obtained after DOPC
582 and DOPC/PA (36:1) injection in CgA solution, we observed an increase due to an
583 exothermic process related to the binding of CgA with DOPC that could result from hydrogen
584 bonding and/or electrostatic force (18). We also noticed that the maximum of enthalpy is
585 reached after 14 injections of DOPC SUVs while it is reached only after 6 injections of
586 DOPC/PA (36:1) SUVs (Fig. 4 C). In addition, for the exothermic process, the calculated
587 enthalpy ratio $\Delta H_{DOPC/PA(36:1)}^{exo}/\Delta H_{DOPC}^{exo}$ is close to 7, the enthalpy ratio
588 $\Delta H_{DOPC/PA(36:1)}^{endo}/\Delta H_{DOPC}^{endo}$ is equal to 12, and the stoichiometry (n) for both processes is lower
589 with DOPC/PA (36:1) SUVs (Table 1). Moreover, the endothermic phase leads to enthalpy
590 decrease (Fig. 4 C) and the stoichiometric ratio, which is the ratio between the total number of
591 lipids and the number of lipids on the outer surface of the SUV, is close to 1.8, suggesting a
592 reorganization of the protein/lipid complex in the SUV membrane. Together, these results
593 clearly show that PA favors the incorporation of CgA in the SUV membrane.

594

595

596 **Chromogranin A induces the remodeling of supported membrane bilayers enriched with**
597 **Golgi/secretory granule PA**

598 Using atomic force microscopy (AFM), we studied the impact of CgA on supported bilayers
599 composed of DOPC or DOPC/PA (36:1). Addition of 0.6 or 1.2 μM CgA during 15 min (T1)
600 and 75 min (T2) on DOPC membranes revealed small alterations of their topographical
601 profile corresponding to protrusions with a maximal height of 4 nm (Fig. 5 A). In contrast,
602 addition of 0.6 or 1.2 μM CgA on DOPC/PA (36:1) membranes led to local profile changes of
603 the membrane with a height of the protrusions above 4 and 16 nm, respectively (Fig. 5 B). Of
604 note, the grain size analysis of 11-15 images obtained with 1.2 μM CgA during 75 min
605 revealed that the total number of CgA-induced domains increases by a factor of 3 after PA
606 (36:1) addition in DOPC bilayers (41 ± 23 domains per $100 \mu\text{m}^2$ DOPC bilayer (Fig. 6 A)
607 versus 120 ± 41 domains per $100 \mu\text{m}^2$ DOPC/PA (36:1) bilayer (Fig. 6 B)). Moreover, domains
608 with an area greater than $20 \mu\text{m}^2$ were clearly more abundant in presence of PA (36:1) in the
609 supported bilayer, and their height increased with their area to reach a mean of 21 ± 3 nm (Fig.
610 6 B). These results suggest that CgA interacts with PA-enriched lipid bilayers and that this
611 interaction modifies membrane topology, in a concentration and time-dependent manner.

612

613 **Chromogranin A PABD is required for secretory granule biogenesis**

614 To analyze the role of the predicted CgA PABD in the formation of secretory granules, we
615 expressed CgA where the PABD was deleted (CgA Δ PABD-GFP) in COS7 and AtT20 cells.
616 This specific CgA alteration significantly reduced secretory granule formation as compared to
617 native CgA (Fig. 7, A and B). Indeed, although CgA Δ PABD-GFP and CgA-GFP both
618 colocalized with the Golgi marker GM130, the number of CgA positive granules is reduced
619 when the PABD is deleted from CgA in COS7 cells (Fig. 7 A), but also in ATt20 cells
620 although transfection efficiency was lower (Fig.7 B). Using deconvoluted time-gated
621 continuous wave stimulated emission depletion (gCW STED) nanoscopy to overcome the
622 noise at the Golgi level (Supplemental Fig. 2), we confirmed a reduction of CgA-containing
623 structures in the Golgi area of both cell types expressing CgA Δ PABD-GFP (Fig. 7, C and D).
624 Altogether, these results support the idea that the interaction of CgA with PA through its
625 PADB is important for granule biogenesis.

626

627

628 **Inhibition of PLD-mediated PA synthesis alters the biogenesis of secretory granules**

629 To study the implication of PA in Golgi membranes during the biogenesis of CgA-containing
630 secretory granules, we altered PLD-mediated PA synthesis since PLD appears to be the most
631 important provider of PA in the Golgi (37). First, secretory cells were incubated with the PLD
632 pan-inhibitor FIPI, which provoked a significant reduction in the number of CgA-containing
633 granules in COS7-CgA cells (Fig. 8 A) and a smaller reduction in PC12 cells (Fig. 8 B),
634 without any apparent effect on the levels of three major granins (CgA, CgB, SgII).
635 Interestingly, in chromaffin cells obtained from mice lacking *Pld1* gene (*Pld1*^{-/-}), we observed
636 by electron microscopy a significant reduction in the number of secretory granules (Fig. 8, C
637 and D), associated to a decrease in their dense core diameter (Fig. 8, C and E). Thus,
638 pharmacological and genetic invalidation of PLD activity and expression supported the notion
639 that PLD1 is an important provider of PA in Golgi membranes (38), and showed that PLD1-
640 generated PA is required for the biogenesis of secretory granules containing CgA.

641

642 **DISCUSSION**

643 Although considerable efforts have been deployed to identify the molecular mechanism at the
644 origin of secretory granule formation, many aspects of this fundamental process remain to be
645 elucidated. The biogenesis of secretory granules depends on TGN membrane dynamics that
646 rely on interactions of cytosolic and luminal proteins with molecular components of the TGN
647 membrane. Among TGN luminal proteins, members of the granin protein family have been
648 long considered as major actors of secretory granule biogenesis but direct mechanistic
649 evidence is still lacking (39).

650 Here, we first uncovered the ability of the coiled-coil glycoprotein CgA to bind acidic
651 phospholipids, in particular PA, using a lipid-overlay assay. Since PA was suspected to play a
652 role in secretory granule biogenesis (40), we overexpressed the cytosolic phosphodiesterase
653 PDE4A1, a *bona fide* PA binding protein in Golgi membranes characterized previously (21)
654 to test this possibility. We observed a decrease in the number of CgA-containing secretory
655 granules in PC12 cells in the presence of PDE4A1-PABD, presumably acting as a competitor
656 for endogenous PA, suggesting that luminal CgA requires TGN membrane PA to generate
657 secretory granules. As most enzymes involved in PA synthesis are found in the cytoplasmic
658 leaflet of intracellular membranes, it is expected that most PA is generated in these membrane
659 cytosolic leaflets. However, passive PA flip/flop or active PA-transfer by flippase, floppase
660 and scramblase could efficiently alter this translayer asymmetry for PA. Accordingly, we

661 observed that PA was readily detected on the inner leaflet of the plasma membrane of PC12
662 cells expressing the PA binding protein Spo20p-GFP less than one minute after cell
663 incubation with 10 μ M of PA micelles (unpublished observation). Additionally, *in vitro*
664 experiments have suggested that intrinsic flipping of PA is affected by various parameters
665 including pH and the geometry of specific PA species (41, 42). Thus, it is likely that
666 significant levels of PA are present in the luminal leaflet of the TGN and therefore CgA/PA
667 interaction could be effective at this location.

668 *In silico* identification of the CgA(364-381) sequence as a PABD further supported the
669 possibility of CgA/PA interaction, which was confirmed using PA-enriched liposomes, as this
670 sequence specifically binds PA. Interestingly, CgA PABD binds PA at a level similar to that
671 observed for PDE4A1. In contrast, shorter fragments, corresponding to the expected cleavage
672 of CgA occurring during secretory granule maturation to generate catestatin, exhibited only a
673 weak interaction with PA. We can therefore anticipate that CgA/PA interaction might be
674 regulated during secretory granule maturation and CgA processing. It is tempting to speculate
675 that CgA and PA interact early in the TGN to trigger secretory granule formation, but that this
676 interaction is disrupted later by proteolysis after budding to generate catestatin and to ensure
677 secretory granule homeostasis. Such a sequential role has previously been demonstrated for
678 the granin 7B2 in the regulation of the proteolytic enzyme PC2 and hormone sorting and
679 secretion in endocrine cells (43). Moreover, the CgA PABD sequence may allow hydrophobic
680 residue insertion into lipid bilayers and direct ionic interaction between basic residues and
681 several PA molecules. Thus, CgA may participate to TGN remodeling by interacting with
682 TGN predominant PA species that could induce the formation of PA-enriched microdomains.
683 Indeed, CgA represents 40 to 80% of the protein content of secretory granules in chromaffin
684 cells and the concentration is in the order of millimolar, representing around 5,000 CgA
685 molecules per granule (44, 45). On the other hand, molecular dynamic simulation of the
686 interaction of PA with the yeast protein Spo20p PABD revealed that up to 6 PA molecules
687 bind to one Spo20p molecule (46). Based on the number of positively charged residues in the
688 CgA PABD, it is possible that one CgA binds and sequesters up to 5 PA. Therefore, this can
689 yield an extremely high density of PA, with up to 25,000 molecules accumulating with CgA
690 on specific locations of the inner leaflet of the TGN membrane. Accumulation of the conical-
691 shaped PA enables membrane bending (47), and this phenomenon could generate TGN
692 membrane curvature preceding secretory granule budding.

