Endosomal Phosphatidylinositol 3-Kinase Is Essential for Canonical GPCR Signaling

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ABSTRACT

G protein–coupled receptors (GPCRs), the largest family of signaling receptors, are critically regulated by endosomal trafficking, suggesting that endosomes might provide new strategies for manipulating GPCR signaling. Here we test this hypothesis by focusing on class III phosphatidylinositol 3-kinase (Vps34), which is an essential regulator of endosomal trafficking. We verify that Vps34 is required for recycling of the β2-adrenoceptor (β2AR), a prototypical GPCR, and then investigate the effects of Vps34 inhibition on the canonical cAMP response elicited by β2AR activation. Vps34 inhibition impairs the ability of cells to recover this response after prolonged activation, which is in accord with the established role of recycling in GPCR resensitization. In addition, Vps34 inhibition also attenuates the short-term cAMP response, and its effect begins several minutes after initial agonist application. These results establish Vps34 as an essential determinant of both short-term and long-term canonical GPCR signaling, and support the potential utility of the endosomal system as a druggable target for signaling.

Introduction

Many signaling receptors, including G protein–coupled receptors (GPCRs), enter the endosomal system after ligand-induced activation at the plasma membrane (PM), and the endosomal network is known to have myriad effects on cellular GPCR signaling (Pierce et al., 2002; Sorkin and von Zastrow, 2009; von Zastrow and Williams, 2012; Iranejad et al., 2015). Internalized receptors are sorted after endocytosis for recycling back to the PM or subsequent transport to lysosomes for degradation. This sorting decision, by determining whether regulated endocytosis maintains or depletes the surface receptor complement, has been believed to exert long-term control over cellular signaling responsiveness under conditions of prolonged or repeated ligand application (Hanyaloglu and von Zastrow, 2008). In addition, endosomes themselves can function as sites of receptor-mediated signal initiation, which potentially affects both short-term and long-term cellular signaling activities (Murphy et al., 2009; Irannejad and von Zastrow, 2014).

Phosphatidylinositol (PI) 3-phosphate (PI3P) is a phosphorylated inositol lipid (phosphoinositide) that is found mainly in endosomal membranes (Gillollo et al., 2000). This lipid, although present in relatively small amounts, is a defining feature of endosomal membranes that regulates many aspects of endosome biogenesis, organization, and trafficking by recruiting PI3P-binding proteins (Schink et al., 2013). A major pathway of PI3P synthesis is mediated by class III phosphatidylinositol 3-kinase (Vps34), which specifically phosphorylates PI to produce PI3P in endosomal membranes (Shin et al., 2005; Raiborg et al., 2013). β2-adrenoceptors (β2ARs) are widely considered to be prototypic GPCRs (Lefkowitz, 2007). β2-adrenoceptors internalize rapidly after ligand-induced activation via clathrin-coated pits and have the ability to recycle to the PM with remarkably high efficiency via a retromer-dependent pathway (Goodman et al., 1996; Temkin et al., 2011). Several components of this machinery require 3-phosphorylated phosphoinositides for membrane attachment (Cullen and Korswagen, 2011), and a previous study (Awad et al., 2007) has implicated Vps34 in promoting β2AR recycling. Accordingly, we hypothesized that

ABBREVIATIONS: ANOVA, analysis of variance; AR, adrenoceptor; β2ARs, β2-adrenoceptors; DEMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethylsulfoxide; FKBK, FK906 binding protein; FRB, FKBP12-rapamycin–binding domain; GDC-0941, 4-[2-[(1H-indazol-4-yl)-6-[4-(methylsulfonyl)pheno]azo]-1-yl]methyl]thieno[3,2-d]pyrimidin-4-yl]morpholine; GFP, green fluorescent protein; GPCR, G protein–coupled receptor; HEK, human embryonic kidney; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PM, plasma membrane; SAR405, (8S)-9-[5-chloropyridin-3-yl]methyl]-2-[3R]-3-methylmorpholin-4-yl]-8-(trifluoromethyl)-7,8-dihydro-6H-pyrimidino[1,2-a]pyrimidin-4-one; UCSF, University of California, San Francisco; Vps34, class III phosphatidylinositol 3-kinase; VPS34-1N1, 1-(2-[[2-[(chloropyridin-4-yl)]amino]-4-[(cyclopropyl(ethyl)]-4,5,5’-bipyrindim-2’-yl)amino]-2-methylpropan-2-ol; WM, wortmannin; WT, wild type; YM201636, 6-amino-N-[3-(4-morpholin-4-ylpyrido[2,3]furo[2,4-b]pyrimidin-2-yl]phenyl]pyridine-3-carboxamide.
Vps34 represents an endosomal target that controls β2AR signaling. Chemical inhibitors used in previous studies of β2AR trafficking (Sorensen et al., 1999; Naga Prasad et al., 2001; Awad et al., 2007) [wortmannin (WM) and LY294002] have additional cellular targets (Knight, 2010). In the current study, we employed more specific manipulations to examine the trafficking and signaling effects of endosomal PI3P and Vps34, and revealed previously unrecognized roles of this critical endosomal kinase in GPCR signaling. These results demonstrate that both short-term and sustained GPCR signaling can be manipulated by targeting the endosomal system.

**Materials and Methods**

**Cell Culture, cDNA Constructs, and Transfection.** Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum [University of California, San Francisco (UCSF) Cell Culture Facility, San Francisco, CA]. A plasmid-encoding Flag-tagged β2AR was previously described (Cao et al., 1999). A plasmid encoding enhanced green fluorescent protein (GFP)-tagged Hrs 2xFYVE was a gift from Harald Stenmark (Oslo University Hospital, Oslo, Norway) (Gillooly et al., 2000). mCherry-FKBP-myotubularin 1 (MTM1) [wild type (WT)] and iRF-P-FRB (FKBP12-rapamycin–binding domain)-Rab5 plasmids were obtained from Tamas Balla (National Institutes of Health, Bethesda, MD) through Addgene (Cambridge, MA) (Hammond et al., 2014). A phosphatase-dead mutant of mCherry-FKBP-MTM1 (C375S) was generated by using Quikchange Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Nb37-GFP plasmid was previously described (Irannejad et al., 2013). DNA transfection was performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer instructions, and cells were used for subsequent experiments 24 hours after transfection, unless otherwise indicated.

Cells stably expressing with Flag-tagged β2AR were created as previously described (Lauffer et al., 2010).

**Inhibitors.** PIK-III was synthesized according to the published protocol (Honda et al., 2015). The commercial sources of other inhibitors used in this study are as follows: WM (Sigma-Aldrich, St. Louis, MO); VPS34-IN1 (1-(2-(2-chloropyridin-4-yl)amino)-4-(cyclopentylmethyl)-1H-[4,5-bipyrimidin]-2'-ylamo)-3-methylpropan-2-ol; MedKoo Biosciences, Chapel Hill, NC; SAR405 (8S)-9-[5-(chloropyridin-3-yl)ethyl]-2-(3R)-3-methylmorpholin-4-yl-8-(trifluoromethyl)-7,8-dihydro-6H-pyrido[1,2-al]pyrimidine-4-one; AxisBio, Houston, TX); GDC-0941 (4-(2-1H-indazol-4-yl)-6-(4-methyllauryl)pyrazine-1-yl)methyllhino[3,2-d][3,4-bipyrimidin]-yl)morpholine; Selleck Chemicals, Houston, TX); and YM201636 (YM201636, 6-amino-N-[3-[4-[morpholin-4-yl]pyrido][2,3]furo][2,4,5-bipyrimidin-2-yl]phenyl]pyridine-3-carboxamide; Cayman Chemical, Ann Arbor, MI). Unless otherwise indicated, cells were pretreated with these inhibitors in serum-free DMEM for either 1 hour (100 nM WM, 3 μM PIK-III, 1 μM VPS34-IN1, 3 μM SAR405, or 1 μM GDC-0941) or 3 hours (800 nM YM201636) before agonist application. Trafficking and signaling assays were performed in the presence of the indicated inhibitors.

