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# Cytoskeleton protein Filamin A is required for efficient Somatostatin receptor type 2 internalization and recycling through Rab5 and Rab4 sorting endosomes in tumor somatotroph cells.

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**Running Title:** FLNA regulates $SST_2$ intracellular sorting

**Abstract**

The high expression of somatostatin receptor 2 ($SST_2$) in growth hormone (GH)-secreting tumors represents the rationale for the clinical use of somatostatin analogs (SSAs) in acromegaly. Recently, the cytoskeletal protein Filamin A (FLNA) has emerged as key modulator of the responsiveness of GH-secreting pituitary tumors to SSAs by regulating $SST_2$ signaling and expression.

The aim of this study was to explore FLNA involvement in $SST_2$ intracellular trafficking in tumor somatotroph cells.

By biotinylation assay we found that FLNA silencing abolished octreotide-mediated $SST_2$ internalization in rat GH3 cell line (28.0±2.7% vs 4±4.3% $SST_2$ internalization, control vs FLNA siRNA cells, respectively, $P<0.001$) and human GH-secreting primary cultured cells (70.3±21.1% vs 24±19.2% $SST_2$ internalization, control vs FLNA siRNA cells, respectively, $P<0.05$). In addition, confocal imaging revealed impaired $SST_2$ recycling to the plasma membrane in FLNA silenced GH3 cells.

Co-immunoprecipitation and immunofluorescence experiments showed that FLNA, as well as β-arrestin2, is timely-dependent recruited to octreotide-stimulated $SST_2$ receptors both in rat and human tumor somatotroph cells. Although FLNA expression knock down did not prevent the formation of β-arrestin2-$SST_2$ complex in GH3 cells, it significantly impaired efficient $SST_2$ loading into cytosolic vesicles positive for the early endocytic and recycling markers Rab5 and Rab4,

respectively (33.7±8.9% down to 25.9±6.9%, $p<0.05$, and 28.4±7.4% down to 17.6±5.7%, $p<0.01$, for $SST_2$-Rab5 and $SST_2$-Rab4 colocalization, respectively, in control vs FLNA siRNA cells).

Altogether these data support an important role for FLNA in the mediation of octreotide-induced $SST_2$ trafficking in GH-secreting pituitary tumor cells through Rab5 and Rab4 sorting endosomes.

**Introduction**

The G protein-coupled receptor (GPCR) somatostatin receptor type 2 ($SST_2$) is the most widely distributed somatostatin receptor in human tissues and neuroendocrine tumors [1,2]. Due to its well-established inhibitory effects on hormone release and cell proliferation, and its high levels of expression in these tumors, $SST_2$ is considered the main molecular target for long-acting somatostatin analogs (SSAs) treatment in growth hormone (GH)-secreting pituitary tumors [3,4]. However, despite high expression of $SST_2$, a consistent subset of patients harboring a GH-secreting pituitary tumor is not successfully controlled by the medical therapy, showing poor or lacking GH and IGF-1 normalization [5,6]. In the last decades, several studies have attempted to characterize new molecular factors responsible for this variability in the response to the SSAs octreotide and lanreotide [7,8].

Intracellular endosome trafficking has emerged as a critical aspect of GPCRs regulation, able to affect receptor cell surface availability and signaling, with consequences for the responsiveness to physiological ligands and drugs [9,10].

A pleiotropic role of the cytoskeleton protein filamin A (FLNA) in the intracellular trafficking of several membrane receptors has been demonstrated [11]. From a structural point of view, FLNA is a 280 kDa homodimeric protein composed of an N-terminal actin-binding domain and 24 Ig-like repeats of about 96 amino acids. FLNA stabilizes cortical three-dimensional actin filament networks and links them to cellular membranes. Furthermore, it acts as a scaffold platform for intracellular molecules involved in different signaling pathways [11,12]. Previous studies showed that FLNA integrates cell signaling events and cell shape changes that ultimately dictate pituitary tumors drug

responsiveness and invasiveness [13-15]. In particular, in tumor somatotroph cells, $SST_2$ directly binds FLNA and this interaction is essential for $SST_2$-mediated antiproliferative effects and for $SST_2$ protein levels stability after prolonged agonist stimulation [14]. Moreover, in CHO cells transfected with human $SST_2$, FLNA modulates receptor mobility at the plasma membrane and actively cooperates in ligand-induced $SST_2$ clusters formation, alignment on actin filaments and internalization [16].

