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Monitoring endosomal trafficking of the G protein-coupled receptor somatostatin receptor 3

Cristy Tower-Gilchrist[1], **Melanie L. Styers**[2], **Bradley K. Yoder**[3], **Nicolas F. Berbari**[3], and **Elizabeth Sztul**[3]

[1]Department of Cell Biology, Emory University, Atlanta, GA 30322

[2]Department of Biology, Birmingham-Southern College, Birmingham, AL 35254

[3]Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL 35294

Abstract

Endocytic trafficking of G protein-coupled receptors (GPCRs) regulates the number of cell surface receptors available for activation by agonists and serves as one mechanism that controls the intensity and duration of signaling. Deregulation of GPCR-mediated signaling pathways results in a multitude of diseases, and thus extensive efforts have been directed toward understand the pathways and molecular events that regulate endocytic trafficking of these receptors. The general paradigms associated with internalization and recycling, as well as many of the key regulators involved in endosomal trafficking of GPCRs have been identified. This knowledge provides goalposts to facilitate the analysis of endosomal pathways traversed by previously uncharacterized GPCRs. Some of the most informative markers associated with GPCR transit are the Rab members of the Ras-related family of small GTPases. Individual Rabs show high selectivity for distinct endosomal compartments, and thus co-localization of a GPCR with a particular Rab informs on the internalization pathway traversed by the receptor. Progress in our knowledge of endosomal trafficking of GPCRs has been achieved through advances in our ability to tag GPCRs and Rabs with fluorescent proteins and perform live cell imaging of multiple fluorophores, allowing real-time observation of receptor trafficking between subcellular compartments in a cell culture model.

Keywords

Somatostatin receptor 3; endosomal trafficking; live cell imaging; G protein-coupled receptor (GPCR); Rab GTPases; epitope tag; fluorescent proteins

1. INTRODUCTION

GPCRs are comprised of a large class of seven transmembrane-spanning receptors with key roles in regulation of cellular physiological responses. Disruption of GPCR function or trafficking can lead to perturbation in the activity of downstream signaling pathways and can

Corresponding Author: Elizabeth Sztul, Ph.D., Professor of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL 35294, Phone: 205-934-1465, Fax: 205-975-9131, esztul@uab.edu.

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result in a multitude of diseases, including cancer, congestive heart failure, nephrogenic diabetes insipidus, retinitis pigmentosa, hypo- and hyperthyroidism, and fertility disorders (Lappano and Maggiolini, 2012; Schöneberg et al., 2004; Sorkin and von Zastrow, 2009).

GPCRs bind ligands at the cell surface and signal by coupling to different types of heterotrimeric Gαβγ proteins (Lappano and Maggiolini, 2012). This coupling event catalyzes the exchange of GDP for GTP on the Gα subunit, leading to dissociation of Gα from Gβγ, and the subsequent activation of downstream effectors by the Gα and the Gβγ moieties (reviewed in Lappano and Maggiolini, 2012). GPCRs can exist in different active conformations that are preferentially stabilized by specific agonists, and thus, binding of different ligands can result in the same GPCR coupling to different signaling pathways. In addition, GPCR pairs have been shown to interact and form heterocomplexes *in vitro* and *in vivo*, which may contribute to functional specificity of GPCR signaling (Gurevich and Gurevich, 2008; Hubbard and Hepler, 2006; Milligan, 2010).

Following ligand binding and signaling, many GPCRs are rapidly phosphorylated by GPCR kinases (GRKs) that selectively phosphorylate agonist-activated receptors. Phosphorylation promotes the binding of β-arrestin, which prevents the interaction of GPCRs with G proteins and terminates signaling. Arrestin binding also promotes endocytosis of GPCRs, leading to the elimination of the GPCR from cell surface. The pathways taken by GPCRs after endocytosis differ; some are targeted for lysosomal degradation, while others are recycled back to the cell surface through either rapid or slow traffic from endosomes (Jean-Alphonse and Hanyaloglu, 2011; Sorkin and Von Zastrow, 2002). Thus, the plasma membrane and the endosomal pathway together represent a sophisticated communication and homeostatic device used by the cell to regulate GPCR-mediated signaling. Considering the extreme clinical importance of GPCRs, it is not surprising that elucidating the intracellular pathways traversed by diverse GPCRs and the molecular machinery regulating passage of GPCRs through these compartments is under active investigation.

Rab GTPases belong to a large family of Ras-related small GTPases and have been shown to regulate membrane trafficking by influencing multiple steps, including vesicle formation, tethering and fusion (Stenmark, 2009). Importantly, Rabs have been shown to localize to specific vesicular compartments and can be used as spatial markers to monitor passage of GPCRs through the endosomal pathway (reviewed in (Jean-Alphonse and Hanyaloglu, 2011; Stenmark, 2009). Within the endocytic pathway, Rab4 and Rab5 localize to early endosomes, Rab7 localizes to late endosomes, and Rab11 localizes to recycling endosomes. Even within single compartments, early endosomal Rabs show spatial and temporal separation, as Rab4 and Rab5 show extensive co-localization, but also areas of separation (Sönnichsen et al., 2000). Therefore, Rabs represent useful molecular markers to monitor endosomal trafficking of GPCRs by measuring the dynamics of GPCR movement through specific compartments and sub-compartments defined by each endosomal Rab.

