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NPC1 inhibitor treatments and immunoblotting of whole-cell lysates from cell culture systems

In 1 collection

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We use this protocol and it's working

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Abstract

This is a protocol for assessing abundance of autophagy proteins after inhibition of the lysosomal cholesterol transprorter NPC1 via the U18666A inhibitor by Western blotting from whole-cell lysates derived from HeLa cell culture systems.

Troubleshooting

Safety warnings

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Cell Culture and Treatments

5m

1 Seed HeLa TMEM192-3xHA cells of desired genotypes into 6-well plates.

2 Upon reading desired confluency (~50 - 70%, depending on the duration of inhibitor treatment), treat cells according to the following four treatments:
1. Fed
2. NPC1 inhibitor U18666A (2µM)

3 Treat cells for 1 to 3 days (depending on the experiment).
Change media and inhibitor daily.

4 Aspirate growth media, wash twice with 1xPBS and harvest cells in ice-cold PBS on ice by scraping cells from the wells.

SDS-PAGE and immunoblotting

5m

5 For whole-cell lysates, prepare samples following standard protocols, and final samples should be in LDS buffer with DTT or similar. Incubate samples at 80 °C for 00:05:00 . 5m

6 Load samples into a NuPAGE Novex® 4-12% Bis-Tris Midi Protein Gels and separate by electrophoresis in 1xTris/Glycine/SDS buffer.

7 Transfer proteins to PVDF or nitrocellulose membranes by standard wet transfer in 20% methanol Tis/Glycine buffer.

8 Block membrane in blocking buffer (5% non-fat dry milk or 3% BSA in TBST) at Room temperature for 01:00:00 . 1h

9 Incubate membrane in primary antibody solution (blocking solution plus primary antibody at 1:500-1:1,000, depending on the primary antibody) at 4 °C for 12:00:00 to 16:00:00 . 1d 4h

Primary antibodies:
NCOA4
FTH1

SQSTM1 / p62
LC3B
panGARBARAP
Actin

10 Wash membrane six times with TBST for 00:05:00 each wash. 5m

11 Incubate membrane in secondary antibody solution (blocking solution plus secondary antibody conjugated to HRP at 1:5,000-1:10,000) at Room temperature for 01:00:00 . 1h

12 Wash membrane four times with TBST for 00:05:00 each wash. 5m

13 Apply Western Lightning Plus Chemiluminescence substrate (Revvity) to membrane and acquire blot images using a ChemiDoc MP imager.

14 Process raw image files with Image Lab software (Bio-Rad).