



HAL
open science

Role of the Sec22b/E-Syt complex in neurite growth and ramification

Alessandra Gallo, Lydia Danglot, Francesca Giordano, Bailey Hewlett,
Thomas Binz, Christian Vannier, Thierry Galli

► **To cite this version:**

Alessandra Gallo, Lydia Danglot, Francesca Giordano, Bailey Hewlett, Thomas Binz, et al.. Role of the Sec22b/E-Syt complex in neurite growth and ramification. *Journal of Cell Science*, 2020, 10.1242/jcs.247148 . hal-02928155

HAL Id: hal-02928155

<https://hal.science/hal-02928155>

Submitted on 2 Oct 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Role of the Sec22b/E-Syt complex in neurite growth and ramification**

2

3 Alessandra Gallo^{1,2}, Lydia Danglot¹, Francesca Giordano³, Bailey Hewlett¹, Thomas Binz⁴, Christian
4 Vannier¹, Thierry Galli^{1,5}

5

6 ¹ Université de Paris, Institute of Psychiatry and Neuroscience of Paris (IPNP), INSERM, Membrane
7 Traffic in Healthy & Diseased Brain, Paris, France

8 ² Ecole des Neurosciences de Paris (ENP), Paris, France

9 ³ Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Paris-Sud University, Paris-Saclay
10 University, Gif-sur-Yvette cedex, 91198, France

11 ⁴ Medizinische Hochschule Hannover, Institut für Physiologische Chemie OE4310, 30625,
12 Hannover, Germany

13 ⁵ GHU PARIS psychiatrie & neurosciences, F-75014 Paris, France

14 * to whom correspondence should be addressed: Thierry Galli, INSERM U1266, 102-108 rue de la
15 Santé, 75014 Paris, France, thierry.galli@inserm.fr

16

17

18 **Summary**

19 Axons and dendrites are long and often ramified neurites that need particularly intense plasma
20 membrane (PM) expansion during the development of the nervous system. Neurite growth depends
21 on non-fusogenic Sec22b–Stx1 SNARE complexes at endoplasmic reticulum (ER)-PM contacts.
22 Here we show that Sec22b interacts with the endoplasmic reticulum lipid transfer proteins (LTPs)
23 Extended-Synaptotagmins (E-Syts) and this interaction depends on the Longin domain of Sec22b.

24 Overexpression of E-Syts stabilizes Sec22b-Stx1 association, whereas silencing of E-Syts has the
25 opposite effect. Overexpression of wild-type E-Syt2, but not mutants unable to transfer lipids or
26 attach to the ER, increase the formation of axonal filopodia and ramification of neurites in developing
27 neurons. This effect is inhibited by a clostridial neurotoxin cleaving Stx1, expression of Sec22b
28 Longin domain and a Sec22b mutant with extended linker between SNARE and transmembrane
29 domains. We conclude that Sec22b-Stx1 ER-PM contact sites contribute to PM expansion by
30 interacting with LTPs such as E-Syts.

31

32

33 **Introduction**

34 Tissue and organism expansion is supported by the growth of each cell after each cell division.
35 Plasma membrane (PM) and intracellular membranes growth support cell growth. Cell growth is
36 particularly dramatic in highly polarized cells like neurons. During their development, neurons
37 elaborate processes extending hundreds of microns to meters from the cell body, requiring an
38 increase in their PM surface by 20% per day (Pfenninger, 2009). Hence, compared to other cell
39 types, developing neurons have to face a formidable challenge of adding new membrane to
40 appropriate locations in a manner that requires both high processivity and fine regulation.

41 Membrane expansion during neuronal development has been thought to be mediated by soluble N-
42 ethylmaleimide-sensitive attachment protein receptor (SNARE)-dependent fusion of secretory
43 vesicles with the PM (Wojnacki and Galli, 2016). SNAREs are transmembrane proteins mediating
44 membrane fusion in all the trafficking steps of the secretory pathway. In order for fusion to occur, a
45 *trans*-SNARE complex, composed of three Q-SNAREs (on the acceptor compartment) and one R-
46 SNARE (on the opposing membrane), assemble to bring the opposite lipid bilayers in close proximity
47 and trigger their fusion (Jahn and Scheller, 2006; Südhof and Rothman, 2009). In mammals, the R-
48 SNAREs Syb2/VAMP2, VAMP4 and TI-VAMP/VAMP7 have been implicated in neurite extension
49 (Alberts et al., 2003; Grassi et al., 2015; Martinez-Arca et al., 2001; Schulte et al., 2010). However,
50 single knockouts (KO) mice for VAMP7 (Danglot et al., 2012) or VAMP2 (Schoch et al., 2001) display

51 no major defects in neuronal development, and apparent redundant pathways of neurite outgrowth,
52 mediated by VAMP2, VAMP4 and VAMP7, have been described (Gupton and Gertler, 2010;
53 Racchetti et al., 2010; Schulte et al., 2010). These evidences raised the possibility that several
54 secretory vesicles equipped with different R-SNAREs, as well as complementary non-vesicular
55 mechanisms, could contribute to neurite extension during brain development. Indeed, we previously
56 found that the R-SNARE Sec22b, a conserved endoplasmic reticulum (ER)-localized R-SNARE
57 involved in vesicle fusion within the early secretory pathway (Xu et al., 2000), had an additional and
58 unexpected function in promoting PM expansion during polarized growth. Sec22b concentrates in
59 neuronal growth cones, where it interacts with the neuronal Stx1. Sec22b-Stx1 complex does not
60 mediate fusion, but it rather creates a non-fusogenic bridge between ER and PM. In addition, we
61 showed that increasing the distance between ER and PM, by the insertion of a rigid spacer in
62 Sec22b, reduced neuronal growth, and in budding yeast, the orthologue Sec22/Sso1 complexes
63 contained oxysterol transfer proteins (Petkovic et al., 2014). Based on biophysical experiments with
64 synaptic SNAREs (Li et al., 2007; Zorman et al., 2014), incompletely zippered Sec22b/Stx1 complex
65 would tether ER and PM at distances between 10 and 20 nm, corresponding to the narrowest ER-
66 PM contact sites (Gallo et al., 2016).

67 The critical role of the ER in PM growth is based on its central function in lipid synthesis (Jacquemyn
68 et al., 2017). Once synthesized in the ER, lipids travel to the PM along the secretory pathway or they
69 can be directly transferred at ER-PM contact sites. The ER-integral membrane protein Extended-
70 Synaptotagmin (E-Syt) family mediate lipid transfer at ER-PM contact sites. E-Syts are ER-anchored
71 proteins defined by the presence of a cytosolic synaptotagmin-like mitochondrial lipid-binding protein
72 (SMP) domain and multiple Ca²⁺-binding C2 domains (Giordano et al., 2013; Min et al., 2007).
73 Besides their classical function in tethering ER and PM membranes (Giordano et al., 2013), E-Syts
74 transfer lipids via their SMP domains at ER-PM contact sites (Fernández-Busnadiego et al., 2015;
75 Reinisch and De Camilli, 2016; Saheki et al., 2016; Schauder et al., 2014). On one hand, triple KO
76 of E-Syts 1-3 did not show a major morphological phenotype in neurons suggesting that most
77 function associated with these proteins might be redundant with that of other LTPs (Sclip et al.,
78 2016). On the other hand, overexpression of drosophila E-Syt leads to synaptic overgrowth (Kikuma

79 et al., 2017) and knock out in drosophila led to major growth defect of the pupa (Nath et al., 2019).
80 Therefore, E-Syts may be limiting factors in PM growth and their function may be linked to specific
81 features of neuronal differentiation. Based on these data, we hypothesized that E-Syts might interact
82 with Sec22b-Stx1 complexes, which in turn could enable bulk ER to PM transfer of lipids responsible
83 for specific features of neurite growth. Here we found a novel interaction between the Sec22b-Stx1
84 SNARE complex and members of the E-Syt family. We showed that E-Syts were required to stabilize
85 Sec22b-Stx1 association at ER-PM contact sites and that their overexpression in developing
86 neurons promoted axonal growth and ramification which depended on the presence of the SMP and
87 membrane anchoring domains. Furthermore, this E-Syt-mediated morphogenetic effect was
88 inhibited by botulinum neurotoxin C1, which cleaves Stx1, and the expression of Sec22b Longin
89 domain or a mutant with extended SNARE to transmembrane domain linker. These findings support
90 the conclusion that the ternary association between the LTPs E-Syts, Sec22b and Stx plays an
91 important role in plasma membrane expansion leading to axonal growth and ramification.

92

93

94 **Results**

95 **1. Sec22b and Stx1, 3 interact with the lipid transfer proteins E-Syt2 and E-Syt3.**

96 First, we asked whether Sec22b and PM Stx could interact with LTPs. We focused on the E-Syt
97 family of ER-resident LTPs because of their well established presence at ER-PM contact sites and
98 role in glycerophospholipid transfer (Fernández-Busnadiego et al., 2015; Saheki et al., 2016;
99 Schauder et al., 2014; Yu et al., 2016). We performed GFP-trap precipitation experiments on lysates
100 from different cell lines expressing GFP-tagged PM Stx1 and 3 and Sec22b and tested the presence
101 of E-Syt family members (Fig. 1).

102 HeLa cells were used (Fig. 1A-C), which lack neuronal Stx1 but express the closely related
103 homologue Stx3 (Bennett et al., 1993). Cells were transfected with the GFP-tagged Stx3 (eGFP-
104 Stx3), together with FLAG-Sec22b and either Myc-E-Syt2 (Fig.1A) or Myc-E-Syt3 (Fig. 1C), and cell
105 lysates were subjected to GFP-trap. GFP-Stx3 was able to bring down FLAG-Sec22b, further
106 extending previous results obtained with Stx1 (Petkovic et al., 2014). eGFP-Stx3 was also able to

107 precipitate Myc-E-Syt2 (Fig. 1A) as well as Myc-E-Syt3 (Fig. 1C). A mirror trap experiment using
108 pHluorin (pHL)-tagged Sec22b (Fig. 1B) comparing the association of full length E-Syt2 and SMP
109 domain-lacking E-Syt2, shows that removal of the SMP domain did not impair binding to Sec22b of
110 E-Syt2 thus suggesting that Sec22b might interact with the C-terminus part of E-Syts. We then tested
111 whether a Sec22b-Stx-E-Syts association also occurred in a neuronal-like context. First, we
112 immunoprecipitated endogenous Syntaxins 1 and 3 from E18 rat brains, both of which localize at the
113 neuronal plasma membrane (Darios and Davletov, 2006). We found that endogenous Sec22b and
114 E-Syt2 co-immunoprecipitated (Fig. 1D). PC12 cells were then chosen for further studies because
115 they express neuronal Stx1 and, when treated with NGF, they extend processes similar to those
116 produced by cultured neurons (Greene and Tischler, 1976). Lysates of NGF-differentiated PC12
117 cells transfected with Myc-E-Syt2 and Sec22b-pHL were subjected to GFP-trap precipitation.
118 Sec22b-pHL co-immunoprecipitated Myc-E-Syt2 (Fig. 1E, first lane). In addition, Sec22b-pHL
119 precipitated small amounts of endogenous Sec22b, Stx1 and SNAP25. To assess the extent of the
120 specificity of the interaction between E-Syt2 and Sec22b, we performed GFP-trap in lysates of NGF-
121 differentiated PC12 cells co-expressing Myc-E-Syt2 and various pHL- or GFP-tagged v- and t-
122 SNAREs, i.e. Sec22b Δ L-pHL, the mutant Sec22b lacking the N-terminal Longin domain, GFP-
123 SNAP25, Stx1-pHL, VAMP2-pHL, VAMP4-GFP, and GFP alone as indicated (Fig. 1E). A trace
124 amount of E-Syt2 was found to co-precipitate with GFP and reduced amounts were found in the
125 precipitates of GFP-SNAP25, Stx1-pHL, VAMP2-pHL, VAMP4-GFP (Fig. 1F). The amount of co-
126 precipitated E-Syt2 was greatly reduced while the amount of SNAP25 was increased in the case of
127 Sec22b Δ L-pHL mutant, as compared to wild-type Sec22b (Fig. 1G). These data strongly suggest
128 that Sec22b association with E-Syt2 is specific and requires the N-terminal Longin domain of
129 Sec22b. These results are compatible with Longin domain-dependent presence of Stx1 in Sec22b/E-
130 Syt2 complexes. The fact that Sec22b Δ L-pHL precipitated more SNAP25 than Sec22b-pHL
131 suggests that the Longin domain may prevent SNAP25 from entering the Stx1/Sec22b complex. It
132 is interesting to note, as positive control, that Stx1-pHL and GFP-SNAP25 co-precipitated large
133 amounts their endogenous synaptic SNARE complex partners (Stx1, SNAP25, VAMP2).

134 Taken together GFP-trap experiments support the notion that Sec22b-PM Stx complexes associate
135 with members of the E-Syt family of LTPs and that this interaction occurs both in a non-neuronal and
136 neuronal context. Comparison with the typical synaptic SNARE complex suggests that Stx1 would
137 associate primarily with SNAP25 and VAMP2 whereas complexes with Sec22b would represent a
138 minor pool of Stx1, in agreement with their restricted occurrence of ER-PM contact sites.

139 To go further into the characterization of the Sec22b Δ L mutant, we used surface staining to detect
140 the presence of wild type or mutant Sec22b at the plasma membrane with ectoplasmic antibodies
141 added to living cells. We used again Sec22b-pHL and Sec22b Δ L-pHL chimera in which Sec22b and
142 its mutant version are C-terminally-tagged with pHL, so that anti-GFP antibody present in the
143 medium would bind and reveal only Sec22b that reached the cell surface. N-terminally tagged
144 Sec22b served as a negative control in these experiments, since its GFP tag should never be
145 exposed extracellularly thus be undetectable with anti-GFP antibody without permeabilization. As a
146 positive control for fusion, we used VAMP2-pHL (Fig. S1A). In contrast to the strong positive signal
147 given by VAMP2, we found no detectable Sec22b at the plasma membrane, consistent with its non-
148 fusogenic function (Petkovic et al., 2014) (Fig. S1B-C). On the contrary, significant amounts of
149 Sec22b Δ L were detected at the cell surface (Fig. S1B-C). Thus, Sec22b Δ L was able to mediate
150 fusion to a certain degree with the plasma membrane. In addition, in COS7 cells, Sec22b Δ L mutants
151 did not display the ER localization typical of Sec22b wild type, but it was also found in vesicular-like
152 structures (Fig. S1D), leading to the hypothesis that it might in part escape into secretory vesicles
153 that eventually fuse with the PM because Sec22b Δ L was able to bind both Stx1 and SNAP25 (Fig.
154 1D) These data further suggest the involvement of the Longin domain of Sec22b in the formation of
155 the non-fusogenic Sec22b/Stx1 complex.

156 **2. E-Syt2 and Sec22b are in close proximity in neurites and growth cones.**

157 To determine whether the observed association between Sec22b and E-Syts could also be observed
158 in cells *in situ*, we used *in situ* Proximity Ligation Assay (PLA), live cell imaging and Stimulated
159 Emission Depletion (STED) super-resolution microscopy.

160 Fixed HeLa cells and hippocampal neurons at 3DIV were reacted with Sec22b and E-Syt2 antibodies
161 and processed for PLA. As specificity control of the PLA signal given by Sec22b and E-Syt2, we also
162 tested the proximity of Sec22b with Calnexin, another ER-resident protein not supposed to interact
163 with Sec22b (Fig. 2). Distinct PLA dots were observed in HeLa cells labeled for endogenous Sec22b
164 and E-Syt2 (7.18 ± 0.60 per cell) (Fig. 2A). The PLA signal decreased to an average of 3.30 ± 0.40
165 in cells stained for Sec22b and Calnexin and to an average of 1.15 ± 0.14 and 1.20 ± 0.11 in negative
166 controls, performed by incubating cells with only Sec22b and E-Syt2 antibodies, respectively (Fig.
167 2B). To further confirm these results, we performed live cell imaging in HeLa cells co-expressing
168 mCherry-Sec22b and GFP-E-Syt2. We found co-localization in discrete puncta of Sec22b and E-
169 Syt2, prominently localized in the periphery of the cell. Interestingly, such puncta appeared to be
170 less dynamic compared to corresponding whole protein populations, suggesting that they could
171 represent hot-spots of membrane contact sites populated by Sec22b and E-Syt2 (Movie 1). In
172 neurons, the amount of PLA signal per cell was normalized for the area of neurites, growth cones or
173 cell body (Fig. 2C-F). Interestingly, dots revealing Sec22b-E-Syt2 proximity were more numerous in
174 neurites ($5.53\% \pm 0.60\%$) (Fig. 2D) and growth cones ($7.64\% \pm 1.49$) (Fig. 2E) compared to cell
175 bodies ($2.85\% \pm 0.45\%$) (Fig. 2F), suggesting a preferential interaction of the two proteins in growing
176 processes. Similarly, the PLA signal between Sec22b and Calnexin and in the negative control was
177 strongly reduced as compared to that of Sec22b-E-Syt2.

178 Close proximity between Sec22b-E-Syt2 was confirmed by STED microscopy visualizing Sec22b-
179 pHL and endogenous E-Syt2 localization in growth cones at high resolution (Fig. 3A, C, Movie 2).
180 Spatial distribution analysis of E-Syt2 and Sec22b was done using "Icy SODA" plugin and Ripley's
181 function (Lagache et al., 2018) (Movie 2). When statistically associated, both Sec22b and E-Syt2
182 were detected in growth cones at an average distance of $84.33 \text{ nm} \pm 9.64 \text{ nm}$ of each other. Shorter
183 distances between the two proteins were measured in all the analyzed growth cones. Furthermore,
184 we measured the distance between these two molecules and the PM (Fig. 3D). To this end, we
185 labelled the PM with the Wheat Germ Agglutinin (WGA), which allows the detection of glycoconjugates,
186 via N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) residues, on cell membranes. We
187 found that the median distance d (Fig. 3D) between PM and E-Syt2 coupled to Sec22b-pHL was

188 33.6 nm (4 independent growth cones, 4226 clusters) suggesting that when E-Syt2 and Sec22b
189 are associated they populate ER-PM contact sites to a large extent.