In this chapter, we use the somatostatin 3 receptor (SSTR3), a GPCR involved in signal transmission initiated by the neuropeptide somatostatin, as a model protein to describe the methodology to monitor endosomal trafficking of GPCRs. SSTR3 is one of five somatostatin receptors (somatostatin receptors 1–5; SSTR1–5) found in many different

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tissues that exhibit different expression patterns during development (Barnett, 2003 ; Handel et al., 1999). Previous histological and immunofluorescence studies localized SSTR3 to many regions of the rodent brain and demonstrated its localization to the primary cilium in hippocampal neurons (Berbari et al., 2007; Berbari et al., 2008; Handel et al., 1999). Biochemical and molecular studies document that the binding of somatostatin induces SSTR3 internalization from the cell membrane via clathrin-coated vesicles in a process that requires β-arrestin 2 and adaptor protein 2 (Jacobs and Schulz, 2008; Tulipano et al., 2004). Internalized SSTR3 has been detected in recycling endosomes, as evidenced by colocalization with Rab11 and endocytosed transferrin, two markers of recycling endosomes (Kreuzer et al., 2001). In addition, internalized SSTR3 also localizes to early endosomal compartments lacking transferrin. However, the exact pathway traversed by SSTR3 upon internalization and the dynamics of its passage through individual endosomal compartments can only be assessed by dynamic live cell imaging.

To investigate the endosomal trafficking of GPCRs in live cells, a number of key parameters must be considered. First, it is essential that the receptor be tagged with a fluorescent moiety (usually the green fluorescent protein GFP or a spectral derivative of GFP) and that the tagged receptor is expressed at levels sufficient for direct imaging. Second, the tag must not affect the ability of the receptor to bind ligand or signal at a level comparable to that of the endogenous receptor. Third, the tagged receptor must traffic to the correct cellular compartment based on current knowledge regarding the distribution and/or function of the endogenous untagged receptor. Fourth, fluorescently-tagged markers must be available to label the organelle through which transport will be monitored. Fifth, the expression levels of the fluorescently tagged receptor and the organellar marker within the cell should be in the low-to-medium range to allow the detection of small trafficking intermediates formed from highly labeled endosomal compartments. These five criteria should be assessed prior to live cell imaging.

In this chapter, we discuss approaches and provide examples of methodology used to express epitope-tagged SSTR3 and Rabs in mammalian cells, assess levels of co-localization between the SSTR3 and different Rabs, and measure dynamic passage of SSTR3 relative to different Rabs by real-time microscopy. Techniques to be discussed include tagging a GPCR with a fluorescent protein, mammalian tissue culture on glass and plastic, transfection of DNA constructs into mammalian cells by electroporation, selection of stable cell lines expressing the tagged GPCR, and methods for live imaging and image processing to define parameters associated with endosomal transport of the GFP-tagged GPCR relative to red fluorescent protein (RFP)- and mCherry-tagged organellar markers.

2. DEVELOPMENT OF CELL LINES STABLY EXPRESSING SSTR3

A variety of methods can be used to generate cell lines stably expressing fluorescent fusion proteins. Here we describe a method of selection of stable clones expressing the exogenous gene after transient transfection. We describe in detail the generation and characterization of the EGFP-SSTR3 cell line that has previously been used to analyze the endosomal trafficking of SSTR3 (Tower et al., 2011). The major steps we will cover include generation

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of the tagged SSTR3 construct, transfection of target cells, and clonal selection and characterization. This entire process requires approximately 4 to 8 weeks.

2.1. Generation of a tagged SSTR3 construct

The goal of this procedure is to generate a construct that drives expression of SSTR3 fused to enhanced green fluorescent protein (EGFP) in mammalian cells. The pEGFP-N3 plasmid (Clontech, Mountain View, CA) was selected as the expression vector, as it allows for fusion of EGFP at the C-terminus of SSTR3. Although there are commercially available vectors that can be used to fuse EGFP to the N-terminus of GPCRs (pEGFP-C series, Clontech), we recommend the use of the C-terminal EGFP tag when possible. Use of the N-terminal EGFP tag requires additional steps be taken to ensure proper membrane threading of the seven transmembrane domain GPCR, as previously described (Huang and Willars, 2011).