As for most other GPCRs, agonist-stimulation promotes $SST_2$ endocytosis via causing its phosphorylation by G protein-coupled receptor kinases at serine and threonine residues located at its C-terminus, followed by β-arrestin recruitment [17-20]. In the context of acromegaly, controversial data are present in the literature regarding the correlation between β-arrestins and $SST_2$ expression and responsiveness of GH-secreting pituitary tumors to SSAs [21-23]. Although FLNA is a known β-arrestin binding partner in mammalian cells [24,25], a contribution of FLNA in the β-arrestin-recruitment of $SST_2$ has not been investigated yet. Once in the cytoplasm, $SST_2$ does not follow a degradation route, as no detectable lysosome or proteasome-mediated degradation of $SST_2$ has been observed in HEK293 or the rat GH-secreting pituitary cell line GH3. Indeed, only GH3 cells lacking FLNA- $SST_2$ interaction showed receptor downregulation after prolonged agonist stimulation [14,17,26].

Receptor sorting within endosome trafficking is regulated by members of the Rab family of small GTPases whose functions are membrane budding, docking, and interaction with the cytoskeleton [27,28]. Rab4, Rab21, and Rab11 have been identified as key regulators of $SST_3$ trafficking [29], however no detailed data are available in the literature about $SST_2$ dynamics inside the cell.

Since in many cases receptor trafficking is facilitated by actin filaments dynamics [30,31], here we tested a role of FLNA in the postendocytic sorting and recycling of $SST_2$ both in rat and human tumor somatotroph cells.

## Materials and Methods

### Cell culture

Rat tumoral pituitary GH3 cells (ATCC CCL-82.1) and GH4C1 cells (ATCC CCL-82.2) were cultured in F12K and F10 medium, respectively, supplemented with 15% Horse Serum (HS), 2.5% Fetal Bovine Serum (FBS), 2 mM glutamine and antibiotics. Human pituitary cells were obtained by the transphenoidal route from a total of 10 patients with GH-secreting tumors. Tissues were subjected to an enzymatic dissociation in DMEM containing 2 mg/mL collagenase at 37 °C for 2 h, as previously described [14]. Dispersed cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, and antibiotics. The study was approved by the local ethics committee and informed consent was obtained from patients involved in the study.

### Plasmids transfection and FLNA silencing

Expression vectors coding for human HA-$SST_2$ (influenza hemagglutinin-tagged $SST_2$), SNAP-$SST_2$ ($SST_2$ fused to SNAP tag, a 20-kDa protein derived from the enzyme $O^6$-alkylguanine-DNA alkyltransferase), EGFP-β-arrestin2 (enhanced green fluorescent protein-tagged β-arrestin2) and FLNA truncated mutants (FLNA 19-20, FLNA 21-24) were previously described [14,16,32]. These vectors were transiently transfected in GH3 and GH4C1 cells using Lipofectamine 2000 as reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Transfection efficiency was evaluated by fluorescence microscopy in each experiment. Only experiments with transfection efficiency > 60% were accepted. Corresponding empty vectors were used in each experiment as negative controls. FLNA gene silencing was performed in GH3 cells and GH-secreting tumor primary cells using rat or human FLNA predesigned small interfering RNAs (siRNAs), respectively, and Dharmafect transfection agent 2 (Dharmacon, GE Healthcare Life Sciences, Chicago, IL) according to the instruction of the manufacturer. A negative control (scramble) siRNA was used in each experiment. The optimal concentration of siRNAs and the kinetics of silencing of FLNA were established in preliminary experiments and 72 h of silencing was chosen as optimal time point. For

simultaneous plasmids transfections and FLNA gene silencing, cells were co-transfected for 72 h with vectors of interest and FLNA siRNAs using Lipofectamine 2000 as transfection agent following the manufacturer's instructions.