693 To further support the role of PA in secretory granule formation, we analyzed and
694 compared the PA composition in Golgi and secretory granule membranes from COS7-CgA
695 cells by mass spectrometry, and found a strong similarity in the PA species present in the
696 membranes of these two compartments. Among these PA species, we observed a
697 predominance of several common mono- and poly-unsaturated forms, *i.e.* PA (36:1) and PA
698 (40:6). Interestingly, these PA species were also identified in the membrane of secretory
699 granules from neuroendocrine PC12 cells, suggesting that specific PA species occur at the
700 Golgi membrane of CgA-expressing cells to induce the biogenesis of secretory granules, and
701 that one or some of these species have a key role in this process. Using liposome models
702 enriched with distinct phospholipids, we observed that CgA preferentially interacts with PA
703 (36:1) and PA (40:6), and that addition of this granin generates deformation of PA (36:1)- or
704 PA (40:6)-enriched artificial membranes in a dose-dependent manner. Of note, budding
705 occurs in an outward direction from PA-enriched giant liposome membrane towards CgA,
706 contrasting with the required budding towards the cytosol required for secretory granule
707 formation. This apparent discrepancy is probably due to the fact that native membranes are
708 more complex than artificial membranes in terms of lipid variety and asymmetric distribution
709 between the two leaflets, in addition to the presence of other membrane proteins and the
710 impact of cytosolic and luminal membrane environment (pH, ion concentration, cytosolic
711 proteins). The hydrophobic face of CgA PABD helix was proposed to partially insert into
712 artificial membrane, stressing nearby lipid packing, creating local defects, and promoting
713 membrane curvature. In any case, the fact that generated buds stay attached to the GUV
714 surface is consistent with the absence of cytosolic proteins involved in vesicle fission, such as
715 dynamin. The analysis of the thermodynamic characteristics of CgA/PA (36:1) interaction by
716 ITC consolidated the observation that CgA strongly interacts with PA (36:1)-enriched
717 membranes to promote membrane deformation. Atomic force microscopy on supported
718 membrane bilayers revealed that PA (36:1) enrichment increased the number, the height and
719 the surface of CgA-induced domains resulting from either CgA aggregation, membrane
720 deformation, microdomain formation, or any combination of these. These data support a
721 central role for PA in the formation of CgA-induced domains as observed by confocal
722 microscopy and ITC. Altogether, these results are in line with the idea that CgA/PA (36:1) or
723 PA (40:6) interaction could play a key role in the membrane remodeling process necessary for
724 secretory granule budding.

725 In accordance, we showed that expression of CgA deprived from its PABD leads to a
726 significant decrease in the number of CgA granules, with a reduction of CgA granule number
727 in whole cell but also in the Golgi area. The CgA-PABD deletion mutant is apparently acting
728 as a negative-dominant impairing CgA-induced granule formation, probably by disrupting the
729 molecular interactions normally occurring between CgA and PA at Golgi membranes.
730 Therefore, these data support the notion that CgA-PABD is involved in granule biogenesis
731 and that CgA/PA interaction through this domain is critical for the initiation of the secretory
732 process in neuroendocrine cells. Interestingly, the C-terminal sequence of catestatin
733 (CgA(352-372)) including the PABD contains several single nucleotide polymorphisms
734 associated with metabolic and cardiovascular disorders in humans (48, 49), suggesting a
735 potential link between the onset of these diseases and the disruption of hormone release
736 related to an alteration of CgA secretory granule biogenesis.

737 Moreover, several studies demonstrated that the enzymatic activity of PLD1
738 converting PC to PA is an important source of Golgi PA (38), which is required for secretory
739 granule budding (50), but also involved in secretory granule fusion with the plasma
740 membrane (51). Pharmacological inhibition of PLD in COS7-CgA and PC12 cells, and *Pld1*
741 knockout in mouse chromaffin cells, both reduced secretory granule biogenesis. The smaller
742 reduction in PC12 could be due to the endogenous expression of additional granin family
743 members endowed with a granulogenic effect and which could act through PLD/PA-
744 independent manner. Interestingly, this reduction in secretory granule number was also
745 accompanied by an alteration in the shape of these organelles, very similarly to what was
746 found in CgA-knockout chromaffin cells (52), indicating that PLD1 depletion partially
747 phenocopies CgA deficiency regarding the impact on secretory granule biogenesis. These
748 observations strongly suggest again that CgA and PLD1-generated PA cooperate to trigger the
749 formation of secretory granules in neuroendocrine cells.

750 Finally, it must be pointed out that a recent study based on *in vitro* reconstitution
751 assays and molecular modeling reported additional functions of specific species of PA during
752 COPI vesicle fission (53). Indeed, PA generated by lysophosphatidic acid acyltransferase type
753 gamma promotes the early stage of COPI vesicle fission whereas PA generated by
754 phospholipase D type 2 promotes the late stage of fission. Intriguingly, PA with shorter fatty
755 acid chains better supported the later function of PA, most likely by triggering the activity of
756 BARS (53). It is of note that our lipidomic analysis indicates that these shorter forms of PA
757 are scarce in Golgi or secretory granule membranes suggesting that only a tiny fraction of PA

758 could trigger the fission of budding vesicle, while larger proportion of PA is required for
759 budding. A recent study revealed that PI4P in the cytosolic leaflet of the TGN membrane
760 recruits cytosolic proteins such as GOLPH3 through its membrane curvature ability (54),
761 suggesting that PA could also initiate this process by activating Golgi PIP-kinase to produce
762 PI4P in the cytosolic leaflet.

763 Altogether, our data show that CgA binds to specific species of PA which in turn
764 could act as lipid cofactors (55) at the TGN to induce membrane remodeling and curvature in
765 order to initiate secretory granule budding, concomitantly or prior to the recruitment of
766 cytosolic proteins such as GOLPH3 (54), arfaptins (56, 57), the acto-myosin 1b complex (58)
767 or the membrane-associated myristoylated protein HID1 (59), but also small GTPase of the
768 Arf family (60), all of which are also essential actors in this fundamental biological process
769 necessary for regulated secretion. Further studies are now needed to evaluate the connection
770 of CgA/PA interaction with the additional machinery involved in secretory granule
771 biogenesis, in living neuroendocrine cells, and the potential implication of this interaction in
772 diseases related to hormone secretion deregulation.

773

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784

785 **AUTHOR CONTRIBUTIONS**

786 OC, FL and LR: designed and performed most of the experiments, analyzed the data and
787 contributed to writing of the paper. CD-D: performed and analyzed lipid overlay assays. QW:
788 performed some immunofluorescence and electron microscopy experiments and associated
789 quantifications. ET and TT: performed the experiments related to liposome flotation and

790 binding assays. LJ: managed cell line culture and secretion experiments. DC: contributed to
791 CgA-GFP and CgA Δ PABD-GFP cloning. AMH: performed electron microscopy experiments
792 and associated quantifications. LF: performed the lipidome analysis using LC-MS/MS. OR,
793 AH and P-YR: produced CgA coupled to Alexa Fluor 488 dye. DS and LG: performed super-
794 resolution observations using STED nanoscope. YG: synthesized recombinant CgA. SA:
795 designed AFM and ITC setup, and analyzed AFM and ITC data. NV: designed liposome
796 flotation and binding assays, analyzed data from electron microscopy experiments, performed
797 PA-binding domain prediction, and contributed to writing of the paper. YA: contributed to
798 experiment design, data analysis and interpretation, and writing of the paper. MM-H:
799 coordinated the study, designed, analyzed and interpreted data, and wrote the paper.

800

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998

999 **FIGURE LEGENDS**

1000 **Figure 1.** CgA interacts with PA through a putative PABD and Golgi PA is involved in CgA
1001 secretory granule biogenesis.

1002 A Protein–lipid overlay assay in the presence of recombinant CgA (500 ng/ml) using
1003 commercial membrane strips where 100 pmol/spot of the following lipids are adsorbed:
1004 triglycerides, diacylglycerol (DAG), phosphatidic acid (PA), phosphatidylserine (PS),
1005 phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG),
1006 cardiolipin, phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI(4)P),
1007 phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), phosphatidylinositol 3,4,5-trisphosphate
1008 (PI(3,4,5)P₃), cholesterol, sphingomyelin, or 3-sulfogalactosylceramide (sulfatide). After
1009 the overlay, the membrane was immunostained for CgA using the anti-WE-14 antibody,
1010 and revealed using a chemiluminescence kit. Plotted are means of CgA binding intensity
1011 expressed as percentage normalized to control (blank) \pm s.e.m. (n=3). *** $P < 0.001$, Anova
1012 one-way analysis of variance test with Bonferroni’s comparison test.

1013 B Involvement of Golgi PA in the formation of CgA-containing secretory granules in PC12
1014 cells. Cells expressing wild-type PDE4A1 coupled with GFP (PDE4A1(PABD)) or PABD-
1015 mutated PDE4A1 coupled with GFP (PDE4A1(PABD Mut)) are surrounded by a dashed
1016 line. Cells were immunolabelled with anti-CgA antibody and their fluorescence was
1017 examined using confocal microscopy. Representative confocal microscopy sections
1018 throughout the cells are shown. Values for the intensity of CgA staining and the number of
1019 granules per cell are plotted as the means \pm S.D. (n=2; 40 cells per condition). ** $P < 0.01$,
1020 Mann-Whitney test. The scale bar represents 20 μ m. Western blots show expression of
1021 CgA and α -tubulin as loading control.

1022 C Human CgA (hCgA) sequence showing a region of 18 amino acids (364-381),
1023 encompassing a positive charge cluster and hydrophobic residues that could adopt an α -
1024 helical conformation, delimited by arrows, suggesting its function as PA-binding domain
1025 (PABD).

1026 D PA binding profile of the putative human CgA-PABD. Amphipathic α -helix projection of
1027 the core 18 amino acids of the PABD of CgA obtained with Heliquest software. Arrow
1028 indicates hydrophobic moment. Red N and C indicate the beginning and the end of PABD
1029 amino acid sequence.

1030 E Model of the putative human CgA-PABD. Ribbon corresponds to the α -helix. The
1031 structure was determined using MacPyMOL (v1.74).

1032 F Characterization of the PA binding capacity of human CgA through the putative PABD.
1033 Semi-quantitative fluorescent liposome assays with fluorescent liposomes (5% PE-NBD,
1034 95% DOPC) or PA-containing fluorescent liposomes (5% PE-NBD, 85% DOPC, 10% PA
1035 mix) and GST-CgA-PABD constructs linked to GSH-sepharose beads. The binding of
1036 liposomes with CgA-PABD constructs was monitored by fluorimetry. Results are
1037 presented as means \pm S.D. (triplicate measurements; n=3).

1038

1039 **Figure 2.** Same predominant PA species are found in Golgi and secretory granule membranes
1040 from COS7-WT, COS7-CgA and PC12 cells.