** Trafficking Assays.** Unless otherwise indicated, trafficking assays were performed at 37°C using HEK293 cells stably expressing Flag-β2AR. Cells plated on coverslips were used to assess receptor localization by fluorescence microscopy. To examine agonist-induced receptor internalization, cells were treated with 10 μM isoproterenol (a β2AR agonist; Sigma-Aldrich) for 25 minutes. To examine recycling after agonist removal, cells were first treated with isoproterenol for 25 minutes and then washed with phosphate-buffered saline (PBS) and treated with 10 μM alpenrolon (a β2AR antagonist; Sigma-Aldrich) for 45 minutes. Antagonist was used to prevent any residual agonist effects in the recycling period. Cells were fixed by 4% paraformaldehyde in PBS for 20 minutes, quenched with Tris-buffered saline for 20 minutes, and subjected to immunocytochemistry, as described below. For acute PI3P depletion by MTM1 recruitment, cells transfected with mCherry-FKBP-MTM1 and iRF-P-FRB-Rab5 plasmids were used, and 1 μM rapamycin (Sigma-Aldrich) was applied to cells either 20 minutes before isoproterenol treatment (for internalization) or with the same timing as for alpenrolon (for recycling).

Cells were plated on 12-well plates to analyze β2AR trafficking by fluorescence flow cytometry. Both agonist-induced internalization and recycling after agonist removal were tracked quantitatively by determining surface β2AR levels. For internalization, cells were treated with 1 μM isoproterenol for the indicated times. For recycling, cells were first treated with 1 μM isoproterenol for 25 minutes and then with 10 μM alpenrolon for 45 minutes. Surface β2AR receptors were then labeled with Alexa Fluor 488–conjugated M1 anti-Flag antibody (Sigma-Aldrich) at 4°C. The mean fluorescence intensity of each sample (2000–10,000 cells/sample) was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The percentage of recycled receptors was calculated as follows: 100 × [(intensity after the recycling period) – (intensity after the internalization period)]/[(intensity without agonist addition) – (intensity after the internalization period)].

To measure β2AR recycling in the continuous presence of agonist, a previously established method (Tsao and von Zastrow, 2000) was used with modifications. Briefly, surface β2ARs were first labeled for 10 minutes with Alexa Fluor 647–conjugated M1 anti-Flag antibody, which requires calcium to bind the Flag epitope. Cells were then treated with 1 μM isoproterenol for 25 minutes to induce receptor internalization. At this point, cells were washed with calcium, and magnesium-free PBS with 0.4% EDTA to dissociate antibodies from surface receptors and specifically label internalized receptors. Cells were then incubated in EDTA-supplemented PBS with 1 μM isoproterenol for 5 minutes. Control samples without this incubation period were also made. Cells were then chilled on ice and subjected to flow cytometry, as described above, to measure the antibody efflux in the 5-minute period. Because antibodies bound to receptors that were recycled to the PM in this period eluted immediately, the antibody efflux was correlated to the degree of β2AR recycling.

**Immunocytochemistry and Fixed-Cell Imaging.** Permeabilization and blocking were performed for 20 minutes with 0.1% Triton X-100 and 3% skimmed milk in PBS. Cells were then incubated with anti-Flag (M1) antibody (1:1000; Sigma-Aldrich) and Alexa Fluor 488–conjugated secondary antibody (1:1000; Molecular Probes, Carlsbad, CA) in the blocking buffer for 1 hour each. Fixed samples were mounted with ProLong Gold (Molecular Probes). Cells were imaged with an inverted microscope (TE-2000; Nikon, Tokyo, Japan) with a numerical aperture 1.45 objective. Images were collected using an electron-multiplying CCD camera (iXon 897; Andor Technology, Belfast, UK) operated in the linear range controlled by Micro-Manager software (https://www.micro-manager.org).

**Live-Cell Confocal Imaging.** Cells were imaged in DMEM without phenol red (UCSF Cell Culture Facility) with 30 mM HEPEs (pH 7.4). For MTM1 recruitment experiments, HEK293 cells stably expressing Flag-β2AR were used and imaged with the aforementioned confocal microscope. For Nb37 localization experiments, HEK293 cells were transiently transfected with Flag-β2AR and Nb37–enhanced GFP plasmids. Cells were then pretreated with dimethylsulfoxide (DMSO) or PIK-III, and then treated with 10 μM isoproterenol. Receptor-expressing cells were randomly chosen for assessing Nb37 localization and imaged after 15–30 minutes of isoproterenol treatment. Cells were imaged with another spinning disk confocal microscope (Ti-E microscope, Nikon) in the Nikon Imaging Center at UCSF (with CSU22 confocal scanner unit; Yokogawa) using a 100× numerical aperture 1.45 objective. Images were collected using an electron-multiplying CCD camera (Evolv Delta; Photometrics, Tucson, AZ) operated in the linear range controlled by Micro-Manager software.
**Image Analysis.** Images were saved as 16-bit TIFF files and analyzed using Fiji software (Schindelin et al., 2012). Colocalization between Flag-β2AR and FRB-Rab5 was estimated by calculating Pearson’s correlation coefficients between the two channels using the Coloc 2 plug-in in Fiji. Line-scan analysis was performed with the plot profile function, and the obtained values were normalized to the maximum value of each channel. The intensities of Nb37 and β2AR were measured by a custom-written program created by one of the authors (D.J.), which works on MATLAB (MathWorks, Natick, MA). The script is shown in the Supplemental Material. Briefly, after selecting a background region in cytosol and β2AR-containing endosomes, the program created donut-shaped regions (3 pixels wide), which include endosome-limiting membranes, and calculated the average fluorescence intensity of each donut. Five endosomes per cell were randomly chosen for the analysis, and endosomes with negative Nb37 intensities were excluded from the analysis. The Nb37 intensity of each endosome was then normalized to the β2AR intensity.

**Luminescence-Based Real-Time cAMP Assay.** The experimental procedure was performed as described previously, except for using WT HEK293 cells (Irannejad et al., 2013). Briefly, cells were transiently transfected with pGloSensor-20F (Promega, Madison, WI), which encodes a cyclic-permuted luciferase cAMP reporter construct. For short-term signaling, cells were treated with luciferin (GoldBio, St. Louis, MO) in serum-free media for 1 hour in a 24-well plate, and luminescence values in the experimental wells were obtained after adding 1 μM isoproterenol. Reference wells were made in the same columns in the 24-well plate as the experimental wells and were treated with 5 μM forskolin (Sigma-Aldrich). The luminescence values obtained in experimental wells were normalized to the maximum and minimum values in the reference wells (i.e., the maximum and minimum values of the reference wells were set to 100% and 0%, respectively). For repeated signaling, cells were lifted and seeded on a 12-well plate 1 d after transfection, and the experiments were performed on the next day. Cells were treated with DMSO or 5 μM PIK-III and luciferin in serum-containing media for 1 hour, and then treated with 1 μM isoproterenol for 1 hour (first treatment). Cells were washed three times with serum-containing media without isoproterenol, and 10 minutes after washout were re-challenged with 1 μM isoproterenol (second treatment). Luminescence was recorded after both the first and second treatment, and the increase in luminescence values after the second treatment was normalized to that after the first treatment in the same well.