**Biotinylation assay**

To perform quantitative analysis of $SST_2$ internalization, GH3 and GH4C1 cells were seeded at the cell density of 1 x $10^6$ cells in 10 cm petridish, transiently co-transfected with FLNA siRNA and HA-$SST_2$ the following day. GH-secreting tumor primary cells were plated at the cell density of 5 x $10^6$ cells in p6 multiwell plate and silenced for FLNA. Biotinylation of cell surface-receptors was assessed accordingly to the previously described protocol [16] and glutathione (Sigma-Aldrich, St. Louis, MO) was used to release the biotin label from proteins at the cell surface. $SST_2$ internalization was promoted by stimulation with 100 nM octreotide for 30 min. For immunoprecipitation, 200 µg of total cellular protein extract was mixed with 1 µg $SST_2$ (yI-17) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight on a rotating device at 4 °C. Twenty µl of protein G Plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added afterwards. Ice-cold PBS (Thermofisher, Rockfor, IL), was used to wash the pellet and 45 µl of Blue loading buffer (Cell Signaling Technology, Danvers, MA) for resuspension. Eluted proteins were separated by SDS-PAGE under nonreducing conditions. Anti-FLNA antibody (1:1000, Abnova, Taiwan, RC) was used to test FLNA silencing efficiency and only experiments with silencing efficiency > 80% were considered. Biotinylated proteins were detected with a 1:500 diluted horseradish peroxidase-linked antibody specific for biotin (Cell Signaling Technology, Danvers, MA). The presence of equal $SST_2$ amounts in the immunoprecipitates was confirmed by stripping and reprobing with an antibody against $SST_2$ (1:1000; UMB-1, Abcam, Cambridge, UK) and using an anti-mouse secondary antibody covalently coupled to horseradish peroxidase (1:2000). The obtained bands were analyzed with the NIH ImageJ software. Experiments were performed in triplicate in rat cell lines and in primary cultures from 3 different GH-secreting pituitary tumors.

**Co-immunoprecipitation of $SST_2$-βarrestin2-FLNA**

To test FLNA and β-arrestin2 recruitment to $SST_2$, GH3 cells were seeded at the cell density of 3,3 x $10^5$ cells in p6 multiwell plate for 18 h at 37 °C, then simultaneously silenced for FLNA and transiently co-transfected with EGFP-β-arrestin2 and HA-$SST_2$. Cells were incubated in presence or absence of octreotide (100 nM) at 37 °C for the indicated times (2, 5, 10, 30 min) and then lysed. The homogenates were centrifuged and 150 µg of supernatant incubated with 1 µg of $SST_2$ (yI-17) antibody, followed by 20 µl volume of Protein G PLUS-Agarose beads addition, as above described. After final wash, the pellet was resuspended in 45 µl of Blue loading buffer containing reducing agents and analyzed by Western blot. FLNA and EGFP-β-arrestin2 were detected by anti-FLNA (1:1000, Abnova, Taiwan, RC) and anti-GFP primary antibodies (Clonetech, CA, USA, diluted 1:1000), respectively, and anti-mouse and anti-rabbit secondary antibodies (diluted 1:2000), respectively. Experiments were repeated three times.

**Immunofluorescence**

GH3 and human GH-secreting pituitary cultured cells were plated on 13-mm poly-L-lysine coated coverslips at a density of $125 \times 10^3$ cells/well in 24-well plates and grown at 37 °C for 18 h, and subjected to transient transfection or gene silencing the following day. In GH3 cells SNAP-$SST_2$ receptors were labeled with AlexaFluor™ 647-BG (ThermoFisher Scientific, CA) 1 µM for 20 min at 37 °C, as previously described [16]. Cells were incubated with octreotide 100 nM at 37°C for 5, 15 or 30 min for β-arrestin2 mobilization, $SST_2$-Rab4/5 colocalization, and $SST_2$ internalization analysis, respectively. For wash out experiments cells were stimulated for 30 min at 37 °C with 100 nM octreotide, then extensively washed with warm medium and finally let at 37° for 30 min in fresh medium. Cells were fixed for 10 min at room temperature with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO), followed by three washes with PBS and incubated 1 h at room temperature with blocking buffer (5% FBS, 0.3% Triton™X-100, in PBS). The overnight incubation with anti-

