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Endogenous tagging of the YIPF4 gene with mNEON Green and imaging of these cells by microscopy v2

Forked from a private protocol

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We use this protocol and it's working

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Abstract

This protocol describes a method to create cells in which the YIPF4 gene has been endogenously tagged with mNEON fluorescent protein. CRISPR-based gene editing was used to introduce mNEON Green into the N-terminus of YIPF4 in WT and FIP200-/- HEK293T cells. We also describe a protocol for imaging of mNEON-YIPF4 in Golgi using the Golgi marker GOLGB1.

Materials

pX459 (Addgene #62988)
HEK293 (ATCC CRL-1573, RRID: CVCL_0045)
anti-YIPF4 (Sino Biological 202844-T46)
anti-GOLGB1/ Giantin (abcam ab37266)
PBS; Phosphate buffered saline: ThermoFisher (#14040133)
PhosSTOP (Sigma-Aldrich, 4906845001)
NuPAGE™ LDS Sample Buffer (4X): Invitrogen (#NP0007)
DTT (dithiothreitol): Thermo-Fisher (#R0861)
Pierce™ Rapid Gold BCA Protein Assay Kit: Thermo Scientific (#A53226)
Immobilon®-P Membrane, PVDF: Milipore (#IPVH85R)
Protease inhibitor cocktail: Sigma-Aldrich (#P8340),
PhosSTOP: Sigma-Aldrich (#4906845001)
Invitrogen, NuPAGE™ MOPS SDS Running Buffer (#NP0001)
TBST, TRIS-buffered saline (TBS, 20X), with 2% Tween 20 (#J60497.K3)
Ponceau S, Electrophoresis Grade, Thermo Scientific™ (#J60744.18)
Benzonase Nuclease HC: Millipore (#71205-3)
Urea: Sigma (#U5378)
Dulbecco's MEM (DMEM), high glucose, pyruvate (Gibco / Invitrogen, 11995)
AlexaFluor 594 Goat anti-Rabbit IgG, ThermoFisher (#A-11012)
Vectashield H-1000: Cole-Parmer (#3304766)
repair template containing an mNEON Green cassette flanked by homology arms (pSMART-mNEON-YIPF4): Insert sequence:

Troubleshooting

Cell Culture

1 HEK293 (human embryonic kidney, fetus, ATCC CRL-1573, RRID: CVCL_0045) cells were grown in Dulbecco's modified Eagle's medium (DMEM, high glucose and pyruvate) supplemented with 10% fetal calf serum and maintained in a 5% CO_2 incubator at 37^{O}C. Cells were maintained at <80% confluency throughout the course of experiments.

CRISPR editing and validation

2 The gRNA duplex DNA with the top strand sequence 5′ TCGCCGCGAGATGCAGCCTC 3′ was cloned into pX459 (previously digested with XX) to create a vector for Cas9-based cutting adjacent to the start codon of the YIPF4 gene.

3 A synthetic gene block containing Left and Right homology arms from near the N-terminus of YIPF4 flanking an mNEON cassette was cloned into pSMART. The insert sequence is provided in MATERIALS and the plasmid is available at Addgene (#XXX).

4 Transfect HEK293 WT or HEK293-FIP200$^{-/-}$ cells with the pX459-gRNA-YIPF4 N-terminal tagging plasmid (Addgene #XXX) by Lipofectamine 3000. After 7 days, a population of cells for both genotypes was sorted for the same level of mNEON Green signal.

5 Cell populations were then subjected to immunoblotting with anti-YIPF4 antibodies. Cells were cultured in the presence of the corresponding stress to 60-80% confluency in 6-well plates, 10 cm or 15 cm dishes. After removing the media, the cells were washed with PBS three times.

5.1 To lyse, urea buffer (8M urea, 50 mM TRIS 7.5, 150 mM NaCl, containing mammalian protease inhibitor cocktail (Sigma), Phos-STOP, and 20 unit/ml Benzonase (Millipore)) was added directly onto the cells. Cell lysates were collected by cell scrapers and sonicated on ice for 10 seconds, and lysates were cleared by centrifugation (15000 rpm, 10 min at 4 ^{O}C).

5.2 The concentration of the supernatant was measured by BCA assay.

5.3 The whole cell lysate was denatured by the addition of LDS sample buffer supplemented with 100 mM Dithiothreitol, followed by boiling at 95^{O}C for 5 minutes. 10-20 µg of each

lysate was loaded onto a 4-12% NuPAGE Bis-Tris gel (Thermo Fisher Scientific), followed by SDS-PAGE with MOPS SDS running buffer (Thermo Fisher Scientific), respectively.

5.4 For westerns, gels were electro-transferred to PVDF membranes (0.45 µm, Millipore), and then the total protein was stained using Ponceau (Thermo Fisher Scientific). The membrane was then blocked with 5% non-fat milk (room temperature, 60 min) incubated with rabbit anti-YIPF4 (Sino Biological 202844-T46) primary antibodies (1:1000; 4^{o}C, overnight), washed three times with TBST (total 30 min), and further incubated with fluorescent IRDye 800CW Goat anti-Rabbit IgG secondary antibody at (1:10,000) at room temperature for 1 hour. After thorough wash with TBST for 30 min, near infrared signal was detected using OdysseyCLx imager and quantified using ImageStudioLite (LI-COR).

5.5 The mNEON Green-YIPF4 fusion protein was detected at 50 KDa, as anticipated and the endogenous YIPF4 in control (unedited) cells was detected at ~25 KDa.

Imaging of mNEON-YIPF4 cells to demonstrate localization in the Golgi

6 **Confocal Microscopy:**
Cells were plated onto 18 or 22 mm-glass coverslips (No. 1.5, 22×22 mm glass diameter, VWR 48366-227) for imaging. OPTIONAL: for some experiments, it is possible to employ nutrient deprivation. In such cases, DMEM was removed and cells were washed three times with PBS, followed by resuspension in EBSS with or without the appropriate inhibitor(s) (SAR405, BafA) for the desired time period (e.g. 3 hours).

6.1 Cells were then fixed using 4% PFA followed by permeabilization with 0.5% Triton-X100.

6.2 Cells were blocked in 3% BSA for 30 minutes, followed by incubation in primary antibodies (e.g. anti-GOLGB1; 1:1000) for 1 hour at room temperature. Cells were washed 3 times with PBS + 0.02% tween-20, followed by incubation in secondary (alexafluor conjugated secondary antibodies such as AlexaFluor 594 Goat anti-Rabbit IgG) for 1 hour at room temperature.

6.3 Coverslips were then washed 3 times with DPBS + 0.02% tween-20 and mounted onto glass slides using mounting media (Vectashield H-1000) and sealed with nail polish.

6.4 The cells were imaged by con-focal microscopy. We employ a Yokogawa CSU-W1 spinning disk confocal on a Nikon Ti motorized microscope equipped with a Nikon Plan Apo 100x/1.40 N.A objective lens, and Hamamatsu ORCA-Fusion BT CMOS camera. For the analysis, the equal gamma, brightness, and contrast are applied for each image using FiJi software. We observe strong colocalization of mNEON-YIPF4 with GOLGB1 staining, consistent with the localization of YIPF4 primarily in the Golgi.