

### Single-Molecule Microscopy Reveals Dynamic FLNA Interactions Governing SSTR2 Clustering and Internalization

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1 **Single-Molecule Microscopy Reveals Dynamic FLNA Interactions Governing SSTR2**

2 **Clustering and Internalization**

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41

42

43

44 **Abstract**

45 The cytoskeletal protein filamin A (FLNA) has been suggested to play an important role in the  
46 responsiveness of GH-secreting pituitary tumors to somatostatin receptor subtype 2 (SSTR2)  
47 agonists, by regulating SSTR2 expression and signaling. However, the underlying mechanisms  
48 are unknown. Here, we use fast multi-color single-molecule microscopy to image individual  
49 SSTR2 and FLNA molecules at the surface of living cells with unprecedented spatiotemporal  
50 resolution. We find that SSTR2 and FLNA undergo transient interactions, which occur  
51 preferentially along actin fibers and contribute to restraining SSTR2 diffusion. Agonist  
52 stimulation increases the localization of SSTR2 along actin fibers and, subsequently, SSTR2  
53 clustering and recruitment to clathrin-coated pits (CCPs). Interfering with FLNA–SSTR2 binding  
54 with a dominant-negative FLNA fragment increases SSTR2 mobility, hampers the formation and  
55 alignment of SSTR2 clusters along actin fibers, and impairs both SSTR2 recruitment to CCPs  
56 and SSTR2 internalization. These findings indicate that dynamic SSTR2–FLNA interactions  
57 critically control the nanoscale localization of SSTR2 at the plasma membrane and are required  
58 for coupling SSTR2 clustering to internalization. These mechanisms explain the critical role of  
59 FLNA in the control of SSTR2 expression and signaling and suggest the possibility of targeting  
60 SSTR2–FLNA interactions for the therapy of pharmacologically resistant GH-secreting pituitary  
61 tumors.

62 **Keywords: Scaffolding proteins; cytoskeleton; GPCR endocytosis; TIRF microscopy**

63

## 64 **1. Introduction**

65 Somatostatin (SS) is a peptide hormone that exerts key regulatory functions on the endocrine,  
66 neuronal and gastrointestinal systems. These actions are mediated by a family of five G-protein-  
67 coupled receptors (GPCRs) known as SSTR1–5 (1, 2), and include inhibition of both cell  
68 proliferation and hormone secretion (3–6). Somatostatin receptor type 2 (SSTR2) – one of the  
69 most expressed receptor subtypes in GH-secreting pituitary adenomas – is the main target of  
70 somatostatin analogs (SSAs), which are widely used to treat acromegalic patients (7, 8).  
71 However, a relevant subset of patients is not successfully controlled by medical therapy with  
72 SSAs (9–11), and several studies have attempted to clarify the molecular mechanisms underlying  
73 the pharmacological resistance to SSAs (12–15). More recently, increasing attention has been  
74 directed towards the role of scaffolding proteins and cytoskeletal elements in mediating the  
75 formation of specialized signaling subdomains at the plasma membrane and facilitating receptor  
76 internalization (16–19). Thus, a better understanding of these mechanisms appears crucial to  
77 develop innovative pharmacological therapies for acromegaly and other human diseases.

78 Filamin A (FLNA) is a large cytoskeletal protein characterized by an actin binding domain  
79 located at its N-terminus and multiple binding sites for molecules involved in different signaling  
80 pathways, which are distributed along the rest of its flexible structure. The primary function of  
81 FLNA is to cross-link actin filaments (F-actin) into a three-dimensional network that defines and  
82 controls cell shape. In addition, thanks to its ability to anchor transmembrane proteins to the actin  
83 cytoskeleton and its scaffolding role for intracellular proteins, FLNA is emerging as an important  
84 regulator of G-coupled receptor (GPCR) expression, subcellular localization, trafficking and  
85 signaling (20, 21). In particular, previous studies suggested that FLNA directly interacts with  
86 SSTR2 and that this interaction might be required for the SSTR2-mediated biological effects of

87 SSAs (17, 22). However, how these interactions affect SSTR2 organization at the plasma  
88 membrane and its internalization was unknown.

89 Here, we used innovative single-molecule imaging methods based on total internal reflection  
90 fluorescence (TIRF) microscopy (23-26) to investigate the involvement of FLNA in the spatial  
91 arrangement, mobility and internalization of SSTR2s with unprecedented spatio-temporal  
92 resolution. Our findings indicate that FLNA plays an important role in controlling the  
93 arrangement and mobility of SSTR2s at the plasma membrane by providing a physical link with  
94 actin fibers, which facilitates the clathrin-mediated internalization of SSTR2s.

## 95 **2. Materials and Methods**

### 96 **Plasmids and constructs**

97 A plasmid encoding the human wild-type SSTR2 was kindly provided by Dr. Stefan Schulz. A  
98 plasmid encoding the SSTR2 with a FLAG sequence followed by a SNAP tag at its N-terminus  
99 (SNAP-SSTR2) was cloned by inserting the SSTR2 receptor sequence into a plasmid containing  
100 the SNAP tag directly after the FLAG sequence (23). A plasmid encoding FLNA with a CLIP tag  
101 inserted in its first hinge region (CLIP-FLNA) was generated by replacing eGFP with a CLIP tag  
102 in a construct coding for eGFP-FLNA (27), kindly provided by Dr. Anna M. Aragay. Plasmids  
103 expressing the FLNA repeats 19-20 or 17-18 (FLNA 19-20, FLNA 17-18) were previously  
104 described (17). A plasmid encoding eGFP-AP2 (28) was kindly provided by Dr. Emanuele  
105 Cocucci and Dr. Tom Kirchhausen. The plasmid coding for LifeAct-GFP (29) was kindly  
106 provided by Prof. Antje Gohla (Institute for Pharmacology and Toxicology, University of  
107 Würzburg).

### 108 **Cell culture**

109 Chinese hamster ovary K1 (CHO-K1) cells were maintained in Dulbecco's modified Eagle's  
110 medium/nutrient mixture F-12 (DMEM/F12) supplemented with 10% (v/v) fetal bovine serum  
111 (FBS), 100 U/ml penicillin and 100 µg streptomycin. Human embryonic kidney 293A  
112 (HEK293A) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented  
113 with 10% (v/v) FBS, 2mM glutamine, 100 U/ml penicillin and 100 µg streptomycin. Cells were  
114 maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

### 115 **cAMP measurements**

116 To assess the functionality of the SNAP-SSTR2 construct, HEK293A cells were transiently  
117 cotransfected for 48 h with 1 µg of wild-type or SNAP-tagged SSTR2 and 1 µg of a fluorescence  
118 resonance energy transfer (FRET) sensor for cAMP (Epac1-camps) (30, 31) using the Effectene  
119 reagent (QIAGEN, Hilden, Germany), and according to the instructions of the manufacturer.  
120 Ratiometric FRET measurements of intracellular cAMP levels, before and after incubation with  
121 increasing concentration of the selective SSTR2 agonist BIM23120 (Ypsen, Milan, IT), were  
122 performed on an Axiovert 200 inverted microscope (Zeiss; Jena, Germany), equipped with an oil-  
123 immersion objective (plan-NEOFLUAR 63×/1.25), a 505 dextr beam splitter (Visitron Systems;  
124 Puchheim, Germany), a high-speed polychromator system (Visitron Systems) and an iXon Ultra  
125 EMCCD camera (Andor; Belfast, UK) (32).

### 126 **Total Internal Reflection Fluorescence Microscopy**

127 For single-molecule experiments, CHO cells were seeded on 24-mm clean glass coverslips at a  
128 density of  $3 \times 10^5$  cells per well, in complete phenol-red-free medium in order to minimize  
129 autofluorescence. On the following day, cells were transiently transfected with 2 µg DNA and 4-  
130 6 µL of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), according to the  
131 manufacturer's instructions. Cells were analyzed 4–12 h after transfection to achieve low

132 expression levels. CHO cells, transfected with SNAP-SSTR2 and CLIP-FLNA were labeled with  
133 1  $\mu$ M Alexa647-BG (Alexa Fluor 647-SNAP Surface; New England Biolabs, UK) and 1  
134  $\mu$ MTMR-BC (CLIP-Cell TMR-Star; New England Biolabs, UK), respectively. Labeling was  
135 performed in complete phenol-red-free medium for 20 min at 37 °C 5% CO<sub>2</sub>. At the end of the  
136 incubation, cells were washed three times with complete phenol-red-free medium, each time  
137 followed by 5 min incubation at 37 °C, and immediately imaged. These conditions resulted in  
138 optimized labeling of cell-surface SNAP-tagged receptors and intracellular CLIP-FLNA  
139 particles. A custom total internal reflection fluorescence (TIRF) microscope based on an Eclipse  
140 Ti (Nikon) equipped with four EMCCD cameras (iXon DU897, Andor), 405 nm, 488 nm , 561  
141 nm and 640 nm diode lasers (Coherent), and a 100 $\times$  oil-immersion objective (CFI Apo TIRF  
142 100x N.A. 1.49, Nikon) was used. Cells were first searched using bright field illumination and  
143 then a fine focus adjustment was performed switching to TIRF mode, always keeping the  
144 intensity of the laser power as low as possible (3% laser power). This procedure minimized  
145 photobleaching before image acquisition. Afterwards, laser power was set to 30% and image  
146 sequences (300–400 frames) were acquired with an exposure time of 30 ms, resulting in an  
147 interval between frames of 61.9 ms. The penetration depth of the evanescent field was  $\sim$ 100 nm.  
148 The microscope was equipped with an incubator and a temperature control unit. Experiments  
149 were performed at  $20.5 \pm 0.3$  °C. Only cells with less than 0.57 receptor particle/ $\mu$ m<sup>2</sup> were  
150 analyzed.