FLNA (1:200, Abnova, Taiwan, RC) and anti-Rab4 (1:200, ThermoFisher Scientific, CA) or anti-Rab5 (1:200, Cell Signaling Technology, Danvers, MA) antibodies was performed at 4 °C. The anti-$SST_2$ antibody was used to stain $SST_2$ receptor in human primary cultured cells (UMB1, Abcam, Cambridge, UK, 1:100). After that, anti-mouse Alexa Fluor™ -546-conjugated secondary antibody (1:500, ThermoFisher Scientific, CA), or anti-rabbit Alexa Fluor™ -488-conjugated secondary antibody (1:1000, ThermoFisher Scientific, CA), and anti-rabbit Alexa Fluor™ -647-conjugated secondary antibody (1:1000, ThermoFisher Scientific, CA) was incubated at room temperature for 1 h. All antibodies were diluted in Antibody Diluent Reagent Solution (LifeTechologies, ThermoFisher, CA). Coverslips were mounted on glass slides with ProLong Diamond Antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, CA) for subsequent observation.

**Confocal fluorescence microscopy and colocalization analysis**

Images of representative intracellular optical section were acquired at Leica TCS SP2 laser scanning confocal microscope equipped with Ar 488 nm, HeNe 543 nm and 635 nm lines and a 63× objective (HCX PL APO 63X/1.4-0.60 OIL) (Leica Microsystems, Wetzlar, Germany). Acquisition was conducted at 1240 × 1240 pixels. Images were obtained using sequential scanning for each channel in order to avoid possible ‘‘cross-talk’’ of chromophores and photomultiplier gain adjusted to limit background noise and avoid saturated pixels. After background correction, RGB profile intensity and Manders’ correlation coefficient (MCC) was used to measure the degree of colocalization between $SST_2$ and Rab5 or Rab4 with the JACoP tool in the NIH ImageJ software.

**Statistical Analysis**

All statistical analyses were performed using the Prism 7 software (GraphPad, San Diego, CA). Data were analyzed by two-tailed paired Student *t* test. The results are expressed as the mean ± SD and $P < 0.05$ was accepted as statistically significant.

## Results

### 1. Efficient octreotide-induced $SST_2$ internalization requires FLNA

Recent experiments published by our group had demonstrated that the interaction of FLNA with $SST_2$ is essential for $SST_2$ recruitment to clathrin coated pits and subsequent internalization in CHO cells treated with the $SST_2$ selective ligand, BIM23120 [16]. Here, we tested whether FLNA was involved in $SST_2$ endocytosis in tumor somatotroph cells. By siRNA techniques we knocked down FLNA expression in rat GH-secreting pituitary tumor GH3 cells which we stimulated for 30 min to induce $SST_2$ internalization. Biotinylation experiments showed a statistically significant decrease of octreotide-induced $SST_2$ internalization in FLNA siRNA transfected cells compared with control cells (28.0±2.7% vs 4±4.3% $SST_2$ internalization, control vs FLNA SiRNA cells, respectively, $P<0.001$) (Fig.1A). In agreement, comparable data were obtained in rat GH4C1 cells (data not shown). Moreover, similar results were observed in human GH-secreting pituitary tumor cultured cells from 5 patients, where FLNA silencing impaired $SST_2$ internalization upon octreotide stimulation (70.3±21.1% vs 24±19.2% $SST_2$ internalization, control vs FLNA SiRNA cells, respectively, $P<0.05$) (Fig.1B).

### 2. FLNA is required for $SST_2$ recycling to the plasma membrane

Receptor recycling back to the plasma membrane represents a crucial aspect of receptor resensitization. To evaluate $SST_2$ recycling, we performed immunofluorescence experiments both in GH-secreting pituitary primary cultured cells and GH3 cells. 30-min cells exposure to octreotide firstly induced $SST_2$ to translocate from cell surface into the cytoplasm. Subsequently, 30 min of octreotide removal allowed internalized $SST_2$ receptor pool to almost completely recycle back to the plasma membrane (Fig. 2A&B). We then asked whether FLNA is required for receptor recycling. To address this question, GH3 cells were silenced for FLNA and simultaneously transfected with SNAP-$SST_2$ and then treated as above described. In agreement to that observed in human tumor somatotroph

cells, cell surface recycling of $SST_2$ was detected during wash-out experiments in control cells. On the contrary, 30-min octreotide incubation only partially induced $SST_2$ internalization in FLNA-silenced cells. Moreover, under this condition of FLNA depletion the internalized $SST_2$ pool remained sequestered into intracellular compartment after the wash/out step, demonstrating that, without FLNA, $SST_2$ fails to reach to the plasma membrane (Fig. 2B).

