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Proximity biotinylation of ATG8 proteins and selective autophagy receptors V2

Forked from Proximity biotinylation of ATG8 proteins and selective autophagy receptors

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ABSTRACT

Autophagy involves the formation of an autophagosome around a cellular cargo, and once encapsulated in this double membrane structure, the autophagosome fuses with the lysosome to facilitate degradation of the cargo. Membrane-bounded organelles such as mitochondria are frequent targets. This protocol concerns the use of proximity biotinylation with the engineered peroxidase APEX2 (doi.org/10.1038/nprot.2016.018) fused with either ATG8 proteins or autophagic cargo receptors in mammalian cells. There are six ATG8 proteins in mammals (MAP1LC3A, B, and C & GABARAP, L1, and L2). These proteins bind LC3 interacting motifs (LIR motifs) on cargo receptors. Therefore, proximity biotinylation can be used to identify proteins that are nearby ATG8 proteins in response to activation of selective autophagy in cells. This protocol uses nutrient stress as the autophagy activator. The protocol builds on a previously published method (Hung et al., 2016)

MATERIALS

biotin phenol (LS-3500.0250, Iris Biotech)

Chemicals, Peptides, and Recombinant Proteins

- Protease Inhibitor Cocktail **Roche Catalog #11873580001**
- PBS (10x) **Santa Cruz Catalog #sc-24947**
- tris(2-carboxyethyl)phosphine (TCEP) **Gold Biotechnology Catalog #TCEP2**
- Formic Acid (FA) **Sigma – Aldrich Catalog #94318**
- Acetonitrile (ACN) **Sigma – Aldrich Catalog #34851**

Protocol status: Working
We use this protocol and it's working

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Sodium Chloride **Sigma – Aldrich Catalog #S9888**

MOPS **Sigma – Aldrich Catalog #M1254**

Sequencing grade Trypsin **Promega Catalog #V5111**

Lys-C **Wako Chemicals Catalog #129-02541**

EPPS **Sigma – Aldrich Catalog #E9502**

2-Chloroacetamide **Sigma – Aldrich Catalog #C0267**

Critical Commercial Assays

Tandem Mass Tags **Thermo Fisher Scientific Catalog #90406**

Bio-Rad Protein Assay Dye Reagent Concentrate **BIO-RAD Catalog #5000006**

Other

Sep-Pak C18 1cc Vac Cartridge 50 mg **Waters**

Empore™ SPE Disks C18 **Contributed by users Catalog #2215**

- Orbitrap Fusion Lumos Mass Spectrometer, ThermoFisher Scientific, Cat#IQLAAEGAAPFADBMBHQCat#IQLAAEGAAPFADBMBHQ
- high-pH reverse-phase HPLC - LC1260, Agilent

Other reagents

1. KCl: Sigma (#P9541)
2. EPPS buffer (50 mM EPPS, pH 8.5)
3. PBS; Phosphate buffered saline: ThermoFisher (#14040133)
4. EBSS (Sigma- Aldrich Cat#E3024).
5. Dulbecco's MEM (DMEM), high glucose, pyruvate (Gibco / Invitrogen, 11995)
6. RIPA buffer: 50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0 mM EDTA, 0.1% (w/v) SDS and 0.01% (w/v) sodium azide at a pH of 7.4.
7. Trolox: Cayman Chemical (#53188-07-1)
8. Bafilomycin A1; Cayman Chemical (#88899-55-2)
9. streptavidin-coated agarose beads: Pierce (#88817)
10. $NaCO_3$: Sigma (#223530)

Workflow for proximity biotinylation of autophagy proteins w...

1 The choice of cells to employ for proximity biotinylation depends on the experimental design. Here HeLa (ATCC CCL-2, RRID: CVCL_0030) cells gene edited to lack either MAP1LC3B or GABARAPL2 (made in Vaites LP et al Mol Cell Biol. 2017, 38:e00392-17) were reconstituted with pHAGE-APEX2-MAP1LC3B or pHAGE-APEX2-GABARAPL2, respectively. Analogous workflows were also performed with HeLa cells expressing pHAGE-APEX2-YIPF3.

1.1 HeLa cells harboring these deletions 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.

1.2 The appropriate HeLa cells were transduced with pHAGE-APEX2-MAP1LC3B or pHAGE-APEX2-GABARAPL2 and cells selected using 1.5 µM puromycin for 48 hours.

1.3 DMEM was removed and cells were washed 3 times with PBS followed by resuspending cells in EBSS containing BafA (500 nM). The investigator can select the length of time for starvation but this is typically 3 hours in our experiments.

2 To induce proximity labeling in live cells, cells were incubated with 500 µM biotin phenol for 1 hr and treated with 1 mM H_2O_2 for 1 min, and the reaction was quenched with three washes of 1× PBS supplemented with 5 mM Trolox, 10 mM sodium ascorbate and 10 mM sodium azide.

3 Cells were then harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer.

4 To enrich biotinylated proteins, ~2mg of cleared lysates was subjected to affinity purification by incubating with the streptavidin-coated agarose beads for 1.5 hours at room temperature. Beads were subsequently washed twice with RIPA buffer, once with 1M KCl, once with 0.1 M $NaCO_3$,

once with PBS and once with H_2O.

5 For proteomics, biotinylated protein bound to the beads were reduced using TCEP (10mM final concentration) in EPPS buffer at room temperature for 30 minutes.

6 Samples were then alkylated with the addition of Chloracetamide (20mM final concentration) for 20 minutes.

7 Beads were washed three times with water. Proteins bound to beads were then digested with LysC (0.5µl) in 100ul of 0.1 M EPPS (pH 5) for 2 hours at 37^{o}C, followed by trypsin overnight at 37^{o}C (1 µl)

8 To quantify the relative abundance of individual proteins across different samples, each digest was labeled with 62.5 µg of TMT11 reagents for 2 hours at room temperature, mixed, and desalted with a C18 StageTip (packed with Empore C18; 3M Corporation) before SPS-MS3 analysis on an Orbitrap Fusion Lumos Tribrid Mass Spectometer (Thermo Fisher Scientific) coupled to a Proxeon EASY-nLC1200 liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were separated on a 100 µm inner diameter microcapillary column packed with ~35 cm of Accucore150 resin (2.6 µm, 150 Å, ThermoFisher Scientific, San Jose, CA) with a gradient consisting of 5%–21% (Acetonitrile, 0.1% Fomic Acid) over a total 150 min run at ~500 nL/min as described in McAlister, G.C. et al., Anal Chem 86, 7150-7158 (2014).

9 Peptide identification and TMT reporter ion analysis is performed using user-selected platforms.