**Biochemical cAMP Assay.** After the indicated time of isoproterenol or forskolin treatment, cells were washed with ice-cold PBS and lysed by 0.1 M HCl for 10 minutes at room temperature. The cAMP concentration in the lysate was determined by using the Direct cAMP ELISA kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer instructions. The cAMP concentration was normalized

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**Fig. 1.** Endosomal PI3P is required for β2AR recycling, but not for endocytosis. (A) Schematic of the rapamycin (rapa)-induced recruitment of MTM1 to Rab5-containing endosomes. (B) Representative images of live cells expressing GFP-2xFYVE (a PI3P probe protein), mCherry-FKBP-MTM1 [either WT or a phosphatase-dead mutant (C375S)] and iRFP-FRB-Rab5. Cells were imaged before and after 5 minutes of rapa treatment by confocal microscopy. Scale bar, 10 μm. (C and D) Trafficking of β2AR examined by immunofluorescence microscopy. (C) Cells stably expressing Flag-β2AR were transfected with mCherry-FKBP-MTM1 (WT or C375S) and iRFP-FRB-Rab5. In the agonist condition, cells were treated with rapa for 20 minutes, and then with a β2AR agonist isoproterenol (iso) for 25 minutes. In the agonist-to-antagonist condition, cells were first treated with isoproterenol for 25 minutes, and then with a β2AR antagonist alprenolol and rapa for 45 minutes. After fixation, cells were stained for Flag epitope. Representative confocal images are shown. Insets show the boxed areas at higher magnification. Arrows show β2AR localization to Rab5-containing endosomes. Scale bar, 10 μm. (D) Quantification of the results. Pearson’s correlation coefficients between β2AR and FRB-Rab5 were calculated (n = 11 [WT, agonist], 11 [C375S, agonist], 21 [WT, agonist to antagonist], or 19 [C375S, agonist to antagonist] cells from two independent experiments, ***P < 0.001 by two-tailed t test). Error bars represent the S.D.
Fig. 2. Vps34 is required for efficient β2AR recycling, but not for endocytosis. (A) Representative images of HEK293 cells expressing GFP-2xFYVE. Cells were treated with PIK-III or DMSO for 1 hour, fixed, and imaged by confocal microscopy. Scale bar, 10 μm. (B) Effects of PI3K inhibitors on basal surface β2AR levels. Cells stably expressing Flag-tagged β2AR were treated with the indicated inhibitors for 1 hour. Surface receptor levels were then determined by flow cytometry and were expressed as a percentage of the levels in DMSO-treated cells (n = 4 independent experiments). (C) Effects of PI3K inhibitors on agonist-induced reduction of surface β2AR levels. After 1 hour of pretreatment of inhibitors, cells were treated with isoproterenol for the indicated periods, and surface receptor levels were determined. Basal receptor levels shown in (B) were included in a graph as “0 minutes” for comparison, and data were expressed as a percentage of the basal receptor levels in DMSO-treated cells. Data are from n = 3 (5–40 minutes) or n = 4 (0 minutes) independent experiments. PIK-III or WM treatment significantly reduced β2AR levels compared with DMSO control at 10, 20, and 40 minutes after isoproterenol treatment (***P < 0.001, ***P < 0.01, ***P < 0.001 by two-way ANOVA and Tukey’s post hoc tests). (D) The efficiency of β2AR endocytosis was estimated from the percentage reduction in surface receptor levels after the shortest (5 minutes) isoproterenol application in (C). Data were from n = 3 independent experiments. (E) Direct measurement of β2AR recycling after agonist removal and antagonist treatment. After 1 hour of treatment of the indicated inhibitor, cells were treated with isoproterenol for 25 minutes, and then with alprenolol for 45 minutes. The percentage of recycled receptors was calculated as described in Materials and Methods and is shown in bar graphs (n = 3 independent experiments, **P < 0.01 compared with DMSO control by one-way ANOVA and Tukey’s post hoc tests). (F) Recycling defect in PIK-III–treated cells was verified by fluorescence microscopy. After 1 hour treatment with PIK-III or DMSO, cells were treated with isoproterenol for 25 minutes (in the agonist condition) and then with alprenolol for 45 minutes (in the agonist-to-antagonist condition). After fixation, cells were stained for the Flag epitope. Representative confocal images are shown. Scale bars, 10 μm. (G) β2AR recycling in the continuous presence of agonist. Surface receptors were labeled with Alexa Fluor 647–conjugated anti-Flag antibody, and then internalized by 1 μM isoproterenol for 25 minutes. After stripping antibodies bound to residual surface receptors by calcium-depleted medium, cells were further incubated for 5 minutes in calcium-depleted medium. β2AR recycling was then estimated by measuring the antibody efflux in this 5-minute period (n = 3 samples from one experiment, °°P < 0.01 by two-tailed t test. Similar results were obtained in two other independent experiments). Error bars indicate the S.D.
samples (Fig. 1D), suggest that endosomal PI3P is specifically required for β2AR recycling but not endocytosis.

To examine the role of Vps34 activity, we used PIK-III, a potent chemical inhibitor of Vps34 that has high selectivity for Vps34 over other kinases (Dowdle et al., 2014; Honda et al., 2015). PIK-III application produced a pronounced redistribution of the 2xFYVE probe from endosomes to the cytoplasm (Fig. 2A). We then quantified the effects of specific Vps34 inhibition on β2AR trafficking using fluorescence flow cytometry. PIK-III did not detectably change basal surface β2AR levels over the time interval required for our experiments, similar to the broad-spectrum PI 3-kinase (PI3K) inhibitor WM (Fig. 2B). These inhibitors also had little effect on isoproterenol-induced reduction of β2ARs from the PM measured 5 minutes after agonist application (Fig. 2, C and D), a time point at which net β2AR internalization is dominated by endocytic rate. However, both PIK-III and WM significantly reduced surface concentration of β2ARs at later time points (Fig. 2C), suggesting a selective inhibition of receptor recycling. We verified this by direct measurement of β2AR recycling after agonist removal (Fig. 2E). The similar degree of inhibition produced by PIK-III and WM suggests that Vps34 is the major PI3K isoform controlling β2AR trafficking in this cell system. We also confirmed the PIK-III effects by immunofluorescence microscopy. PIK-III caused clearly detectable retention of β2AR in intracellular structures after agonist removal (Fig. 2F), consistent with the recycling defect observed by flow cytometry.