**3. FLNA and β-arrestin2 are recruited to cell surface activated $SST_2$**

FLNA has been shown to directly bind β-arrestins through its C-terminal repeat 22 [25]. The GH3 cell line expresses both β-arrestin1 and β-arrestin2 mRNA [21]. However, in contrast to β-arrestin2, β-arrestin1 is almost undetectable by western blot analysis (data not shown). Therefore, we investigated the recruitment kinetics of FLNA and β-arrestin2 to cell-surface $SST_2$. As shown in figure 3A, under basal conditions $SST_2$ was exclusively located at the plasma membrane where it partially colocalized with FLNA, whereas β-arrestin2 was widely distributed in the cytoplasm. A 2-min octreotide exposure triggered a rapid β-arrestin2 recruitment to the cell surface where it colocalized with $SST_2$ and FLNA. As expected, after 5-10 min of octreotide stimulation, β-arrestin2 and $SST_2$ appear together as internalizing punctate clusters and almost complete endocytosis of $SST_2$-β-arrestin-2 complexes was induced by prolonged agonist stimulation (30 min). Interestingly, FLNA translocation from the plasma membrane to the cytosol was visualized after 30 min. To confirm these observations we used a biochemical approach. Representative western blot images of co-immunoprecipitation experiments reported in figure 3B show that FLNA and β-arrestin2 interact with $SST_2$ to form a complex, and that octreotide-induced FLNA and β-arrestin2 binding to $SST_2$ is maximal at 2 min.

Furthermore, a similar redistribution of FLNA and β-arrestin2 signals was observed in human primary cells obtained from GH-secreting pituitary tumors and treated with octreotide (Fig. 3C).

**4. β-arrestin2 binding to $SST_2$ is FLNA-independent**

As efficient $SST_2$ internalization requires FLNA, we investigated whether FLNA could play a role in facilitating or stabilizing β-arrestin2 association with $SST_2$, being this a critical step of ligand-induced GPCR endocytosis. To this aim, we silenced FLNA expression in GH3 cells and stimulated cells for 0, 5 or 30 min with octreotide. As shown by confocal images, 5 min after $SST_2$ activation, β-arrestin2 translocated from the cytoplasm to the plasma membrane both in control and FLNA-deficient cells, and $SST_2$-β-arrestin2 complexes colocalized regardless of FLNA expression (Fig. 4A). Accordingly, co-immunoprecipitation results demonstrated that $SST_2$-β-arrestins2 interaction upon receptor activation occurs either in the presence or absence of FLNA (Fig. 4B), suggesting that FLNA is not essential for the formation of $SST_2$-β-arrestins2 complex. The occurrence of FLNA-independent β-arrestin2 recruitment to $SST_2$ was further confirmed by using dominant negative FLNA mutants FLNA 19-20 and FLNA 21-24 [14] that selectively interfere with the ability of endogenous FLNA to bind $SST_2$ and β-arrestin-2, respectively (Suppl.Fig.1).

**5. $SST_2$ intracellular endosomal sorting is regulated by FLNA.**

Next, we tested whether FLNA might be directly involved in the intracellular route followed by $SST_2$ once endocytosed. To this purpose, we analyzed the extent of $SST_2$ colocalization with Rab5 and Rab4, two well-defined endosomal markers of early endosomes and a fast recycling compartment, respectively, in GH3 cells treated with octreotide for 15 min. Interestingly, Manders' coefficient analysis performed on confocal images revealed that the fraction of $SST_2$ colocalizing with Rab5 and Rab4-positive endosomes observed in control cells was significantly reduced in the absence of FLNA (33.7 ± 8.9% down to 25.9 ± 6.9% and 28.4 ± 7.4% down to 17.6 ± 5.7%, MCC for $SST_2$-Rab5 and $SST_2$-Rab4 colocalization in control vs FLNA silenced cells, respectively) (Fig. 2A&B).

These results suggest that FLNA might contribute to the regulation of $SST_2$ trafficking to early endosomes and the Rab4 recycling compartment.

**Discussion**

With the present study we describe a crucial role of the cytoskeleton protein FLNA in $SST_2$ internalization and trafficking along the endocytic pathway both in human and rat tumor GH-secreting pituitary cells.