190 **3. E-Syts favor the occurrence of Sec22b-Stx complexes.**

191 As previously reported, E-Syts function as regulated tethers between the ER and the PM and their
192 overexpression stabilizes and increases the density of ER-PM contact sites (Giordano et al., 2013).
193 The question as to whether E-Syts overexpression stabilizes Sec22b-Stx complexes at MCSs was
194 therefore addressed. To do so the PLA technique was used to evaluate Sec22b/Stx3 association in
195 non-transfected or in E-Syt3-overexpressing HeLa cells (Fig. 4A, B). We found that the number of
196 PLA puncta significantly increased from 4.08 ± 0.67 per cell dots counted in non-transfected cells to
197 6.66 ± 0.91 in cells where E-Syt3 is overexpressed (Fig. 4C). We then investigated to which extent
198 impairing the formation of ER-PM contact sites would influence the interaction between Sec22b and
199 Stx3. To do this, we inhibited expression of the three E-Syt isoforms (E-Syt1, E-Syt2 and E-Syt3)
200 simultaneously using siRNAs targeting the mRNAs of these proteins (Fig. 4D, E). Interestingly, the
201 total number of PLA dots given by Sec22b and Stx3 was strongly decreased in the E-Syt-deficient
202 HeLa cells as compared to control cells (Fig. 4F).

203 Taken together, our results indicate that increasing the amount of or abolishing the tethering activity
204 of E-Syts correlates with the probability to observe proximity between Sec22b and Stx3. Thus, E-
205 Syts likely promote the formation of ER-PM contact sites populated by Sec22b-Stx3 complexes.

206 **4. E-Syts overexpression promotes filopodia formation and ramifications in developing** 207 **neurons**

208 At the neuromuscular junction of *Drosophila melanogaster*, overexpression of *Esy1* orthologue,
209 enhances synaptic growth (Kikuma et al., 2017). This prompted us to test the effect of E-Syts
210 overexpression in the growth of cultured neurons. Therefore, rat hippocampal neurons were
211 nucleofected with Myc-E-Syt2 and cultured until 3DIV, a time when axonal polarization is achieved
212 and the major axonal process is distinguishable from the minor dendritic neurites (Dotti et al., 1988).
213 eGFP co-transfection was included to appreciate neuronal morphology of transfected neurons.
214 Overexpression of E-Syt2 induced the formation of actin-positive filopodia and ramifications,

215 particularly in the growing axons (Fig. 5A; Fig. S2). Comparison of the phenotype of neurons
216 expressing Myc-E-Syt2, and neurons expressing E-Syt2 mutant versions, lacking either the SMP
217 (Myc-E-Syt2 Δ SMP[119-294]) or the membrane spanning domain (Myc E-Syt2 Δ MSD[1-72]) was
218 undertaken using the Myc-Empty vector as negative control (Fig. 5B). As expected, Myc E-Syt2
219 Δ MSD[1-72]) appeared soluble and Myc-E-Syt2 Δ SMP[119-294]) still appeared on intra-cellular
220 membranes but with less proximity to Sec22b than WT (Fig. S3). Quantification of morphological
221 parameters of transfected cells showed that neurons overexpressing Myc-E-Syt2 displayed a ~1.5-
222 and a ~2-fold increase in the total neurite length and in the number of branching, respectively, as
223 compared to E-Syt2 mutants or negative control (Fig. 5C). Surprisingly, despite a tendency
224 measured as a ~35% increase, the major neurite was not significantly longer in Myc-E-Syt2
225 overexpressing neurons (Fig. 5C). Noteworthy, E-Syt2 mutants exhibited no significant differences
226 in the parameters analyzed when compared to the negative control. Thus, elevating the expression
227 level of wild type but not mutant E-Syt2 in developing neurons clearly promoted total neurite growth
228 and ramification.

229 **5. E-Syts overexpression stimulates membrane growth in HeLa cells**

230 To assess whether the increase in growth observed in neurons is a general feature resulting from E-
231 Syt2 overexpression, we compared the phenotype elicited by expression of Myc-E-Syt2 and its
232 deleted mutants in non-neuronal HeLa cells, following an experimental paradigm similar to that used
233 in the previous experiment. Briefly, we transfected HeLa with Myc-E-Syt2, Myc-E-Syt2 Δ SMP[119-
234 294], Myc E-Syt2 Δ MSD[1-72] or Myc-Empty vector together with eGFP. Two days after transfection,
235 cell were fixed and the eGFP immunostaining was used to allow a phenotype comparison in the
236 different conditions (Fig. 6A, B). Consistent with the observations in developing neurons, a clear
237 morphogenetic effect was observed. Myc-E-Syt2 overexpressing HeLa cells displayed an enhanced
238 filopodia formation as compared to control and mutants overexpression, as measured by the
239 percentage of the PM spikes area over the total cell surface (Fig. 6C). Taken together, these results
240 provide additional evidence of the involvement of E-Syts in membrane growth, both in neuronal and
241 non-neuronal contexts.

242 **6. E-Syts-mediated morphogenetic effect depends on Stx1**

243 Next, the role of the ternary assembly of E-Syt2, Sec22b and Stx1 in the phenotype of enhanced
244 neuronal growth elicited by E-Syt2 was investigated. To this end, we designed experiments aimed
245 at impairing Stx1 in neurons overexpressing Myc-E-Syt2. Enzymatic disruption of the SNARE
246 Sec22b-Stx1 complex was achieved using botulinum (BoNT) neurotoxins, zinc-endoproteases
247 known to cleave SNARE proteins (Binz, 2013; Binz et al., 2010; Sikorra et al., 2008). For this study,
248 purified BoNT/A was used to cleave SNAP25, BoNT/C1 to cleave both Stx1 and SNAP25, and
249 BoNT/D to cleave VAMP2 (Fig. 7A). We nucleofected rat hippocampal neurons with Myc-E-Syt2
250 and, before fixation at 3DIV, we incubated cells with BoNTs (as indicated in figure legend). Cells
251 incubated with the toxins-diluting culture medium were used as negative control. Since exposure to
252 BoNT/C1 at high concentrations and for long incubation periods causes degeneration of neurons in
253 culture (Igarashi et al., 1996; Osen-Sand et al., 1996), various concentrations and incubation times
254 were tested, and a 4-hour treatment of neurons with 1nM BoNTs was chosen to avoid such
255 deleterious effects. This extremely low concentration of toxins, associated with a relatively short
256 incubation period, is sufficient to induce SNARE cleavage, as shown by reduced protein signals
257 detected after Western blotting (Fig. 7B, C). Assuming that Sec22b, Stx1 and E-Syt2 form a complex,
258 only BoNT/C1 was expected to prevent the E-Syt2-induced phenotype of growth, since this would
259 occur following a cleavage of Stx1. BoNT/A and D, acting on SNAP25 and VAMP2 respectively,
260 should have no effect. Noticeably, the cleavage of Stx1, occurring after BoNTC/1 incubation, was
261 found to cause a ~50% decrease in the extent of branching, resulting in a ~20% reduction of the
262 total neurite length, as compared to neurons incubated with the other tested BoNTs and negative
263 control (Fig. 7D, E). No significant decrease in the length of the major neurite was observed in cells
264 after BoNT/C1 incubation.

265 These results support the conclusion that Stx1 is required for E-Syt2 to promote neuronal growth,
266 as particularly stressed out by ramifications and filopodia formation.

267 **7. E-Syts-mediated morphogenetic effects depend on the close apposition of ER to PM**
268 **mediated by Sec22b-Stx1 complexes**

269 In view of the data reported above, it was necessary to investigate whether the distance between
270 ER and PM of contact sites formed by Sec22b and Stx1 was a determining factor for the acquisition
271 of the E-Syt2-mediated morphological phenotype. To this end, the GFP-Sec22b-P33 mutant was
272 used as previously described (Petkovic et al., 2014). In GFP-Sec22b-P33 the SNARE and
273 transmembrane domains of Sec22b are linked by a stretch of 33 prolines (Fig. 8A). Electron
274 tomography analysis showed that GFP-Sec22b-P33 expression resulted in a 6-nm increase of the
275 ER to PM distance at contact sites, without changing Sec22b localization and its interaction with
276 Stx1 (Petkovic et al., 2014). Co-expression of the GFP-Sec22b-P33 mutant with Myc-E-Syt2 was
277 found to prevent the increase in branching observed in neurons overexpressing E-Syt2 (Fig. 8B, C).
278 This data confirmed and extended results obtained by impairing Stx1 via BoNT/C1. They support
279 the notion that, for E-Syt2 to perform its function in membrane growth, the strict structure of the
280 Sec22b-Stx1 complex is required at ER-PM contact sites.

281 To gain further insight in the requirement of Sec22b in E-Syts mediated neurite growth, the effect of
282 co-expressing the Sec22b Longin domain with E-Syt2 was tested because we showed previously
283 (Fig. 1) that the Longin domain was important for Sec22b/E-Syt2 interaction. Similarly to what was
284 previously reported for the Sec22b-P33 mutant, the expression of the Sec22b Longin domain was
285 found to reduce branching and the overall neurite length in Myc-E-Syt2 overexpressing hippocampal
286 neurons (Fig. 8B, C).

287

288 **Discussion**

289 In the present study, we report on a novel interaction between two ER-resident membrane proteins,
290 the R-SNARE Sec22b and members of the Extended-Synaptotagmin (E-Syts) family and show the
291 functional relevance of this interaction in neuronal differentiation. Indeed we found 1/ an interaction
292 between Sec22b and E-Syt2 which required the Longin domain of Sec22b, 2/ this interaction
293 occurred at ER-PM contact sites particularly in neurites, 3/ overexpression of wild type E-Syt2 but
294 not mutants devoid of lipid transfer or membrane anchoring domains increased filopodia formation,

295 neurite growth and ramification, and 4/ this morphological effect of E-Syt2 overexpression required
296 functional Stx1 and Sec22b and was regulated by the Longin domain of Sec22b.

297 **LTPs as partners of SNARE complexes at MCSs**

298 GFP-trap pull down experiments using Sec22b as a bait systematically captured E-Syts. The N-
299 terminal Longin domain of Sec22b had a prominent role in this interaction because the Longin
300 deletion mutant of Sec22b had a reduced ability to capture E-Syts. PM Stx1 and Stx3 also
301 precipitated Sec22b and E-Syts suggesting a ternary complex. This complex however likely
302 corresponded to a small pool of Stx1 in PC12 cells, when compared to the synaptic SNARE complex.
303 Notably, SNAP25 coprecipitated with Sec22b at a very low level and deletion of the Longin domain
304 increased the amount of coprecipitated SNAP25. This is consistent with the lack of SNAP proteins
305 in Stx1/Sec22b complexes (Petkovic et al., 2014) and the notion that the Longin domain may play a
306 role to exclude SNAP25. Moreover, the 1:1 complex Stx1 and SNAP25 is very abundant and it
307 precedes the recruitment of VAMP2 leading to the formation of the ternary synaptic SNARE complex
308 essential for neurotransmitter release (Fasshauer and Margittai, 2004; Weninger et al., 2008).
309 Hence, we are led to propose a model whereby the binding of a pre-assembled, ER-resident,
310 Sec22b/E-Syts complex to a PM-resident Stx1/SNAP25 complex might displace SNAP25 from Stx1
311 leading to the formation of a non-fusogenic ternary assembly of Sec22b, Stx1 and E-Syts and this
312 hypothetical sequence of event would depend on the presence of the Longin domain (Fig. S4). Other
313 LTPs are expected to interact with Sec22b and PM Stx, as in yeast we previously found Osh2/3
314 associated with Sso1 (Petkovic et al., 2014). Furthermore, in mammalian cells, both Sec22b and
315 Stx1 were shown to interact with the ER-resident VAMP-associated protein VAP-A. (Weir et al.,
316 2001). VAP-A mediates stable ER-PM tethering (Loewen et al., 2003) and binds a wide number of
317 LTPs, such as oxysterol-binding protein (OSBP)-related proteins (ORPs) and ceramide transfer
318 protein (CERT). Identifying the full catalogue of LTP associated with PM Stx and Sec22b will thus
319 be an important future direction to understand in greater details the molecular mechanisms occurring
320 at ER-PM contact sites.

321 **Interdependence of E-Syts and Sec22b-Stx complexes for the establishment of ER-PM** 322 **junctions operating in membrane expansion**

323 The association of Sec22b to PM Syntaxins was dependent upon E-Syts expression because it was
324 increased upon E-Syts overexpression and reduced in the absence of the three E-Syts isoforms. By
325 promoting Sec22b/PM Stx interaction, E-Syts may increase the abundance of close contact sites
326 between ER and PM because SNARE complexes mediate a ~10nm distance. This shortening of the
327 distance between the ER and the PM may further enhance the LTP activity of E-Syts as it was shown
328 using DNA-origami *in vitro* (Bian et al., 2019). In addition, E-Syts interaction may take Sec22b away
329 from its main function of mediating fusion events within the anterograde and retrograde membrane
330 trafficking between ER and Golgi, in association with SNARE partners such as Stx5 or Stx18 (Burri
331 et al., 2003; Liu and Barlowe, 2002). As an ER-resident protein, Sec22b can diffuse over the entire
332 ER network, and thus is expected to visit areas of cortical ER where it can be trapped by E-Syts
333 eventually engaged in an ER-PM tethering (Fig. S4). This in turn increases the probability to bind
334 the PM-residing Syntaxins and form a complex in *trans*-configuration. In this view, E-Syts would have
335 a dual function. Firstly, they would be responsible for promoting the formation of a non-fusogenic
336 membrane-tethering complex with PM containing Sec22b and PM Stx. Secondly, the lipid transfer
337 activity E-Syts would be enhanced by the formation of the minimal ternary complex E-Syts-Sec22b-
338 Stx because the distance between the ER and the PM would be smaller at these interaction sites.
339 This dual activity of E-Syts could be viewed as the generation of a MCS specialized for lipid transfer
340 functioning in membrane growth.

341 **Morphogenetic effect of E-Syt overexpression**

342 Our data showed that E-Syt2 overexpression elicited membrane expansion. It is known that proteins
343 harbouring C2 domains could potentially promote non-specific effects on neurite growth when
344 overexpressed (Park et al., 2014). Here, this possibility can be excluded because the morphological
345 effects of elevated E-Syt2 was abolished in cells expressing versions of the protein lacking the SMP
346 or the membrane-anchoring domains, but still harboring the three C2 modules. The increased neurite
347 growth and filopodia formation induced by E-Syt2 overexpression depended on the presence of a
348 functional Stx1 in neurons because it was prevented by treatment with BoNTC1 but not BoNTs A or
349 D. This morphological phenotype also depended on Sec22b since expression of 33P mutant and the
350 Longin domain alone both reversed the effect of E-Syt2 overexpression. It is thus very clear that E-

351 Syts, Stx1 and Sec22b interact both biochemically and functionally. Interestingly, growth was not
352 impaired in cells expressing E-Syt2 mutants, as we could not detect significant differences in
353 membrane expansion as compared to control cells. Therefore, expression of these mutants did not
354 act in a dominant negative manner. These results suggest that other LTPs share redundant function
355 with E-Syts. Furthermore, *Esyts* triple knockout mice display no major defects in neuronal
356 development and morphology (Sclip et al., 2016) whereas recent data showed that dE-Syt knockout
357 in the fly led to major growth defect (Nath et al., 2019). Taken together, these evidences suggest
358 that a functional redundancy exists among LTPs in promoting membrane growth in mammals, as
359 the removal of one class of such proteins can be compensated by the activity of the others. The
360 precise contribution of each class LTPs, as well as their mutual interplay, will require further studies.
361 The effect of E-Syt overexpression suggest that LTP may be limiting factors and that fine tuning of
362 their expression level may be critical for their function. FGFR was shown to regulate the expression
363 of E-Syt2 in *Xenopus* embryos (Jean et al., 2010). Therefore, it will be particularly interesting to
364 search if growth factors which promote axonal filopodia formation (Menna et al., 2009) might also
365 control the expression level of LTPs such as E-Syts. Since actin instability is important for axon
366 formation (Bradke and Dotti, 1999), and actin dynamics is regulated by lipidic rafts (Caroni, 2001), it
367 will also be particularly interesting to characterize the lipidic composition of E-Syt-generated filopodia
368 plasma membrane using dyes in living cells.

369 In conclusion, the protein complex between E-Syts and Sec22b unraveled here appears as an
370 important model for further studies aimed at understanding how lipid transfer at MCS between the
371 ER and PM could participate to the development of the neuronal cell shape. As exemplified in the
372 case of other SNAREs like VAMP7 (Daste et al., 2015), our results point to central regulatory function
373 of the Longin domain of Sec22b.

374

375

376 **Materials and Methods**

377 **Antibodies**

378 Primary antibodies used in this study were: mouse monoclonal anti-Sec22b 29-F7 (Santa Cruz, sc-
379 101267); rabbit polyclonal anti-ESYT2 (Sigma-Aldrich, HPA002132); rabbit polyclonal anti-Calnexin
380 (Cell Signaling, 2433S); mouse monoclonal anti-GFP (Roche, 11814460001); mouse monoclonal
381 anti-c-myc 9E10 (Roche, 11667203001); mouse monoclonal anti-FLAG M2 (Sigma-Aldrich); mouse
382 monoclonal anti-Stx1 HPC-1 (Abcam, (ab3265); mouse monoclonal anti-SNAP25 (Synaptic
383 Systems, 111011); rabbit polyclonal anti-VAMP2 (Synaptic Systems, 104 202); rabbit polyclonal anti-
384 β 3 tubulin (Synaptic Systems, 302 302); chicken polyclonal anti-MAP2 (AbCam, ab5392); goat
385 polyclonal anti-GFP (AbCam, ab6673); Alexa Fluor™ 633 Phalloidin (ThermoFisher, A22284).
386 Rabbit polyclonal anti-Sec22b (Clone TG51) and rabbit polyclonal anti-Syntaxin3 (Clone TG0) were
387 produced in the laboratory.

388 **Plasmids and cDNA clones**

389 Plasmids encoding eGFP-ESyt2, eGFP-ESyt3, myc-ESyt2 and myc-ESyt3 were obtained from P.
390 De Camilli (Giordano et al, 2013). mCherry-Sec22b, the pHluorin-tagged forms of Syntaxin1,
391 Sec22b, and VAMP2 and GFP-tagged forms of Sec22b-P33 mutant, of Sec22b-Longin (Ribault et
392 al., 2011. Petkovic et al., 2014), of VAMP4 and of SNAP25 (Mallard et al., 2002, Martinez Arca et al.
393 2000) were previously described. cDNA clones of human Sec22b and Syntaxin2 and Syntaxin3 were
394 obtained from Dharmacon (GE Healthcare) (Sec22b: Clone3051087, Accession BC001364;
395 Syntaxin2: Clone 5296500, Accession BC047496; Syntaxin3: Clone 3010338, Accession
396 BC007405).