Internalized β2ARs are efficiently recycled not only after agonist removal, but also in the continuous presence of agonist (Morrison et al., 1996; Tsao and von Zastrow, 2000). To analyze β2AR recycling in the presence of agonist, we employed a previously established “loss of internal receptor” assay (Tsao and von Zastrow, 2000). In this assay, Flag-tagged β2ARs are labeled with fluorochrome-conjugated anti-Flag antibody and are subsequently internalized by isoproterenol application. The recycling of Flag-tagged β2AR is then estimated by the efflux of anti-Flag antibody to calcium-depleted medium. PIK-III essentially abolished this antibody efflux, providing evidence that PIK-III blocks β2AR recycling in the presence of isoproterenol (Fig. 2G).

To further confirm the role of Vps34 in β2AR recycling, we used two additional recently reported selective Vps34 inhibitors, VPS34-IN1 (Bago et al., 2014) and SAR405 (Ronan et al., 2014). Both inhibitors suppressed β2AR recycling after agonist removal as well as PIK-III (Fig. 3A). In marked contrast, a selective inhibitor of class I PI3Ks, GDC-0941 (Folkes et al., 2008), did not affect β2AR recycling, indicating that class I PI3Ks are not required for β2AR recycling (Fig. 3A).

Notably, PI3P is a precursor for producing another phosphoinositide, phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2), which also regulates trafficking and ion homeostasis at endosomes (McCartney et al., 2014). The reduction of PI3P levels thus potentially affects PI(3,5)P2 production, and, moreover, MTM1 has a phosphatase activity toward PI(3,5)P2 as well as PI3P (Fili et al., 2006). Thus, we considered the possibility that Vps34 inhibition affects β2AR recycling through depleting PI(3,5)P2 rather than PI3P itself. To test this, we used YM201636 to selectively block PIKfyve, which generates PI(3,5)P2 from PI3P (Jefferies et al., 2008), and measured β2AR recycling by flow cytometry. YM201636, in contrast to PIK-III, did not significantly affect β2AR recycling (Fig. 3B). These observations verify that PI3P is the key phosphoinositide product mediating the Vps34 effect on β2AR recycling.

We next investigated whether Vps34 affects β2AR signaling. The key biochemical step initiating canonical β2AR signaling is receptor-mediated activation of the heterotrimeric G protein Gs (Rasmussen et al., 2011). Ligand-activated β2ARs activate Gs first in the PM and then in the endosome-limiting membrane after endocytosis, with both events occurring sequentially over a period of several minutes (Irannejad et al., 2013). Accordingly, we reasoned that Vps34 might affect canonical β2AR signaling by enhancing or inhibiting the endosome phase of Gs activation. To test this, we applied a previously described conformational biosensor, Nb37-GFP, which detects Gs activation in intact cells by local recruitment (Irannejad et al., 2013). The recruitment of Nb37-GFP, which is indicative of Gs activation, was clearly evident at β2AR-containing endosomes (Supplemental Fig. 1A) and was localized to the limiting membrane (Supplemental Fig. 1A, insets, line scans). PIK-III did not detectably change this behavior (Supplemental Fig. 1A). We quantified the intensity of Nb37-GFP signal in endosome-limiting membranes and revealed that Nb37-GFP was similarly recruited, irrespective of PIK-III application (Supplemental Fig. 1B). This suggests that Vps34 is not essential for Gs activation by β2AR at endosomes.

After β2AR activation, an initial phase of cytoplasmic cAMP accumulation occurs from the PM and then receptors internalize to initiate a second signaling phase from endosomes (Irannejad et al., 2013; Tsvetanova and von Zastrow, 2014). The cellular cAMP response is desensitized by prolonged agonist exposure (Pierce et al., 2002), and recycling of internalized β2ARs to the PM after agonist removal promotes reactivation of the cellular cAMP response to subsequent agonist challenge (Yu et al., 1993; Pippig et al., 1995; Odley et al., 2004). Accordingly, to investigate the potential signaling consequences of Vps34 activity, we investigated the effects of specific Vps34 blockade, and used three different experimental protocols to examine both short-term and long-term β2AR-mediated cAMP responses.

We first examined the isoproterenol-induced cAMP response elicited within seconds after short-term application, which we
reasoned would primarily sample signaling from the PM. To do so, we used a biosensor (GloSensor; Promega) that offers rapid detection without cell lysis for sample preparation, as is required for standard biochemical determination of cAMP (Fan et al., 2008). Blocking Vps34 activity with PIK-III did not detectably affect this immediate signal (Fig. 4A). We next examined the isoproterenol response elicited after a 60-minute interval of isoproterenol pre-exposure followed by washout, which we reasoned would sample the resensitized β2AR pool present in the PM. Vps34 blockade significantly reduced this response (Fig. 4B). Together, these results are consistent with an essential role of Vps34 in β2AR recycling and the previously established role of recycling in promoting β2AR resensitization.

We then investigated the effect of Vps34 blockade on the short-term isoproterenol-induced cAMP response when measured 5–10 minutes after initial application, which we reasoned could include signaling initiated from both the PM and endosomes. It was possible to use a standard biochemical determination of cAMP over this time scale, facilitating a more direct and quantitative comparison of Vps34 effects on the cAMP response than using the GloSensor. PIK-III had no detectable effect on the cAMP response measured 1 minute after isoproterenol application, which is consistent with the GloSensor results. However, when measured after 5–10 minutes, PIK-III caused a pronounced inhibition (Fig. 4C). Another selective Vps34 inhibitor, VPS34-IN1, caused essentially the same effects as PIK-III.

Fig. 4. Vps34 supports not only long-term cellular β2AR signaling but also the short-term signaling response. (A) Luminescence-based cAMP assay examining the initial accumulation of cytoplasmic cAMP induced by isoproterenol (iso) application. WT HEK293 cells were transiently transfected with pGloSensor-20F. Cells were pretreated with PIK-III or DMSO, and then iso or forskolin (fsk) was added (time = 0 seconds). cAMP responses in iso-treated cells are shown. Data are normalized to the luminescence of fsk-treated wells (n = 6 independent experiments). (B) Luminescence-based cAMP assay examining the resensitization of the cellular cAMP response after prolonged iso treatment and washout. Cells were pretreated with PIK-III or DMSO, and then treated with iso for 1 hour. After a 10-minute washout period, cells were rechallenged with iso. Luminescence increased by the second stimulation was normalized to that of the first stimulation. Normalized luminescence is significantly reduced in PIK-III–treated cells after 70–150 seconds of iso application compared with DMSO-treated cells (n = 3 independent experiments; *P < 0.05 by two-tailed t test). (C and D) Biochemical determination of cAMP levels. HEK293 cells were pretreated with the indicated inhibitor, and then treated with iso (C) or fsk (D) for the indicated times. Cells were then lysed, and cAMP concentration in the lysates was determined by enzyme-linked immunosorbent assay and normalized to protein concentration. In (C), data are expressed as a percentage of the cAMP levels in cells pretreated with DMSO and then with iso for 1 minute (n = 8 [DMSO] or n = 4 [PIK-III and VPS34-IN1] independent experiments; *P < 0.05, **P < 0.01 compared with DMSO control by one-way ANOVA and Tukey’s post hoc tests). In (D), data are expressed as a percentage of the cAMP levels in cells pretreated with DMSO and then fsk for the indicated times (n = 4 independent experiments). Error bars indicate the S.E.M.
Since it was first identified in yeast as a gene required for vacuolar protein sorting (Herman and Emr, 1990), Vps34 has long been recognized to regulate endosomal trafficking. In line with this, a previous study (Awwad et al., 2007) showed that broad-spectrum PI3K inhibitors (LY294002 and WM), which block Vps34 and other PI3Ks, suppress β2AR recycling. However, since such inhibitors are not specific for Vps34, it was not possible previously to interrogate the role of Vps34 or endosomal PI3P specifically. In this study, we verify the role of Vps34 and PI3P by using recently developed specific manipulations. We demonstrate that both short-term PI3P depletion in endosomal membranes (Fig. 1) and specific Vps34 inhibition (Figs. 2 and 3) suppress β2AR recycling. In addition, we show that a highly selective inhibitor of class I PI3Ks, which are blocked by broad-spectrum inhibitors, does not affect β2AR recycling (Fig. 3A). Together, our results unambiguously show that Vps34 is essential for β2AR recycling. Although Vps34 might have additional kinase-independent effects, our kinase inhibitor and short-term PI3P depletion data that are presented here indicate that the kinase activity of Vps34 and endosomal PI3P are required for β2AR recycling.