### 151 **MSD analysis**

152 Single-molecule image sequences were analyzed as previously described (23–26), including  
153 automated single particle detection and tracking, which were performed using the *utrack* software  
154 in MATLAB (MathWorks) environment (33). Receptor diffusion was calculated on the basis of a



155 mean square displacement (MSD) analysis of individual trajectories derived from TIRF image  
156 sequences as previously described (24). MSD data were fitted with the following equation:

$$157 \quad \text{MSD}(t) = 4Dt^\alpha + 4\sigma_l^2 \quad (\text{Eq. 1})$$

158 where  $t$  indicates time and  $\alpha$  is the anomalous diffusion exponent.  $\sigma_l$  is the standard deviation of  
159 the localization error, which was estimated to be approximately 23 nm. Only trajectories lasting  
160 at least 70 frames were analyzed. Since this analysis revealed heterogeneity among particles,  
161 trajectories were then classified according to the diffusion parameters  $D$  and  $\alpha$ . We considered  
162 particles with  $D < 0.01 \mu\text{m}^2.\text{s}^{-1}$  to be confined. Particles with  $D \geq 0.01 \mu\text{m}^2.\text{s}^{-1}$  and  $0.75 \leq \alpha \leq$   
163  $1.25$  were considered to have normal diffusion. Particles were considered to have sub- or super-  
164 diffusion in case of  $D \geq 0.01 \mu\text{m}^2.\text{s}^{-1}$  and  $\alpha < 0.75$  or  $\alpha > 1.25$ , respectively.

#### 165 **Colocalization index analysis based on single-molecule localizations**

166 To analyze the relationship between localizations of SSTR2 obtained by single-molecule  
167 microscopy and the signals obtained in the actin channel, we adapted the method developed by  
168 Ibach et al. (34), as previously described (26). The method is based on a modification of  
169 Manders' colocalization coefficients (35). Briefly, in our study, we generated a binary mask  
170 corresponding to the fibers the actin channel and we calculated the number of SSTR2  
171 localizations where the mask in the actin channel is equal to 1. This was used to calculate a  
172 colocalization index, whose values can range from -1 in case of perfect anti-correlation to +1 in  
173 case of perfect correlation/colocalization, whereas a value of 0 indicates no colocalization.

#### 174 **Immunofluorescence and confocal fluorescence microscopy**

175 CHO cells were plated on 13-mm coverslips at a density of  $1.5 \times 10^5$  cells per well in 24-well  
176 plates and grown at 37 °C for 18 h. Cells were then cotransfected with LifeAct-GFP, SNAP-

177 SSTR2 and FLNA17-18 or FLNA19-20. Receptors were labeled 24-48 h after transfection and  
178 stimulated with saturating concentration (100 nM) of BIM23120 up to 10 min to observe receptor  
179 clusters alignment with actin and colocalization with AP-2, for 15, 30, and 60 min to follow  
180 receptor internalization, at 37°C. After that, cells were fixed with 4% paraformaldehyde for 10  
181 min at room temperature, and washed three times in PBS. Cells cotransfected with SNAP-SSTR2  
182 and FLNA fragments only were permeabilized with 0.3% Triton X-100 in PBS for 5 min,  
183 incubated with 10% FBS in PBS (Thermofisher, Rockfor, IL) for 30 min and then incubated with  
184 anti AP-2 antibody (Thermofisher, Rockfor, IL) for 2 h at room temperature. After 3 washes with  
185 PBS containing 0.05% Tween 20, cells were stained with Alexa Fluor 488 conjugated secondary  
186 antibody (Thermofisher, Rockfor, IL) for 1 h at room temperature and extensively washed. Both  
187 primary and secondary antibodies were diluted in an antibody dilution buffer containing 1%  
188 BSA, 0.3% Triton X-100 in PBS. All coverslips were mounted on glass slides with ProLong  
189 Diamond Antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Life  
190 Technologies, Carlsbad, CA). Image acquisition was performed on a Leica TCS SP2 laser  
191 scanning confocal microscope equipped with Ar 488 nm, HeNe 543 nm and 635 nm lines and a  
192 63× objective (HCX PL APO 63X/1.4-0.60 OIL) (Leica Microsystems, Wetzlar, Germany).

### 193 **Colocalization analysis based on confocal images**

194 For colocalization analysis based on confocal microscopy, confocal images in the different  
195 channels were acquired separately, upon adjusting the photomultiplier gain for each channel to  
196 minimize background noise and avoid saturated pixels. Only the optical section corresponding to  
197 the plasma membrane was analyzed. The degree of colocalization between SSTR2 and actin and  
198 between SSTR2 and AP-2 were measured on raw images, by calculating Pearson's correlation  
199 coefficient (PCC) and Manders' colocalization coefficients (MCC), respectively, with the JACoP

200 tool in the NIH ImageJ software. SSTR2 clusters analysis was performed by including clusters of  
201 area between 0.01 and 0.3  $\mu\text{m}^2$ .

## 202 **Quantification of SSTR2 internalization**

203 To quantify SSTR2 internalization, about 8-12 equatorial confocal sections from each cell body  
204 were sequentially collected to ensure a scan thickness of  $\sim 500$  nm. The mean fluorescence  
205 density ( $F$ ) in two distinct regions corresponding to the plasma membrane and the cytosol were  
206 determined densitometrically in one representative focal plane for each cell. The mean plasma  
207 membrane to cytosol fluorescence ratio ( $f_R$ ) was then calculated according to the following  
208 equation:  $f_R = [F(\text{membrane}) - F(\text{background})] / [F(\text{total}) - F(\text{background})]$  with NIH ImageJ, as  
209 previously described (36). At least 30 cells for each group, from three independent transfections  
210 were analyzed, and results were plotted as mean value  $\pm$  SEM expressed as % of basal condition.

211 An assay based on reversible biotinylation of cell surface-proteins was used to quantify receptor  
212 internalization. CHO cells transiently expressing FLNA fragments and wild-type SSTR2 were  
213 washed three times with ice-cold PBS, followed by a 30-min incubation with 500  $\mu\text{g}/\text{ml}$   
214 cleavable EZ-Link sulfo-NHS-SS-biotin (Thermofisher, Rockfor, IL) at 4  $^{\circ}\text{C}$ . Unreacted biotin  
215 was blocked and removed by three washes with cold Tris-buffered saline containing 10 mM  
216 glycine. Biotinylated cells were incubated in pre-warmed medium with or without 100 nM  
217 BIM23120 at 37  $^{\circ}\text{C}$  for 30 min, and then chilled on ice to stop endocytosis. Glutathione (Sigma-  
218 Aldrich, St. Louis, MO) was used to release the biotin label from proteins at the cell surface: cells  
219 were washed twice with cold glutathione strip buffer (50 mM glutathione, 75 mM NaCl, 75 mM  
220 NaOH, 10% FBS in  $\text{H}_2\text{O}$ ), at 4  $^{\circ}\text{C}$  for 20 min. Excess glutathione was then quenched by 30 min  
221 incubation with 50 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO) in PBS, 1% BSA, pH 7.4  
222 at 4  $^{\circ}\text{C}$ . Cells were lysed with 35  $\mu\text{l}$  lysis buffer (Cell Signaling Technology, Danvers, MA) and

223 60 µg of total cellular protein extract was incubated with 1 µg SSTR2 (yI-17) antibody (Santa  
224 Cruz Biotechnology, Santa Cruz, CA) in a total volume of 100 µl of lysis buffer over night at 4  
225 °C on a rotating device, for immunoprecipitation. 20 µl of protein A/G Plus-Agarose (Santa Cruz  
226 Biotechnology, Santa Cruz, CA) was then added, and tubes were incubated for 3 h at 4 °C under  
227 constant rotation. After 5 washes with ice-cold PBS, the pellet was resuspended in 45 µl of Blue  
228 loading buffer (Cell Signaling Technology, Danvers, MA). Eluted proteins were separated by  
229 SDS-PAGE under nonreducing conditions. To detect biotinylated proteins, a 1:500 dilution of a  
230 horseradish peroxidase-linked antibody specific for biotin (Cell Signaling Technology, Danvers,  
231 MA) was used. The presence of equal receptor amounts in the immunoprecipitates was confirmed  
232 by stripping and reprobing with an antibody against SSTR2 (1:1000; UMB-1, Abcam,  
233 Cambridge, UK ) and using an anti-mouse secondary antibody covalently coupled to horseradish  
234 peroxidase (1:2000). The resulting bands were analyzed with the NIH ImageJ software.  
235 Experiments were performed in triplicate.