As far as GPCRs expressed in pituitary cells are concerned, $SST_2$ represents the main molecular target for the medical therapy with SSA of GH-secreting tumors in acromegalic patients. Previous data obtained by our group showed that FLNA is a key modulator of $SST_2$ stabilization and signaling in tumor somatotroph cells [14,33], and that transient $SST_2$-FLNA interactions occur in living CHO cells to preferentially localize receptors along actin fibers, allowing efficient ligand-promoted $SST_2$ recruitment to clathrin-coated pits and internalization [15].

Here, we firstly demonstrate that FLNA is required for correct octreotide-induced $SST_2$ internalization in rat GH-secreting pituitary tumor GH3 cells. Accordingly, SST2 internalization was affected by FLNA knock down in primary cultures from 5 dispersed GH-secreting pituitary tumors. In this regard, the structural and functional properties of FLNA have been extensively investigated and found implicated in the internalization process of a variety of transmembrane receptors, such as dopamine receptor type 2 and 3 (DRD2 and DRD3) [34-36], mu opioid receptor [37], furin receptor [38] and chemotactic cytokine receptor 2B (CCR2B) [39], in different cell types. Moreover, we show that $SST_2$ receptors almost completely reach the plasma membrane after washout of octreotide both in rat and human tumor GH-secreting pituitary cells. Similar results have been obtained by previous studies in HEK293 cells and hippocampal neurons [17,40,41]. Importantly, our data in GH3 cells demonstrate that FLNA is required to sustain efficient $SST_2$ recycling. Indeed, the absence of FLNA resulted in $SST_2$ accumulation in the perinuclear region of the cell and a failure in reaching the plasma membrane during washout experiments. This finding likely indicates that $SST_2$ is redirected to lysosomes for degradation, in accordance with our previous results of an increased lysosome-mediated $SST_2$ downregulation observed after prolonged agonist exposure in GH3 cells lacking $SST_2$-FLNA binding [14]. It is worth noting that, under resting condition, FLNA depletion does not affect $SST_2$ correct localization at the plasma membrane in somatotroph cells, this being in line with the

literature [14]. Such observation allowed to establish an initial setpoint that would not compromise the analysis of receptor internalization and recycling of FLNA silenced cells.

In an attempt to unveil the molecular mechanisms governing FLNA effects on $SST_2$ internalization and recycling, here we excluded the involvement of FLNA in the recruitment of the predominant β-arrestin isoform expressed in GH-secreting pituitary tumors and GH3 cells [21], β-arrestin2, to ligand-activated $SST_2$. Our imaging and co-immunoprecipitation data show that both FLNA and β-arrestin2 are immediately recruited to octreotide-activated $SST_2$ at the cell surface (2 min after $SST_2$ stimulation), this being followed by their interaction with $SST_2$ clusters (5-10 min after $SST_2$ stimulation), subsequent β-arrestin2-$SST_2$ internalization and FLNA redistribution to the cytoplasm (30 min after $SST_2$ stimulation). However, β-arrestin2 still retains the capacity to bind $SST_2$ in the absence of FLNA in GH3 cells. Similarly, the lack of FLNA did not prevent β-arrestin2 recruitment to DRD3 in M2 cells [24]. The biological implication of such β-arrestin2-$SST_2$ complexes, regardless FLNA expression, still deserves further investigations.

Moreover, our immunofluorescence experiments show that 15 min of cells exposure to octreotide promotes $SST_2$ accumulation at both Rab5-positive early endosomes and Rab4-positive recycling compartment, suggesting that $SST_2$ might undergo rapid recycling. Typically, fast recycling of receptors to the plasma membrane promotes functional resensitization. To the best of our knowledge, this is the first evidence showing the intracellular routes followed by human $SST_2$ in tumor somatotroph cells. Upon stimulation, rat $sst_{2A}$ has been shown to accumulate at intracellular vesicles containing endocytosed transferrin, a well-characterized marker of fast recycling, both in transfected HEK293 cells and hippocampal neurons [17,41]. Interestingly, in FLNA silenced GH3 cells, octreotide treatment fails to properly induce $SST_2$ localization at such endosomal domains. This finding suggests that FLNA might play a crucial cargo adaptor function to sustain efficient $SST_2$ internalization and trafficking through the endosomal compartments. Moreover, it indicates a different mechanism underlying the impairment of $SST_2$ endocytosis in FLNA depleted cells beside β-arrestin2 mobilization to activated $SST_2$.