1041 A The level of distinct PA species was measured in fractions of secretory granule (SG) and
1042 Golgi membranes from COS7-CgA cells by duplicate UPLC/MS/MS analysis of two
1043 samples (each containing 200 μ g of protein) from each fraction. The acyl chain
1044 composition of each species is shown on the x axis. The y axis shows the abundance of
1045 each species as a percentage of total PA in the sample.

1046 B PA levels were measured in fractions of Golgi membranes from COS7-WT or COS7-CgA
1047 cells by duplicate UPLC/MS/MS analysis of two samples (each containing 200 μ g of
1048 protein) from each fraction. The acyl chain composition of each species is shown on the x
1049 axis. The y axis shows the abundance of each species as a percentage of total PA in the
1050 sample.

1051 C PA levels were measured in secretory granule-containing fractions from PC12 or COS7-
1052 CgA cells by duplicate UPLC/MS/MS analysis of two samples (each containing 200 μ g of
1053 protein) from each fraction. The acyl chain composition of each species is shown on the x
1054 axis. The y axis shows the abundance of each species as a percentage of total PA in the
1055 sample.

1056

1057 **Figure 3.** CgA interacts with PA species identified in Golgi and secretory granule membranes
1058 and provokes membrane deformation of PA-enriched GUVs.

1059 A Schematic representation of the liposome flotation assay approach used to study the
1060 interaction of CgA from COS7-CgA cell extracts with PA species.

1061 B Detection of CgA in the different fractions (Top (T), Medium (M), Bottom (B)) obtained
1062 from liposome assays. COS7-CgA cell extracts were incubated with liposomes containing
1063 5% PE-NBD, 85% of DOPC, and 10% of the indicated phosphatidylcholine (PC),
1064 phosphatidylserine (PS), phosphatidylinositol-4,5-bisphosphate (PIP2), a commercial

1065 phosphatidic acid mix (PA) or distinct species of PA (PA(36:0), PA(36:1) or PA(40:6)).
1066 Thirty µg of protein from each fraction were run on SDS-PAGE and immunoblotted with
1067 the WE-14 antibody.

1068 C Formation of fluorescent PA-containing GUVs by PVA-assisted swelling. Stable
1069 fluorescent GUVs (95% DOPC, 4% PA(36:1) and 1% PE-NBD) were generated and
1070 observed by live confocal microscopy during 1 min. Representative confocal microscopy
1071 sections throughout the GUVs are shown. The scale bar represents 30 µm.

1072 D Effect of CgA on fluorescent PA-containing GUVs. Fluorescent GUVs (95% DOPC, 4%
1073 PA(36:1) or PA(40:6) and 1% PE-NBD) were incubated with 2 µM CgA. Deformation of
1074 GUV membrane (arrows) was observed by live confocal microscopy during 1 min.
1075 Representative confocal microscopy sections throughout the GUVs are shown. The scale
1076 bar represents 30 µm.

1077 E Quantification of generated vesicles following membrane deformation of PA-containing
1078 GUVs. GUVs (95% DOPC, 4% PA(36:1), 1% PE-NBD) were incubated with 2, 4 and 6
1079 µM CgA (n=3; 35 videos per condition). *** $P < 0.001$, Mann-Whitney test. Means \pm s.e.m.
1080 are plotted.

1081 F Distribution of CgA-Alexa488 on PA-containing GUVs. GUVs (96% DOPC, 4%
1082 PA(36:1)) were incubated with 2 µM CgA-Alexa488. Staining and budding (arrows) of
1083 GUV membrane was observed by live confocal microscopy during 1 min. Representative
1084 confocal microscopy sections throughout the GUVs are shown. The scale bar represents
1085 20 µm.

1086

1087 **Figure 4.** ITC analysis reveals that PA favors membrane incorporation of CgA.

1088 A DOPC SUVs and CgA binding measured by titrating 5 µM CgA in the chamber with 10
1089 mM vesicle suspension in the syringe. Top panel, raw heating power over time obtained
1090 with DOPC SUV injection in water; bottom panel, raw heating power over time obtained
1091 with DOPC SUV injection in CgA solution.

1092 B DOPC/PA(36:1) SUVs and CgA binding measured by titrating 5 µM CgA in the chamber
1093 with 10 mM vesicle suspension in the syringe. Top panel, raw heating power over time
1094 obtained with DOPC/PA(36:1) SUV injection in water; bottom panel, raw heating power
1095 over time obtained with DOPC/PA(36:1) SUV injection in CgA solution.

1096 C Fit curves of the integrated energy values obtained from A and B traces in bottom panels
1097 using A and B traces in top panels as blank, respectively. Experiments were carried out at
1098 10°C, and each value is the mean of three independent titrations.

1099

1100 **Figure 5.** CgA induces the remodeling of PA-enriched membrane bilayers.

1101 A Atomic force microscopy topographical images of mica supported bilayer surfaces from
1102 DOPC GUVs, incubated without or with 0.6 and 1.2 μM CgA, during 15 min (T1) and 75
1103 min (T2). The scale bar represents 2 μm . The scheme corresponds to a topographical
1104 profile through an aggregate (enlarged view) showing a difference of height of 6 nm with
1105 the continuous phase.

1106 B Atomic force microscopy topographical images of mica supported bilayer surfaces from
1107 DOPC/PA(36:1) (9:1, mol ratio) GUVs, incubated without or with 0.6 and 1.2 μM CgA,
1108 during 15 min (T1) and 75 min (T2). Arrow heads indicate aggregates that appeared at T1
1109 and are maintained at T2; arrows indicate aggregates that appeared between T1 and T2.
1110 The scale bar represents 2 μm . The scheme corresponds to topographical profiles through
1111 aggregates (enlarged views) showing a difference of height of 6 nm (a) and 17 nm (b) with
1112 the continuous phase.

1113

1114 **Figure 6.** Number, surface and height of CgA-induced domains depend on PA enrichment of
1115 membrane bilayers.

1116 A Scatter plot showing the number, area and height of domains obtained after incubation of
1117 supported bilayer surfaces from DOPC GUVs with 1.2 μM CgA during 75 min. Insert
1118 corresponds to enlarged view of the delimited zone emphasizing domains with height
1119 below 4 nm and area below 6,000 nm^2 (within blue surrounded zone). Analysis of domains
1120 was performed on 15 images and domains detected on an image are of the same color.

1121 B Scatter plot showing the number, area and height of domains obtained after incubation of
1122 supported bilayer surfaces from DOPC/PA(36:1) (9:1, mol ratio) GUVs with 1.2 μM CgA
1123 during 75 min. Insert corresponds to enlarged view of the delimited zone emphasizing
1124 domains with height below 4 nm and area below 6,000 nm^2 (within blue surrounded zone).
1125 Analysis of domains was performed on 11 images and domains detected on an image are
1126 of the same color.

1127

1128 **Figure 7.** CgA PABD is required for secretory granule biogenesis.

1129 A COS-7 cells transfected with plasmids encoding full-length CgA-GFP or CgA PABD-
1130 mutated form (CgA Δ PABD)-GFP were examined for GFP fluorescence and GM130
1131 immunoreactivity using confocal microscopy. Representative confocal microscopy
1132 sections throughout the cells are shown and were used to quantify automatically the
1133 granules with a diameter > 200 nm. Values for the number of granules/cell are plotted as
1134 the means \pm s.e.m. (n=3; 65 cells per condition). *** P < 0.001, Mann-Whitney test. The
1135 scale bar represents 20 μ m. Western blots show expression of CgA-GFP or CgA Δ PABD-
1136 GFP fusion proteins and α -tubulin as loading control.

1137 B AtT20 cells transfected with plasmids encoding full-length CgA-GFP or CgA PABD-
1138 mutated form (CgA Δ PABD)-GFP were examined for GFP fluorescence and GM130
1139 immunoreactivity using confocal microscopy. Representative confocal microscopy
1140 sections throughout the cells are shown and were used to quantify automatically the
1141 granules with a diameter > 200 nm. Values for the number of granules/cell are plotted as
1142 the means \pm s.e.m. (n=3; 70 cells per condition). *** P < 0.001, Mann-Whitney test. The
1143 scale bar represents 20 μ m. Western blots show expression of CgA-GFP or CgA Δ PABD-
1144 GFP fusion proteins and α -tubulin as loading control.

1145 C Golgi area of COS-7 cells transfected with plasmids encoding full-length CgA-GFP or
1146 CgA PABD-mutated form (CgA Δ PABD)-GFP was examined for GFP fluorescence using
1147 gCW STED. Representative sections are shown after deconvolution. The scale bar
1148 represents 1 μ m.

1149 D Golgi area of AtT20 cells transfected with plasmids encoding full-length CgA-GFP or CgA
1150 PABD-mutated form (CgA Δ PABD)-GFP was examined for GFP fluorescence using gCW
1151 STED. Representative sections are shown after deconvolution. The scale bar represents
1152 1 μ m.

1153

1154 **Figure 8.** Inhibition of PLD-mediated PA synthesis alters secretory granule biogenesis.

1155 A COS7-CgA cells were treated or not with 75 nM FIPI for 6 h, fixed and immunolabelled
1156 with anti-GM130 and anti-CgA antibodies. Representative confocal microscopy sections
1157 throughout the cells are shown. The scale bar represents 20 μ m. Values for the number of
1158 CgA secretory granules are plotted as the means \pm s.e.m. (n=3; 60 cells per condition).

1159 *** $P < 0.001$, Mann-Whitney test. Western blots show expression of CgA and α -tubulin as
1160 loading control.

1161 B PC12 cells were treated or not with 75 nM FIPI for 6 h, fixed and immunolabelled with
1162 anti-GM130 and anti-CgA antibodies. Representative confocal microscopy sections
1163 throughout the cells are shown. The scale bar represents 10 μ m. Values for the number of
1164 CgA secretory granules are plotted as the means \pm s.e.m. (n=3; 60 cells per condition).
1165 Mann-Whitney test. Western blots show expression of CgA, CgB and SgII proteins, and α -
1166 tubulin as loading control.