We then demonstrate a fundamental role of Vps34 in the cAMP signaling response triggered by β2AR activation. Figure 5 summarizes our proposed model regarding this. The short-term cAMP response by single agonist application reaches a maximum before 5 minutes at the latest, but then is rapidly desensitized. After agonist washout, cellular responsiveness is recovered at least in part by β2AR recycling to the PM. Vps34, by promoting β2AR recycling, supports this resensitization (Fig. 5A). Our data also reveal an additional role of Vps34 in promoting the short-term cellular cAMP response, and they resolve a second component of the short-term response that is specifically Vps34 dependent (Fig. 5B).

What is the underlying mechanism of this Vps34-dependent component? We previously showed that β2AR recycling requires Vps34, and, in line with this, Vps34 supports the repeated cAMP response. (B and C) According to our data showing Vps34 inhibition reduces cAMP levels after 5–10 minutes of short-term agonist application, the short-term cAMP response seems to be divided into the following two phases: the Vps34-independent initial phase and the Vps34-dependent later phase. Our models regarding the role of Vps34 in the short-term cAMP response are shown in (C). In the initial phase, β2ARs activate cAMP synthesis in the PM and endosomes, but are not recycled yet. In the later phase, the recycling of β2AR begins in a Vps34/PI3P-dependent manner, and the recycled receptors are subjected to the next round of receptor cycling between the PM and endosomes (indicated by red arrows). The continuous cycling of β2ARs, which is driven by Vps34, would enhance cAMP production from both locations in the later phase. It is also possible that Vps34 directly enhances endosomal cAMP production downstream of Gs activation (indicated by another red arrow with a question mark).

(Fig. 4C) This effect is specific to the isoproterenol-induced cAMP response because Vps34 inhibition did not attenuate the cAMP response caused by receptor-independent adenyl cyclase activation with forskolin (Awad et al., 1983) (Fig. 4D). Thus, Vps34 activity not only supports long-term cellular β2AR signaling, as indicated by its effect on resensitization, but it also plays an essential role in supporting the short-term signaling response.
Vps34 inhibition increases the endosomal β2AR pool (Fig. 2C) and reduces the cAMP response after 10 minutes of isoproterenol application (Fig. 4C). One interpretation of these data is that the endosomal β2AR pool cannot activate the cAMP response as strongly as the surface β2AR pool. Indeed, our previous report suggested that endosomal β2AR activation makes a relatively small, but functionally distinct, contribution to the cellular cAMP response. To confirm this idea, we determined the kinetics of receptor recycling through Vps34 blockade and found that the cAMP response is mediated by a canonical GPCR cascade. Although class I PI3Ks are well known to promote signaling at the PM (Marat and Haucke, 1996), Vps34 has been reported to mediate signaling from endosomes (Tskazaki et al., 2013). It is also possible that the endosome signaling component is transiently present, as is the surface component, so that inhibiting recycling through Vps34 blockade causes the net response to delocalize. It will be interesting to investigate these possibilities in future studies.

The present results show that the endosomal system can indeed be targeted to manipulate canonical GPCR signaling, and identify an essential role of the endosomal PI3K Vps34 in promoting both short-term and long-term cellular responses mediated by a canonical GPCR cascade. Although class I PI3Ks are well known to promote signaling at the PM (Marat and Haucke, 1996), Vps34 has been reported to mediate signaling from endosomes (Tskazaki et al., 2013). It is also possible that the endosome signaling component is transiently present, as is the surface component, so that inhibiting recycling through Vps34 blockade causes the net response to delocalize. It will be interesting to investigate these possibilities in future studies.


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Supplementary Data

Endosomal phosphatidylinositol 3-kinase is essential for canonical GPCR signaling

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Supplementary Fig. 1.

Supplementary Fig. 1. VPS34 is not essential for Gs activation by β2AR at endosomes. (A) Representative images of live HEK293 cells transiently expressing Flag-tagged β2AR and Nb37-GFP. Cells were pre-treated with PIK-III or DMSO for 1 h, and then treated with iso. Cells were imaged after 15-30 min of iso treatment by confocal microscopy. Insets show the boxed areas at higher magnification. Fluorescence intensity profiles of Flag-β2AR and Nb37-GFP from the lines in the insets were shown in the right graphs. Arrows indicate the enrichment of Nb37 signal in the limiting membrane of β2AR-containing endosomes. Scale bars, 10 μm. (B) Quantification of the results in (A). The intensities of Nb37 and β2AR in endosome limiting membranes were calculated, and the Nb37 intensity was normalized to the β2AR intensity. n = 99 (DMSO) or 92 (PIK-III) endosomes from three independent experiments, n.s., not significant by two-tailed t test. Error bars mean SD.
Supplementary Text
Program script for measuring fluorescence intensity in endosomal limiting membranes

Bold letters show the part used for the actual analysis in this study. The ‘width of linescan’ was set to 3 pixels.

This program allows a 3-clicks quantification of the "tubule index", enrichment of the marker in the tubule relative to the endosome.

Rotative linescan to detect enrichment in the endosomal membrane relative to the tubule position

Input is a Tif image +/- a region file
Output is an excel file with the quantification and a .txt region file

Two parameters can be set:
size of the window that pops up "a", line 158
number of steps for the rotative linescan "step" line 343

This program won't run on mac Matlab because of Excel, minor modifications can be done to export files with the final matrixes. No other bug known so far.

Run the program by entering “Tubulindex” on the command window, and select the .tif image.
You can use the sliders to change the contrast, playing with them once the analysis is started will stop the program.
If you want to create new regions, a message box will remind you to start by selecting a background region (user defined rectangle).
A dialog box will then pop up for the user to enter the desired width of the linescan.
I recommend entering an uneven number, but in all cases this must be an integer > 1!