1. Isolate total RNA from postnatal day 30 mouse whole brain using Trizol reagent according to the manufacturer's protocol (#15596-026, Life Technologies, Carlsbad, CA).
2. Synthesize complementary DNA (cDNA) using Superscript II reverse transcriptase according to the manufacturer's recommended instructions (#18064-022, Life Technologies). We recommend 5 μg of total RNA be used as a template for the reaction.
3. Design primers that bind sequences located in the untranslated regions (UTRs) of SSTR3 to ensure successful amplification of the entire coding region. The National Center for Biotechnology Information (NCBI) has a useful website for primer design (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).
4. Amplify the coding sequence of mouse SSTR3 via the polymerase chain reaction (PCR) using mouse cDNA as a template. The Platinum® *Taq* DNA Polymerase High Fidelity kit (Invitrogen, Carlsbad, CA), or a similar high fidelity polymerase kit, should be used according to manufacturer instructions. Genomic DNA may also be used as a template for the reaction, as the SSTR3 gene, like many GPCRs, does not contain introns.
5. Subclone the resulting PCR product, containing the entire open reading frame of SSTR3, into a TA cloning vector, such as pSTBlue-1 (Novagen, San Diego, CA), according to manufacturer instructions.
6. Confirm that the sequence of SSTR3 is correct by DNA sequencing. This open reading frame will serve as a template for further subcloning.
7. Amplify SSTR3 by PCR for insertion into the pEGFP-N3 vector. Primers for the PCR reaction must add restriction enzyme sites compatible with the vector (Xhol and Kpnl). Because pEGFP-N3 contains a C-terminal enhanced GFP (EGFP) tag, the reverse primer must also remove the SSTR3 stop codon in order to allow fusion with the tag in the vector.
8. The resulting PCR product can be digested with both Xhol and Kpnl and ligated into the pEGFP-N3 vector digested with the same enzymes. Detailed experimental

procedures for subcloning have been described and can be found at http://www.scribd.com/doc/23261720/Molecular-Cloning-A-Laboratory-Manual-On-The-Web-Maniatis.

2.2. Transfection of IMCD-3 cells via electroporation

IMCD-3 cells, derived from mouse inner medullary collecting duct cells, were chosen to generate a stable line expressing SSTR3-EGFP because they retain many characteristics of the original kidney epithelial tissue (Rauchman et al., 1993). These cells can be grown in monolayers on plastic and glass or as monolayers on filters, where they show basolateral polarity (Goel et al., 2006). IMCD-3 cells are refractory to some common methods of transfection, such as the use of Lipofectamine 2000 (Invitrogen; Carlsbad, CA) and FuGENE (Promega; Madison, WI). For this reason, we have found that electroporation is the preferred and most efficient method.

1. IMCD-3 cells (ATCC, Manassas, VA) should be cultured in Dulbecco's Modified Eagle Medium/Ham's F12 50/50 (DMEM:F12) media supplemented with 10% Fetal Bovine Serum (FBS), 1.2 g/L sodium bicarbonate, 0.5 mM sodium pyruvate, 2.5 mM L-glutamine, and 100 units/mL penicillin/streptomycin (Invitrogen; Carlsbad, CA). Cells should be maintained at 37°C in a humidified incubator containing 5% CO_2.

2. To prepare for transfection, split the cells in a 1:3 dilution into a 100 mm culture dish.

3. When confluent (2–3 days later), collect the IMCD-3 cells from the culture dish by first washing cells with 10 mL sterile phosphate-buffered saline (PBS; Invitrogen), and then incubating cells in 1 mL 0.25% trypsin/EDTA (Invitrogen) for approximately 5 minutes at room temperature. Rinse cells off of the plate using 10 mL DMEM/F12, and pipette up and down (5×). Collect cells by centrifugation for 2 min at $200 \times g$ in a table-top centrifuge.

4. Remove the media, and resuspend 5×10^6 cells in 800 μl of cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM HEPES, 2 mM EGTA, 4 mM $MgCl_2$, pH adjusted to 7.6 with KOH), which mimics the cytoplasmic cellular environment (van den Hoff et al., 1992), supplemented with fresh 2 mM ATP and 5 mM glutathione. Gently mix the cell suspension by pipetting up and down three times.

5. To a 1.5 mL sterile, nuclease-free microcentrifuge tube, add 10 μg of the pEGFP-N3-SSTR3 DNA construct described above. The DNA-containing tubes, as well as the electroporation cuvettes (4 mm gap cuvette, Fisher, Freemont, CA), should be maintained on ice.

6. To each DNA sample, add 400 μL of the cell suspension prepared in step 3. Gently mix the DNA with the cells by briefly pipetting up and down. Transfer the DNA-cell suspension mixture to a labeled 4 mm electroporation cuvette, and place on ice.

7. Electroporate the cells using BioRad Gene Pulser II (Hercules, CA) using the following parameters: 3200 volts, 950 μF.

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8. Remove the cuvette from the ice, and quickly add 0.5 mL of fresh culture media. Resuspend the cells using a Pasteur pipette. The cells should be gently mixed three times before addition to culture dishes.

9. Transfer the transfected cells to a 100 mm plate containing 10 mL IMCD-3 media (described above), and incubate for 24–48 hours.

2.3. Selection of IMCD-3 cells stably expressing SSTR3

This procedure describes the isolation of single clones of IMCD-3 cells stably expressing SSTR3-EGFP. Use of cells derived from a single clone is preferable for imaging studies because it alleviates the need for co-transfection of multiple constructs and allows for better consistency in expression levels of the tagged protein. An alternative approach to what is described here would be to use flow cytometry to sort the stably transfected cells and select cells expressing the desired levels of the EGFP-tagged protein before subculturing.