1167 C Electron microscopy analysis of chromaffin cells from WT (control) and *Pld1*^{-/-} mice.
1168 White arrows indicate representative secretory granules. The scale bars represent 2 μ m.

1169 D Quantification of the number of secretory granules (SG) shown in panel (A) (n=6 mice per
1170 condition; 50 cells per mouse adrenal medulla). * $P < 0.05$, Mann-Whitney test.
1171 Means \pm s.e.m. are plotted.

1172 E Quantification of the dense core diameter of secretory granules (SG) shown in panel (A)
1173 (n=6 mice per condition; 950 granules). * $P < 0.05$, Mann-Whitney test. Means \pm s.e.m. are
1174 plotted.

Table 1.

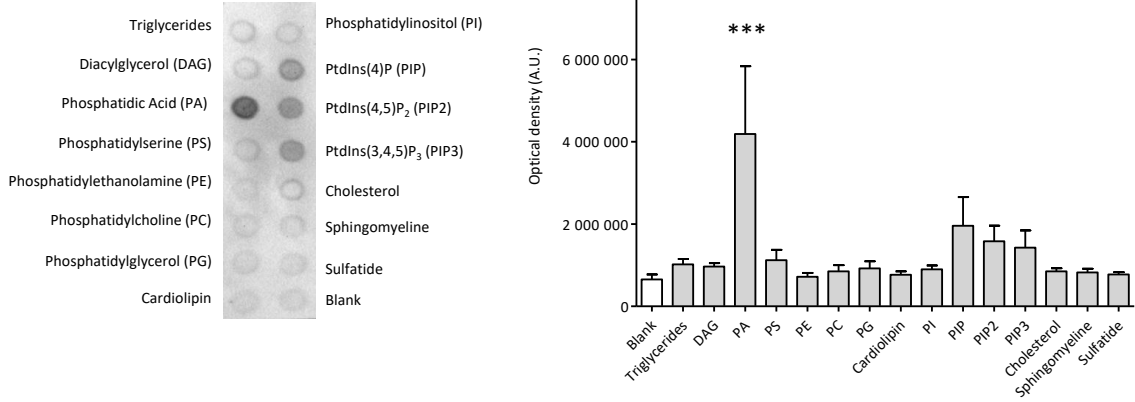
Thermodynamic parameters for CgA binding on DOPC and DOPC/PA(36:1) SUVs. The values are plotted in Fig. 4C.

	DOPC		DOPC / PA(36 :1)	
	exothermic	endothermic	exothermic	endothermic
Kd (M)	$1.5 \cdot 10^{-4} \pm 0.5 \cdot 10^{-4}$	$2 \cdot 10^{-5} \pm 1 \cdot 10^{-5}$	$1.16 \cdot 10^{-3} \pm 0.03 \cdot 10^{-3}$	$3 \cdot 10^{-4} \pm 0.1 \cdot 10^{-4}$
n	259 ± 30	460 ± 40	199 ± 5	355 ± 5
ΔH (kJ/mol)	-1.82 ± 0.09	0.640 ± 0.06	-12.9 ± 0.3	7.65 ± 0.15
ΔS (J/mol.K)	67.3 ± 3.2	93.4 ± 4.8	10.6 ± 1.3	94.5 ± 0.8

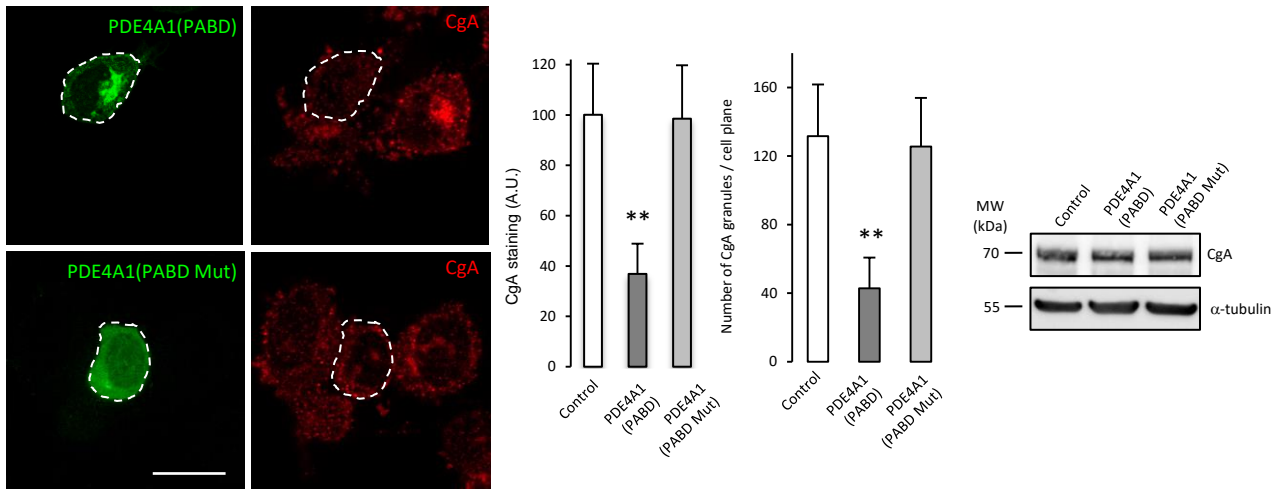
Experiments were carried out at 10°C, and each value is the mean \pm standard deviation from the fitted data of three independent experiments. Kd, dissociation constant; n, number of binding sites; ΔH , enthalpy change; ΔS , entropy change.

Figure 1

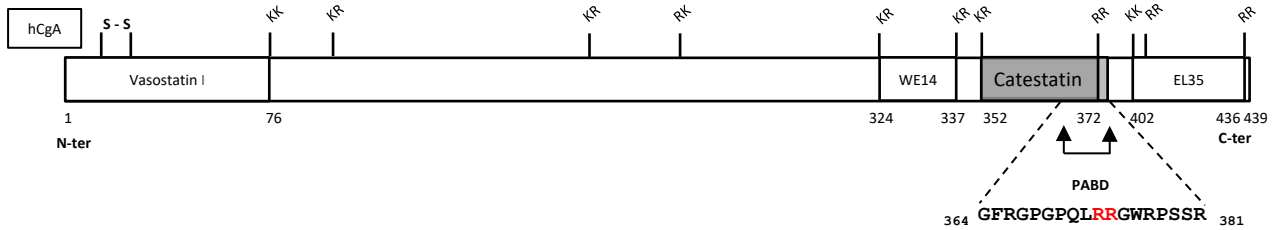
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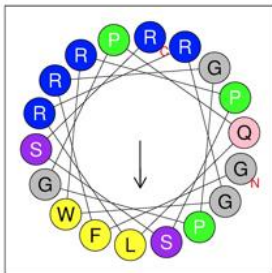
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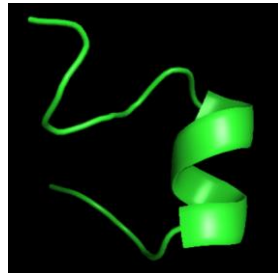
C



D



E



F

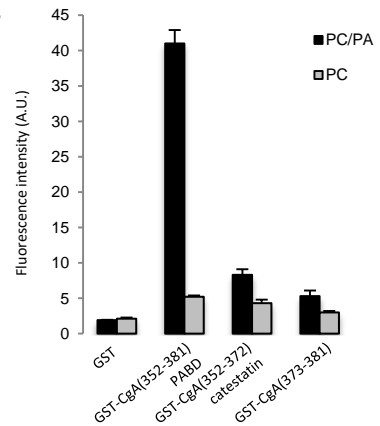
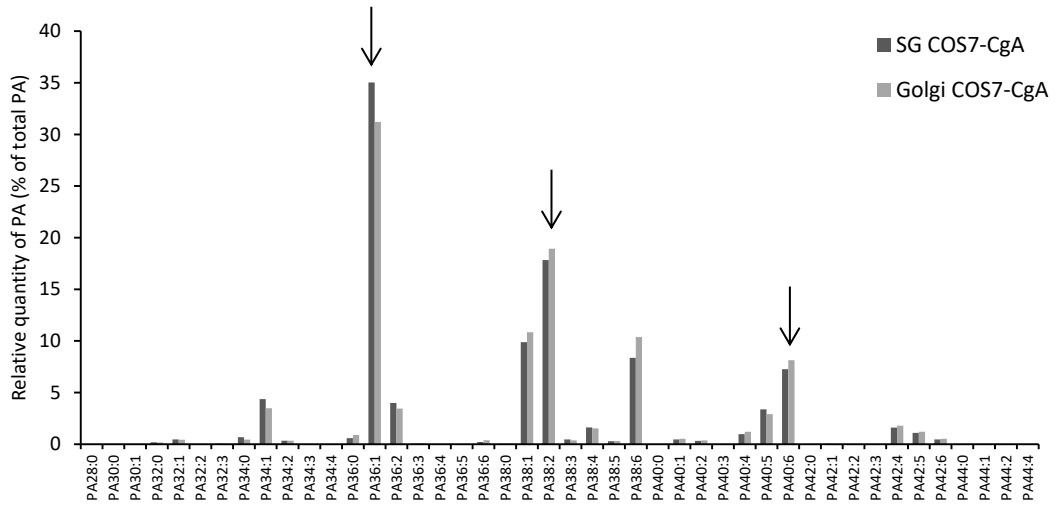