### 236 **Statistical analysis**

237 All statistical analyses were performed using the Prism 7 software (GraphPad, San Diego, CA).  
238 Unless otherwise stated, data were analyzed by two-tailed paired Student *t* test or Chi square test,  
239 as indicated.  $P < 0.05$  was accepted as statistically significant.

## 240 **3. Results**

### 241 **Single-molecule microscopy captures individual SSTR2 and FLNA molecules in living cells**

242 To visualize individual SSTR2 molecules at the plasma membrane of living cells, we generated a  
243 SSTR2 construct carrying a SNAP-tag at its N-terminus (SNAP-SSTR2) (37). The functional  
244 activity of SNAP-SSTR2 was confirmed by testing its ability to inhibit adenylyl cyclase activity

245 in transfected HEK293A cells incubated with increasing concentrations of the selective SSTR2  
246 agonist BIM23120 (Figure 1A and Supplemental Fig. 1). To allow simultaneous imaging of  
247 SSTR2 and FLNA, we additionally generated a FLNA construct carrying a CLIP-tag within its  
248 first hinge region (CLIP-FLNA) (Figure 1B) (38). The CLIP-FLNA construct was validated in  
249 transfected CHO cells for its capacity to induce correct stress fibers organization and colocalize  
250 with actin filaments (Supplemental Fig. 2). These constructs were subsequently transiently  
251 transfected in CHO cells, covalently labeled with selective SNAP and/or CLIP fluorescent  
252 substrates and imaged by fast one- or two-color TIRF microscopy (Figure 1C, D). Individual  
253 SSTR2 and FLNA particles were then automatically detected and tracked using the *utrack*  
254 software (33) (Figure 1E, F, G, H).

### 255 **Single-molecule analysis of SSTR2 reveals heterogeneous receptor dynamics at the plasma** 256 **membrane**

257 The spatio-temporal dynamics of SSTR2 at the plasma membrane of living cells was first  
258 analyzed in terms of receptor lateral mobility. To this end, CHO cells, which do not express  
259 endogenous SSTR2 (data not shown), were transiently transfected to express SNAP-SSTR2 at  
260 low physiological densities ( $0.57 \pm 0.07$  particle/ $\mu\text{m}^2$ ) and imaged by TIRF microscopy before  
261 and up to 5 min after stimulation with 100 nM of BIM23120. A mean-square displacement  
262 (MSD) analysis (23, 26) was used to estimate the diffusion coefficients of the SSTR2 particles  
263 based on their trajectories (Figure 2A and B). The results of this analysis revealed a high  
264 heterogeneity among SSTR2 particles, both under basal and stimulated conditions. The results of  
265 the MSD analysis were also used to classify the SSTR2 trajectories into four groups  
266 corresponding to receptors that were either virtually immobile or were characterized by a  
267 confined, directed or Brownian motion (sub-, super-, normal diffusion, respectively) (Figure 2A

268 and C).

269 Prior to stimulation, the mobile fraction was 94.6%. Short-term stimulation with BIM23120  
270 caused a statistically significant increase in the fraction of virtually immobile SSTR2s compared  
271 to basal conditions (16.5% vs. 5.4 %, respectively) and a corresponding reduction of around 3%  
272 for the remaining fractions (Figure 2C). This was accompanied by a general reduction of the  
273 average diffusion coefficient ( $D$ ) values estimated for the three mobile fractions compared to  
274 basal conditions (from 0.062 to 0.077  $\mu\text{m}^2\cdot\text{s}^{-1}$ , from 0.099 to 0.114  $\mu\text{m}^2\cdot\text{s}^{-1}$  and from 0.115 to  
275 0.130  $\mu\text{m}^2\cdot\text{s}^{-1}$  for sub-, normal and super-diffusion fractions, respectively) (Figure 2D).

276 To investigate the possible involvement of the cortical actin cytoskeleton in anchoring and/or  
277 limiting the mobility of SSTR2s (23, 26, 39), we simultaneously imaged actin fibers via co-  
278 transfection of LifeAct-GFP (29) (Figure 2E). We found that SSTR2s were preferentially  
279 localized along actin fibers, as indicated by positive colocalization index values (for details about  
280 the analysis see ref. 26). The preferential localization of SSTR2s along actin filaments was  
281 further enhanced by BIM23120 stimulation (Figure 2F). These data suggested that SSTR2s were  
282 either directly or indirectly interacting with the underlying actin cytoskeleton.

### 283 **Filamin A controls SSTR2 mobility at the plasma membrane**

284 The subcortical cytoskeleton has been shown to provide anchor points for receptors and other  
285 membrane proteins as well as barriers to their diffusion – a concept known as the fence-and-  
286 picket model (39), ultimately resulting in the formation of subdomains at the plasma membrane  
287 (26, 39). Since FLNA is a major actin binding protein and has been previously suggested to  
288 interact with SSTR2 based on *in vitro* results (17, 22), we investigated whether FLNA-SSTR2  
289 interactions occur in living cells and play a role in SSTR2 spatial arrangement and mobility.

290 For this purpose, we co-expressed SNAP-SSTR2 and CLIP-FLNA in CHO cells at low

291 physiological levels and simultaneously imaged individual SSTR2 and FLNA molecules by fast  
292 two-color TIRF microscopy followed by automated single particle tracking. In a subset of  
293 experiments, we additionally labeled actin fibers via co-transfection of LifeAct-GFP. Importantly,  
294 we observed individual SSTR2s transiently stopping at sites on actin fibers where a FLNA  
295 molecule was also located (Figure 3A and Supplemental Fig. S3, Supplemental Movie 1 and  
296 Supplemental Movie 2). These results revealed that SSTR2 undergo transient interactions with  
297 FLNA lasting approximately 0.521 seconds, which resulted in a preferential localization of  
298 SSTR2s along actin fibers.

299 We then explored the overall contribution of SSTR2–FLNA interactions on SSTR2 mobility at  
300 the plasma membrane. In order to interfere with SSTR2–FLNA interactions, we co-expressed a  
301 FLNA fragment corresponding to domains 19 and 20 (FLNA 19-20), which has been previously  
302 suggested to exert a dominant negative effect on the binding of SSTR2 to endogenous FLNA  
303 (17). The FLNA fragment encompassing repeats 17 and 18 (FLNA 17-18) was used as control  
304 (17). Individual SSTR2 particles retained their heterogeneous diffusion dynamics, independently  
305 of the presence of the dominant negative fragment (FLNA 19-20), as shown by a MSD analysis  
306 (Figure 3B). However, the average diffusion coefficients measured with FLNA 19-20 under basal  
307 conditions were overall higher than with the control fragment (FLNA 17-18) and these  
308 differences reached statistical significance for the super-diffusing particles, suggesting that the  
309 previously observed dynamic SSTR2-FLNA interactions on actin fibers contributed to slowing  
310 down SSTR2 diffusion at the plasma membrane (Figure 3C).

### 311 **Disrupting SSTR2–FLNA interactions hampers agonist-dependent SSTR2 clustering**

312 Since interactions with the actin cytoskeleton have also been suggested to play a possible role in  
313 receptor clustering (40, 41), we simultaneously imaged SSTR2, FLNA and actin at later time

314 points. TIRF images acquired in CHO cells stimulated with BIM23120 for 10 min showed the  
315 formation of SSTR2 clusters, which were absent under basal conditions (Figure 4A, arrowheads).  
316 Interestingly, we found that these clusters had a tendency to be aligned along actin fibers and that  
317 FLNA was often present together with SSTR2 in these clusters. Similar results were also  
318 obtained in confocal microscopy experiments (Figure 4B), in which we used higher SSTR2 and  
319 FLNA expression levels to facilitate the detection of the clusters. Co-expression of the dominant  
320 negative FLNA fragment (FLNA 19-20) caused a statistically significant reduction of the  
321 colocalization of SSTR2 clusters with actin (Figure 4B and C). This was accompanied by a  
322 statistically significant reduction in the size of SSTR2 clusters (Figure 4D) and a tendency  
323 towards a reduction of their number (Figure 4E). These findings indicate a role of FLNA in the  
324 formation and correct spatial arrangement of SSTR2 clusters along actin fibers.