1. After allowing electroporated cells to recover for 24–48 hours, replace the standard media with media containing 400 μg/μl G418 (Invitrogen; Carlsbad, CA). This step will select cells that have stably incorporated SSTR3-EGFP into the genomic DNA.

2. Carefully change the media in the plate daily, replacing the media with media containing 400 μg/μl G418. Massive cell death should be expected, and the vast majority of the cells will detach from the plate, leaving colonies of stably transfected cells behind.

3. After approximately two weeks of selection, detach all cells by digesting with 1 mL 0.25% trypsin/EDTA (Invitrogen; Carlsbad, CA) for approximately 5 min at 37°C. Add the cells to 9 mL media containing 400 μg/μl G418, and plate on a 15 cm tissue culture dish. Grow for 2–5 days until visible colonies of cells form.

4. Isolate individual colonies using cloning rings attached to the dish with autoclaved vacuum grease. To select a colony, aspirate medium within the ring, and then detach cells within a cloning ring by digesting with 1 mL 0.25% trypsin/EDTA for approximately 5 min at 37°C. Transfer cells from a single ring to a 6-well plate containing media with 400 μg/μl G418. Multiple colonies should be selected to ensure that a colony with the desired expression level of the tagged protein is obtained.

5. When the cells reach confluence, wash with sterile PBS, and then detach with 100 μl of trypsin-EDTA for approximately 5 min at room temperature. Plate 50 μl of the cells into one well of a 6-well plate and 50 μl into one well of a 12-well plate containing a 12 mm glass coverslip (Fisher Scientific, Pittsburgh, PA).

6. After 24 to 48 hours, process the coverslips using fluorescence microscopy to identify clones that express properly localized SSTR3-EGFP. SSTR3 has previously been localized to cilia (Berbari et al., 2008; Handel et al., 1999). We show that EGFP-SSTR3 localizes to cilia in IMCD-3 cells (Figure 1).

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7. To ensure that full-length SSTR3-GFP is expressed in the selected clonal lines, an α-GFP antibody (ab290; Abcam; Cambridge, MA) can be used to perform a Western immunoblot on lysates from the selected cells.
8. Passage confirmed clones from the 6-well plate into a minimum of two dishes: one for freezing as Passage 0, and one for further passaging (if the line is already clonal).
9. Once a clone(s) of interest has been identified, a lower concentration of G418 (200 μg/mL) may be used for maintaining the cells.

3. LIVE IMAGING OF SSTR3 AND RABS IN MAMMALIAN KIDNEY CELLS

3.1. Transfection of Rabs into IMCD-3 cells

This procedure describes the transfection of IMCD-3 cells stably expressing EGFP-SSTR3 (SSTR3#1 IMCD-3) with expression vectors for markers of endosomal compartments, including Cherry-Rab4, mRFP-Rab5, Ds-Red-Rab7, or Ds-Red Rab11. SSTR3#1 IMCD-3 cells were generated as described in section 2. Use of a clonal cell line stably expressing SSTR3 is preferable because this alleviates the need to electroporate two DNA constructs at once to achieve co-expression of both SSTR3 and Rab within a single cell. Also, the use of a stable cell line allows for better control of SSTR3 expression levels across experiments for more accurate comparisons.

1. Grow SSTR3#1 IMCD-3 cells in plastic dishes in DMEM/F12 culture media supplemented with 200 μg/mL G418.
2. Coat glass bottom plates (Warner Instruments, Hamden, CT) by adding 200 μL filtered 0.1% gelatin diluted in molecular grade or sterile distilled water (a volume sufficient to cover only the glass) to the bottom of each dish. Aspirate any excess solution. This step helps cells to adhere to the glass, but not to the sides of the dish, following electroporation.
3. Transfect SSTR3#1 IMCD-3 cells with each DNA construct (empty vector and vector containing the epitope-tagged wild-type or mutant Rab; mutant constructs described below) by electroporation as described above in section 2.2.
4. Add each transfected cell suspension to a single 0.1% gelatin-coated, 35 mm glass-bottom dish containing 2.5 mL fresh pre-warmed media.
5. Place cells in an incubator in 5% CO_2 at 37°C. After 4–6 hours, change the media to fresh DMEM/F12 containing 10% FBS, 100 units/mL penicillin/streptomycin, 1.2 g/L sodium bicarbonate, 0.5 mM sodium pyruvate, and 200 μg/mL G418. This step appears to decrease the toxicity associated with electroporation.
6. Change the media after 24 hours. It is normal to have ~25% of cells detached and floating after 24 hours. Live imaging can be performed 24–48 hours after transfection, depending upon the expression levels of the transfected Rab.

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3.2 Microscope set-up

Imaging can be performed on any microscope equipped to acquire rapid fluorescence images. A warming stage and a CO_2 chamber are beneficial but not essential. Although multiple microscope setups can be utilized, here we describe our imaging studies performed using the PerkinElmer Ultra-VIEWERS 6FE-US spinning disk confocal attached to a Nikon TE2000-U with either a CFI Plan APO 63× or 100× oil immersion objective, equipped with laser and filter sets for GFP, TRITC, Cy5, and DAPI fluorescence. In addition, this microscope has a temperature adjustable stage-top (for our studies the temperature was set to 37°C) incubation system equipped with a CO_2 gas mixer. A Hamamatsu C9100-50 camera was used for image acquisition.