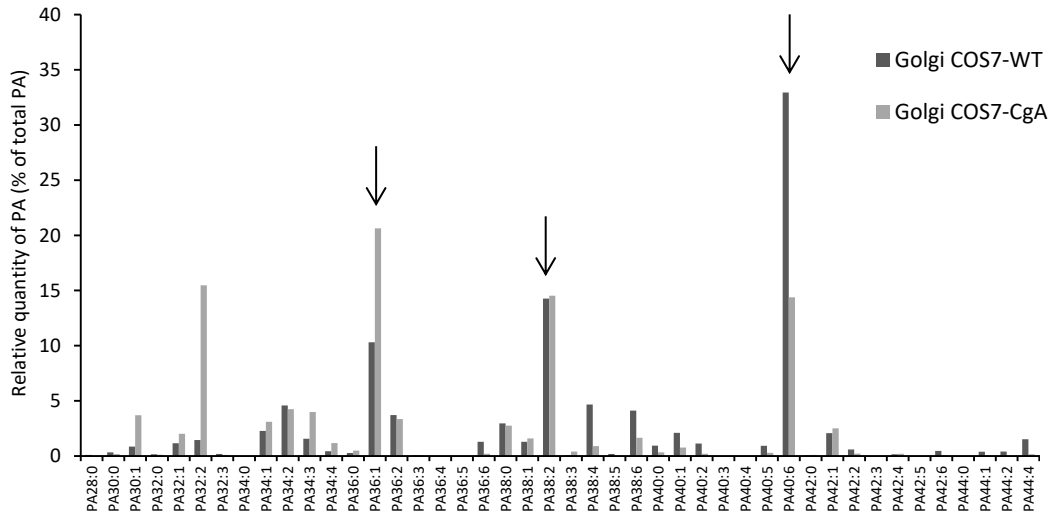
Figure 1

Figure 2

A



B



C

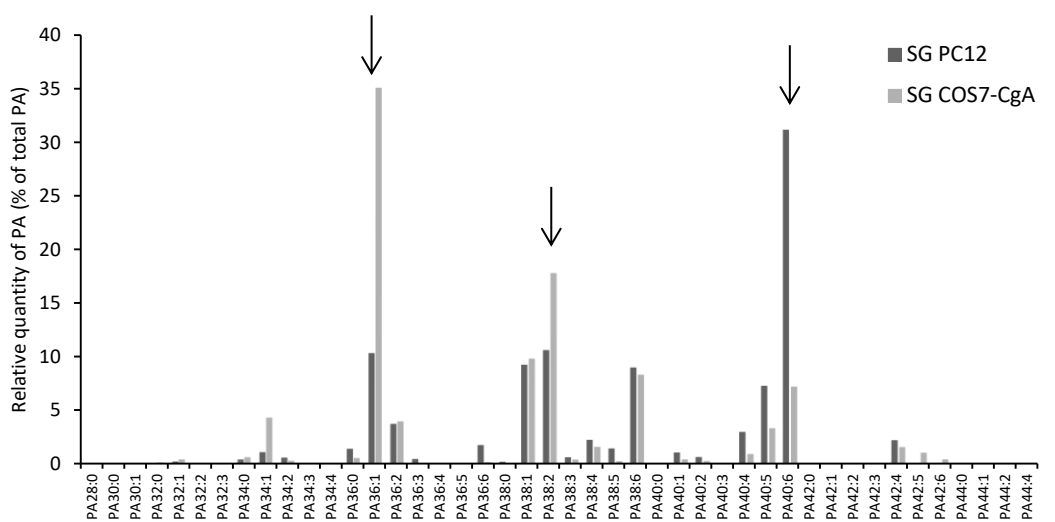
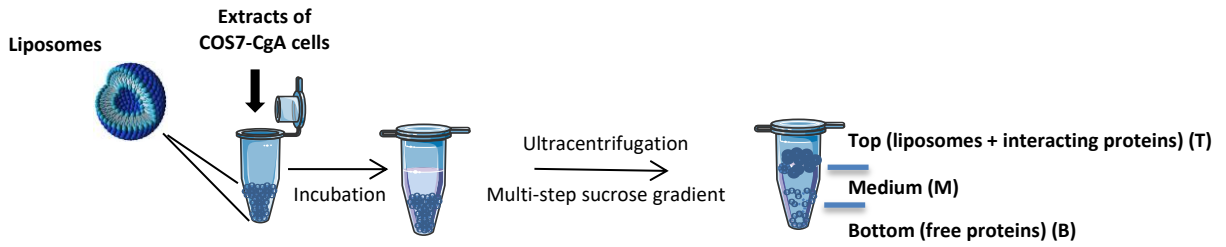
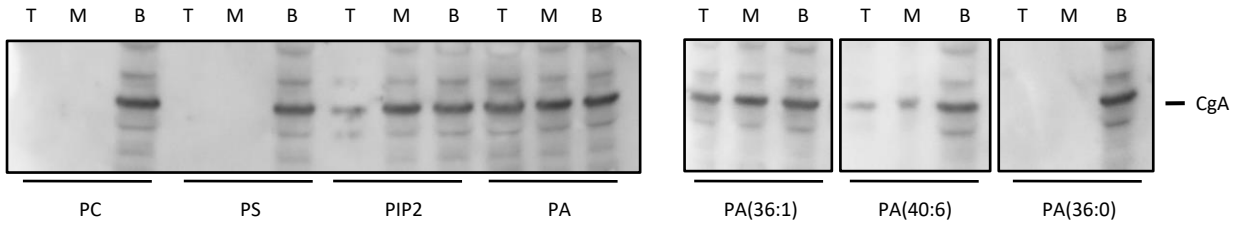


Figure 3

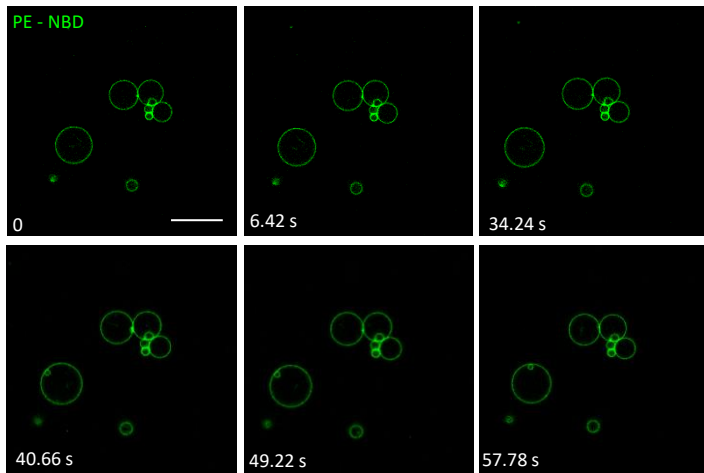
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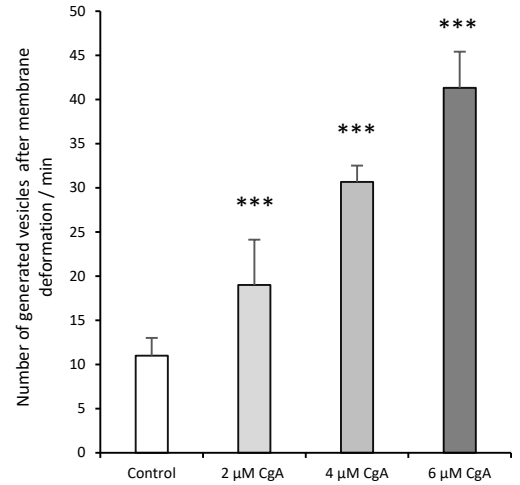
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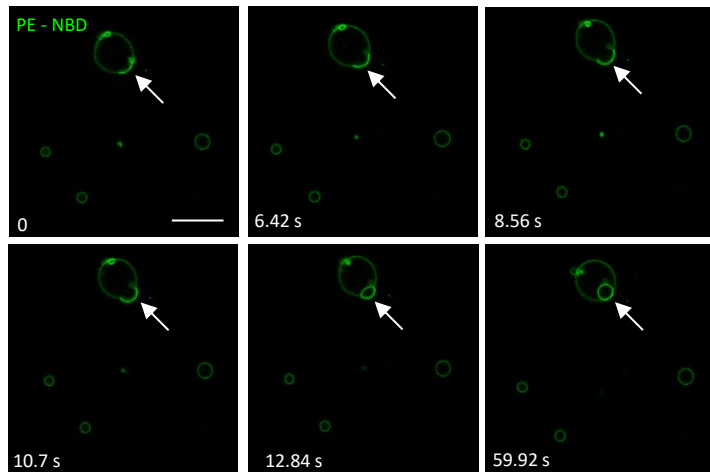
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E