### 325 **FLNA is required for efficient clathrin-mediated endocytosis of SSTR2**

326 Like for many other GPCRs, prolonged SSTR2 stimulation leads to its internalization, mainly via  
327 clathrin-mediated endocytosis (CME) (42, 43). Since actin and FLNA have been implicated in  
328 CME (40, 44–49), we investigated whether FLNA played a role in SSTR2 internalization. For  
329 this purpose, we simultaneously imaged the adaptor protein complex 2 (AP-2), which participates  
330 in both clathrin coated pit (CCP) initiation and recruitment of receptors to nascent CCPs, and is a  
331 widely used marker of CME (28). Confocal microscopy showed that, in cells expressing the  
332 FLNA17-18 control fragment and stimulated with BIM23120 for 10 min, a relevant fraction of  
333 SSTR2 clusters contained AP-2 (Figure 5A, white spots). These structures containing SSTR2s in  
334 nascent CCPs were observed to a remarkably lesser extent in FLNA 19-20 expressing cells  
335 (Figure 5A). Indeed, Manders' coefficient analysis demonstrated that the degree of SSTR2  
336 colocalization with AP-2-positive pits (MCC1) was significantly reduced (from  $37.1 \pm 8.7\%$  to



337 18.9 ± 9.9%) in the presence of the dominant-negative FLNA19-20 fragment. This was  
338 accompanied by a similar reduction in the colocalization of AP2 with SSTR2 (MCC2; Figure  
339 5B). These data suggested that whereas interfering with SSTR2–FLNA interactions had  
340 significant but modest effects on the formation of SSTR2 clusters – consistent with the results of  
341 Figure 4B – it largely impaired their coupling to CCPs and, thus, the recruitment of SSTR2 to  
342 CCPs.

### 343 **Interfering with SSTR2–FLNA interactions impairs SSTR2 internalization**

344 Given our observation that FLNA is required for SSTR2 recruitment to CCPs, we further studied  
345 the impact of FLNA–SSTR2 interactions on SSTR2 internalization. For this purpose, we  
346 analyzed the subcellular localization of SSTR2 by confocal microscopy in CHO cells transiently  
347 cotransfected with SNAP-SSTR2 and either FLNA17-18 or FLNA19-20 fragments and incubated  
348 with or without the agonist BIM23120 for up to 60 min. Under basal conditions, SSTR2 was  
349 virtually exclusively located at the plasma membrane, both in the presence of FLNA17-18 and  
350 FLNA19-20. As expected, BIM23120 induced a robust, time-dependent internalization and  
351 accumulation of SSTR2 in vesicles scattered throughout the cytoplasm in the presence of the  
352 control FLNA17-18 fragment (amount of internalized receptor of 54.8%, 68.7% and 71.4% after  
353 15, 30 and 60 min, respectively, corresponding to a fR of 0.45, 0.31 and 0.27, respectively)  
354 (Figure 6A and B). In contrast, SSTR2 internalization was significantly impaired in the presence  
355 of the dominant negative FLNA19-20 fragment (amount of internalized receptor of 41.5%,  
356 45.4% and 53% after 15, 30 and 60 min, respectively, corresponding to a fR of 0.58, 0.55 and  
357 0.47, respectively) (Figure 6A and B).

358 An assay based on biotinylation of cell-surface receptors further showed that SSTR2  
359 internalization was impaired in cells expressing the dominant negative FLNA19-20 fragment

360 compared to cells expressing the control FLNA17-18 fragment ( $39.1 \pm 6.7\%$  vs.  $9.0 \pm 2.9\%$ ,  
361 respectively; \*,  $P < 0.01$  vs. FLNA 17-18 expressing cells) (Figure 6C).

## 362 **4. Discussion**

363 The present study investigated the spatiotemporal dynamics of SSTR2 at the plasma membrane,  
364 revealing a crucial active role of the cytoskeletal adaptor protein FLNA in coordinating SSTR2  
365 diffusion dynamics and internalization. Our major findings suggest a model whereby FLNA  
366 molecules transiently interact with agonist-activated SSTR2s, facilitating their loose attachment  
367 to subcortical actin fibers and, thus, controlling their spatial arrangement and mobility. By  
368 controlling the localization of SSTR2s relative to the actin cytoskeleton, FLNA-SSTR2  
369 interactions promote SSTR2 internalization via facilitating the coupling between receptor  
370 clustering and accumulation in CCPs, both processes that occur with intervention of the actin  
371 cytoskeleton (45–49) (Figure 7).

372 Our single-molecule data indicate that SSTR2 lateral diffusion is modulated by agonist  
373 stimulation. The very high percentage (94.6%) of the mobile fraction seen under basal conditions  
374 is in agreement with fluorescence recovery after photobleaching (FRAP) results obtained with  
375 murine SST2a in living hippocampal neurons (50). Interestingly, our data show a significant  
376 increase in the fraction of virtually immobile receptors and lower diffusion coefficients within the  
377 mobile fractions in stimulated CHO cells in comparison with the basal state. These findings  
378 indicate that only a minor fraction of SSTR2s is associated with the cytoskeleton in the resting  
379 state, whereas such cytoskeletal interactions occur more frequently when the receptors are  
380 activated (Figure 2). Few controversial data are present in the literature regarding the role of  
381 FLNA in regulating the diffusion of cell surface proteins (51, 52). Our MSD analysis shows that  
382 in the presence of FLNA19-20, used to interfere with SSTR2-FLNA interactions (17, 22),

383 SSTR2s are more mobile compared to control cells (Figure 3). Overall, our findings are in  
384 agreement with the fence-and-picket model of the plasma membrane (53, 54), according to which  
385 integral membrane proteins (“pickets”) and barriers provided by the subcortical cytoskeleton  
386 (“fences”) compartmentalize the plasma membrane into small domains where receptors are  
387 loosely trapped. Our recently published data indicate that this phenomenon contributes to the  
388 formation of hot spots where receptors preferentially accumulate and signal (26). The findings of  
389 the present study suggest the FLNA might act as a scaffold to preferentially recruit ligand-  
390 activated receptors at specific actin-rich regions of the plasma membrane, which, in turn, would  
391 facilitate SSTR2 recruitment in CCPs and their internalization.

392 Previous studies have implicated the actin cytoskeleton in the maintenance of discrete sites of  
393 CCP assembling on the plasma membrane (44–49). When we investigated SSTR2 dynamics at  
394 higher receptor expression levels (obtained after 48 h transfection) than the ones achieved in  
395 previous single molecule experiments (obtained after 4-18 h transfection), we observed receptor  
396 clustering upon prolonged stimulation. However, both the colocalization between SSTR2 clusters  
397 and actin filaments and the size of SSTR2 clusters were significantly reduced in the presence of  
398 FLNA19-20, the dominant negative fragment of FLNA (Figure 4). These findings suggest that  
399 FLNA acts by linking SSTR2 clusters, CCPs and subcortical actin fibers. A similar clathrin–actin  
400 linking role has been previously suggested for other actin binding proteins such as the huntingtin-  
401 interacting protein 1 related protein (Hip1R) (46). Among the different proteins that cooperate in  
402 the formation of CCPs, AP-2 is one of the key co-factors that promotes CCP initiation at the  
403 plasma membrane to then disengage from CCPs immediately before vesicle internalization (55).  
404 In particular, the association of membrane-bound AP-2 with cytosolic clathrin triskelions favors  
405 cargo protein capture by the activated  $\mu$ 2 subunit of AP-2 (28, 56). Intriguingly, our data indicate

406 that interfering with SSTR2-FLNA interactions reduces the colocalization between agonist-  
407 induced SSTR2 clusters and AP2-containing CCPs, further supporting our hypothesis of a role  
408 for FLNA in the spatial coordination of receptor clustering and recruitment into nascent CCPs  
409 (Figure 5).

410 It has been previously postulated that the actin cytoskeleton might also play a mechanical role in  
411 CME, providing the force to drive invagination and translocation of the nascent vesicles into the  
412 cytoplasm (57, 58). Our data suggest that interactions with FLNA are required to initiate and  
413 sustain the overall process of clathrin-dependent SSTR2 internalization (40, 41, 52, 59–64). Our  
414 imaging and biochemical data show that SSTR2 is rapidly and efficiently internalized in CHO  
415 cells (about 70% internalization after 30 min of agonist exposure), in agreement with previous  
416 observations (42, 65–67). However, SSTR2 internalization was strongly impaired when  
417 FLNA–SSTR2 association was inhibited (Figure 6). This is in accordance with our previous  
418 observation that FLNA–SSTR2 binding is not required for SSTR2 expression and  
419 membrane localization in GH-secreting tumor cells but is rather involved in SSTR2 signaling and  
420 downregulation (17).