1. Turn on the power to the microscope a minimum of 15 to 20 min before beginning the experiment to allow the laser and CCD camera to stabilize.
2. Add a drop of immersion oil (Cargille Labs, Cedar Grove, NJ) to the 100×-objective, and place the glass-bottom plate of transfected cells in the center of the heated stage above the objective. Cover the heated stage with the CO_2 chamber. Raise the objective to the bottom of the plate.
3. Start up the image acquisition software on the computer, and input parameters appropriate for the microscope used. We utilized Volocity software (Volocity 5.2, Perkin Elmer, Shelton, CT) for the experiments described. However, newer software (version 6.1) is now available. The software package selected should be appropriate for the microscope and camera and should allow rapid acquisition of live images.
4. To acquire images, set up the light pathways by selecting appropriate laser/filter combinations for GFP and RFP for dual color imaging. Filters should be managed at maximum speed for live imaging. Set the z step size to 0.3 mm.
5. Set the exposure time for each channel so that the image intensity fits well within the dynamic range of the camera (maximum intensity of around 5000–10000 for this set-up). Utilize a fast sequential time-lapse imaging setting. We do not recommend the use of auto contrast and recommend a sensitivity of approximately 155 for image acquisition.
6. Adjust the intensity of each laser according to the cell selected for imaging. We recommend that the laser intensity be set to a lower level (<55% for this set-up). Use of lower laser intensities and shorter exposure times while acquiring images helps to minimize photobleaching of the fluorophores in the cell. The ideal cell is one in which the GFP signal and RFP or mCherry signal are bright enough to be detected with similar laser settings.
7. For each image, set the top and bottom of the z stack after finding an appropriate co-transfected cell and before initiating time-lapse image acquisition.
8. Acquire images every 5 seconds for 10–20 minutes.

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3.3. Confocal microscopy co-localization of SSTR3 and Rabs

1. Pre-warm DMEM/F12 culture medium in a 37°C water bath for 10 minutes. Remove the 35 mm glass-bottom plates containing SSTR#3 IMCD-3 cells transfected with Rabs from the incubator, and replace the media with 2 mL pre-warmed fresh media.
2. Add 50 μL 1 M HEPES buffer, pH 7.4 to each 35 mm glass-bottom dish. This step helps in maintaining the pH of the media to ensure longer cell survival during imaging.
3. Place the plate on the heated stage of the microscope inside the humidifying chamber and a CO_2 chamber. Add a single drop of immersion oil to the objective and bring the objective up to the bottom of the plate.
4. To prevent a shift of focal plane during recording caused by thermal drift, after setting up the plate on the microscope, it is best to wait for approximately 15 min before continuous imaging. During this time, scan the field of cells to assess the transfection efficiency and the expression levels of the transfected proteins in the cells.
5. Ensure that the selected cells exhibit moderate expression and correct localization of SSTR3 and Rabs. SSTR3 localizes to cilia (Berbari et al., 2008; Handel et al., 1999), and thus SSTR3#1 IMCD-3 cells showing ciliary localization should be selected for subsequent imaging. The correct distribution of Rabs can be assessed by similarity to published patterns (see Choudhury et al., 2002; Daro et al., 1996; Vonderheit and Helenius, 2005; Ward et al., 2005).
6. Focus on a region of interest (ROI) within the cell. Acquire multiple focal planes for each fluorophore in each ROI within a cell. Acquire images from at least 10 different cells from a minimum of three independent experiments.
7. Merge the images of the fluorophores from a single ROI to obtain a visual representation of the co-localization of SSTR3 and Rabs (Figure 2A–D).
8. Quantify the level of SSTR3/Rab co-localization in all collected ROIs using Volocity or similar software. Calculate the Pearson's correlation coefficients for the z-stacked images. Different endosomes can be selected within ROIs to determine co-localization of each object through time. Co-localization is based on a scale of −1 to 1, where values close to 0 indicate that two fluorophores do not show a linear co-localization relationship. A value of −1.0 indicates a complete negative correlation, and a value of 1 indicates complete co-localization. For our experiments, the no co-localization threshold was set at/below 0.100 (Figure 2E).

4. DYNAMICS OF SSTR3 TRANSIT RELATIVE TO RABS

4.1. Time-lapse dual color imaging of SSTR3 and Rabs

1. Place the plate of SSTR3#1 IMCD-3 cells transfected with a specific Rab on the heated stage of the microscope inside the CO_2 chamber. Add a single drop of

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immersion oil to the 100× objective, and bring the objective up to the bottom of the plate.

2. Select a cell expressing moderate levels of SSTR3 and Rab, and focus on an area of the cell containing a representative distribution of Rab. In order to minimize photobleaching of dual-labeled cells, the laser power should be set as low as possible. In our case, the 488 nm laser intensity was set to 15% and the 561 nm laser to 15%.
3. Acquire z-stack images with a spacing of 0.3 mm every 5 seconds for 10 minutes.
4. Merge the images of the fluorophores at each time to obtain a visual representation of the global localization of SSTR3 relative to each Rab at each time point.
5. To analyze entry and exit of SSTR3 from endosomal compartments, use 100× TIRF oil objective and focus on a single endosome to observe fusion and budding events. Image as described above for 10 minutes (Figure 3 A, B).