397 **Construction of Δ LonginSec22b -pHluorin:**

398 Δ LonginSec22b-pHluorin was generated from ERS24-pQ9 construct and cloned into pEGFPC1-
399 pHluorin using the following primers:

400 Forward NheI Sec22deltaLongin,

401 5'-CGCGCTAGCATGCAGAAGACCAAGAACTCTACATTGAT

402 Reverse EcoRI Sec22deltaLongin,

403 5'-CGGGAATTCCAGCCACCAAAACCGCACATACA

404 **Construction of 3XFLAG-Sec22b, EGFP-Syntaxin2 and EGFP-Syntaxin3:**

405 cDNAs of human Sec22b, Syntaxin2 and Syntaxin3 ORFs were amplified by PCR using the following
406 primers:

407 Forward EcoRI-Sec22b,

408 5'-CAAGCTTC GAATTC ATGGTGTGCTAACAATGATC

409 Reverse BamHI-Sec22b,

410 5'- TCCGATTCTGGTGGCTGTGAGGATCCACCGGTCG

411 Forward Sall-Syntaxin2,

412 5'- ACCG GTCGAC ATGCGGGACCGGCTGCCAGA

413 Reverse SacII- Syntaxin2,

414 5'- ATCCTAGCAACAACATTGTCCTAGCCGCGGCGGT

415 Forward Sall-Syntaxin2,

416 5'- ACCG GTCGAC ATGAAGGACCGTCTGGAGCAG

417 Reverse SacII- Syntaxin2,

418 5'- GACTTTCCGTTGGGCTGAATTAACCGCGGCGGT

419 PCR products were ligated into the p3XFLAGCMV10 (Sigma-Aldrich) and pEGFP-C1 (Clontech)
420 vectors, respectively to generate 3XFLAG-Sec22b, EGFP-Syntaxin2 or EGFP-Syntaxin3.

421 **Construction of Δ SMP myc-ESyt2 and Δ MSD myc-ESyt2:**

422 Δ SMP myc-ESyt2 and Δ MSD myc-ESyt2 mutants were generated by site-directed mutagenesis by
423 deleting respectively fragment [119-294] and [1-72] using the following primers:

424 Forward DeltaSMP,

425 5'-CTGGGTTTCAATTTCCAGACACTGAAAGTGAAGTTCAAATAGCTCAGTTGC

426 Reverse DeltaSMP,

427 5'-CCAACTGAGCTATTTGAACTTCACTTTCAGTGTCTGGAAAATGAACCCAG

428 Forward DeltaMSD,

429 5'-AGATCTCGAGCTCAAGCTTCGAATTCTCGCAGCCGCGGCCTCAAG

430 Reverse DeltaMSD,
431 5'-CTTGAGGCCGCGGCTGCGAGAATTCGAAGCTTGAGCTCGAGATCT

432 **Cell culture, siRNA and transfection**

433 HeLa cells were cultured in DMEM containing GlutaMax (ThermoFisher 35050038) and
434 supplemented with 10% (v/v) Bovine Fetal Calf Serum (Biosera 017BS346.) and 1% (v/v)
435 penicillin/streptomycin (ThermoFisher 15140122) at 37°C and 5% CO₂. Transfections were carried
436 out with Lipofectamine 2000 transfection reagent (ThermoFisher 11668027) according to
437 manufacturer's instructions. For knockdowns, HeLa cells were transfected with control or E-Syts
438 siRNA oligos by using Oligofectamine transfection reagent (ThermoFisher 12252011) according to
439 manufacturer's instructions. Double-stranded siRNAs targeting the three human E-Syts and control
440 siRNAs were as described (Giordano et al. 2013). Routinely, transfected cells were cultured for 24
441 or 48 hours on coverslips prior to analysis.

442 PC12 cells were grown at 37°C and 5% CO₂ in RPMI containing and 10% (v/v) horse serum
443 (ThermoFisher 26050088), 5% (v/v) Bovine Fetal Calf Serum (Biosera 017BS346) and 1% (v/v)
444 penicillin/streptomycin. Cells were coated on plastic dishes with a 1 µg/ml collagen (Sigma C7661)
445 solution. Then, cells were differentiated with hNGF-α (Sigma-Aldrich N1408) at 50 ng/ml for 3–4
446 days. PC12 transfections were carried out with Amaxa Nucleofection Kit V (Lonza VCA-1003)
447 according to manufacturer's instructions.

448 All experiments involving rats were performed in accordance with the directive 2010/63/EU of the
449 European Parliament and of the Council of 22 September 2010 on the protection of animals used
450 for scientific purposes. Hippocampal neurons from embryonic rats (E18) were prepared as described
451 previously (Dotti et al. 1988) and modified (Danglot et al.2012). Cells were grown on onto poly-L-
452 lysine-coated 18-mm coverslips (1 mg/ml) or 30-mm plastic dishes (0.1 mg/ml) at a density of 25,000-
453 28,000 cells/cm² in Neurobasal-B27 medium previously conditioned by a confluent glial feeder layer
454 [Neurobasal medium (ThermoFisher 21103049) containing 2% B27 supplement (ThermoFisher
455 A3582801), and 500 µM L-Glutamine (ThermoFisher 25030024)]. Neurons were transfected before
456 plating by using Amaxa Rat Neuron Nucleofection Kit (Lonza VPG-1003) following manufacturer's

457 instructions. After 3 days *in vitro* (3DIV), neurons were processed for immunofluorescence or lysed
458 for immunoblot assays.

459 **Botulinum Neurotoxins (BoNTs) treatment**

460 BoNT/A and BoNT/C were a kind gift from Dr Thomas Binz (Hannover Medical School, Germany),
461 BoNT/D (described in Schiavo et al., 2000). Toxins working concentration at 1 nM in culture media
462 were prepared from 4 μ M stock solutions.

463 Hippocampal neurons overexpressing Myc-E-Syt2 were treated with BoNT/A, BoNT/C, BoNT/D or
464 naïve culture media at 3DIV and maintained for 4 hours at 37°C and 5% CO₂. After extensive
465 washing with culture media, cells were processed for immunofluorescence or lysed for immunoblot
466 assays.

467 **Immunofluorescence**

468 HeLa cells and hippocampal neurons at 3DIV were fixed on coverslips in 4% paraformaldehyde in
469 PBS for 15 minutes and quenched in 50 mM NH₄Cl in PBS for 20 min. Cells were then permeabilized
470 in 0.1% (w/v) Triton X-100 in PBS for 5 min and blocked in 0.25% (w/v) fish gelatin in PBS for 30
471 min. Primary antibodies were diluted in 0.125% fish gelatin in PBS and incubated overnight at 4°C.
472 After washing, secondary antibodies conjugated with Alexa 488, 568, 594 or 647 were incubated for
473 45 min at room temperature before mounting in Prolong medium (ThermoFisher P36930).

474 **Surface staining**

475 Hippocampal neurons at 3DIV were placed on an ice-chilled metallic plate. Neurobasal medium was
476 replaced with ice cold DMEM with 20 mM Hepes containing primary antibody (mouse GFP). Cells
477 were incubated for 5-10 min on ice. Cells were then washed with PBS at 4°C and fixed with 4%
478 para-formaldehyde sucrose for 15 min at room temperature. Following fixation, cells were subjected
479 to the already described whole cell staining protocol. The total pool of tagged proteins was detected
480 with anti-goat GFP antibody. Images were acquired on the epifluorescent microscope with the same
481 exposure in all conditions. Presence at the plasma membrane was expressed as ratio between total
482 and surface signal.

483 **In situ proximity ligation assays (PLA)**

484 In situ proximity ligation assays (PLA) to quantify protein vicinity in HeLa cells and in neurons on
485 coverslips as indicated in figures were performed using the Duolink In Situ Detection Reagents
486 Orange kit (Sigma-Aldrich, DUO92007). Cells were fixed, permeabilized as described above,
487 blocked in Duolink Blocking solution (supplied with the kit) for 30 min at 37°C in a humidified
488 chamber. This was followed by incubation with the primary antibodies rabbit anti-Stx3 (4µg/ml), or
489 rabbit anti-E-Syt2 (2µg/ml) or rabbit anti-Calnexin (2µg/ml) and mouse monoclonal anti-Sec22b
490 (2µg/ml). For the rest of the protocol the manufacturer's instructions were followed. Briefly, cells were
491 washed in kit Buffer A 3 times for 15 minutes and incubated with the PLA probes Duolink In Situ PLA
492 Probe Anti-Mouse PLUS (Sigma-Aldrich, DUO92001) and Duolink In Situ PLA Probe Anti-Rabbit
493 MINUS (Sigma-Aldrich, DUO92005) for 1 hour at 37°C in a humid chamber followed by two washes
494 of 5 min in Buffer A. The ligation reaction was carried out at 37°C for 30 min in a humid chamber
495 followed by two washes of 5 min in Buffer A. Cells were then incubated with the amplification-
496 polymerase solution for 100 min at 37°C in a dark humidified chamber. After two washings with kit
497 Buffer B for 10 minutes followed by a 1-minute wash with 0.01x Buffer B, cells were mounted using
498 the Duolink In Situ Mounting Medium with DAPI (Sigma-Aldrich, DUO92040).

499 **Microscopy and image analysis**

500 **Confocal imaging:** Z-stacked confocal images of neurons and HeLa cells were acquired in a Leica
501 TCS SP8 confocal microscope (Leica Microsystems CMS GmbH), using a 63x/1.4 Oil immersion
502 objective.

503 **HeLa cells live imaging:** HeLa co-expressing mCherry-Sec22b and GFP-E-Syt2 were transferred
504 to an imaging chamber (Chamlide EC) and maintained in Krebs-Ringer buffer (140mM NaCl, 2.8mM
505 KCl, 1mM MgCl₂, 2mM CaCl₂, 20mM HEPES, 11.1mM glucose, pH 7.4). Time-lapse videos were
506 recorded at 5s intervals for 2min using an inverted DMI6000B microscope (Leica Microsystems)
507 equipped with a 63X/1.4-0.6 NA Plan-Apochromat oil immersion objective, an EMCCD digital camera
508 (ImageEMX2, Hamamatsu) and controlled by Metamorph software (Roper Scientific, Trenton, NJ).
509 To virtually abrogate latency between two channels acquisition, illumination was sequentially

510 provided by a 488nm- and a 561nm-diode acousto-optically shuttered lasers (iLas system; Roper
511 Scientific) and a dualband filter cube optimized for 488/561nm laser sources (BrightLine; Semrock)
512 was used. Environmental temperatures during experimental acquisitions averaged 37°C. FIJI
513 software was used for bleaching correction and for movies montage. Binary mask of particles was
514 generated by applying the wavelet-based spot detector plugin of Icy imaging software
515 (<http://icy.bioimageanalysis.org>) to each channel sequence.

516 **STED imaging:** Neuronal membrane was labeled on live neurons using the lectin Wheat Germ
517 Agglutinin (WGA) coupled to Alexa 488 nm for 10 min at 37°C. Neurons were washed and fixed with
518 a 4% PFA /0,2% Glutaraldehyde mixture and then processed for immunochemistry. Growth cone
519 were imaged with 3D STED microscopy using the 775 nm pulsed depletion laser and motorized
520 collar 93X Glycerol objective. Depletions were carried out on primary antibody to endogenous E-
521 Syt2 labelled with secondary antibodies coupled to Alexa 594. Sec22b-pHL were labelled with
522 primary anti-GFP and ATTO647N secondary antibodies. Acquisitions were done in 3D STED so that
523 the voxel size were isotropic. Typically, we imaged a matrix of 1400x1400 pixels over 20 to 25 z
524 planes to include all the growth cone volume. Growth cone was segmented using Icy “HK means”
525 plugin (<http://icy.bioimageanalysis.org/plugin/hk-means/>). Spatial distribution analysis of E-Syt2-and
526 Sec22 was done using “Icy SODA” plugin (Lagache et al, 2018) and a dedicated LD protocol
527 automation. Briefly, E-Syt2 and Sec22b distribution were analyzed through the Ripley function.
528 Statistical coupling between the two molecules was assessed in concentric target. Over 13784 E-
529 Syt2 clusters analyzed in 4 different growth cones, 20 percent were statistically associated with
530 Sec22b. When associated, the 2 molecules were at 84nm ±9nm, which corresponds to 34% of
531 Sec22b clusters. This coupling is of very high significance since the p value was ranging between
532 10^{-5} and 10^{-24} . Distance to the plasma membrane (d) was measured using the Icy ROI inclusion
533 analysis plugin (Lagache et al, 2018).

534 **PLA signal:** Maximum intensity projections of a confocal z-stack including a whole cell were
535 performed to observe the maximum amount of PLA puncta. The number of puncta per cell was
536 counted using the Cell Counter plugin in Fiji/ImageJ. In neurons, the PLA puncta were separately

537 counted in cell body and neurites and the PLA signals were divided by the area of the corresponding
538 compartments.

539 **Neurite length.** For the analysis of neurite length of cultured neurons, images were analyzed using
540 the NeuronJ plugin in Fiji/ImageJ on the maximal intensity projections of z-stacks of the eGFP
541 channel. Main process and branches were measured for each neuron. We could not detect any
542 association among individual cells thereby we considered each cell a sampling unit.

543 **Area of spikes:** in Myc-E-Syt2 overexpressing HeLa cells, the spikes area was measured on the
544 maximal intensity projections of z-stacks of the eGFP channel. The measurement was carried by
545 subtracting the Region on Interest (ROI) of the cell without spikes, obtained with the tool
546 Filters/Median on Fiji/ImageJ by applying a radius of ~80 pixels, from the ROI of the entire cell.

547 **GFP-Trap pull-down and Immunoblotting**

548 Transfected HeLa or PC12 cells were lysed in TBS (20mM Tris-HCl, pH7.5, 150mM NaCl),
549 containing 2mM EDTA, 1% Triton-X100 and protease inhibitors (Roche Diagnostics). of Clarified
550 lysate was obtained by centrifugation at 16,000xg 15 min, and 1 mg protein was submitted to GFP-
551 Trap pull-down for 1 hour at 4 °C under head-to-head agitation. using 10µl of Sepharose-coupled
552 GFP-binding protein (Rothbauer et al., 2008) prepared in the lab After four washes with lysis buffer,
553 beads were heathed at 95°C for 5 min in reducing Laemmli sample buffer. Soluble material was
554 processed for SDS-PAGE using 10 % acrylamide gels and electrotransferred on nitrocellulose
555 membranes (GE-healthcare). The membranes were blocked with 2.5% (w/v) skimmed milk, 0.1%
556 (w/v) Tween-20 in PBS. Membrane areas of interest were incubated with primary antibodies as
557 indicated in figure legends. After washing, the membranes were blotted with HRP-coupled secondary
558 antibodies. Revelation was carried out by using a ChemiDoc luminescence imager (BioRad).

559 **Co-Immunoprecipitation from brain extract**

560 Rat cortex from E18 embryos was homogenized at 0-2°C in 20mM Tris:HCl pH 7.4, 150mM NaCl,
561 4mM EDTA, 2mM MgCl₂ and protease inhibitors (Roche Diagnostics), then lysed for 30 min after
562 adding 2% (w/v, final concentration) Triton X-100. After centrifugation at 14000xg for 12 min proteins

563 were assayed in the clear lysate. Typically 250 mg of tissue yielded extracts at ~11 mg/ml in 1.3 ml.
564 Five milligrams of total proteins (0.6 ml) were incubated with either 5 µg of protein G-bound Rabbit
565 anti-Syntaxin-3 antibody (clone TG0) (Sepharose beads) or 5 µg of naive rabbit IgGs (final volume
566 0.65 ml), for 1 h at 4°C in 0.8-ml sealed Mobicol columns under end-over-end rotation. After removal
567 of unbound material, beads were washed four times with 0.5 ml of cold 20mM Tris:HCl pH 7.4,
568 150mM NaCl, 4mM EDTA, 0.75% (w/v) Triton X-100. Bound material was eluted by a 5-min heating
569 at 95°C with reducing Laemmli sample buffer. The whole eluate was loaded on a 10%-acrylamide
570 Laemmli gel. Clear lysate (50 µg protein, 6 µl in 20 µl reducing Laemmli sample buffer) was run in
571 parallel. Following Western blotting, samples were incubated with antibodies after cutting when
572 appropriate, during 1 h at room temperature for mAb HPC-1Anti-Syntaxin or overnight at 4°C for
573 biotinylated rabbit anti-Esyt2 antibody. Detection of Sec22b was carried out after a low pH -stripping
574 of the membrane piece used for Syntaxin detection, to eliminate excess of rabbit immunoglobulin
575 light chains, and incubation with rabbit anti-Sec22b antibody (overnight at 4°C). Syntaxin and Sec22b
576 immunoreactivity were revealed by Enhanced chemiluminescence and Esyt2 with Alexa680-
577 conjugated Streptavidine on the Odyssey system (LI-COR Biosciences).

578 **Statistical analysis**

579 Calculations were performed in Microsoft Excel. GraphPad Prism software were used for statistical
580 analyses. For each dataset, at least three independent experiments were considered and all data
581 are shown as mean ± SEM. Data were analyzed using one-way ANOVA followed by Dunn's, Tukey
582 or Dunnet post-hoc tests were applied as indicated in figure legends. Non-parametric tests were
583 used when samples did not follow a normal distribution.

584 **Acknowledgements**

585 Work in our group was funded by grants from Association Française contre les Myopathies
586 (Research Grant 16612), the French National Research Agency (*NeuroImmunoSynapse* ANR-13-
587 BSV2-0018-02; *MetDePaDi* ANR-16-CE16-0012), the Institut National du Cancer (PLBIO 2018-
588 149), the Fondation pour la Recherche Médicale (FRM), *Who am I?* Labex (Idex ANR-11-IDEX-
589 0005-01), awards of the Association Robert Debré pour la Recherche Médicale and Fondation

590 Bettencourt-Schueller, Idex USPC ('Conventional And Unconventional Secretion In Neurite Growth')
591 to TG. We acknowledge the ImagoSeine core facility of the Institut Jacques Monod, member of the
592 France Biolmaging (ANR-10-INBS-04) for their services. We acknowledge the Neurlmag and B&B
593 technological core facilities of IPNP for technical support. We thank Leducq Foundation for funding
594 the LEICA TCS SP8 system.