421 In conclusion, our findings reveal that SSTR2–FLNA undergo transient interactions in living  
422 cells, which dynamically link SSTR2s to the actin cytoskeleton. These interactions with FLNA  
423 and actin fibers regulate SSTR2 spatial arrangement and mobility and are required for coupling  
424 agonist-dependent SSTR2 clustering to its recruitment to CCPs and, ultimately, its  
425 internalization. These results, together with our previous observation that FLNA is involved in  
426 the regulation of SSTR2 signaling and downregulation (17), indicate FLNA as a novel potential  
427 target to modulate the amount of active SSTR2s at the plasma membrane, with possible  
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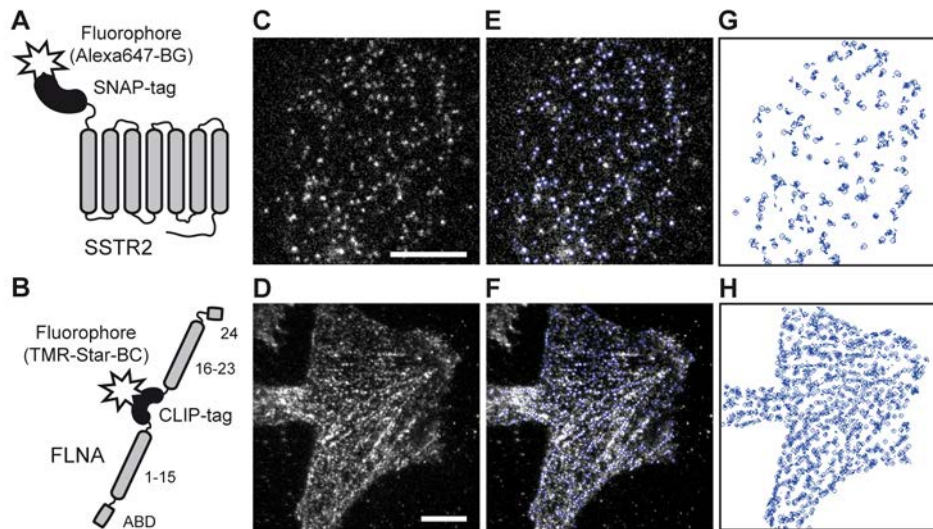
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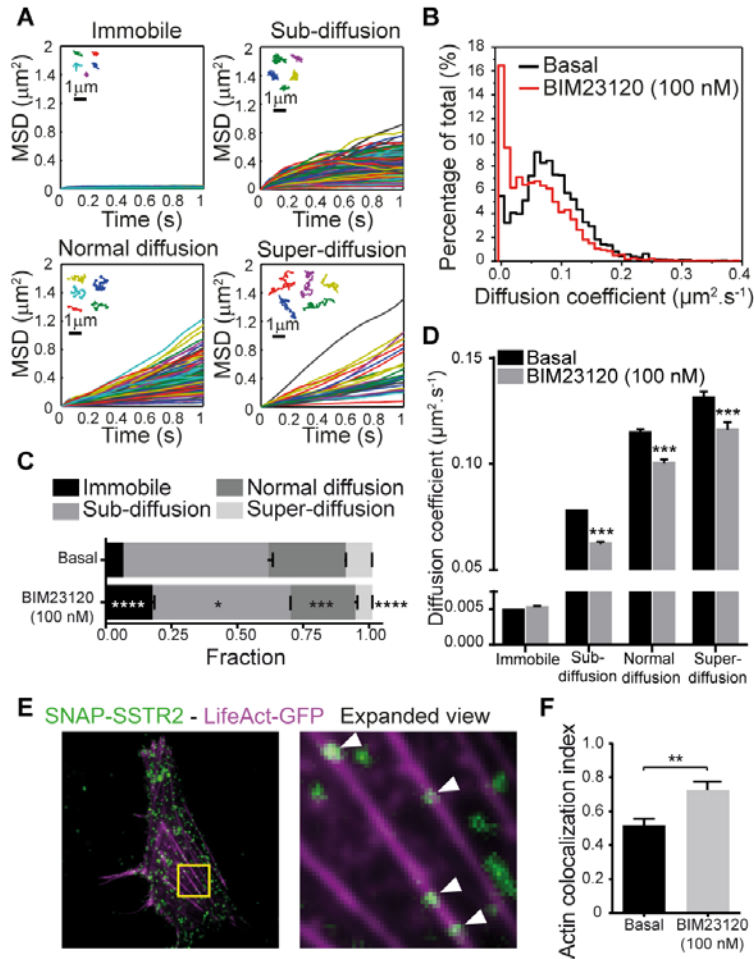
630 **Figure 1.**



631  
632 **Figure 1. Single-molecule visualization and tracking of individual SSTR2 and FLNA**  
633 **particles at the plasma membrane of living cells.**

634 (A and B) Schematic representation of the SNAP-SSTR2 (A) and CLIP-FLNA (B) constructs  
635 used in this study. (C-H) Single-molecule imaging. CHO cells were transfected with SNAP-  
636 SSTR2 (C) or CLIP-FLNA (D), labeled with Alexa647-BG or TMR-Star-BC substrates,  
637 respectively, and imaged by TIRF microscopy. Shown are single frames of representative image  
638 sequences (C and D), the same with overlaid in blue the individual trajectories obtained with the  
639 automated tracking algorithm (E and F) and the trajectories alone (G and H). The current position  
640 of each particle is indicated by a blue circle. Scale bars, 10  $\mu\text{m}$ .

641 **Figure 2.**



642

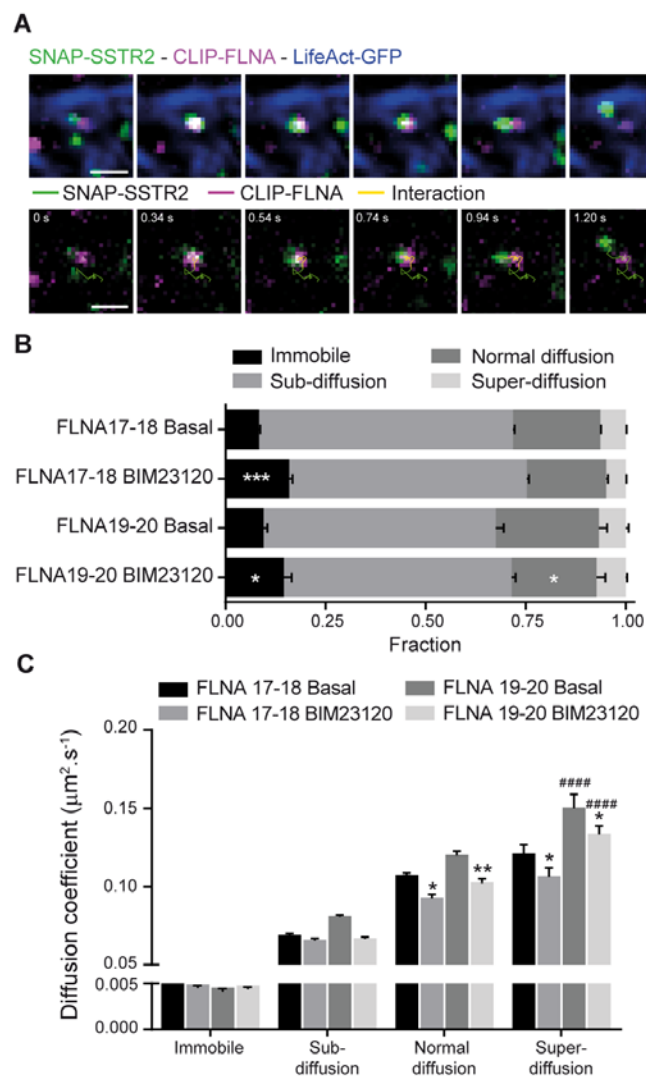
643 **Figure 2. SSTR2 diffusion dynamics at the plasma membrane is modified by agonist**  
 644 **stimulation.**

645 (A) MSD plots of representative SSTR2 trajectories classified into four groups based on their  
 646 mobility. (B) Diffusion coefficient distribution of SSTR2 particles calculated from the MSD  
 647 analysis and reported as percentage of total number of particles under basal (black) or stimulated  
 648 condition (red). (C) Frequency distributions of the trajectories in the four groups under basal and  
 649 stimulated conditions. (D) Average diffusion coefficients of each group shown in (C). Differences  
 650 in C and D are statistically significant by two-way ANOVA. \*,  $P < 0.05$ , \*\*\*,  $P < 0.001$  and \*\*\*\*,  
 651  $P < 0.0001$  vs. corresponding basal condition by Tukey's multiple comparison test. (E)



652 Representative frame from a TIRF image sequence of SSTR2 particles (green) and actin fibers  
 653 (magenta) in basal condition (left) and expanded view of the region marked with the yellow box  
 654 (right). Arrowheads, individual SSTR2s localized along actin fibers. (F) Quantification of SSTR2  
 655 colocalization with actin fibers under basal and stimulated conditions. \*\*, P<0.01 vs. basal  
 656 condition by unpaired t-test. All data are mean  $\pm$  SEM of three independent experiments.