D



F

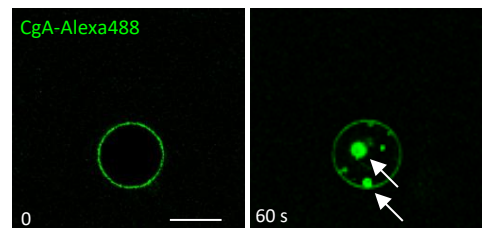


Figure 4

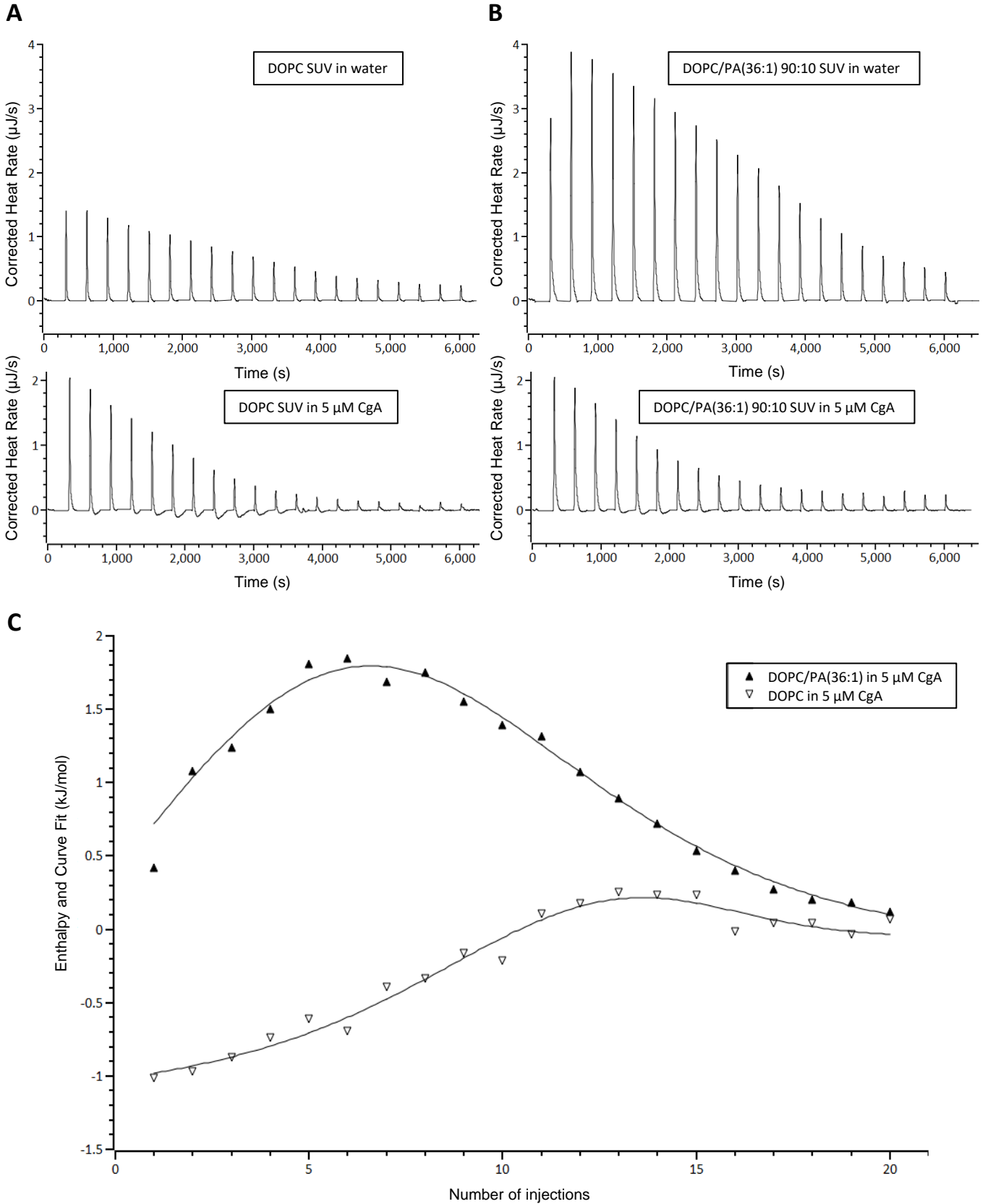


Figure 4

Figure 5

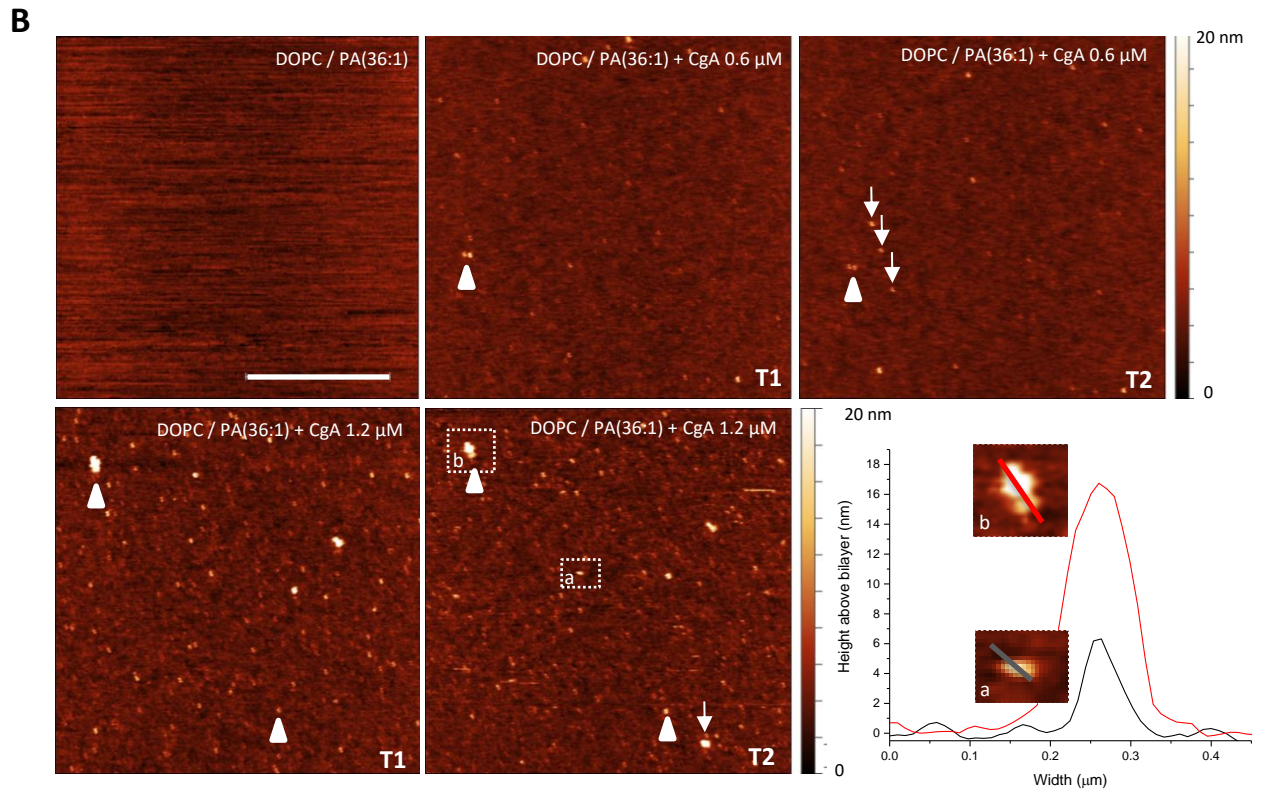
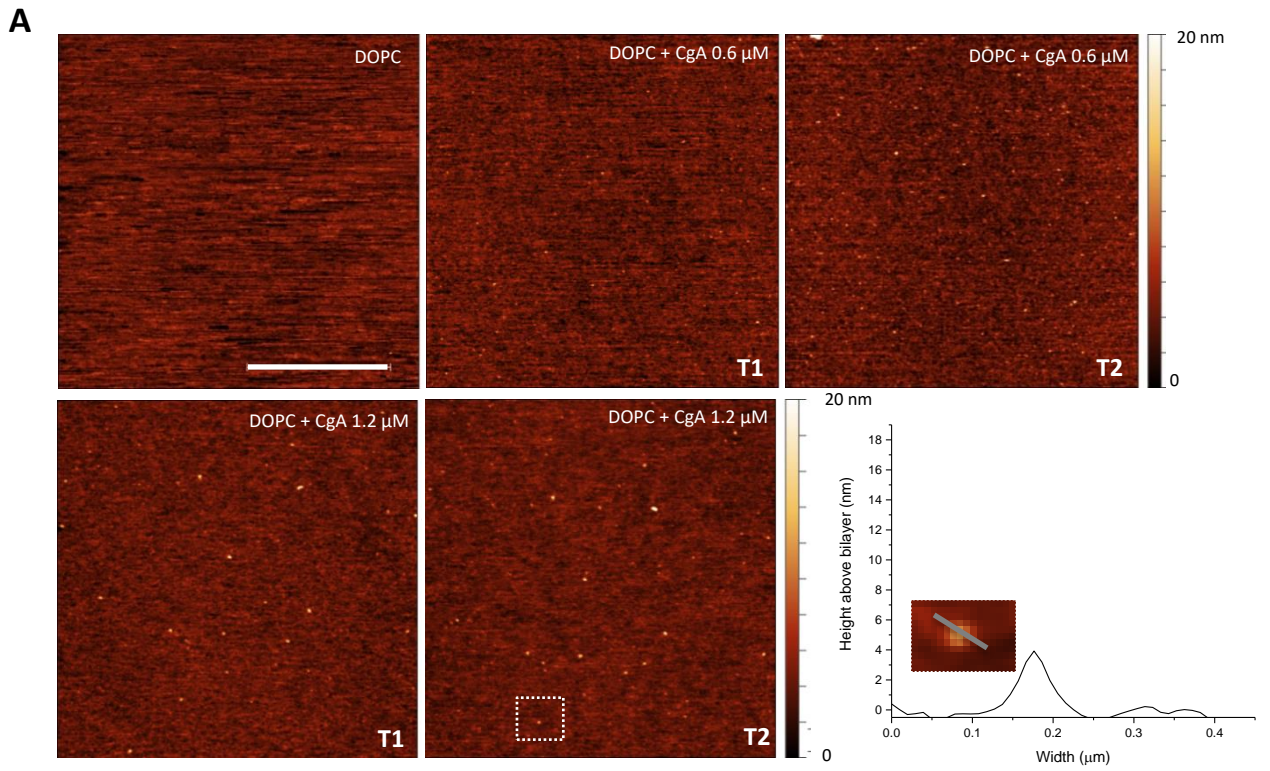
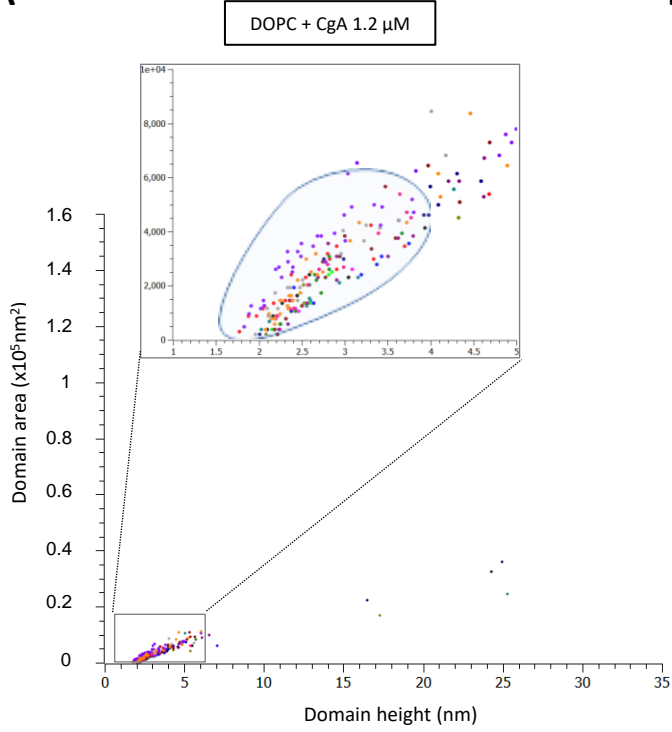