595

596

597 **References**

- 598 **Alberts, P., Rudge, R., Hinners, I., Muzerelle, A., Martinez-Arca, S., Irinopoulou, T., Marthiens,**
599 **V., Tooze, S., Rathjen, F., Gaspar, P., et al.** (2003). Cross talk between tetanus neurotoxin-
600 insensitive vesicle-associated membrane protein-mediated transport and L1-mediated
601 adhesion. *Mol. Biol. Cell* **14**, 4207–4220.
- 602 **Bennett, M. K., García-Arrarás, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hazuka, C.**
603 **D. and Scheller, R. H.** (1993). The syntaxin family of vesicular transport receptors. *Cell* **74**,
604 863–873.
- 605 **Bian, X., Zhang, Z., De Camilli, P. and Lin, C.** (2019). A programmable DNA-origami platform for
606 studying protein-mediated lipid transfer between bilayers. *BioRxiv*.
- 607 **Binz, T.** (2013). Clostridial neurotoxin light chains: devices for SNARE cleavage mediated blockade
608 of neurotransmission. *Curr. Top. Microbiol. Immunol.* **364**, 139–157.
- 609 **Binz, T., Sikorra, S. and Mahrhold, S.** (2010). Clostridial neurotoxins: mechanism of SNARE
610 cleavage and outlook on potential substrate specificity reengineering. *Toxins (Basel)* **2**, 665–
611 682.
- 612 **Bradke, F. and Dotti, C. G.** (1999). The role of local actin instability in axon formation. *Science* **283**,
613 1931–1934.
- 614 **Burri, L., Varlamov, O., Doege, C. A., Hofmann, K., Beilharz, T., Rothman, J. E., Söllner, T. H.**
615 **and Lithgow, T.** (2003). A SNARE required for retrograde transport to the endoplasmic
616 reticulum. *Proc. Natl. Acad. Sci. USA* **100**, 9873–9877.
- 617 **Caroni, P.** (2001). New EMBO members' review: actin cytoskeleton regulation through modulation
618 of PI(4,5)P(2) rafts. *EMBO J.* **20**, 4332–4336.
- 619 **Danglot, L., Zylbersztejn, K., Petkovic, M., Gauberti, M., Meziane, H., Combe, R., Champy, M.-**
620 **F., Birling, M.-C., Pavlovic, G., Bizot, J.-C., et al.** (2012). Absence of TI-VAMP/Vamp7
621 leads to increased anxiety in mice. *J. Neurosci.* **32**, 1962–1968.
- 622 **Darios, F. and Davletov, B.** (2006). Omega-3 and omega-6 fatty acids stimulate cell membrane
623 expansion by acting on syntaxin 3. *Nature* **440**, 813–817.
- 624 **Daste, F., Galli, T. and Taresté, D.** (2015). Structure and function of longin SNAREs. *J. Cell Sci.*
625 **128**, 4263–4272.

- 626 **Dotti, C. G., Sullivan, C. A. and Banker, G. A.** (1988). The establishment of polarity by hippocampal
627 neurons in culture. *J. Neurosci.* **8**, 1454–1468.
- 628 **Fasshauer, D. and Margittai, M.** (2004). A transient N-terminal interaction of SNAP-25 and syntaxin
629 nucleates SNARE assembly. *J. Biol. Chem.* **279**, 7613–7621.
- 630 **Fernández-Busnadiego, R., Saheki, Y. and De Camilli, P.** (2015). Three-dimensional architecture
631 of extended synaptotagmin-mediated endoplasmic reticulum-plasma membrane contact
632 sites. *Proc. Natl. Acad. Sci. USA* **112**, E2004–13.
- 633 **Gallo, A., Vannier, C. and Galli, T.** (2016). Endoplasmic Reticulum-Plasma Membrane
634 Associations: Structures and Functions. *Annu. Rev. Cell Dev. Biol.* **32**, 279–301.
- 635 **Giordano, F., Saheki, Y., Idevall-Hagren, O., Colombo, S. F., Pirruccello, M., Milosevic, I.,
636 Gracheva, E. O., Bagriantsev, S. N., Borgese, N. and De Camilli, P.** (2013). PI(4,5)P(2)-
637 dependent and Ca(2+)-regulated ER-PM interactions mediated by the extended
638 synaptotagmins. *Cell* **153**, 1494–1509.
- 639 **Grassi, D., Plonka, F. B., Oksdath, M., Guil, A. N., Sosa, L. J. and Quiroga, S.** (2015). Selected
640 SNARE proteins are essential for the polarized membrane insertion of igf-1 receptor and the
641 regulation of initial axonal outgrowth in neurons. *Cell Discov.* **1**, 15023.
- 642 **Greene, L. A. and Tischler, A. S.** (1976). Establishment of a noradrenergic clonal line of rat adrenal
643 pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA*
644 **73**, 2424–2428.
- 645 **Gupton, S. L. and Gertler, F. B.** (2010). Integrin signaling switches the cytoskeletal and exocytic
646 machinery that drives neuritogenesis. *Dev. Cell* **18**, 725–736.
- 647 **Igarashi, M., Kozaki, S., Terakawa, S., Kawano, S., Ide, C. and Komiya, Y.** (1996). Growth cone
648 collapse and inhibition of neurite growth by Botulinum neurotoxin C1: a t-SNARE is involved
649 in axonal growth. *J. Cell Biol.* **134**, 205–215.
- 650 **Jacquemyn, J., Cascalho, A. and Goodchild, R. E.** (2017). The ins and outs of endoplasmic
651 reticulum-controlled lipid biosynthesis. *EMBO Rep.* **18**, 1905–1921.
- 652 **Jahn, R. and Scheller, R. H.** (2006). SNAREs--engines for membrane fusion. *Nat. Rev. Mol. Cell*
653 *Biol.* **7**, 631–643.
- 654 **Jean, S., Mikryukov, A., Tremblay, M. G., Baril, J., Guillou, F., Bellenfant, S. and Moss, T.**
655 (2010). Extended-synaptotagmin-2 mediates FGF receptor endocytosis and ERK activation
656 in vivo. *Dev. Cell* **19**, 426–439.
- 657 **Kikuma, K., Li, X., Kim, D., Sutter, D. and Dickman, D. K.** (2017). Extended synaptotagmin
658 localizes to presynaptic ER and promotes neurotransmission and synaptic growth in
659 drosophila. *Genetics* **207**, 993–1006.
- 660 **Lagache, T., Grassart, A., Dallongeville, S., Faklaris, O., Sauvonnet, N., Dufour, A., Danglot,
661 L. and Olivo-Marin, J.-C.** (2018). Mapping molecular assemblies with fluorescence
662 microscopy and object-based spatial statistics. *Nat. Commun.* **9**, 698.
- 663 **Li, F., Pincet, F., Perez, E., Eng, W. S., Melia, T. J., Rothman, J. E. and Tareste, D.** (2007).
664 Energetics and dynamics of SNAREpin folding across lipid bilayers. *Nat. Struct. Mol. Biol.*
665 **14**, 890–896.
- 666 **Liu, Y. and Barlowe, C.** (2002). Analysis of Sec22p in endoplasmic reticulum/Golgi transport reveals
667 cellular redundancy in SNARE protein function. *Mol. Biol. Cell* **13**, 3314–3324.

- 668 **Loewen, C. J. R., Roy, A. and Levine, T. P.** (2003). A conserved ER targeting motif in three families
669 of lipid binding proteins and in Opi1p binds VAP. *EMBO J.* **22**, 2025–2035.
- 670 **Martinez-Arca, S., Coco, S., Mainguy, G., Schenk, U., Alberts, P., Bouillé, P., Mezzina, M.,**
671 **Prochiantz, A., Matteoli, M., Louvard, D., et al.** (2001). A common exocytotic mechanism
672 mediates axonal and dendritic outgrowth. *J. Neurosci.* **21**, 3830–3838.
- 673 **Menna, E., Disanza, A., Cagnoli, C., Schenk, U., Gelsomino, G., Frittoli, E., Hertzog, M.,**
674 **Offenhauser, N., Sawallisch, C., Kreienkamp, H.-J., et al.** (2009). Eps8 regulates axonal
675 filopodia in hippocampal neurons in response to brain-derived neurotrophic factor (BDNF).
676 *PLoS Biol.* **7**, e1000138.
- 677 **Min, S.-W., Chang, W.-P. and Sudhof, T.** (2007). E-Syts, a family of membranous Ca²⁺-sensor
678 proteins with multiple C2domains. *Proc. Natl. Acad. Sci. USA* **104**, 3823–28.
- 679 **Nath, V. R., Mishra, S., Basak, B., Trivedi, D. and Raghu, P.** (2019). Extended synaptotagmin
680 regulates plasma membrane-endoplasmic reticulum contact site structure and lipid transfer
681 function in vivo. *BioRxiv*.
- 682 **Osen-Sand, A., Staple, J. K., Naldi, E., Schiavo, G., Rossetto, O., Petitpierre, S., Malgaroli, A.,**
683 **Montecucco, C. and Catsicas, S.** (1996). Common and distinct fusion proteins in axonal
684 growth and transmitter release. *J. Comp. Neurol.* **367**, 222–234.
- 685 **Park, N., Yoo, J. C., Lee, Y.-S., Choi, H. Y., Hong, S.-G., Hwang, E. M. and Park, J.-Y.** (2014).
686 Copine1 C2 domains have a critical calcium-independent role in the neuronal differentiation
687 of hippocampal progenitor HiB5 cells. *Biochem. Biophys. Res. Commun.* **454**, 228–233.
- 688 **Petkovic, M., Jemaiel, A., Daste, F., Specht, C. G., Izeddin, I., Vorkel, D., Verbavatz, J.-M.,**
689 **Darzacq, X., Triller, A., Pfenninger, K. H., et al.** (2014). The SNARE Sec22b has a non-
690 fusogenic function in plasma membrane expansion. *Nat. Cell Biol.* **16**, 434–444.
- 691 **Pfenninger, K. H.** (2009). Plasma membrane expansion: a neuron's Herculean task. *Nat. Rev.*
692 *Neurosci.* **10**, 251–261.
- 693 **Racchetti, G., Lorusso, A., Schulte, C., Gavello, D., Carabelli, V., D'Alessandro, R. and**
694 **Meldolesi, J.** (2010). Rapid neurite outgrowth in neurosecretory cells and neurons is
695 sustained by the exocytosis of a cytoplasmic organelle, the enlargeosome. *J. Cell Sci.* **123**,
696 165–170.
- 697 **Reinisch, K. M. and De Camilli, P.** (2016). SMP-domain proteins at membrane contact sites:
698 Structure and function. *Biochim. Biophys. Acta* **1861**, 924–927.
- 699 **Saheki, Y., Bian, X., Schauder, C. M., Sawaki, Y., Surma, M. A., Klose, C., Pincet, F., Reinisch,**
700 **K. M. and De Camilli, P.** (2016). Control of plasma membrane lipid homeostasis by the
701 extended synaptotagmins. *Nat. Cell Biol.* **18**, 504–515.
- 702 **Schauder, C. M., Wu, X., Saheki, Y., Narayanaswamy, P., Torta, F., Wenk, M. R., De Camilli, P.**
703 **and Reinisch, K. M.** (2014). Structure of a lipid-bound extended synaptotagmin indicates a
704 role in lipid transfer. *Nature* **510**, 552–555.
- 705 **Schoch, S., Deák, F., Königstorfer, A., Mozhayeva, M., Sara, Y., Südhof, T. C. and Kavalali, E.**
706 **T.** (2001). SNARE function analyzed in synaptobrevin/VAMP knockout mice. *Science* **294**,
707 1117–1122.
- 708 **Schulte, C., Racchetti, G., D'Alessandro, R. and Meldolesi, J.** (2010). A new form of neurite
709 outgrowth sustained by the exocytosis of enlargeosomes expressed under the control of
710 REST. *Traffic* **11**, 1304–1314.

- 711 **Sclip, A., Bacaj, T., Giam, L. R. and Südhof, T. C.** (2016). Extended Synaptotagmin (ESyt) Triple
712 Knock-Out Mice Are Viable and Fertile without Obvious Endoplasmic Reticulum Dysfunction.
713 *PLoS One* **11**, e0158295.
- 714 **Sikorra, S., Henke, T., Galli, T. and Binz, T.** (2008). Substrate recognition mechanism of
715 VAMP/synaptobrevin-cleaving clostridial neurotoxins. *J. Biol. Chem.* **283**, 21145–21152.
- 716 **Südhof, T. C. and Rothman, J. E.** (2009). Membrane fusion: grappling with SNARE and SM
717 proteins. *Science* **323**, 474–477.
- 718 **Weir, M. L., Xie, H., Klip, A. and Trimble, W. S.** (2001). VAP-A binds promiscuously to both v- and
719 tSNAREs. *Biochem. Biophys. Res. Commun.* **286**, 616–621.
- 720 **Weninger, K., Bowen, M. E., Choi, U. B., Chu, S. and Brunger, A. T.** (2008). Accessory proteins
721 stabilize the acceptor complex for synaptobrevin, the 1:1 syntaxin/SNAP-25 complex.
722 *Structure* **16**, 308–320.
- 723 **Wojnacki, J. and Galli, T.** (2016). Membrane traffic during axon development. *Dev. Neurobiol.* **76**,
724 1185–1200.
- 725 **Xu, D., Joglekar, A. P., Williams, A. L. and Hay, J. C.** (2000). Subunit structure of a mammalian
726 ER/Golgi SNARE complex. *J. Biol. Chem.* **275**, 39631–39639.
- 727 **Yu, H., Liu, Y., Gulbranson, D. R., Paine, A., Rathore, S. S. and Shen, J.** (2016). Extended
728 synaptotagmins are Ca²⁺-dependent lipid transfer proteins at membrane contact sites. *Proc.*
729 *Natl. Acad. Sci. USA* **113**, 4362–4367.
- 730 **Zorman, S., Rebane, A. A., Ma, L., Yang, G., Molski, M. A., Coleman, J., Pincet, F., Rothman,
731 J. E. and Zhang, Y.** (2014). Common intermediates and kinetics, but different energetics, in
732 the assembly of SNARE proteins. *Elife* **3**, e03348.

733

734

735 **Figure Legends**

736 **Figure 1. Sec22b and Stx interact with the lipid transfer proteins E-Syt2 and E-Syt3.**

737 (A-C) Immunoblots of material recovered after GFP-Trap pull-down from HeLa cell lysates. Cells
738 were transfected for the co-expression of FLAG-Sec22b and Myc-E-Syt2 (A) or FLAG-Sec22b and
739 Myc-E-Syt3 (C) in the presence of either eGFP-Stx3, or eGFP (negative control). Cell lysates were
740 subjected to GFP-Trap pull-down. Total cells lysate (Input) and trapped material (Bound) were
741 processed for SDS-PAGE and Western blotting. Blots were probed with antibodies directed against
742 the tags (GFP, FLAG and Myc) as indicated. Both Myc-E-Syt2 and Myc-E-Syt3 were selectively
743 recruited by eGFP-Stx3. (B) GFP-Trap using Sec22b-pHL as a bait in cells co-expressing either
744 Myc-E-Syt2 or Myc-E-Syt2ΔSMP. Matrix-bound material was processed as in A, B with the indicated

745 antibodies. (D) Stx1/3 immunoprecipitation from rat embryonic cortex. Naive rabbit IgGs were used
746 as negative control. Total cells lysate (Input) and immunoprecipitated material (IP) were processed
747 for SDS-PAGE and Western blotting. Blots were probed with antibodies directed against the
748 endogenous proteins. Stx1/3 co-immunoprecipitates Sec22b as expected, and E-Syt2. (E)
749 Immunoblots of material recovered after GFP-Trap pull-down from NGF-treated PC12 cell lysates.
750 Cells were transfected for co-expression of Myc-E-Syt2 and the indicated GFP-tagged SNAREs and
751 processed as in A-C. Blots were revealed with antibodies against the indicated six target proteins.
752 Only Sec22b-pHL, but not its Longin-deleted version Sec22b Δ L-pHL or the other tested SNAREs,
753 could recruit Myc-E-Syt2. GFP-Trap pull-down of eGFP was used as control for non-specific binding.
754 (F) Quantification of the ratio between Myc-E-Syt2 signal and the GFP signal given by the
755 immunoprecipitated GFP-tagged vSNAREs (left graph) and tSNAREs (right graph). (G)
756 Quantification of the ratio between Myc-E-Syt2 signal and the GFP signal (left graph) and between
757 endogenous SNAP25 signal and the GFP signal (right graph) given by the immunoprecipitated
758 Sec22b-pHL vs Sec22b Δ L-pHL. One-way ANOVA followed by Dunnett's multiple comparison post-
759 test, $P < 0,05^*$, $P < 0,001^{***}$, $n = 3$ independent experiments. Error bars represent the SEM.

760

761 **Figure 2. E-Syt2 and Sec22b are abundantly in close proximity in neurites and growth**
762 **cones.**

763 Duolink proximity ligation assay (PLA) for protein interactions *in situ* was performed in HeLa cells (A)
764 and 3DIV hippocampal neurons (C). Representative confocal images are shown for the indicated
765 antibody combinations using mouse anti-Sec22b, rabbit anti-E-Syt2, or rabbit anti-Calnexin.
766 Negative controls consisted in using anti-Sec22b or anti-E-Syt2 antibody only. In each field,
767 maximum intensity projection of a confocal z-stacks including a whole cell were performed to observe
768 the maximum amount of PLA dots (red). Nuclei were stained by DAPI (blue). A1-4, PLA dots. Lower
769 panel is a higher magnification of region outlined in A1. C1-3, PLA dots. C4-6, MAP2
770 immunofluorescence staining superimposed to fields shown in C1-3. Lower panels are a higher
771 magnification of regions outlined in C1 and C4. Scale bar, 10 μ m. (B, D, E) Quantification of PLA is

772 expressed as dots per HeLa cell (B), and in 3DIV hippocampal neurons as dots per μm^2 of surface
773 area in neurites (D), growth cones (E) or in cell body (F). The number of individual fluorescent dots
774 is higher in the Sec22b-E-Syt2 pair as compared to Sec22b-Calnexin pair or negative controls both
775 in HeLa and in neurons. It is higher in neurites and growth cones as compared to cell bodies in
776 neurons. One-way ANOVA followed by Dunn's multiple comparison post-test, $P < 0,05^*$, $P < 0.001^{***}$.
777 $n=3$ independent experiments. Error bars represent the SEM.