The user then selects an endosome on the image, a window pops up to define more precisely the region of interest.
The size of this window is fixed in the program, and can be changed by the user if necessary.
If the structure is too big or too small, modify the parameter “a” accordingly (line 343).
On the new window, the user will define the endosomal membrane + tubule in 3 clicks:

- First click is the center of the endosome
%-Second click in the membrane of the endosome at the base of the tubule
%-Third click is the tip of the tubule
%-The user then select another endosome, and so on.
%
%-To stop selecting endosome:
%-When you select the last endosome, use the RIGHT CLICK to select the center of the endosome. The program will recognize it as the last endosome.
%
%-You can load the same endosomes using the Load ROIs button, just select the .txt file containing the info.
%-This is particularly useful for comparison between channels.
%
%-Quantification:
%
%-Code will compute the average fluorescence in a donut centered around the first click.
%-The inner diameter of the donut is the distance between first and second click – width of linescan/2.
%-The outer diameter is distance between first and second click + width of linescan/2.
%-This info is found on the excel sheet TubIndex, column “B”, “Fluo Endo”.
%
%-Code does a rotative linescan. The number of steps for this linescan is defined is the program as 60 steps.
%-You can modify the number of steps line 282, parameter “step”.
%-Basically, if you think of a clock, it will do a linescan of the donut for every second. 12 O’clock is at the opposite side of the tubule.
%-The average fluorescence of the donut linescan is computed, and this information is reported on the sheet “Endo Linescan”.
%-Keep in mind that the first and last values are the same (noon/midnight).
%-The program also plots the average values of this measurement +/- SEM.
%
%-For the tubule, the program draws a line between the second and third click.
%-Then it will draw a certain number of parallel lines centered on the user line.
%-The number of line is the width of the linescan, they are 1 pixel distant.
%-This is why I suggest uneven number, as the center of these lines is the one actually drawn by the user.
%The program does a linescan along every single line, compute the average fluorescence for every line, then the average fluorescence of the different lines.
%This information is found on the excel sheet TubIndex, column “C”, “Fluo Tub”.
%Column D is the “Tubule index”, the ratio of column C/B.

%Once the computation is done
%green circle: circle drawn by the user, endosome
%red line: line drawn by the user, tubule
%white dots: outer diameter of the donuts, the linescan is made between the center of the circle and every single one of these dots, starting from the opposite side of the red line.
%blue line: actual line used for the linescan, starting from the outer diameter of the donut.
%blue rectangle: region used for the linescan before transformation by the rotation matrix
%yellow rectangle: rectangle actually used for the linescan
%red dots: endpoints of the lines used for the linescans.
%Program will display a plot of the rotative linescan average fluorescence +/- SEM.

%If your image is named “MyImage”, the excel file will be named “MyImage_TubIndex.xlsx”.
%The text file containing the endosome coordinates “MyImage_endo.txt”.
%Do not run the program with preexisting files with the same names! It will overwrite.
%You’ll find average and SEM for both sheets.

%Last update on 12/13/2014

%Written by Damien Jullié

function Tubulindex(action)

%Open the image

if nargin == 0
[stk, stkd] = uigetfile('* .tif', 'Choose an image');

M = imread(stk);
M = double(M);

stk = stk(1:end-4);
frame = 1;
figure('name', ['Play ', stk])
map = gray(256);

% Controls for image scaling and user interface

high = double(max(max(max(M))));
low = double(min(min(min(M))));
uMax = find(M > high - 100);
uMin = find(M < low + 100);
MhPix = M;
MhPix(uMax) = low;
McPix = M;
McPix(uMin) = high;
hPix = double(max(max(max(MhPix)))); % the second highest pixel value in Movie
cPix = double(min(min(min(McPix)))); % the second lowest pixel value in Movie
uicontrol('style', 'slider', 'callback', 'Tubulindex scale', 'min', low, 'max', high - 3, 'value', low, ...
'position', [240, 15, 120, 15], 'tag', 'scalelow')
uicontrol('style', 'text', 'position', [210, 15, 30, 15], 'tag', 'low_text')
uicontrol('style', 'text', 'position', [175, 15, 35, 15], 'string', 'Low')
uicontrol('style', 'slider', 'callback', 'Tubulindex scale', ...
'min', low + 3, 'max', high, 'value', high, ...
'position', [240, 30, 120, 15], 'tag', 'scalehigh')
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uicontrol('style','text','position',[210,30,30,15],tag,'high_text')
uicontrol('style','text','position',[175,30,35,15],string,'High')
uicontrol('style','checkbox','position',[370,15,60,15],...
    'string','HotPix','value',0,'tag','hotpix',...
    'callback','Tubulindex scale','userdata',[high,hPix])
uicontrol('style','checkbox','position',[370,30,60,15],...
    'string','ColdPix','value',0,'tag','coldpix',...
    'callback','Tubulindex scale','userdata',[low,cPix])

uicontrol('string','New ROIs','position',[30,50,80,15],...
    'tag','Endo','userdata',[],'callback','Tubulindex NewR')
uicontrol('string','Load ROIs','position',[30,80,80,15],...
    'tag','Largeur','userdata',[],'callback','Tubulindex LoadR')

%%% drawing the figure

set(gcf,'UserData',M,'keypressfcn','Tubulindex key','doublebuffer','on',...
    'colormap',map)
u = image(M(:,:,frame),'cdatamapping','scaled','tag','movi');
set(gca,'clim',[low,high],'tag','moviaxis')

set(gca,'position',[0.13,0.15,0.9,0.75])
h = title([stk,' Frame # = ',num2str(frame)],...
    'interpreter','none');
set(h,'userdata',stk)
axis image
pixvalm
scale

else
    eval(action)
end
%% defining regions

function NewR

M = get(gcf,'userdata');
stk = get(gca,title,'userdata');

% size of the window for picking endosome

a = 20;

% defining background region

msgbox('Select the background region')
waitfor(gcf)

[Xback,Yback,Back,rect] = imcrop;
rect = round(rect);
bx = rect(1); by = rect(2); bw = rect(3); bh = rect(4);
X = [bx,bx,bx+bw,bx+bw,bx];
Y = [by,by+bh,by+bh,by,by];
line('XData',X,'YData',Y,'color','r')

% storing endosomes coordinates

endo = cat(2,[0,0,0],rect);

fluoback = sum(sum(M(by:by+bh,bx:bx+bw)))/((1+bw)*(1+bh));
% width of the linescan, >1
largeur = inputdlg({'width of the quantification line'},'Width');
largeur = str2num(largeur{1});

set(findobj('tag','Largeur'),'UserData',largeur);

button = 1;
endonum = 1;

while button == 1;

xy = ginput(1);
xy = round(xy);

limits = get(gca,'clim');

minx = xy(1)-a;
maxx = xy(1)+a +1;
miny = xy(2)-a;
maxy = xy(2)+a +1;

if xy(1)-a < 1
    minx = 1;
end
if xy(1)+a > size(M,2)
    maxx = size(M,2);
end
if xy(2)-a < 1
    miny = 1;
end
end
if xy(2)+a > size(M,1)
    maxy = size(M,1);
end

MiniM = M(miny:maxy,minx:maxx);

%closup on the selected endosome

figure('name',['Endosome ',num2str(endonum)])
set(gcf,'userdata',MiniM,'colormap',gray(256));
image(MiniM,'cdatamapping','scaled','tag','miniImage')
set(gca,'clim',limits);
line(a+1,a+1,'lineStyle','none','marker','+','markerEdgeColor','b')
drawnow

%three clics selection
if button == 1;

[x,y,button] = ginput(1);

x = round(x);
y = round(y);

line(x,y,'LineStyle','none','marker','+','markerEdgeColor','g')