Figure 5

Figure 6

A



B

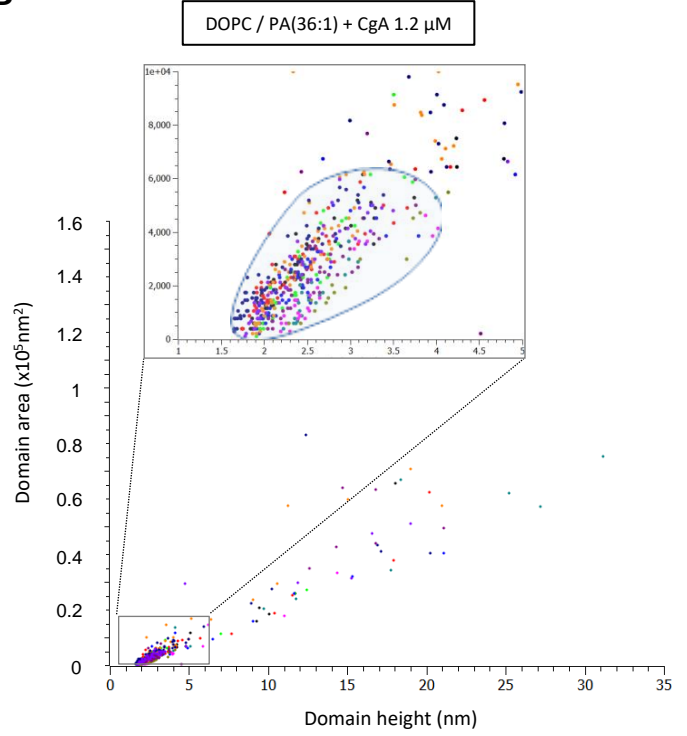


Figure 7

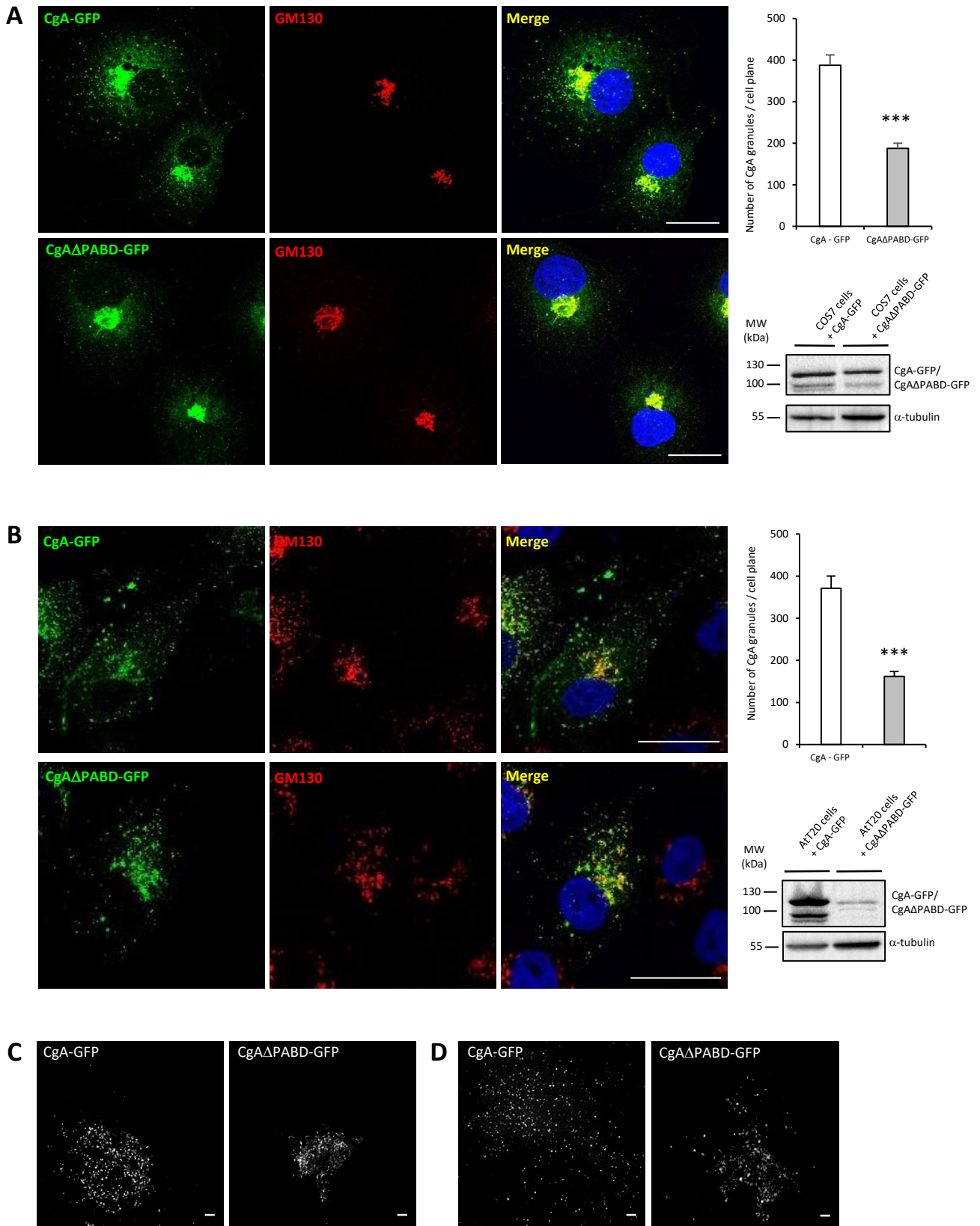


Figure 8

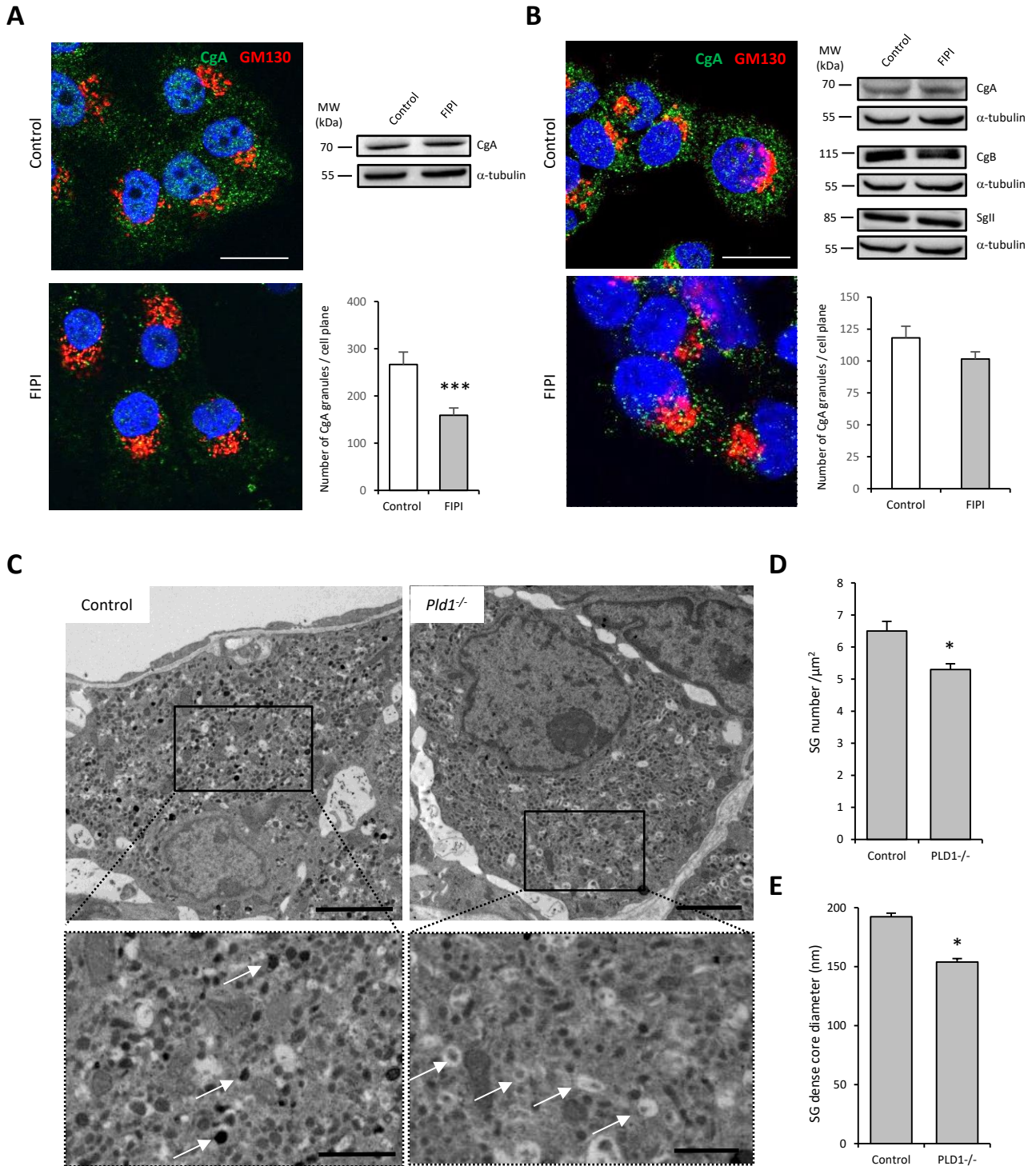


Figure 8

1 SUPPLEMENTAL LEGENDS

2 Supplemental figure 1.

3 A Enrichment of secretory granule membranes from COS7-CgA cells by subcellular
4 fractionation using centrifugation on sucrose gradient. Each fraction was collected and
5 analyzed using SDS-PAGE and SG antibody (anti-RV29.4(CgA), 70 kDa). Surrounding
6 fraction was selected for lipidomic analysis.