778

779 **Figure 3. Analysis of E-Syt2 / Sec22b colocalization using super-resolution microscopy.**

780 (A) Representative confocal images of a 3DIV hippocampal neuron labeled for endogenous E-Syt2
781 (green) and Sec22b-pHL (red) and plasma membrane (gray). Alexa Fluor® 488- conjugated Wheat
782 Germ Agglutinin (WGA) was used to label the plasma membrane (grey). Scale bar, 10 μm . (B)
783 Super-resolution (3D STED) 3D reconstruction of the inset in (A) showing localization of endogenous
784 E-Syt2 and Sec22b-pHL in the growth cone. (C) Statistical association was assessed through spatial
785 distribution analysis using Ripley's function (Icy SODA Plugin). 3D STED reconstruction of Sec22b
786 (in green) and E-Syt2 (in red) particles. Rectangles indicate examples of overlapping puncta: Sec22b
787 statistically associated with E-Syt2 is in yellow; E-Syt2 statistically associated to Sec22b is in orange.
788 (D) Topological scheme illustrating the measured distance (d) between associated Sec22b-E-Syt2
789 puncta and the PM. d was estimated to an average of $67.12\text{nm} \pm 1.22$ in four different growth cones
790 for 4229 clusters and its median was: 33.6nm, indicating that 50% of the clusters were at a distance
791 $\leq d = 33.6\text{nm}$, compatible with ER-PM contact sites.

792

793 **Figure 4. E-Syts favor the occurrence of Sec22b-Stx complexes.**

794 Duolink proximity ligation assay (PLA) for protein interactions *in situ* was performed in HeLa cells
795 either non transfected or overexpressing eGFP-E-Syt3 (A) and in HeLa cells expressing siRNAs
796 targeting E-Syt1, E-Syt2 and E-Syt3 simultaneously (E). Representative confocal images are shown
797 for PLA between Sec22b (Mouse anti-Sec22b) and Stx3 (Rabbit anti-Stx3). Negative controls

798 combined IgG2a and IgGR. In each field, maximum intensity projection of a confocal z-stacks
799 including a whole cell were performed to observe the maximum amount of PLA dots (red). Nuclei
800 were stained by DAPI (blue). Scale bar, 10 μ m. (B) Confocal maximal projection image showing co-
801 localization of Sec22b-Stx3 PLA signal and E-Syt3-positive cortical ER. Scale bar, 10 μ m. (C, F)
802 Quantification of PLA is expressed as dots per HeLa cell. The number of individual fluorescent dots
803 is higher in cells overexpressing eGFP-E-Syt3 as compared to non-transfected cells or negative
804 control (C). It is lower in cells expressing siRNAs targeting the three E-Sys isoforms as compared to
805 cells expressing siCtrl (F). Data are expressed as means \pm SEM. Oneway ANOVA $P < 0,05^*$, Dunn's
806 multiple comparison post-test labeled on graph. $n = 3$ independent experiments. (D) Representative
807 immunoblots from lysates of HeLa cells expressing siRNAs targeting the three E-Sys isoforms.
808 Tubulin was used as a loading control.

809

810 **Figure 5. E-Syts overexpression promotes filopodia formation and ramifications in**
811 **developing neurons**

812 (A) Representative morphology of a nucleofected 3DIV hippocampal neuron expressing Myc-E-Syt2.
813 eGFP co-expression was used to view cell shape. Higher magnification image shows high density
814 of filopodia in a segment of the axonal shaft. Scale bar, 10 μ m. (B) Expression of Myc-E-Syt2 mutated
815 forms. 3DIV neurons co-expressing eGFP and Myc-E-Syt2 or one of the deletion mutants Myc-E-
816 Syt2 Δ SMP and Myc-E-Syt2 Δ MSD, lacking the SMP domain [119-294] and the membrane spanning
817 region [1-72], respectively. The empty Myc vector was used as negative control. Scale bar, 10 μ m.
818 (C) Quantification of morphological parameters in transfected neurons shown in B. Plots were
819 acquired on maximal intensity projections of z-stacks of the eGFP channel. Note that in comparison
820 with the longest neurite length, the number of branching per neuron and the total neurite length are
821 increased in neurons upon Myc-E-Syt2 expression, whereas expression mutants had no effect. Data
822 are expressed as means \pm SEM. Oneway ANOVA $P < 0,001^{***}$, Dunn's multiple comparison post-
823 test labelled on graph. $n = 3$ independent experiments.

824

825 **Figure 6. E-Syts overexpression stimulates membrane growth in HeLa cells**

826 (A) HeLa cells were transfected for co-expression of Myc-E-Syt2 and eGFP (the latter to delineate
827 cell shape). Higher magnifications images show high density of filopodia at the cell periphery. Scale
828 bar, 10 μm . (B) HeLa cells co-expressing eGFP and Myc-E-Syt2 or one of the deletion mutants Myc-
829 E-Syt2 Δ SMP and Myc-E-Syt2 Δ MSD. The empty Myc vector was used as control. Scale bar, 10 μm .
830 (C) Quantification of spike area in transfected cells shown in B, acquired from maximal intensity
831 projections of z-stacks of the eGFP channel. It is expressed as percentage of the total cell surface
832 area. Filopodia formation was enhanced in cells expressing Myc-E-Syt2 as compared to cells
833 expressing the mutant proteins. Data are expressed as means \pm SEM. Oneway ANOVA $P < 0,01^{**}$,
834 Dunn's multiple comparison post-test labelled on graph. $n = 3$ independent experiments.

835

836 **Figure 7. E-Syts-mediated morphogenetic effect depends on Stx1**

837 (A) Schematic of cleavage sites on neuronal SNARE targets of BoNTs. Cleavage can occur on
838 SNAP25 (BoNT/A), on both SNAP25 and Stx1 (BoNT/C1), or specifically on VAMP2 (BoNT/D). (B,
839 C) Cleavage activity of BoNTs. (B) Representative immunoblots from lysates of neurons exposed
840 for 4 h to 1 nM BoNTs. (C) Quantification of ECL signals from B. Ratios of SNAREs to tubulin are
841 plotted. Oneway ANOVA $P < 0,05^*$, followed by post-hoc Tukey HSD test. $n = 3$ independent
842 experiments. Data are expressed as means \pm SEM. (D) 3DIV hippocampal neurons co-expressing
843 Myc-E-Syt2 and eGFP and treated with BoNTs (1 nM, 4h). Scale bar, 10 μm . (E) Quantification of
844 morphometric parameters on treated neurons, measured on maximal intensity projections of z-
845 stacks of the eGFP channel. The specific cleavage of Stx1 by BoNT/C1 reduces the number of
846 ramifications and the total neurite length of Myc-E-Syt2 expressing neurons. Data are expressed as
847 means \pm SEM. Oneway ANOVA $P < 0,01^{**}$ $P < 0,001^{***}$, Dunn's multiple comparison post-test
848 labelled on graph. $n = 3$ independent experiments.

849

850 **Figure 8. E-Syts-mediated morphogenetic effects depend on ER to PM distance**

851 (A) Scheme showing the predicted effect of a polyproline stretch insertion in Sec22b on the ER-PM
852 distance (see text). (B) Hippocampal neurons co-expressing Myc-E-Syt2 and either GFP-Sec22b,
853 GFP-Sec22b-P33 mutant or Sec22b GFP-Longin domain, observed at 3DIV. Scale bar, 10 μ m. (C)
854 Quantification of morphological parameters on treated neurons, measured on maximal intensity
855 projections of z-stacks of the GFP channel. Effect of overexpressed Myc-Esyt2 on number of
856 ramifications and on the sum of neurite lengths is reduced in neurons expressing the GFP-Sec22b-
857 P33 mutant or the GFP-Longin domain. Data are expressed as means \pm SEM. Oneway ANOVA
858 $P < 0,01^{**}$ $P < 0,001^{***}$, Dunn's multiple comparison post-test labelled on graph. $n = 3$ independent
859 experiments.

860

861

862

863

864

Figure 2. E-Syt2 and Sec22b are abundantly in close proximity in neurites and growth cones.

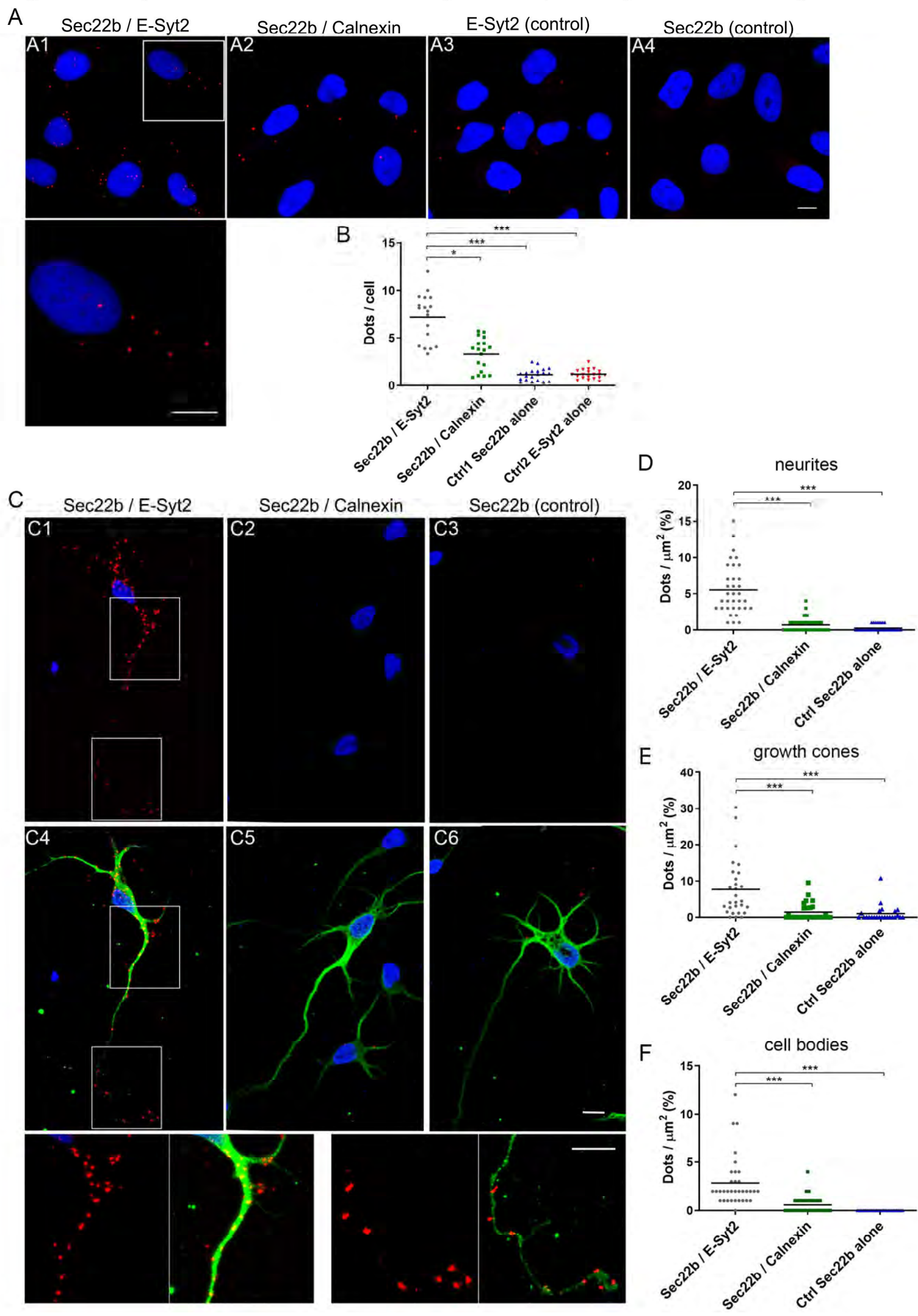
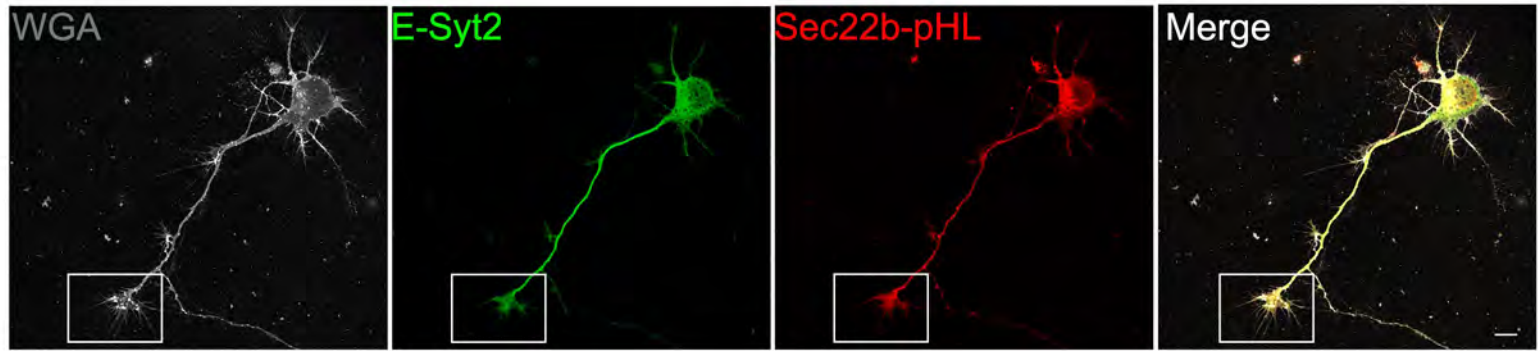
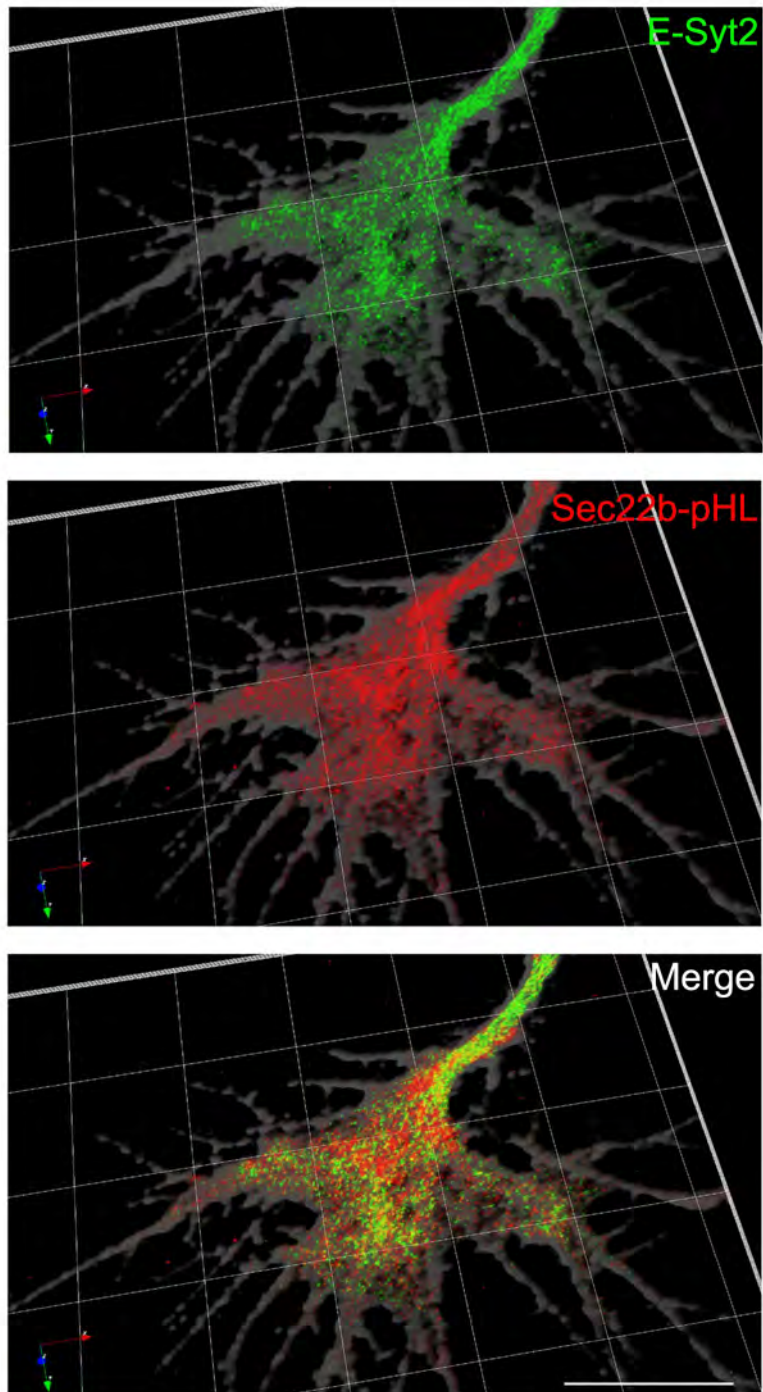


Figure 3. Analysis of E-Syt2 / Sec22b colocalization using super-resolution microscopy

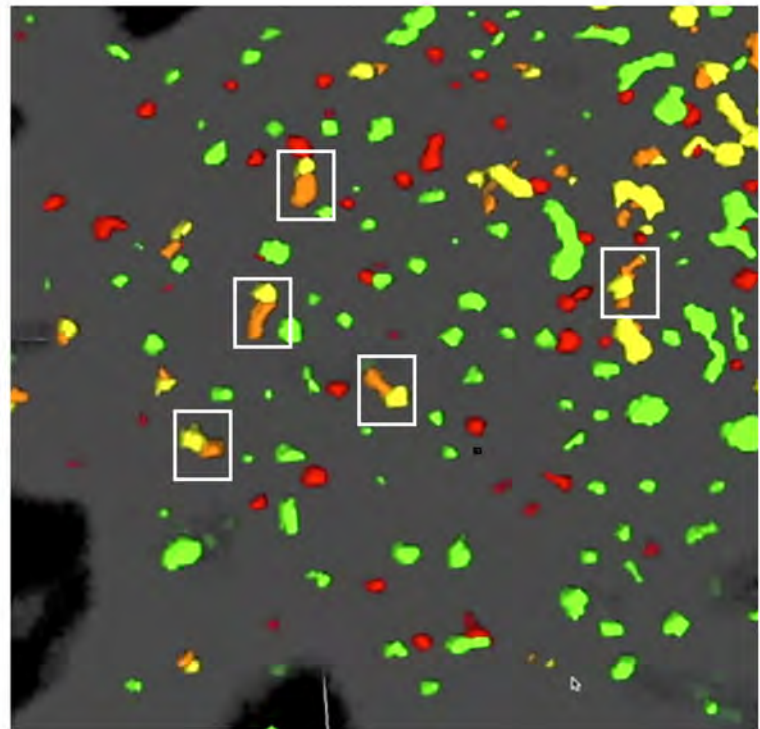
A



B



C



D

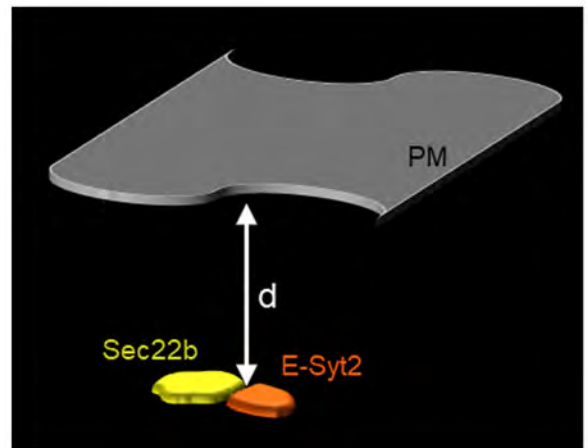


Figure 4. E-Syts favor the occurrence of Sec22b-Stx complexes.