4.2. Analysis of half-time residency and clearance times

For each of these assays, imaging should be performed for at least 10 different cells and at least 20 to 25 different endosomes in each of 3 independent experiments to obtain average transport rates of SSTR3 through a Rab-marked compartment.

1. To assess half-time residency ($t_{1/2}$), collect images over 10 minutes and use them to assess co-localization coefficients between SSTR3 and Rabs at each time point. Based on these values generate $t_{1/2}$ times.
    a. To quantify fusion and fission events, select and analyze 21 to 25 fluorescent objects from different fields of the cell.
    b. Analyze each structure using the Measure Colocalization Application in Volocity (or a similar application) and empirically determine the threshold values. These values can be generated by using automated thresholding (see Volocity 5.2 user manual available at http://cellularimaging.perkinelmer.com/pdfs/manuals/VolocityUserGuide.pdf).
    c. Record the Pearson's correlation coefficient for each object throughout the duration of the time-lapse sequences. As measured by Pearson's correlation coefficient, the changes in co-localization of SSTR3 with endosomes can be used to estimate the time the proteins remain in the same organelle. In our study, the co-localization coefficient was recorded until it decreased to zero, indicating no overlap between the SSTR3-positive structure (green) and the red-labeled endosome.
    d. Plot the percentage of co-localized objects on the y-axis versus the time in seconds on the x-axis. Objects were considered to be colocalized until the Pearson's Correlation Coefficient reached a value less than 0.1.
    e. Calculate the $t_{1/2}$ based on the graph (Figure 4). The $t_{1/2}$ value is defined as the time at which 50% of the objects are no longer co-localized.

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2. To assess clearance, collect images over 10 to 20 minutes, and use these images to assess co-localization correlation coefficients between SSTR3 and Rab-positive endosomes at each time point.

   a. Randomly select 20 to 25 individual Rab/SSTR3 positive endosomes and assess the Pearson's coefficient for each structure over time. When SSTR3 is associated with a Rab-labeled endosome, the structure should be yellow in color and have a high (0.7 to 1.0) Pearson's coefficient. Over time, as SSTR3 is sorted out of the Rab-positive endosome, the yellow structure begins to separate into a red Rab-positive endosome and a green SSTR3-positive structure. The measured Pearson's coefficient of these independent structures should approach 0.

   b. Repeat this analysis for each endosome. Record the time point at which SSTR3 is no longer colocalized with the Rab (Pearson's correlation coefficient < 0.1). This time is considered to be the clearance time because it represents the point at which SSTR3 is no longer associated with the Rab-positive endosome.

5. EFFECTS OF DOMINANT NEGATIVE RABS ON SSTR3 TRAFFICKING

The role of Rabs in trafficking of cargo proteins through the endosomal pathway can be probed based on the availability of mutant forms of Rabs that arrest trafficking within a specific endosomal compartment. Rab GTPases cycle between two nucleotide-bound states: 1) a GDP-bound inactive state and a 2) GTP-bound active state. The inactive form of each Rab can be generated by mutating a single amino acid within the switch region (reviewed in Hutagalung and Novick, 2011; Fukuda, 2010). For the key human endosomal Rabs, the following substitutions generate dominant inactive forms: mutation of serine 22 of Rab4 to asparagine (Rab4/S22N) and of serine 25 of Rab11 to asparagine (Rab11/S25N). When expressed in cells, these proteins inhibit trafficking at early endosomes (Rab4/S22N) or at recycling endosomes (Rab11/S25N) (Bucci et al., 1992; Bucci et al., 1994; Choudhury et al., 2002; Daro et al., 1997; van der Sluijs et al., 1992; Ward et al., 2005)). We first generated a red fluorescently–tagged, dominant inactive mutant of Rab4. We then expressed dominant inactive mutants of Rab4 and Rab11 in SSTR3#1 IMCD-3 cells to determine whether the mutant Rab affected specific SSTR3 trafficking events.

5.1. Generating mutant forms of endosomal Rabs

1. Clone or obtain constructs in which the wild-type Rab is tagged with a red fluorescent protein, such as mCherry or RFP. These constructs will serve as a template for site-directed mutagenesis. We obtained the pmCherry-C2 Rab4 wild-type construct from Dr. James Goldenring (Vanderbilt University Medical Center, Nashville, TN).

2. Perform site-directed mutagenesis using the polymerase chain reaction to substitute conserved Ser for Asn in the α1 helix of wild-type Rab4. There are a number of commercially available mutagenesis kits. We used the QuickChange XL-Site directed mutagenesis kit (# 200516 and 200517, Stratagene, La Jolla, CA) to

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generate pmCherry-C2 Rab4/S22N. The experimental details associated with this procedure are described in http://www.scribd.com/doc/23261720/Molecular-Cloning-A-Laboratory-Manual-On-The-Web-Maniatis.