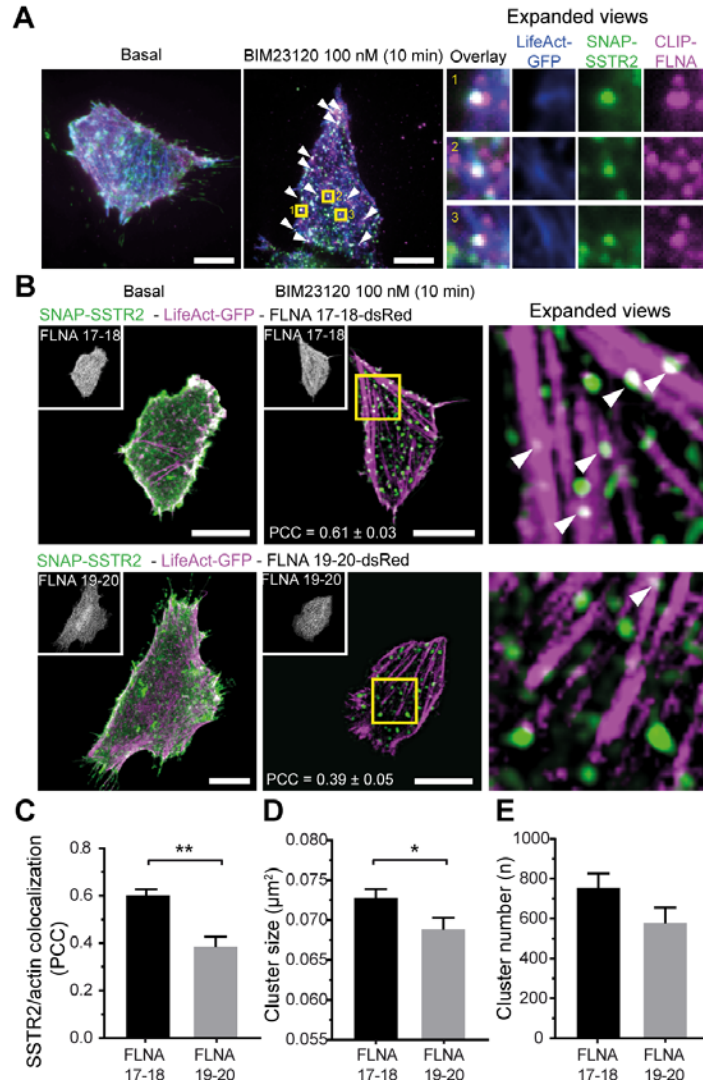
657 **Figure 3.**



658  
 659 **Figure 3. SSTR2 interactions with FLNA and actin at the plasma membrane regulate**  
 660 **SSTR2 mobility.**

661 (A) Top, selected frames from a representative TIRF image sequence acquired in CHO cells co-  
662 expressing SNAP-SSTR2 (green), CLIP-FLNA (magenta) and LifeAct-GFP (blue). Bottom,  
663 corresponding trajectories showing an example of a SSTR2 transiently colocalizing with a FLNA  
664 molecule on an actin fiber (yellow). Scale bars, 1  $\mu$ m. (B) Frequency distributions of the  
665 trajectories classified in the four mobility groups in the presence of FLNA17-18 or FLNA19-20  
666 fragments under both basal and stimulated conditions. (C) Average diffusion coefficients (D)  
667 corresponding to each group shown in (B). Differences in (B) and (C) are statistically significant  
668 by two-way ANOVA. \*,  $P < 0.05$ , \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$  by Tukey's multiple comparison  
669 test vs. basal condition; ####,  $P < 0.0001$  by Tukey's multiple comparison test vs. the  
670 corresponding fraction in the control (FLNA17-18). All data are mean  $\pm$  SEM from three  
671 independent experiments.

672 **Figure 4.**



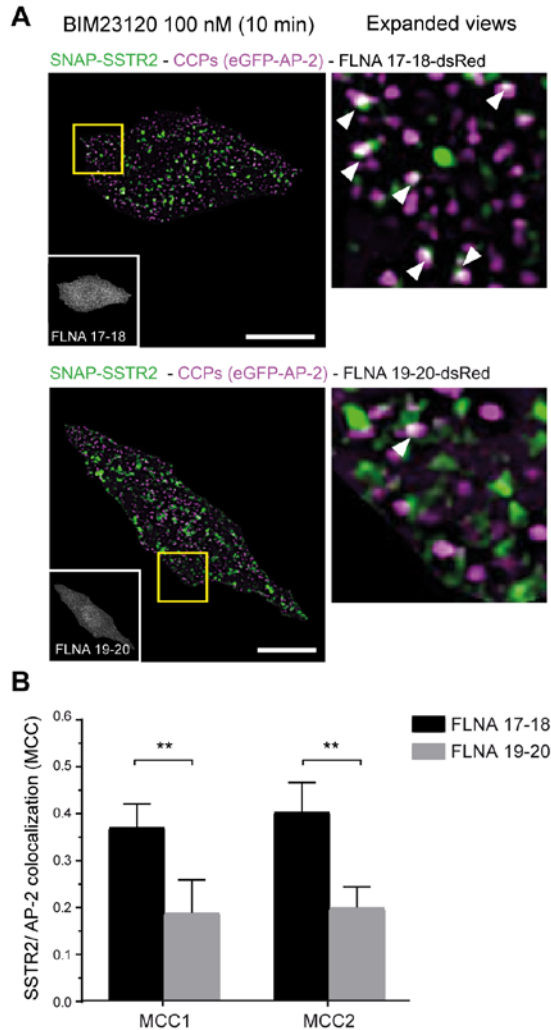
673

674 **Figure 4. Role of FLNA in agonist-dependent SSTR2 clustering.**

675 (A) Preferential alignment of SSTR2 clusters containing FLNA along actin fibers. CHO cells  
 676 were transfected to express LifeAct-GFP (blue) together with single-molecule levels of SNAP-  
 677 SSTR2 (green) and CLIP-FLNA (magenta). Shown are representative average intensity  
 678 projections of TIRF images sequences from three independent experiments under basal  
 679 conditions (left) or after stimulation with BIM23120 for 10 min (middle). Arrowheads, SSTR2  
 680 clusters containing FLNA aligned along actin fibers. Three examples of clusters are shown in  
 681 expanded views (right). Scale bars, 10  $\mu\text{m}$ . (B) Effect of disrupting SSTR2–FLNA interactions on

682 the localization of SSTR2s along actin fibers. Shown are representative confocal optical sections  
683 showing the plasma membrane of CHO cells cotransfected with LifeAct-GFP (magenta), SNAP-  
684 SSTR2 (green) and FLNA17-18 or FLNA19-20 fragments under basal or stimulated conditions.  
685 Insets, images showing high expression of the FLNA fragments. Arrowheads, SSTR2 clusters  
686 aligned along actin fibers. Scale bars, 10  $\mu\text{m}$ . (C-E) Quantitative analyses of images like those  
687 shown in B. Reported are the Pearson's correlation coefficient (PCC) between SNAP-SSTR2 and  
688 LifeAct-GFP images (C) as well as the number (D) and size (E) of SSTR2 clusters.\*\*,  $P < 0.01$   
689 and \*,  $P < 0.05$  vs. FLNA 17-18 transfected cells by unpaired Student's  $t$  test. Data are mean  $\pm$   
690 SEM of 15 cells from three independent experiments.