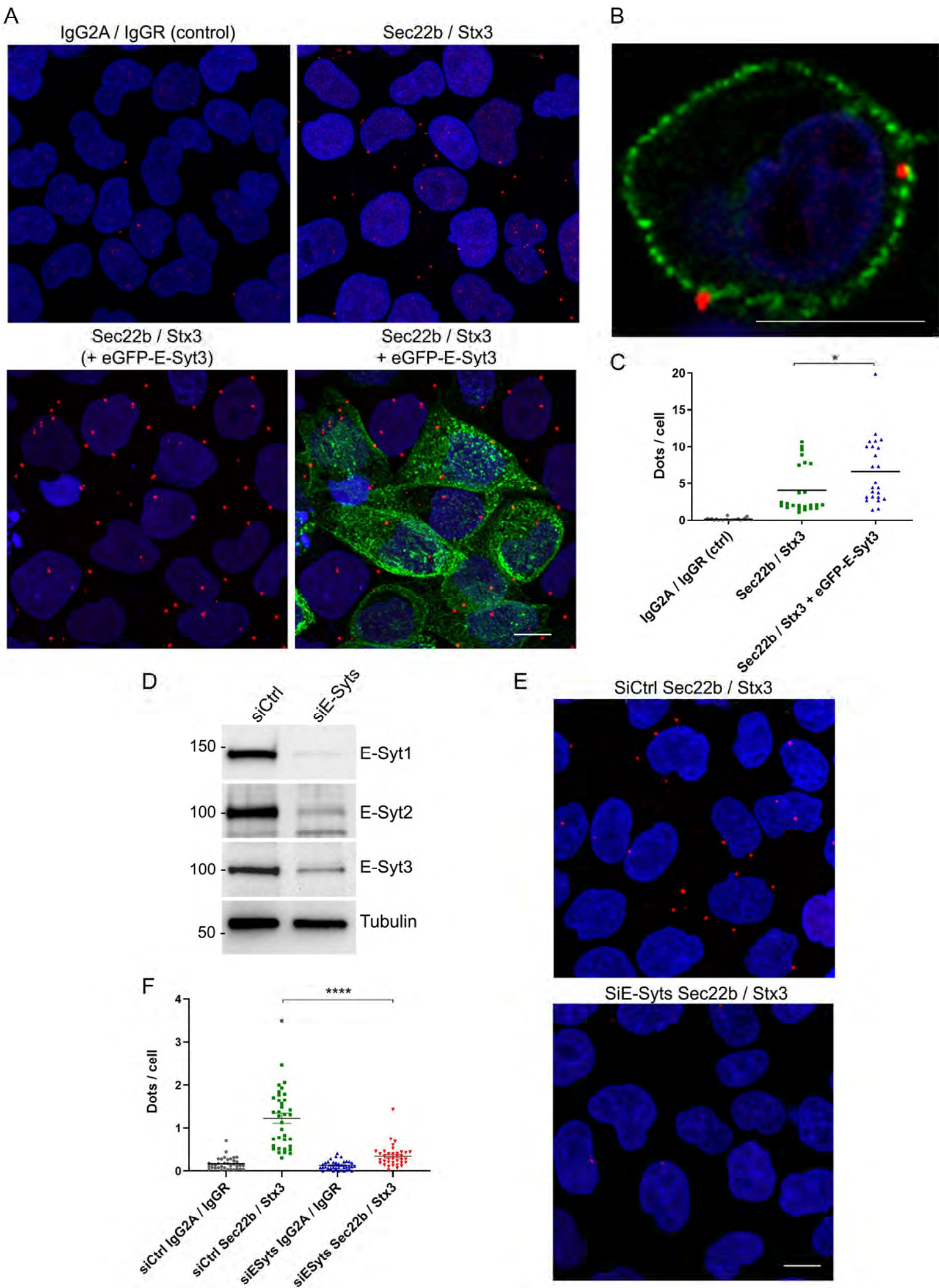
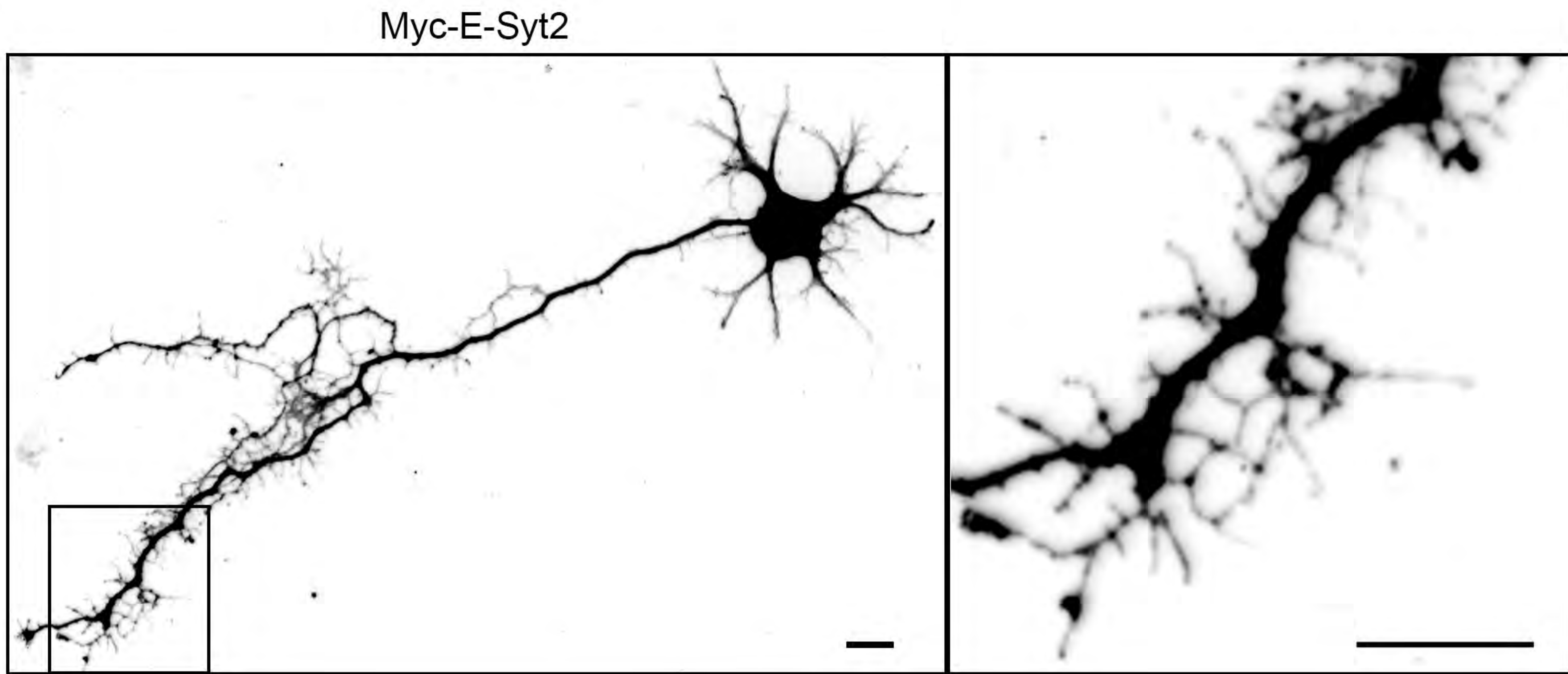
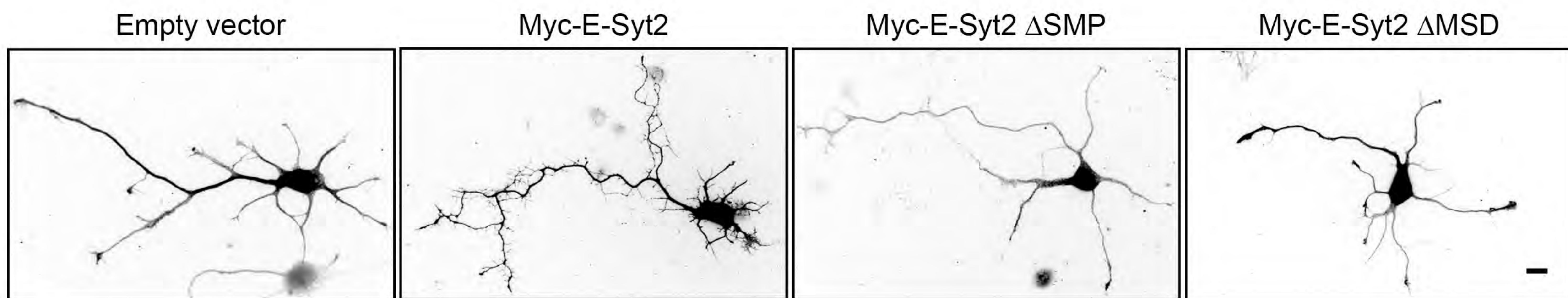


Figure 5. E-Syts overexpression promotes filipodia formation and ramification in developing neurons

A



B



C

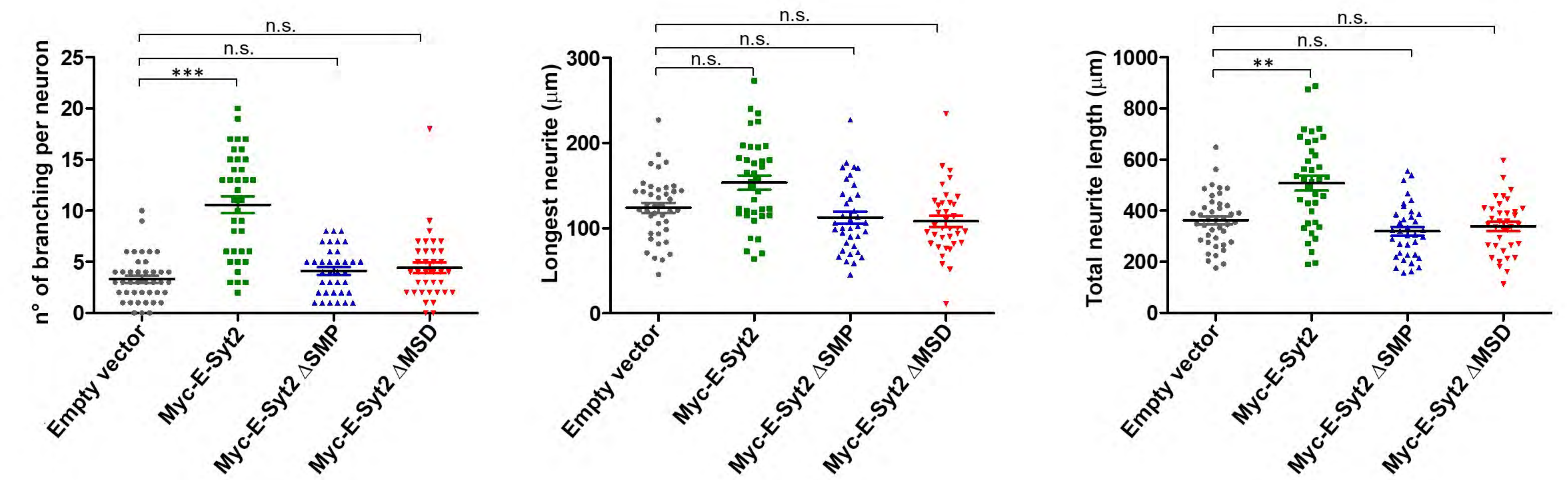
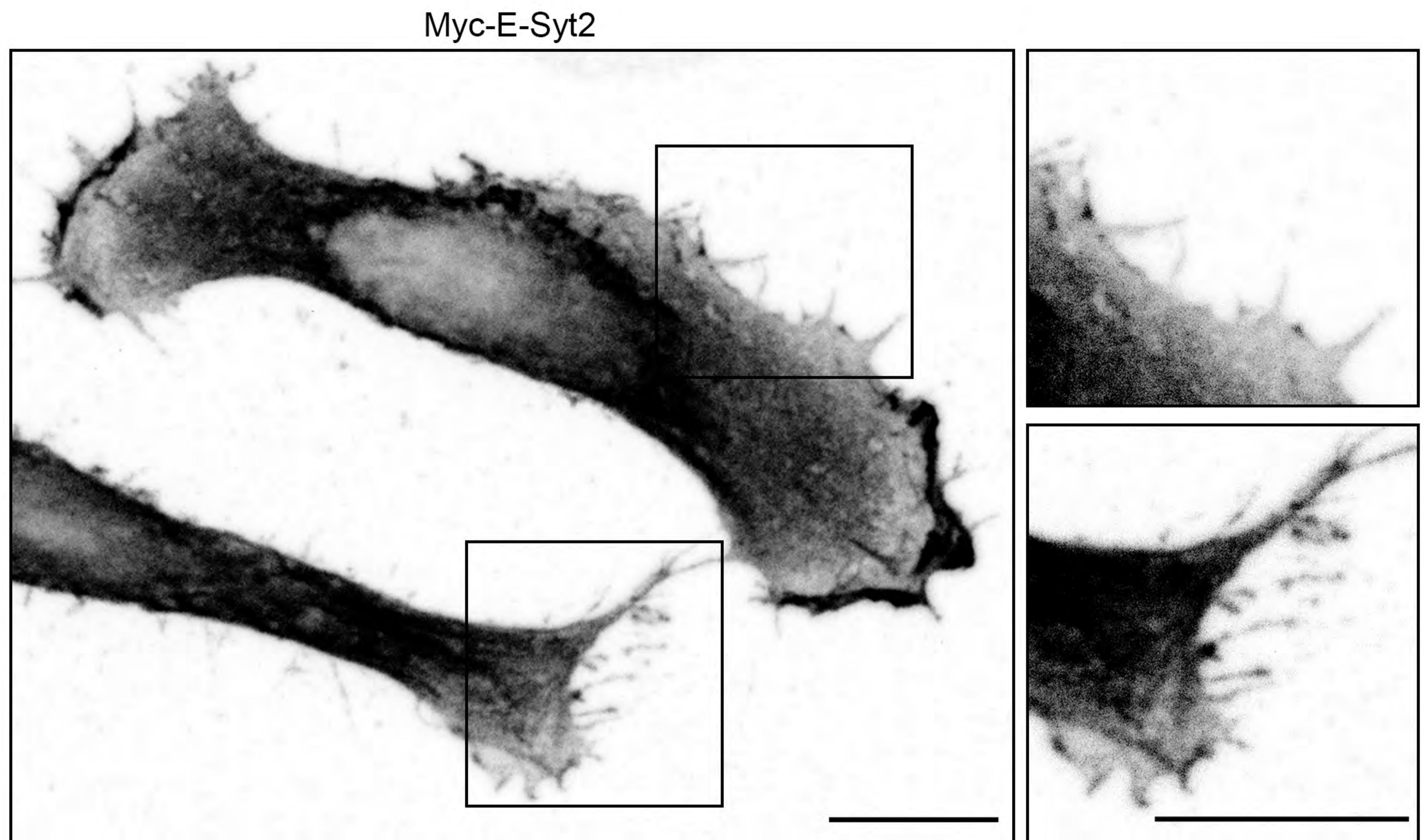
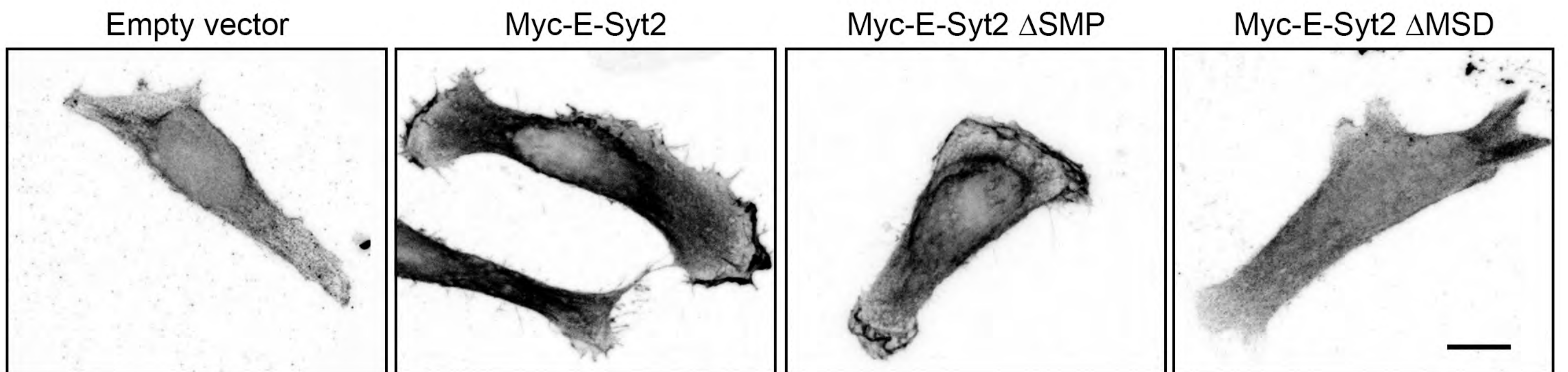