3. If wild-type and dominant inactive forms of the Rab are not available with a red tag but are available in other vectors, they can be subcloned into an appropriate vector containing a red tag. Verify the correct (in frame) cloning and mutagenesis of the new constructs by DNA sequencing.

5.2. Dynamics of SSTR3 traffic in cells expressing mutant Rabs

1. Transfect SSTR3#1 IMCD-3 cells with the inactive (GDP-locked) Rab4/S22N or Rab11/S25N (Addgene, Cambridge, MA, USA) mutants by electroporation (See Section 3.1 for method). Change culture media after 24 hours, and add fresh pre-warmed media supplemented with 200 μg/mL G418.
2. Prior to imaging, add fresh media containing 2 μM HEPES buffer, pH 7.4 and place plate on the heated stage of the humidifying CO_2 chamber.
3. Select the 100× oil immersion objective. Allow the objective to warm before adding drop of oil.
4. Acquire images as described in Section 3. Record the images in the green and red channels for each time point (Figure 3C, D).
5. Process images using Volocity 5.2 or similar image acquisition and processing software. The program calculates the average fluorescence for each endosome (with background subtracted) in every image of the z-series.
6. Export the time series into a QuickTime movie format that plays at five frames per second (Adobe Systems, Inc., San Jose, CA). Monitor individual endosomes containing SSTR3 (yellow) over time to determine the kinetics of SSTR3 (green) clearance from the red-labeled endosome. This data can be analyzed as described in Section 4.2 to assess the length of time SSTR3 remains in endosomes expressing the wild-type or mutant Rab.

6. SUMMARY

Advances in the field of somatostatin receptor trafficking have been impressive; however, further studies need to be conducted to identify additional molecular components that regulate endosomal transport of SSTR3 and to analyze the precise roles of these proteins in distinct steps of endocytosis and recycling of these GPCRs. Importantly, these studies will provide a better understanding of somatostatin physiology and aid in design of therapeutic strategies to target subclasses of GPCRs.

Live cell imaging has proven to be a useful tool to study intracellular trafficking. Over the years, the advancement of technology has led to the development of better means of tagging proteins for live imaging and led to significant improvements in microscopes and image analysis software, allowing for improved visualization of transport events taking place in real time in a single cell or organelle. These techniques can provide spatial and temporal

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information regarding the trafficking events and have proven useful for addressing key questions concerning the endosomal transit of somatostatin receptor 3. In addition, this method permits transport kinetics to be quantified, as well as assessing the effects of specific proteins on kinetic parameters. Instead of simply providing a fixed single snapshot of cellular trafficking events, this procedure provides a window into continuous processes occurring in cells over time, allowing for improved characterization of the coordinated membrane trafficking within the cell.

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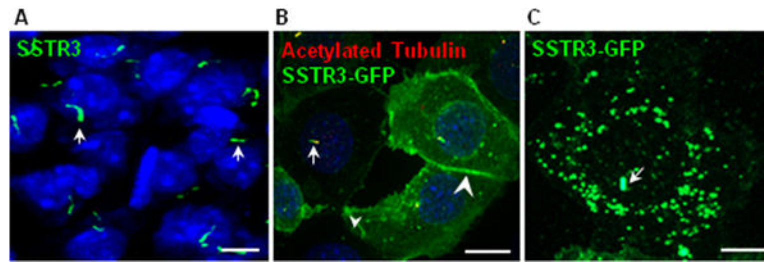

Figure 1. Localization of SSTR3 in IMCD cells

(A) A frozen section of wild-type adult mouse brain was processed for IF with anti-SSTR3 antibodies (sc-11617, 1:500; Santa Cruz, Dallas, TX). The CA3 region of the hippocampus shows cilia specific localization of SSTR3. Draq5 nuclear stain is in blue. (B–C) IMCD-3 cells stably expressing SSTR3-GFP were imaged directly (C), or processed for immunofluorescence using an anti-GFP polyclonal antibody (ab290-50, 1:500; Abcam, Cambridge, MA) (to detect SSTR3) and anti-acetylated tubulin monoclonal antibody (T6793, 1:2000; Sigma, Saint Louis, Missouri Sigma (to detect cilia) (B). SSTR3 localizes to cilia (arrows) and is also detected on the plasma membrane (arrowheads) and in internal endosomes. Scale bars, 10 μm (A, B) and 19 μm (C) Adapted from Figure 1 of Tower-Gilchrist et al. 2011, with permission from Elsevier.