[x2,y2] = ginput(1);
x2 = round(x2);
y2 = round(y2);

line(x2,y2,'LineStyle','none','marker','+','markerEdgeColor','g')
[x3,y3] = ginput(1);
x3 = round(x3);
y3 = round(y3);

line(x3,y3,'lineStyle','none','marker','+', 'markerEdgeColor','r')

diameter = sqrt((x-x2).^2 + (y-y2).^2);

line([x2,x3],[y2,y3],'LineStyle','-','Color','r','LineWidth',largeur)
rectangle('position',[x-diameter, y-diameter, 2*diameter, 2*diameter],...
    'curvature',[1 1],'LineStyle','-','...
    'LineWidth',largeur,'EdgeColor','g')

pause(0.5)
close

x = xy(1)-a + x -1;
y = xy(2)-a + y -1;

x2 = xy(1)-a + x2 -1;
y2 = xy(2)-a + y2 -1;

x3 = xy(1)-a + x3 -1;
y3 = xy(2)-a + y3 -1;

line([x2,x3],[y2,y3],'LineStyle','-','Color','r','LineWidth',2)
rectangle('position',[x-diameter, y-diameter, 2*diameter, 2*diameter],...
    'curvature',[1 1],'LineStyle','-','...
    'LineWidth',largeur,'EdgeColor','g')
endo = cat(1, endo, [endonum,x,x2,x3,y,y2,y3]);

endonum = endonum + 1;

dlmwrite([stk,'_endo.txt'], endo, 'delimiter','	');
set(findobj('tag','Endo'),'UserData',endo);

%export the endosome coordinates file

%run the quantification
QuantTub

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%%If load region is selected, pick a .txt file with endosome coordinates

function LoadR

M = get(gcf,'userdata');

[f,p] = uigetfile('_syn.txt','Choose the region text file');
endo = dlmread([p,f],'	');
set(findobj('tag','Endo'),'UserData',endo);
largeur = inputdlg({'width of the quantification line'},'Width');
largeur = str2num(largeur{1});

set(findobj('tag','Largeur'),'UserData',largeur);

bx = endo(1,4); by = endo(1,5); bw = endo(1,6); bh = endo(1,7);
X = [bx,bx,bx+bw,bx+bw,bx];
Y = [by,by+bh,by+bh,by,by];
line('XData',X,'YData',Y,'color','r')

for i = 2:size(endo,1)
    x = endo(i,2); x2 = endo(i,3); x3 = endo(i,4);
    y = endo(i,5); y2 = endo(i,6); y3 = endo(i,7);
    diametre = sqrt((x-x2).^2 + (y-y2).^2);
    line([x2,x3],[y2,y3],'LineStyle','-','Color','r','LineWidth',2)
    rectangle('position',[x-diametre, y-diametre, 2*diametre, 2*diametre],...
                'curvature',[1 1],'LineStyle','-','LineWidth',largeur,'EdgeColor','g')
end

QuantTub

%%% Quantification of the tubule index
function QuantTub

% Number of steps for the rotative linescan
step = 60;

M = get(gcf,'userdata');
stk = get(get(gca,'title'),'userdata');

children = get(gcf,'children');

endo = get(findobj(children,'tag','Endo'),'UserData');
largeur = get(findobj(children,'tag','Largeur'),'UserData');

bx = endo(1,4); by = endo(1,5); bw = endo(1,6); bh = endo(1,7);
X = [bx,bx,bx+bw,bx+bw,bx];
Y = [by,by+bh,by+bh,by,by];

fluoback = sum(sum(M(by:by+bh,bx:bx+bw)))/((1+bw)*(1+bh));

RotFluo2 = [];
Tubulindex = [];

for i = 2:size(endo,1)
    RotFluo = endo(i,1);
    x = endo(i,2); x2 = endo(i,3); x3 = endo(i,4);
    y = endo(i,5); y2 = endo(i,6); y3 = endo(i,7);
    diametre = sqrt((x-x2).^2 + (y-y2).^2);
\[ [r,t] = \text{meshgrid}(1:\text{size}(M,2),1:\text{size}(M,1)); \]

\[ \text{distance} = \sqrt{(r-x)^2 + (t-y)^2); \]

%define the donut
\[ \text{diametreIn} = \text{diametre} - \text{largeur}/2; \]
\[ \text{diametreOut} = \text{diametre} + \text{largeur}/2; \]

\[ \text{maskIn} = \text{distance} < \text{diametreIn}; \]
\[ \text{maskOut} = \text{distance} < \text{diametreOut}; \]
\[ \text{mask} = \text{maskOut} - \text{maskIn}; \]
\[ \text{Fluocircle} = \text{mask} .* M; \]

% Rotative linescan
\[ \text{stepangle} = 2*\pi/\text{step}; \]
\[ \text{orangle} = \text{acos}((x2-x)/\text{diametre}); \]

for \( k = 0 : \text{step} \):

\[ \text{xk} = x + \text{diametreOut} * \cos(\pi - \text{orangle} + k*\text{stepangle}); \]
\[ \text{yk} = y + \text{diametreOut} * \sin(\pi - \text{orangle} + k*\text{stepangle}); \]

line(xk,yk,'lineStyle','none','marker','*','markerEdgeColor','w')

\[ \text{Rotline} = \text{improfile}(\text{Fluocircle},[x,xk],[y,yk]); \]
\[ \text{CleanRotline} = \text{find}(\text{Rotline} \neq 0); \]
\[ \text{Rotline} = \text{mean}(\text{Rotline}(\text{CleanRotline},1)) - \text{fluoback}; \]
\[ \text{RotFluo} = \text{cat}(2,\text{RotFluo},\text{Rotline}); \]
end

RotFluo2 = cat(1,RotFluo2,RotFluo);

%AvCircle is the backgroud substracted fluo in the donut (Uchida et al. 2016)
AvCircle = sum(sum(Fluocircle))/sum(sum(maskOut-maskIn));
AvCircle = AvCircle - fluoback;

%quantification of the tubule florescence using a rotative rectangle
%This method is used to implement improfile accross multiple paralell lines
if (y2 ~= y3) & (x2 ~= x3)

pentel = (y3 - y2)/(x3 - x2);

if x3 - x2 > 0

mmpc = sqrt(((largeur/2)^2)/(pentel^2 +1));

else

mmpc = - sqrt(((largeur/2)^2)/(pentel^2 +1));

end

x2 = x2 + mmpc;
y2 = y2 + pentel * mmpc;
longueur = sqrt((x3-x2).^2 + (y3-y2).^2);

line([x2,x3],[y2,y3],'LineStyle','-','Color','c','LineWidth',4)

if y3 - y2 < 0

rotationAngle = acos((x3-x2)/longueur);
else
rotationAngle = -acos((x3-x2)/longueur);
end

rotationArray = [cos(rotationAngle), -sin(rotationAngle); sin(rotationAngle), cos(rotationAngle)];

centerx = (x3 - x2)/2;
centery = (y3 - y2)/2;

rectangle('position',[x2 + centerx - longueur/2, y2+ centery - largeur/2, longueur, largeur], 'LineStyle','-','LineWidth',2,'EdgeColor','b')

vertices2 = cat(1,[-longueur/2,-largeur/2],[longueur/2,-largeur/2],[-longueur/2,largeur/2],[longueur/2,largeur/2]);

rotrect2 = vertices2 * rotationArray;

rotrect2(:,1) = rotrect2(:,1) + x2 + centerx;
rotrect2(:,2) = rotrect2(:,2)+ y2 + centery;