Figure 6. E-Syts overexpression promotes membrane growth in HeLa cells

A



B



C

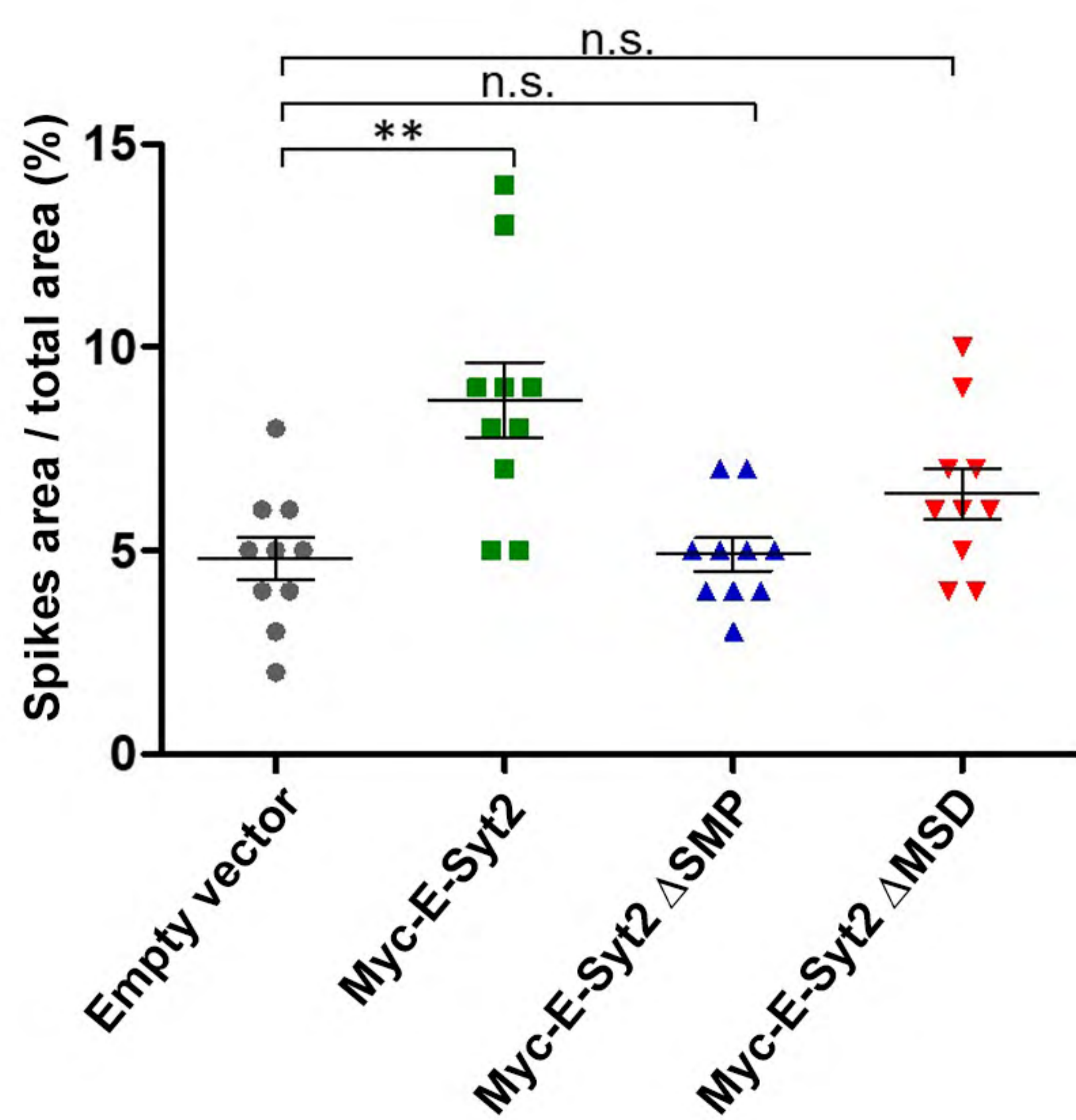


Figure 7. E-Syts-mediated morphogenetic effect depends on Stx1

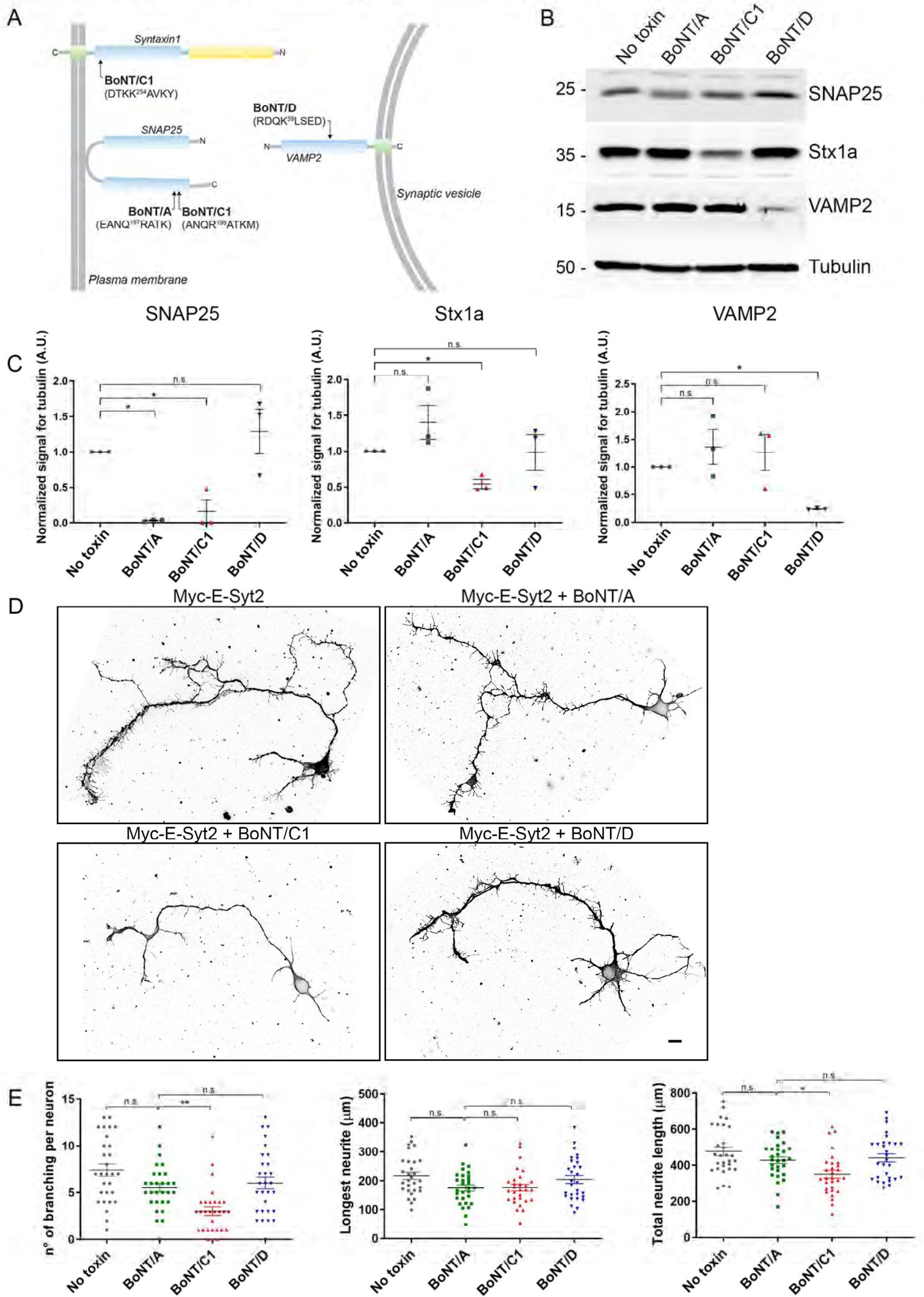
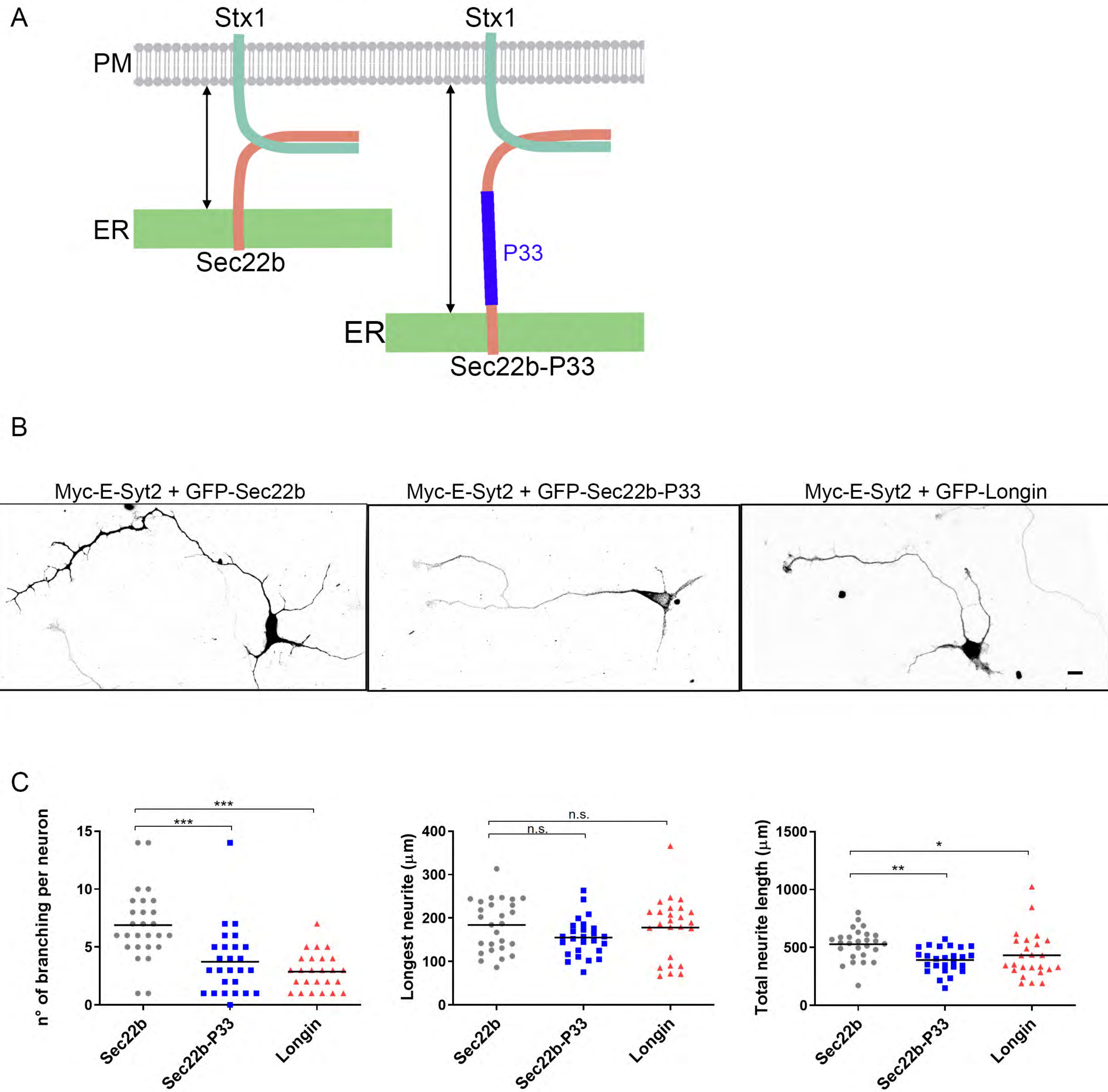


Figure 8. E-Syts-mediated morphogenetic effects depend on the close apposition of ER to PM mediated by Sec22b-Stx1 complexes



1

2 **Figure S1. The Sec22b Δ Longin mutant reaches the cell surface**

3 (A-B) Hippocampal neurons at 3DIV were transfected with Sec22b or Sec22b Δ L mutant tagged with
4 pHluorin at the C-terminal, so that if the Sec22b compartment fuses with the PM, it would be detected
5 by anti-GFP antibody in the medium. N-terminally tagged Sec22b (GFP-Sec22b) has been used as
6 a negative control since its GFP tag should never be exposed extracellularly and detected with anti-
7 GFP antibody, even in the event of membrane fusion. VAMP2-pHL was used as positive control for
8 fusion. For surface staining anti-GFP antibody was allowed to bind for 10 min at 4°C, neurons were
9 fixed and processed for immunochemistry. (A) Scheme of the topology of constructs in the PM if
10 fusion is assumed to occur. (B) Representative images. Scale bar, 10 μ m. (C) Quantitative analysis
11 of surface staining versus total staining expressed as normalized ratio to GFP-Sec22b. Oneway
12 ANOVA $P < 0,0001$ ***, Dunn's multiple comparison post-test labeled on graph. (D) Representative
13 images of COS7 cells expressing Sec22b-HA (left) or Sec22b Δ L-HA (right) and labeled with an anti-
14 HA antibody. Note that the typical reticular localization of Sec22b is partially lost for the Sec22b Δ L
15 mutant.

16

17 **Figure S2. Actin-populated filopodia display clusters of E-Syt2**

18 Representative images of a 3DIV hippocampal neuron labeled for Myc-E-Syt2 (red) and actin
19 (phalloidin labeling, green). Actin is present in filopodia resulting by Myc-E-Syt2 overexpression. In
20 the inset, the immunostaining of Myc-E-Syt2 clearly reveals puncta (arrowheads) which are present
21 in thin actin-positive filopodia.

22

23 **Figure S3. Subcellular localization of E-Syt2 wt and mutants**

24 Representative confocal images of a HeLa cells expressing Myc-E-Syt2 wt (A), Myc-Esyt2 Δ SMP
25 (B) and Myc-Esyt2 Δ MSD (C) and co-labeled for E-Syt2 and endogenous Sec22b. Note that, while

26 the typical localization of E-Syt2 is partially conserved for the Δ SMP mutant, it is completely lost for
27 the Δ MSD mutant, that appears soluble.

28

29 **Figure S4. Hypothetical model for the formation of an E-Syt/SNARE-mediated ER-PM contact**
30 **site**

31 Sec22b diffuses within the ER membrane and is stabilized in the cortical ER after binding to E-Syt
32 where it interacts with the PM-resident Stx1, generating a tight ER-PM junction (~ 10 nm). Sec22b
33 association to the PM-resident Stx1-SNAP25 complex causes SNAP25 displacement from Stx1 and
34 potentially E-Syt, leading to the formation of a non-fusogenic assembly of Sec22b, Stx1 and E-Syts.

35

36 **Movie 1. Dynamics of mCherry-Sec22b and GFP-E-Syt2 in HeLa**

37 Live-microscopy recording of mCherry-Sec22b and GFP-E-Syt2 movements in HeLa cells. The inset
38 shows a region where the colocalized molecules have a notably reduced motility as compared to
39 material with less peripheral distribution.

40

41 **Movie 2: Analysis of E-Syt2 / Sec22b association using super-resolution microscopy.**

42 Comparison is shown between 3D confocal and 3D STED with Sec22b in green and E-Syt2 in red.
43 Sec22b statistically associated with E-Syt2 is in yellow. E-Syt2 statistically associated to Sec22b is
44 in orange.

Figure S1. The Sec22b Δ Longin mutant reaches the cell surface

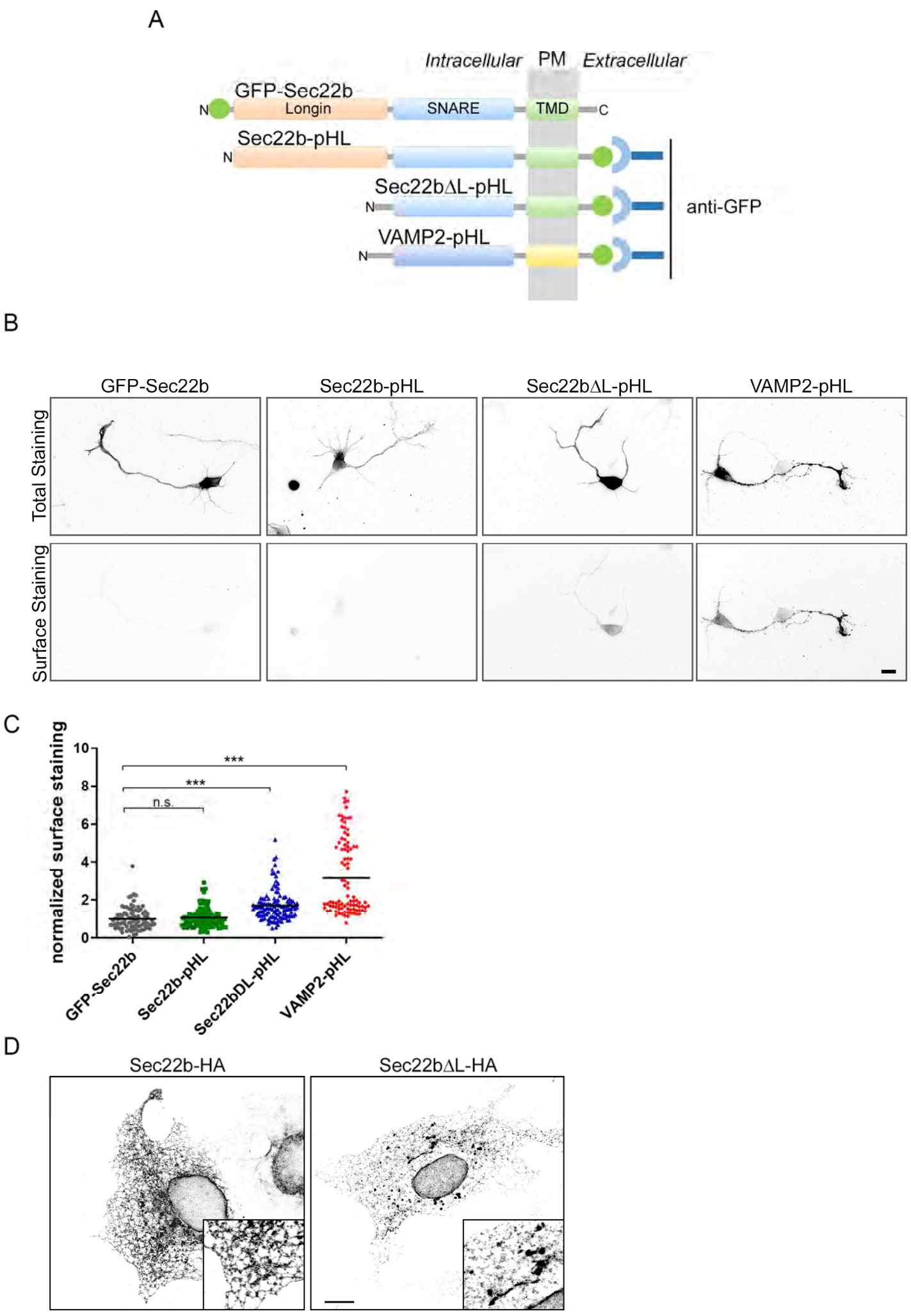


Figure S2. Actin-populated filopodia display clusters of E-Syt2.