A pleiotropic role of FLNA in regulating endosomal trafficking emerged from recent high-speed imaging experiments performed by Pons and colleagues [42]. Their data showed a decreased endosomal motility and shorter distance travelled within the cytoplasm of chemokine receptor CCR2 (CCR2B)-loaded endosomes in FLNA silenced HEK293 cells compared to control cells. Since by single-molecule microscopy we were able to demonstrate that $SST_2$ interactions with FLNA are increased by agonist stimulation and play a role in confining $SST_2$ diffusion on the plasma membrane [16], it is possible to speculate that FLNA might regulate the dynamic behavior of ligand activated-$SST_2$ endosomal trafficking, accordingly. Thus, further experiments are needed to verify this hypothesis.

In conclusion, the present work highlights the importance of FLNA for efficient octreotide-mediated $SST_2$ intracellular trafficking in tumor GH-secreting pituitary cells. At the same time, it shows that FLNA sustains proper endocytic machinery that ultimately influences the number of available $SST_2$ at the cell surface. Further studies are required to uncover the finely regulated molecular mechanisms governing the modulation of $SST_2$-FLNA interactions. Indeed, they might provide insights for the development of innovative therapeutic strategy for patients with GH-secreting pituitary tumors unresponsive to SSA.

**Figure Legends**

**Figure 1. FLNA silencing affects octreotide-mediated $SST_2$ internalization.** Biochemical analysis of $SST_2$ internalization based on biotinylation of membrane receptors in rat (A) and human GH-secreting pituitary tumor cells (B). At 72 h after siRNA transfection (and simultaneous co-transfection with wild-type $SST_2$ for GH3 cells, only), cells were incubated with or without 100 nM octreotide for 30 min. Total proteins were immunoprecipitated by $SST_2$ specific antibody, and the internalized/biotinylated $SST_2$ was detected with an antibody recognizing biotin. The assay shows reduced $SST_2$ internalization in stimulated FLNA silenced cells compared to negative control cells both in rat and human tumor somatotroph cells. Shown are the mean ± SD of three independent

experiments in GH3 cells and five independent experiments in distinct GH-secreting primary cultures. ***, P<0.001, *, P<0.05 vs negative control cells; §§§, P<0.001, §, P<0.05 vs. corresponding basal condition by unpaired Student's t test.

**Figure 2. FLNA sustains $SST_2$ recycling to the plasma membrane**. (A) Human GH-secreting pituitary tumor cells were exposed for 30 min to 100 nM octreotide, extensively washed and incubated with an agonist-free medium for further 30 min. Cells were subsequently fixed and immunostained for endogenous $SST_2$ (magenta) and FLNA (green). Experiments were repeated in two different human primary cultures. (B) Rat GH3 cells were simultaneously transfected with plasmid encoding SNAP-$SST_2$ and siRNA for FLNA (right panel) or negative control (middle panel). After 72 h receptors were labelled (magenta) and cells were treated as above described, fixed and immunostained for FLNA (green). Shown are representative confocal images acquired at the level of the nucleus stained with DAPI (blue). Arrows indicate major $SST_2$ cell localization (plasma membrane or intracellular compartment) for each conditions. For each condition, at least 10 cells from three independent experiments were analyzed. Scale bar, 10 μm.

**Figure 3. Time course kinetics of the recruitment of FLNA and β-arrestin2 to octreotide-activated $SST_2$.** (A) GH3 cells were transiently co-transfected with β-arrestin2 (green) and SNAP-$SST_2$ (magenta) for 48 h. Then receptor were labelled with Alexa-Fluor 647 dye and cells fixed prior or after stimulation with octreotide 100 nM for the indicate times. Immunostaining of endogenous FLNA (red) was performed. DAPI was used to stain the nuclei (blue). The panel show representative confocal images acquired for each channel separately and the overlay, from one of at least three independent experiments. Arrows indicate translocation of both FLNA and β-arrestin2 to the plasma membrane and their colocalization with $SST_2$ at the cell surface, at the compartment below the plasma membrane and at the inner cytosolic compartment, at 2 min, at 5 and 10 min and at 30 min after receptor stimulation, respectively. (B) Representative co-immunoprecipitation experiments showing