%this is to draw the rotated rectangle

line([rotrect2(1,1),rotrect2(2,1)],[rotrect2(1,2),rotrect2(2,2)],'LineStyle','-','Color','y','LineWidth',2)
line([rotrect2(1,1),rotrect2(3,1)],[rotrect2(1,2),rotrect2(3,2)],'LineStyle','-','Color','y','LineWidth',2)
```
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line([rotrect2(2,1),rotrect2(4,1)],[rotrect2(2,2),rotrect2(4,2)],'LineStyle','-','Color','y','LineWidth',2)
line([rotrect2(3,1),rotrect2(4,1)],[rotrect2(3,2),rotrect2(4,2)],'LineStyle','-','Color','y','LineWidth',2)

pente = (rotrect2(3,2) - rotrect2(1,2))/(rotrect2(3,1) - rotrect2(1,1));

b1 = rotrect2(1,2) - pente * rotrect2(1,1);
b2 = rotrect2(2,2) - pente * rotrect2(2,1);

Fluoline2 = [];

for j = 0 : (largeur-1)
    xj1 = rotrect2(1,1) + ((rotrect2(3,1) - rotrect2(1,1))/(largeur-1))*j;
xj2 = rotrect2(2,1) + ((rotrect2(4,1) - rotrect2(2,1))/(largeur-1))*j;

    yj1 = pente * xj1 + b1;
yj2 = pente * xj2 + b2;

    %to draw the dots for the linescans

    line(xj1,yj1,'lineStyle','none','marker','*','markerEdgeColor','r')
    line(xj2,yj2,'lineStyle','none','marker','*','markerEdgeColor','r')

    Fluoline = improfile(M,[xj1,xj2],[yj1,yj2]);
    Fluoline = mean(Fluoline,1);
    Fluoline2 = cat(1,Fluoline2,Fluoline);
end
```
else

% Vertical/horizontal lines (special cases)

    if y2 == y3
    if x3 > x2
        xj1 = x2 + largeur/2;
    else
        xj1 = x2 - largeur/2;
    end
    xj2 = x3;

    line([xj1,xj2],[y2,y3],'LineStyle','-','Color','c','LineWidth',4)

    Fluoline2 = [];

    for j = 0 : (largeur-1)

        yj1 = y2 -(largeur-1)/2 +j;
        yj2 = y3 -(largeur-1)/2 +j;

        line(xj1,yj1,'LineStyle','none','marker','*','markerEdgeColor','r')
        line(xj2,yj2,'LineStyle','none','marker','*','markerEdgeColor','r')

    Fluoline = improfile(M,[xj1,xj2],[yj1,yj2]);
    Fluoline = mean(Fluoline,1);
    Fluoline2 = cat(1,Fluoline2,Fluoline);

    end
else

    if y3 > y2
        yj1 = y2 + largeur/2;
    else
        yj1 = y2 - largeur/2;
    end

    yj2 = y3;

    line([x2,x3],[yj1,yj2],'LineStyle','-','Color','c','LineWidth',4)

    Fluoline2 = [];
    for j = 0 : (largeur-1)

        xj1 = x2 - (largeur-1)/2 +j;
        xj2 = x3 - (largeur-1)/2 +j;

        line(xj1,yj1,'LineStyle','none','marker','*','markerEdgeColor','r')
        line(xj2,yj2,'LineStyle','none','marker','*','markerEdgeColor','r')

    Fluoline = improfile(M,[xj1,xj2],[yj1,yj2]);
    Fluoline = mean(Fluoline,1);
    Fluoline2 = cat(1,Fluoline2,Fluoline);

    end

end
end
FluoTub = mean(FluoLine2,1);
FluoTub = FluoTub - fluoback;

FluoTub = cat(2,endo(i,1),AvCircle,FluoTub,FluoTub/AvCircle);
Tubulindex = cat(1,Tubulindex,FluoTub);

end

%MoyFluo = mean(Tubulindex,1);
%stdmoy=std(Tubulindex,0,1);
%ET=stdmoy./srtm(size(Tubulindex,1));

MoyFluo = mean(Tubulindex,1);
stdmoy=std(Tubulindex,0,1);
ET=stdmoy./sqrtm(size(Tubulindex,1));

MoyRot = mean(RotFluo2(:,2:end),1);
stdRot = std(RotFluo2(:,2:end),0,1);
ETRot = stdRot./sqrtm(size(RotFluo2,1));

normcirc = [0:step]/step;

figure
errorbar(normcirc,MoyRot,ETRot,'-og','MarkerEdgeColor','k');

MoyRot = cat(1,MoyRot,ETRot);
legendRot2 = {'Average';'StDev'};
MoyRot = cat(2,legendRot2,num2cell(MoyRot));

legendRot = cat(2,{'Endosome #'}, num2cell(normcirc));
RotFluo2 = cat(1,legendRot,cell(1,size(RotFluo2,2)),num2cell(RotFluo2),cell(1,size(RotFluo2,2)),MoyRot);
MoyFluo = cat(1,MoyFluo(2:end),ET(2:end));

legend = {'Endosome #','Fluo endo','Fluo Tub','Tubule Index'};
legend2 = {'Average';'StDev'};

legend2 = cat(2,legend2,num2cell(MoyFluo));

Final = cat(1,legend, cell(1,4),num2cell(Tubulindex),cell(1,4),legend2);

xlswrite([stk,'_TubIndex.xlsx'], Final,'TubIndex', 'A1')
xlswrite([stk,'_TubIndex.xlsx'], RotFluo2,'Endo Linescan', 'A1')

function scale
M = get(gcf,'userdata');
children = get(gcf,'children');
low = round(get(findobj(children,'tag','scalelow'),'value'));
high = round(get(findobj(children,'tag','scalehigh'),'value'));
%minlow = get(findobj(children,'tag','scalelow'),'min');
%maxhigh = get(findobj(children,'tag','scalehigh'),'max');
hotPix = get(findobj(children,'tag','hotpix'),'value');
hPix = get(findobj(children,'tag','hotpix'),'userdata');
coldPix = get(findobj(children,'tag','coldpix'),'value');
cPix = get(findobj(children,'tag','coldpix'),'userdata');
%M = get(gcf,'userdata');
if hotPix
    maxhigh = hPix(2);
else
    maxhigh = hPix(1);
end
if coldPix
    minlow = cPix(2);
else
    minlow = cPix(1);
end
if high > maxhigh
    high = maxhigh;
end
if low < minlow
    low = minlow;
end
if high == minlow+1
    high = high +1;
end
if low == maxhigh
    low = low -1;
end

set(gca,'clim',[low,high])
set(findobj(children,'tag','scalelow'),'max',high-1,'min',minlow,...
    'sliderstep',[1/(high-1-minlow),25/(high-1-minlow)]... 
    'value',low)
set(findobj(children,'tag','low_text'),'string',num2str(low));
set(findobj(children,'tag','scalehigh'),'min',low+1,'max',maxhigh,...
    'sliderstep',[1/(maxhigh-(low+1)),25/(maxhigh - (low+1))]... 
    'value',high)
set(findobj(children,'tag','high_text'),'string',num2str(high));
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%End of the code

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