691 **Figure 5.**



692

693 **Figure 5. FLNA is required for efficient SSTR2 recruitment to CCPs.**

694 (A) Effect of interfering with SSTR2–FLNA interactions on agonist-induced SSTR2 recruitment

695 to CCPs. CHO cells were transiently cotransfected with SNAP-SSTR2 (green) and either FLNA

696 17-18 or FLNA 19-20 (insets) and immunostained for AP-2 (magenta). Shown are representative

697 confocal images acquired at the level of the plasma membrane (left) and expanded views of the

698 regions marked with the yellow boxes (right). Arrowheads, SSTR2 clusters colocalizing with AP-

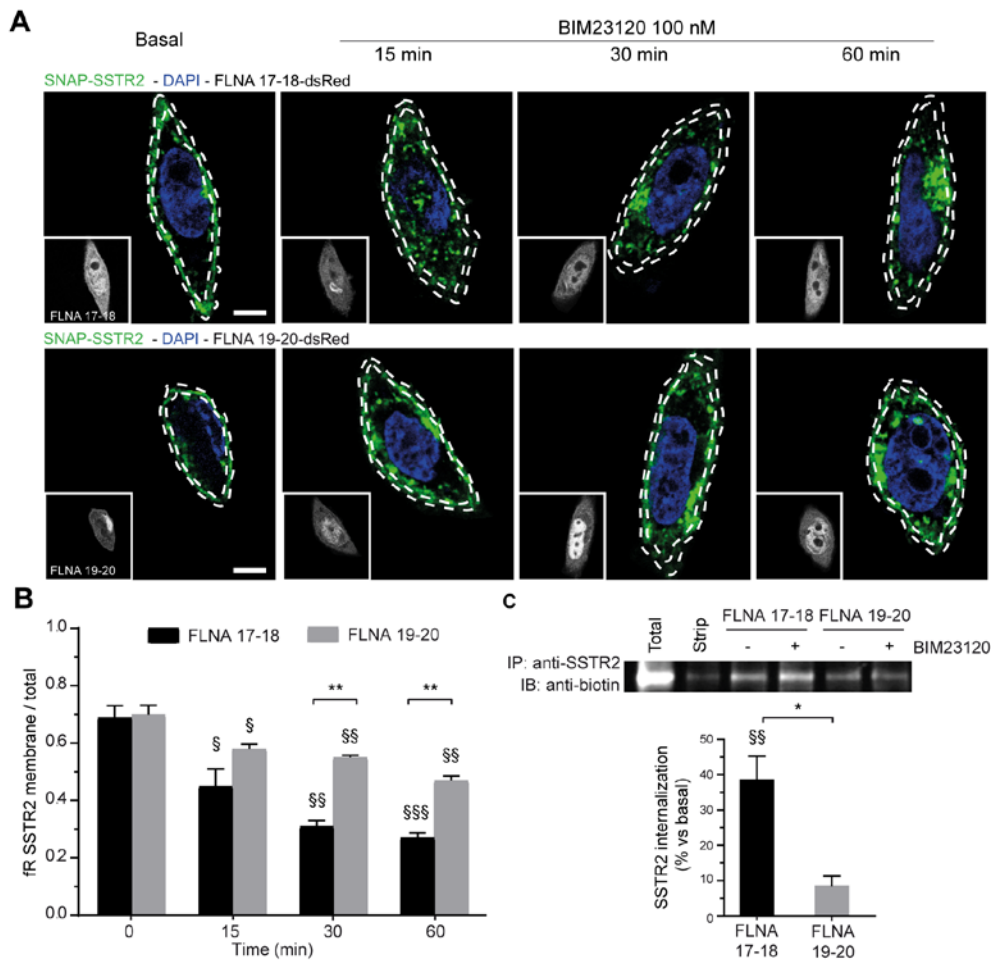
699 2 (white). Scale bars, 10  $\mu$ m. (B) Quantitative analysis of images like those shown in A. The

700 extent of pixel colocalization between SSTR2 and AP-2 is expressed as mean  $\pm$  SEM of Manders'

701 colocalization coefficients (MCC) where MCC1 represents the fraction of SSTR2 overlapping

702 with AP-2 and MCC2 the fraction of AP-2 overlapping with SSTR2 (Menders et al., 1993). For  
 703 each condition, 15 cells from three independent experiments were analyzed. \*\*, P<0.01 vs.  
 704 FLNA 17-18 expressing cells by unpaired Student's *t* test.

705 **Figure 6.**



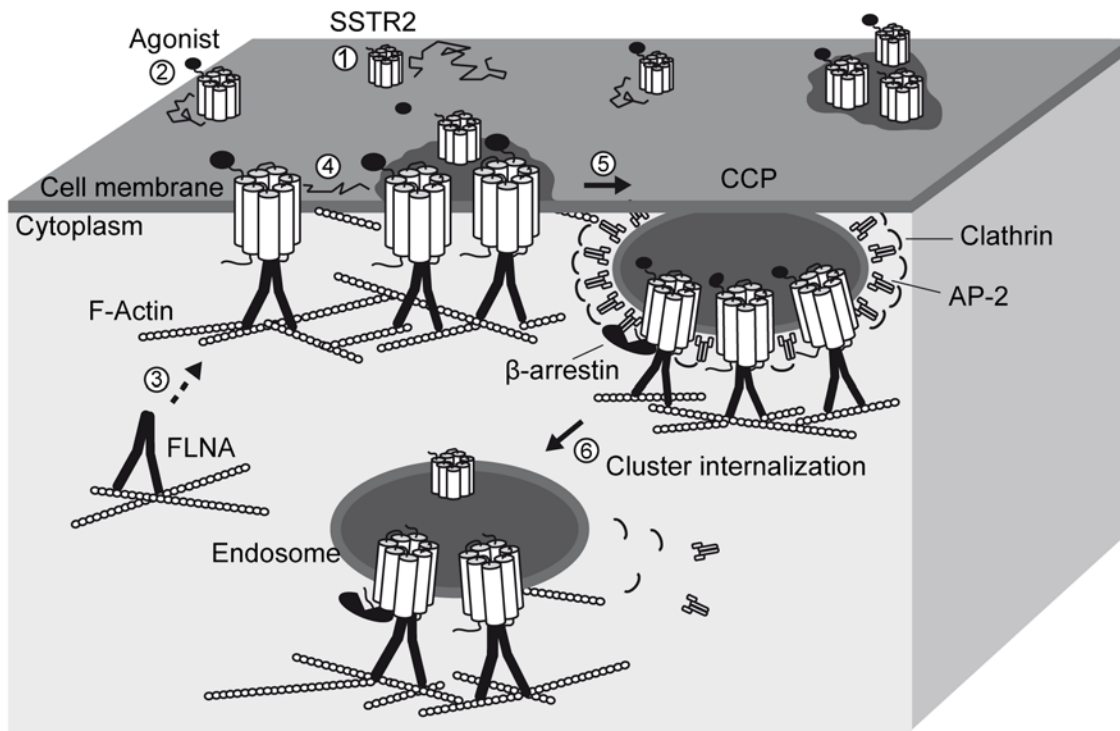
706

707 **Figure 6. Inhibition of FLNA-SSTR2 interactions affects agonist-mediated SSTR2**  
 708 **internalization.**

709 (A) CHO cells coexpressing SNAP-SSTR2 (green) and either FLNA17-18 (top) or FLNA19-20  
 710 (bottom) were incubated with 100 nM BIM23120 for 15, 30, and 60 min. Shown are  
 711 representative confocal images acquired at the level of the nucleus. Hatched white lines represent  
 712 the membrane area. Insets show the corresponding FLNA17-18 (top) or FLNA19-20 (bottom)

713 images. DAPI (blue) was used to stain the nucleus. Scale bars, 5  $\mu\text{m}$ . (B) Quantitative analysis of  
 714 SSTR2 internalization based on confocal images like those in A. For each group, at least 30 cells  
 715 from three independent experiments were analyzed. Data are mean  $\pm$  SEM \*,  $P < 0.05$  and \*\*,   
 716  $P < 0.01$  vs. FLNA 17-18 expressing cells; §,  $P < 0.05$ , §§,  $P < 0.01$ , §§§,  $P < 0.001$  vs. respective  
 717 basal condition by unpaired Student's *t* test. (C) Quantification of SSTR2 internalization based on  
 718 biotinylation of membrane receptors. CHO cells transiently cotransfected with FLNA 17-18 or  
 719 FLNA 19-20 and wild-type SSTR2 were incubated with or without 100 nM BIM23120 for 30  
 720 min. SSTR2 was immunoprecipitated with a specific antibody and the internalized biotinylated  
 721 SSTR2 was detected with an antibody recognizing biotin. Shown are the mean  $\pm$  SEM of three  
 722 independent experiments. \*,  $P < 0.05$  vs. FLNA17-18 transfected cells; §§,  $P < 0.01$  vs.  
 723 corresponding basal condition by unpaired Student's *t* test.