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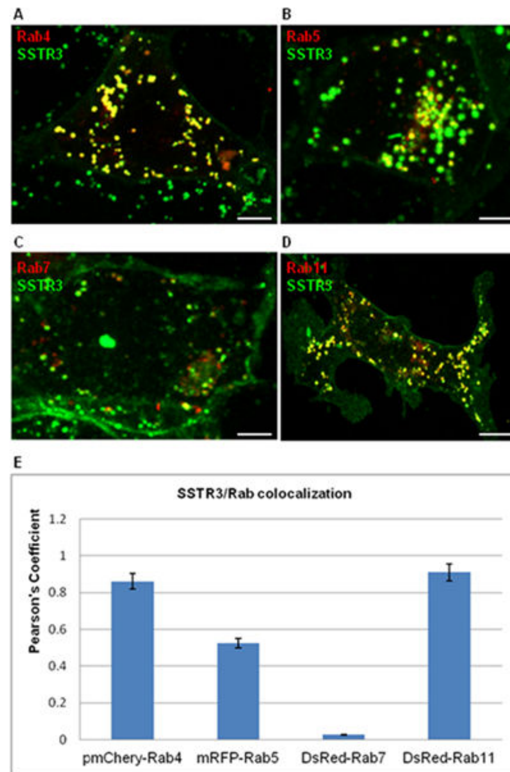

Figure 2. Localization of SSTR3 to a subset of endosomal compartments

(A–D) IMCD-3 cells stably expressing EGFP-SSTR3 were transfected with mCherry-Rab4 (A), mRFP-Rab5 (B), Ds-Red-Rab7 (C), or Ds-Red Rab11 (D). Cells were imaged at 48 hours post-transfection. SSTR3 localizes to Rab4 and Rab5-positive early endosomes, as well as Rab11-positive recycling endosomes, but not to Rab7-positive endosomes. The level of SSTR3 co-localization with each subset of endosomes was measured using Pearson's Correlation Coefficient (E). Scale bars, 19 μm (A–D). Adapted from Tower-Gilchrist et al 2011, with permission from Elsevier.

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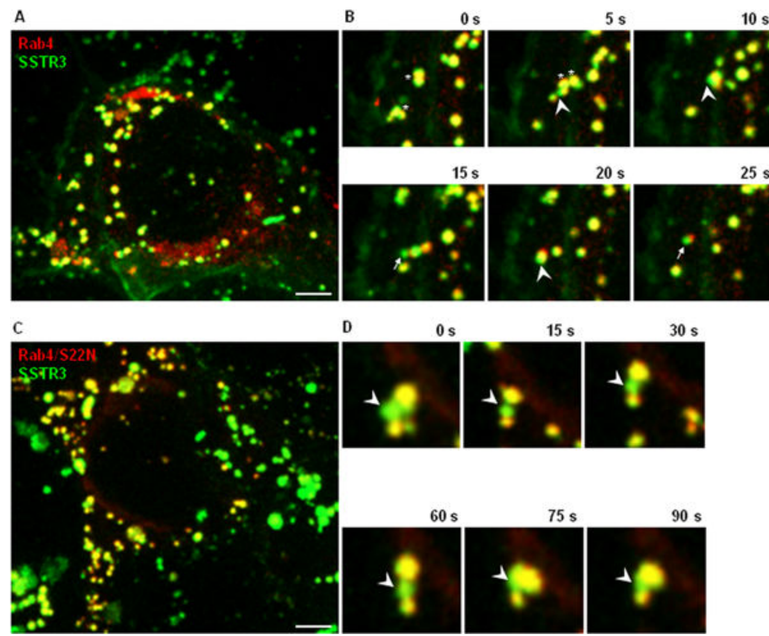

Figure 3. Dynamics of SSTR3 movement through endosomal compartments

IMCD-3 cells stably expressing SSTR3 were transfected with mCherry-Rab4 wild-type (A–B) or with mCherry-Rab4/S22N (C–D). Live imaging was carried out, and still images from movies were selected. (B) Arrowheads point to SSTR3 (green) rapidly budding from Rab4-containing (yellow) endosomes. (D) Arrowheads point to SSTR3 only (green) elements that remain in association with Rab4-containing (yellow) elements for an extended time period. Scale bars, 19 μm (A and C).

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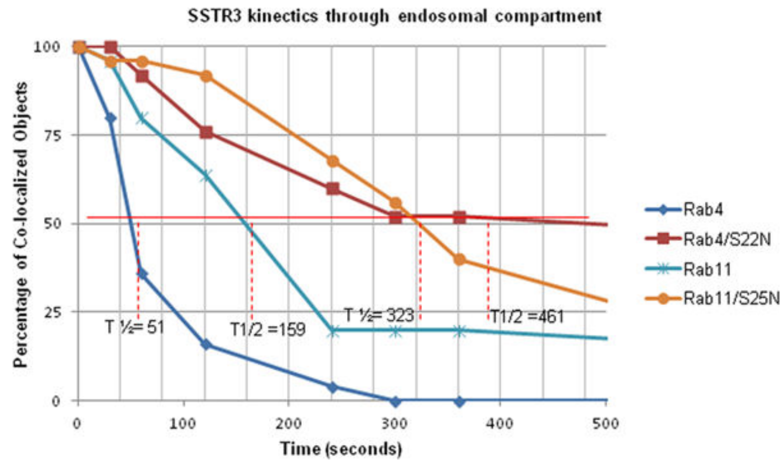

Figure 4. Effects of mutant Rabs on SSTR3 dynamics

The Pearson's Co-localization Coefficients of SSTR3 with wild-type Rab4 and Rab11 or with dominant negative Rab4/S22N and Rab11/S25N were extracted from different times during imaging as in Figure 3 and used to calculate the percentage of co-localized objects at each time point.