the kinetics of FLNA and β-arrestin2 binding to $SST_2$ performed in GH3 cells treated as above described. Lysates were immunoprecipitated (IP) with specific $SST_2$ antibody, and the presence of β-arrestin2, FLNA and $SST_2$ was detected using anti-GFP, anti-FLNA and anti-$SST_2$ antibodies, respectively. Experiments were repeated three times and densitometrical analysis of Western blot images is shown. *, $P<0.05$, vs. corresponding basal condition by unpaired Student's t test. (C) Representative confocal images of human GH-secreting pituitary cells transiently transfected with β-arrestin2 (green) exposed to octreotide for 0, 2 and 30 min, fixed and immunostained for endogenous FLNA (red). Shown are the single channels and the overlay. Arrows indicate yellow region of the cell where FLNA and β-arrestin2 colocalize. Experiments were repeated in three different human primary cultures.

**Figure 4. Effects of FLNA silencing on β-arrestin2 mobilization to cell surface $SST_2$.** (A) GH3 cells were simultaneously transfected with β-arrestin2 (green), SNAP- $SST_2$ (magenta) and negative control (upper panels) or FLNA siRNA (lower panels). After 72 h, SNAP-$SST_2$ were labelled with Alexa-Fluor 647 dye, and cells were stimulated or not with octreotide 100 nM for 5 min and fixed. Endogenous FLNA (red) was immunostained with specific antibody and DAPI was used to stain the nuclei (blue). Shown are representative confocal images acquired at the level of the nucleus from one of at least three independent experiments. Arrows indicate translocation of β-arrestin2 to the plasma membrane where it colocalizes with $SST_2$. Scale bar, 10 μm. (B) Representative co-immunoprecipitation experiment performed in GH3 cells simultaneously transfected with β-arrestin2, HA-$SST_2$ and negative control or FLNA siRNA for 72 h and stimulated with octreotide 100 nM for the indicated times. Total protein extracts were immunoprecipitated (IP) with an antibody recognizing $SST_2$, and the presence of β-arrestin2, FLNA and $SST_2$ was assessed by western blot analysis with anti-GFP, anti-FLNA and anti-$SST_2$ specific antibodies, respectively. Experiments were repeated three times.

**Figure 5. FLNA facilitates $SST_2$ loading in Rab5 and Rab4-positive endosomes.**

Colocalization analysis of $SST_2$-Rab5 (A) and $SST_2$-Rab4 (B) in GH3 cells simultaneously transfected with SNAP-$SST_2$ and negative control or FLNA siRNA. After 72 h, SNAP-$SST_2$ were labelled with Alexa-Fluor 647 dye and cells were exposed to octreotide 100 nM for 15 min. Cells were fixed with 4% PFA and immunostaining of FLNA (red), Rab5 (green) and Rab4 (green) was performed. Left, representative negative control and FLNA siRNA transfected cells from one of three independent experiments and expanded views of the regions marked with the yellow boxes showing individual endosomes and $SST_2$. Scale bar, 10 μm. Middle, representative fluorescence intensity profiles of $SST_2$ (red) together with Rab5 or Rab4 (green) from corresponding selected areas marked with the yellow boxes and analyzed with the tool "RGB profile" in the NIH ImageJ software. Right, graphs showing the quantification of $SST_2$ colocalization with Rab5 or Rab4 in control and FLNA silenced cells, according to Menders' colocalization coefficient (MCC). Results are expressed as mean ± SD *, $P<0.05$, **, $P<0.01$ vs. negative control cells by unpaired Student's t test.

## Statement of Ethics

The study was approved by the local ethics committees.

## Disclosure Statement

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.


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**Author Contribution**

D.T. designed, performed, analyzed the experiments and wrote the manuscript; F.M., E.G., and R.C. contributed to the experiments; M.L. and A.L. provided tumor samples; A.S. provided intellectual input and critically reviewed the manuscript; M.A. provided intellectual input; D.C. critically reviewed the manuscript; G.M. and E.P. supervised the project, provided intellectual input and critically reviewed the manuscript.

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