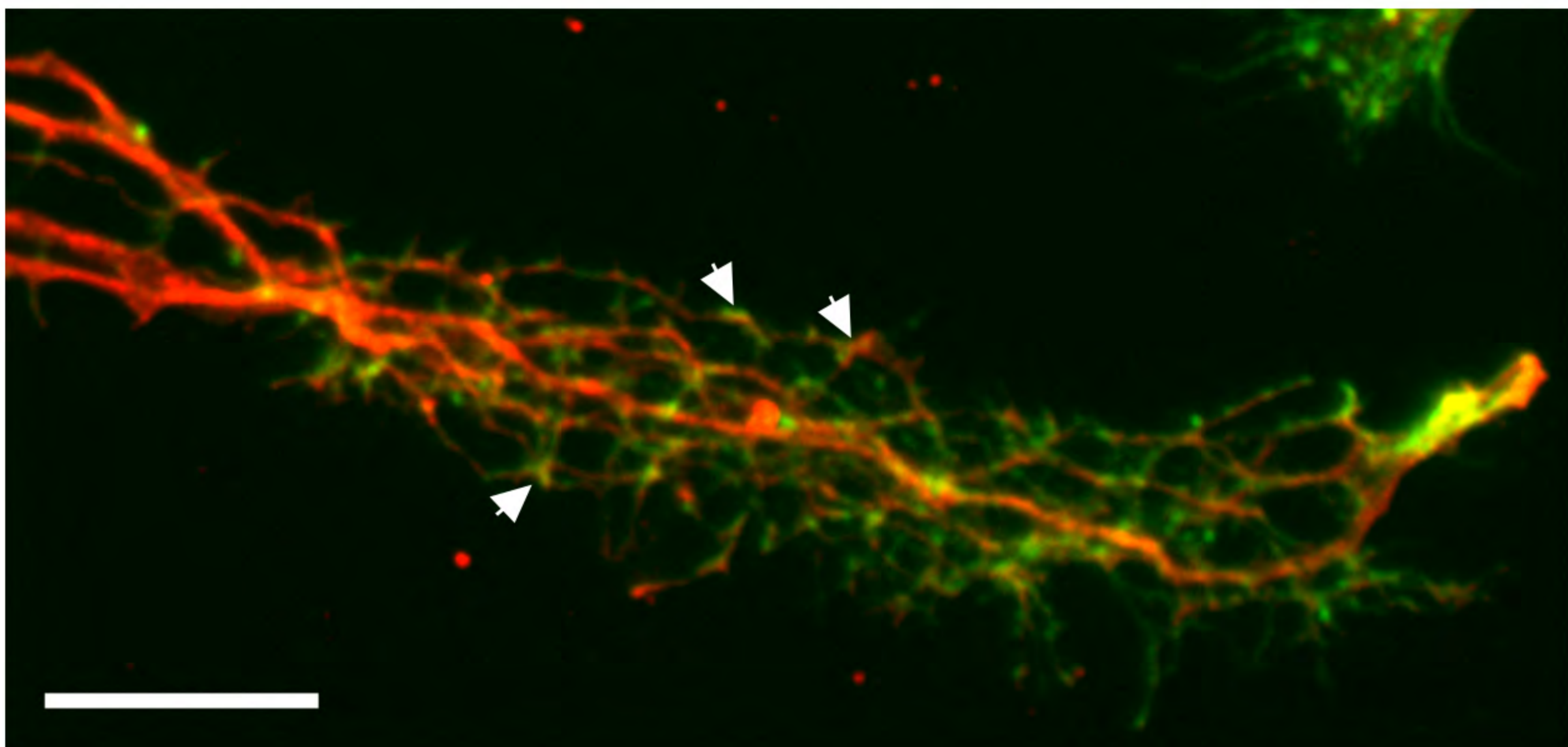
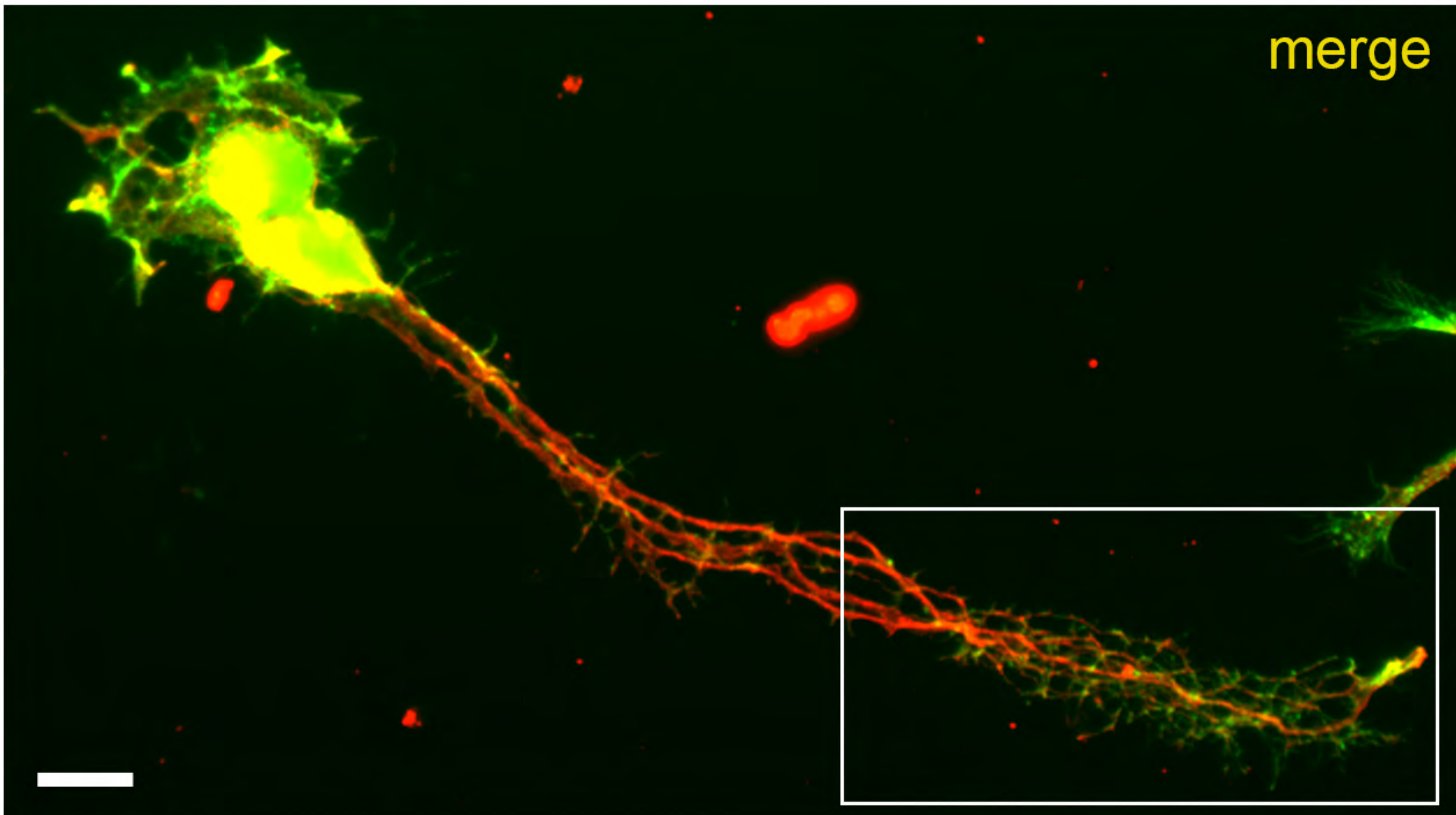
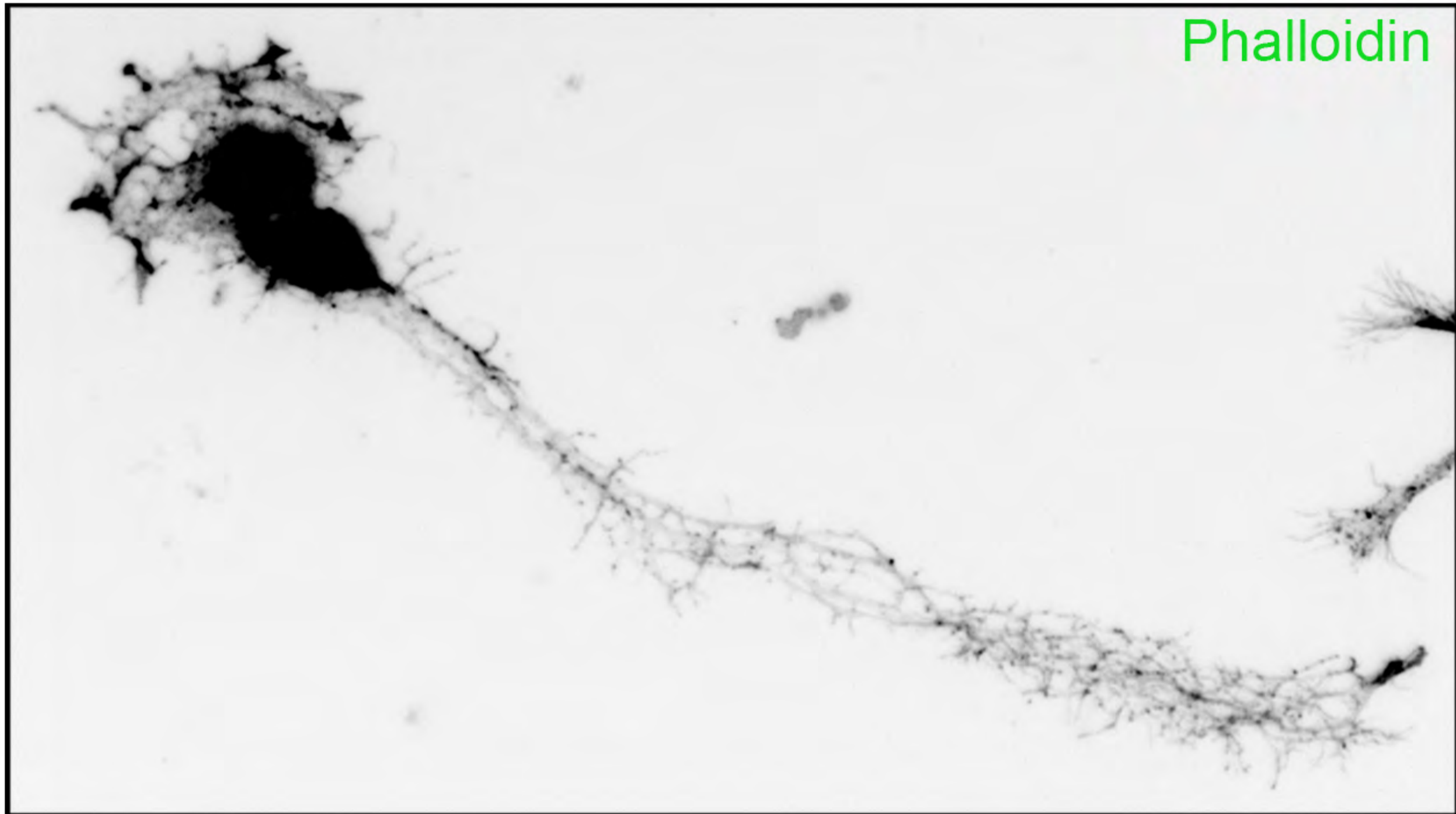
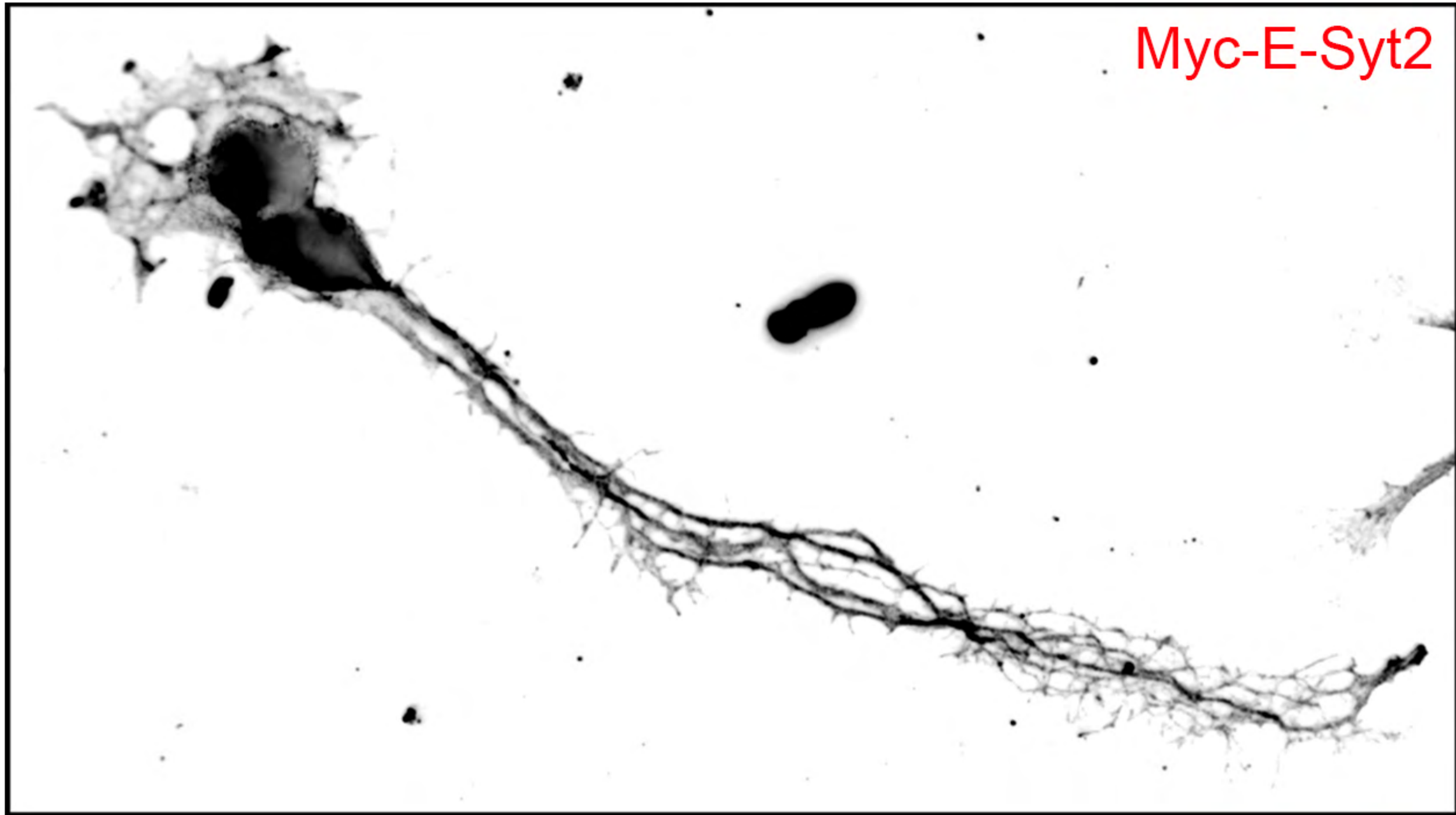


Figure S3. Subcellular localization of E-Syt2 wt and mutants

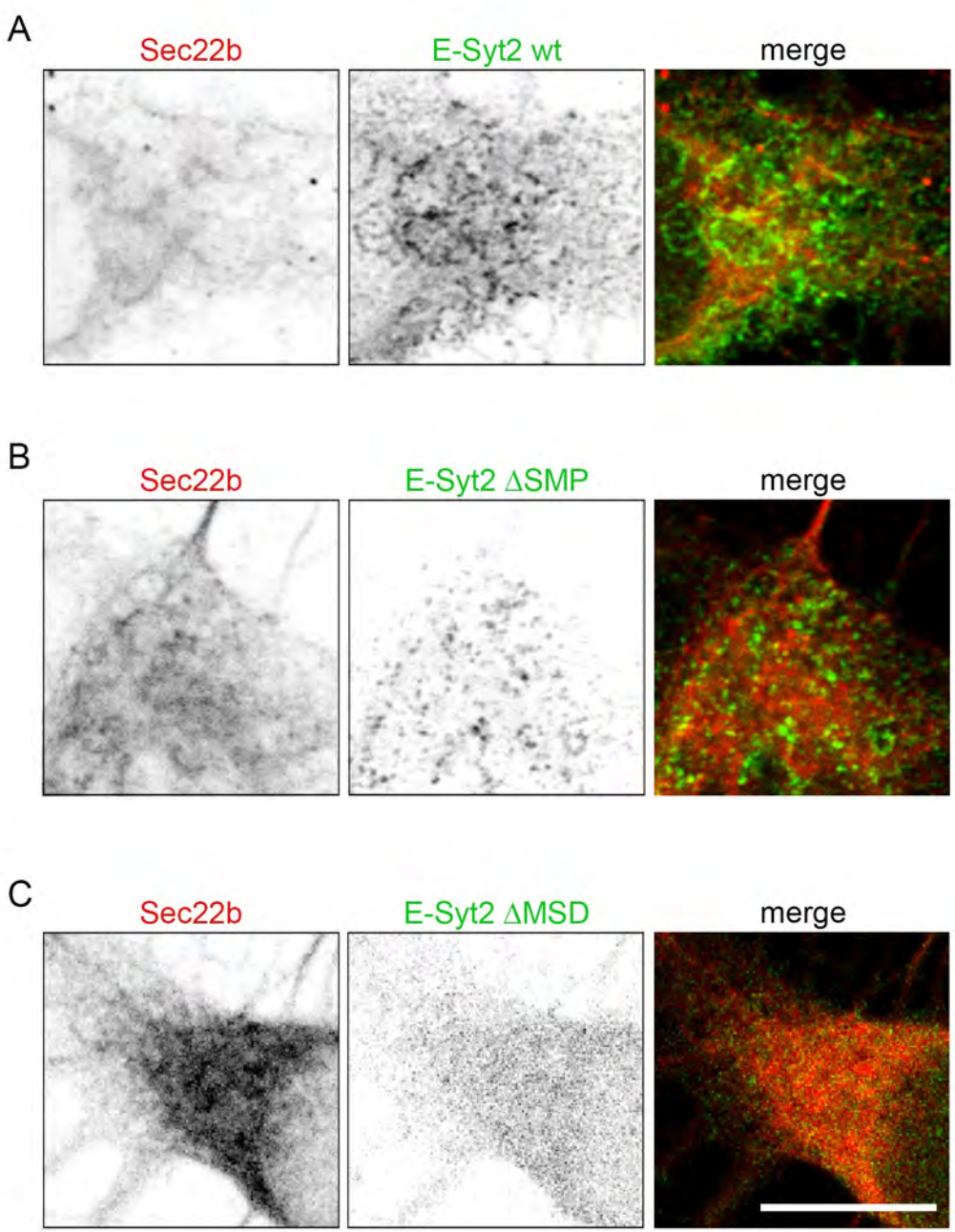


Figure S4. Proposed model for the interaction of Sec22b, Syntaxins and E-Syts.