724 **Figure 7.**

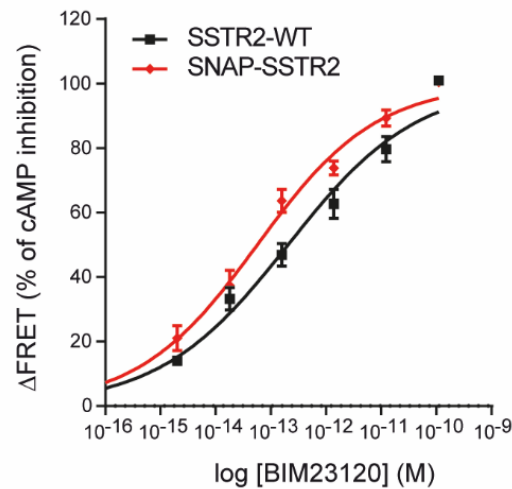


725  
 726 **Figure 7. Proposed model for FLNA role in SSTR2 diffusion dynamics and internalization.**

727 Under resting conditions, SSTR2s diffuse at the cell surface (1). Agonist binding (2) promotes  
728 FLNA recruitment to SSTR2s (3), which increases their interaction with actin fibers. This favors  
729 the formation of SSTR2 clusters and their correct localization in actin-rich regions of the plasma  
730 membrane (4). These events promote the recruitment of SSTR2s to CCPs (5) and, ultimately,  
731 their internalization (6).

732

### 733 **Supplementary Data**



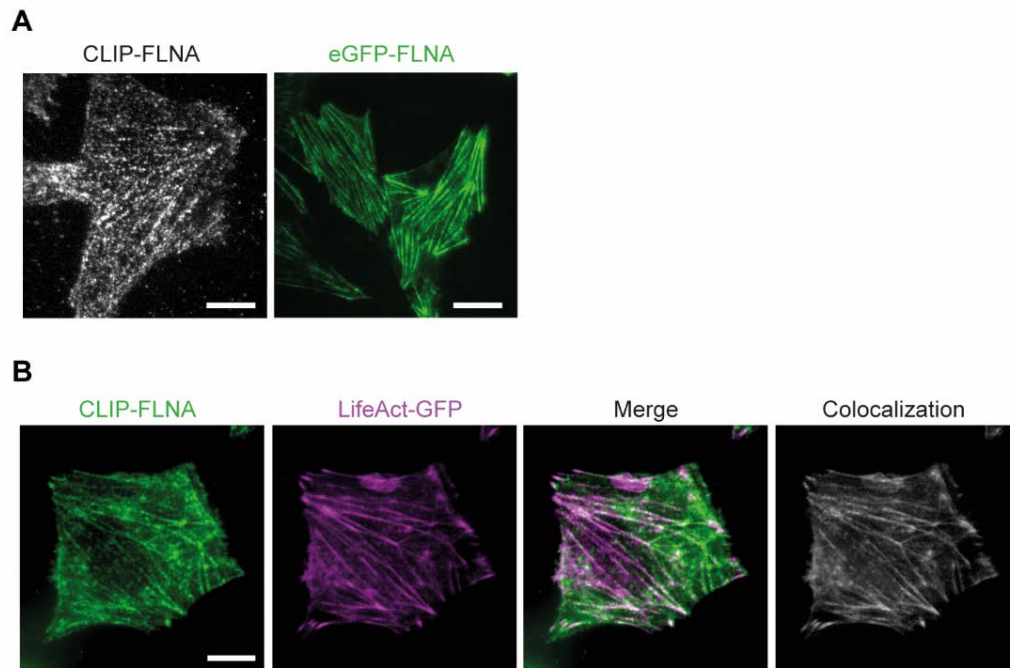
734

### 735 **Supplemental Figure 1.**

736 **Functional characterization of SNAP-SSTR2 construct.** HEK293A cells were co-transfected  
737 with SNAP-SSTR2 or wild-type SSTR2 and the FRET sensor for cAMP Epac1-camps. Cells  
738 were prestimulated with forskolin to activate adenylyl cyclases followed by incubation with  
739 increasing concentrations of the SSTR2-selective agonist BIM23120. The resulting inhibition of  
740 cAMP production was measured in real time by FRET microscopy. The SNAP-SSTR2 construct  
741 is fully functional, as shown by cAMP concentration-response dependencies comparable to those



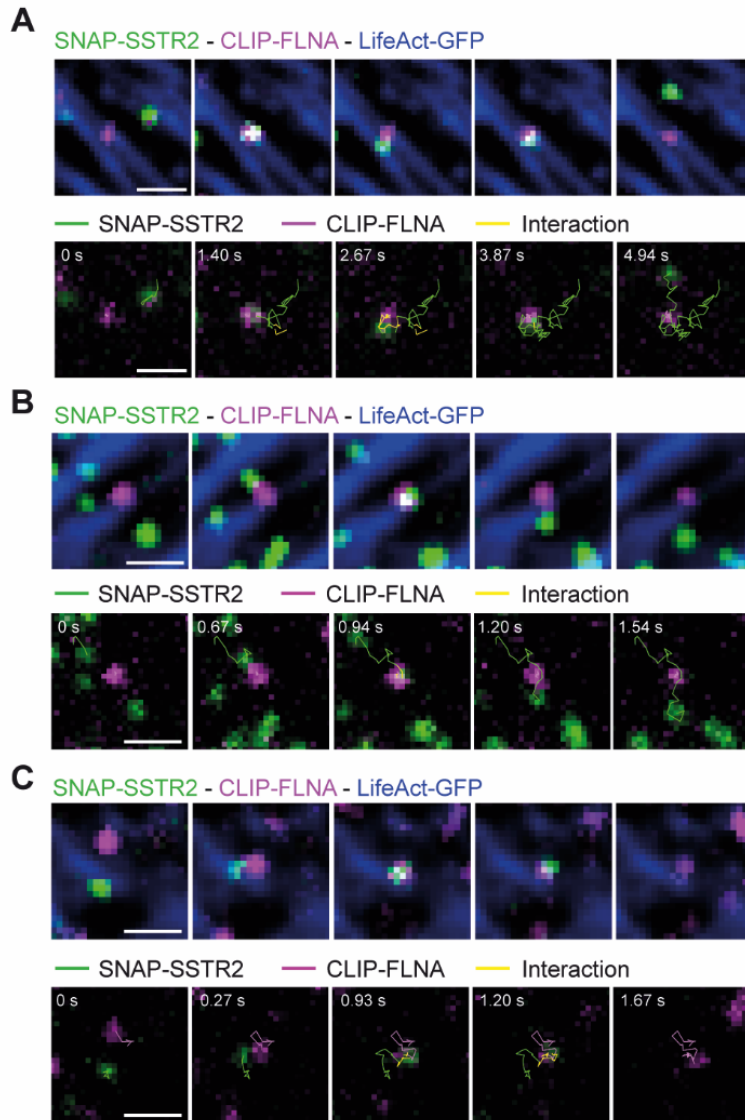
742 observed with wild-type SSTR2. Data are mean  $\pm$  SEM of 10 cells from three independent  
743 experiments.



744

745 **Supplemental Figure 2.**

746 **Validation of CLIP-FLNA construct.** (A) TIRF images of CHO cells transfected with CLIP-  
747 FLNA or eGFP-FLNA, respectively. The CLIP-FLNA construct displays a normal arrangement  
748 along stress fibers, similar to what observed with eGFP-FLNA. (B) TIRF images of CHO cell  
749 cotransfected with CLIP-FLNA (green) and LifeAct-GFP (magenta). The resulting merge image  
750 is shown. Colocalization between CLIP-FLNA and actin filaments was analyzed by NIH ImageJ  
751 software and is shown in white, confirming the actin-binding property of the CLIP-FLNA  
752 construct. Scale bars, 10  $\mu$ m.



753

754 **Supplemental Figure 3.**

755 **Single-molecule visualization of SSTR2 colocalizing with FLNA and actin at the cell**

756 **surface. A-C** Further examples of images and corresponding trajectories from TIRF-M time-

757 lapse sequences acquired in three representative living CHO cells stained for actin (blue) and

758 expressing single-molecule levels of SNAP-SSTR2 (green) and CLIP-FLNA (magenta), labeled

759 with Alexa647-BG and TMR-BC dye, respectively. SSTR2 and FLNA trajectories are depicted

760 in green and magenta, respectively, whilst SSTR2–FLNA apparent interactions are represented  
761 by yellow trajectories. Scale bars, 1  $\mu\text{m}$ .

762 **Supplemental Video 1.**

763 **SSTR2 and FLNA during an apparent interaction on underlying actin fiber.** Shown are  
764 individual SSTR2 (green) and FLNA (magenta) particles undergoing transient interactions over  
765 an actin fiber (blue) in a living CHO cell. Frames were acquired every 61.9 ms.

766 **Supplemental Video 2.**

767 **SSTR2 and FLNA trajectories.** Corresponding trajectories of individual SSTR2 (green) and  
768 FLNA (magenta) particles shown in Movie S1. The trajectories are colored in yellow during the  
769 apparent interaction.

770