7 B Enrichment of Golgi membranes from COS7 and COS7-CgA cells by subcellular
8 fractionation using centrifugation on sucrose gradient. Each fraction was collected and
9 analyzed using SDS-PAGE and immunoblotting with antibodies raised against ER (anti-
10 CALR, 70 kDa), mitochondria (anti-GLUD1, 55 kDa), *cis*-Golgi (anti-GM130, 130 kDa),
11 and *trans*-Golgi (anti-golgin 97, 97 kDa). Surrounding fractions were selected for lipidomic
12 analysis.

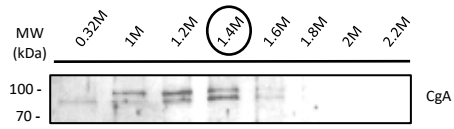
13 **Supplemental figure 2.** Imaging of CgA-GFP positive vesicles in fixed COS7 cells through
14 confocal light scanning microscopy (CLSM) (A) and deconvoluted time-gated STED
15 nanoscopy (STED + Deconvolution) (B). While GFP-CgA-positive structure is described as a
16 single object through CLSM, “STED + Deconvolution” approach reveals the existence of two
17 vesicles (blue arrows). (C) Improvement of lateral resolution (full width at half maximum,
18 FWHM) and signal to noise ratio (SNR) through “STED + Deconvolution” strategy. An
19 intensity profile was measured along a segment drawn on ten vesicles (yellow arrows) and
20 diameter was determined at full width at half maximum (FWHM). Noise (blue squares) and
21 Signal (red circles) were measured in 5 different positions in (A) and (B). Through “STED +
22 Deconvolution” strategy, vesicle diameter is divided by three and SNR is increased sixfold.
23 (D) Graphic representation of the increase in lateral resolution obtained through “STED +
24 Deconvolution” strategy. An intensity profile was measured along a segment and adjusted by
25 using a Gaussian fit. While CgA-GFP positive structure is described as a single object through
26 CLSM (broken line), “STED + Deconvolution” approach reveals the existence of two vesicles
27 (full line).

28 **Supplemental video 1.** Distribution of fluorescence around DOPC/PA/PE-NBD GUVs
29 before CgA addition is homogenous. Confocal live-cell imaging of GUVs containing PE-
30 NBD (green) was registered over a period of 1 min. Still images are shown in Fig. 3C.

31 **Supplemental video 2.** Distribution of fluorescence concentration before membrane
32 deformation of DOPC/PA/PE-NBD GUVs triggered by 2 μM CgA addition is not
33 homogenous. Confocal live-cell imaging of GUVs containing PE-NBD (green) was registered
34 over a period of 1 min. Still images are shown in Fig. 3D.

35

A



B

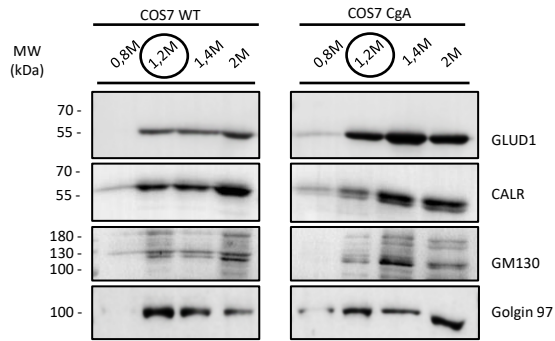
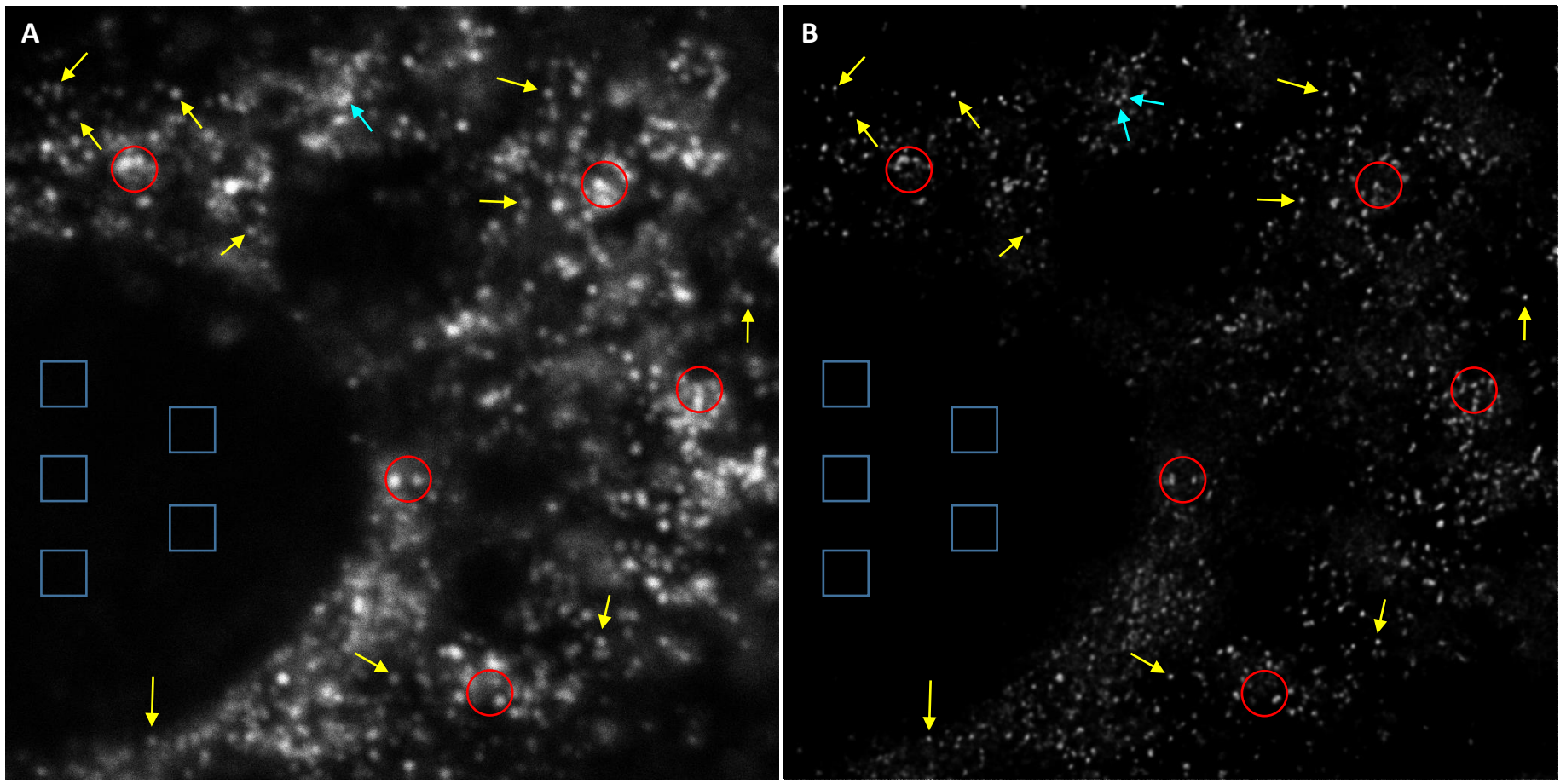


Figure S1



C

	FWHM (nm)	SNR = $\frac{\text{Signal}}{\text{Noise}}$
CLSM	230 ± 17	38 ± 5
STED + Deconvolution	80 ± 9	226 ± 6

D

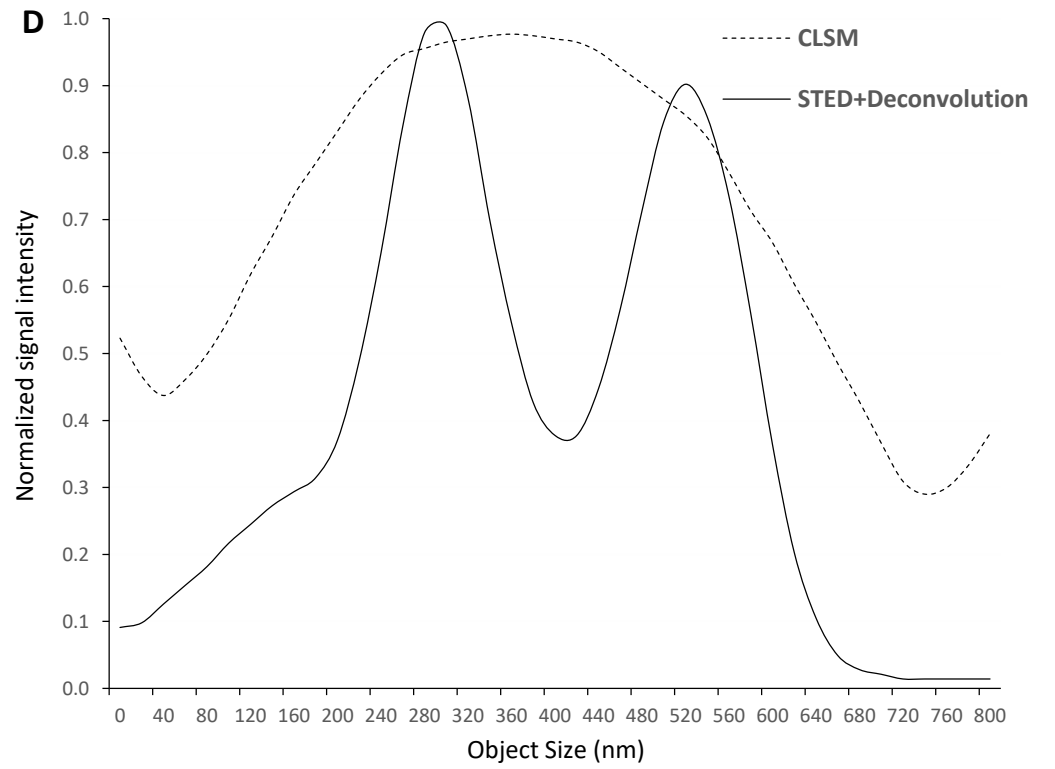


Figure S2