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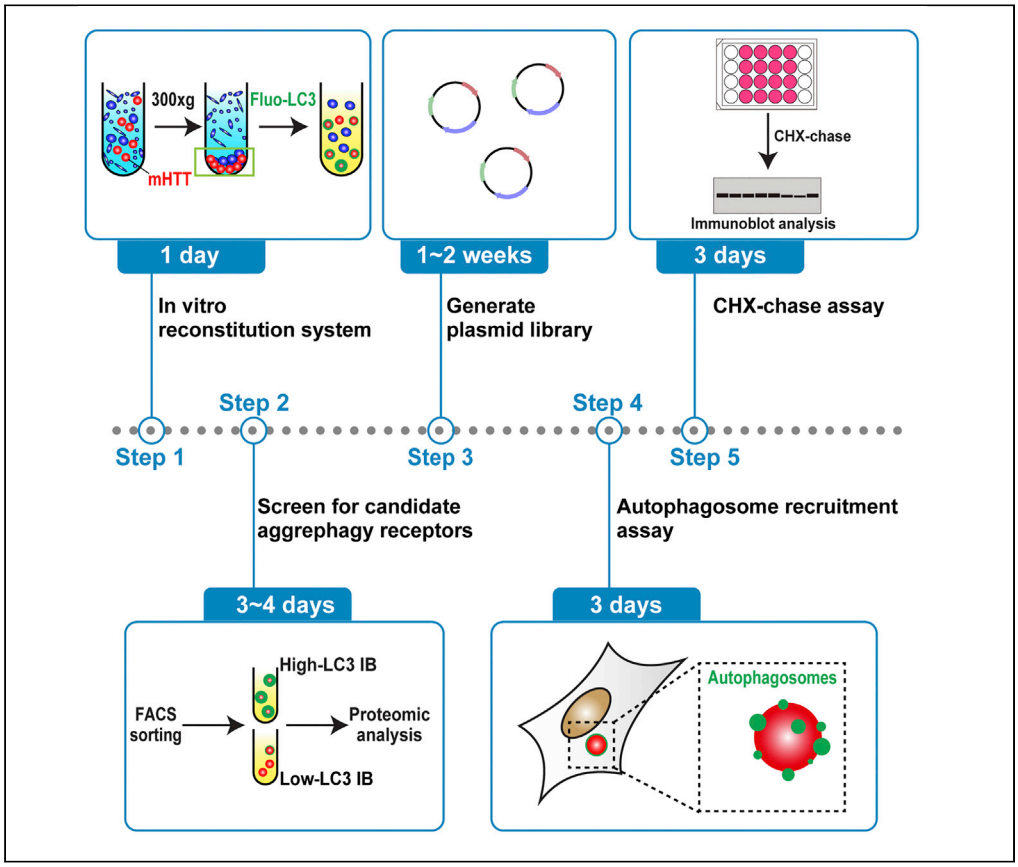
A biochemical reconstitution approach to identify autophagy receptors for aggrephagy in mammalian cells



Aggrephagy is a major way to clear protein aggregates. Here, we describe a pipeline of experiments to find autophagy receptors for aggrephagy. Steps include an *in vitro* reconstitution to recapitulate autophagosome recognizing aggregates and receptor identification steps based on flow cytometry and mass spectrometry. We also describe functional validation steps based on immunofluorescence and immunoblot. The protocol provides a practical way to identify aggrephagy receptors.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

In vitro reconstitution system to mimic autophagic membrane recognition

FACS sorting and proteomic analysis to screen for potential aggrephagy receptors

Immunofluorescence to test potential receptors' function in recruiting autophagosomes

CHX-chase assay to determine the function of receptors in protein aggregates degradation

Ma et al., STAR Protocols *3*, 101662
September 16, 2022
https://doi.org/10.1016/j.xpro.2022.101662

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A biochemical reconstitution approach to identify autophagy receptors for aggrephagy in mammalian cells

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https://doi.org/10.1016/j.xpro.2022.101662

SUMMARY

Aggrephagy is a major way to clear protein aggregates. Here, we describe a pipeline of experiments to find autophagy receptors for aggrephagy. Steps include an *in vitro* reconstitution to recapitulate autophagosome recognizing aggregates and receptor identification steps based on flow cytometry and mass spectrometry. We also describe functional validation steps based on immunofluorescence and immunoblot. The protocol provides a practical way to identify aggrephagy receptors.

For complete details on the use and execution of this protocol, please refer to Ma et al. (2022).

BEFORE YOU BEGIN

Many misfolded proteins can form intracellular protein aggregates. Here, we use the polyQ-HTT aggregates formed by the Huntingtin (HTT) exon1 protein containing expanded polyglutamine (polyQ) track as an example for screening and identifying new aggrephagy receptors. Other aggregation-prone proteins such as Tau P301L, SOD1 G93A, and FUS P525L were also used for functional validation (but not for screening) in our previous study (Ma et al., 2022). The protocol below describes the specific steps for using U2OS cells. However, we have also used this protocol in N2a cells. When using other cells, you need to conduct a preliminary test to calculate the amounts of cells for achieving 1 million inclusion bodies (IBs) in each group to reach sufficient mass spectrometry depth.

Culture cell line

U2OS cell expressing tetracycline-inducible HTT-Q91-mCherry was a gift from Dr. Ron Kopito lab. As instructed, the cell line was generated by co-transfecting pTRE-Tight (Takara Bio Inc. Cat#631059) carrying HTT-Q91-mCherry and linear puromycin marker (Takara Bio Inc. Cat# 631626) in a 20:1 weight/weight ratio into U2OS TET-ON (Takara Bio Inc.) cells. 72 h after transfection, cells were selected in media containing 1 μg/mL puromycin and cloned with limited diluted method. The clonal line exhibiting the highest HTT-Q91-mCherry fluorescence in the presence of dox was co-transfected with pTRE-Tight and pcDNA3.1/Hygro in a 20:1 weight/weight ratio for 72 h and selected in media containing 500 μg/mL hygromycin, and clonal lines exhibiting the highest HTT-Q91-mCherry fluorescence were isolated by limited dilution cloning (Bersuker et al., 2016). The

cells were maintained in DMEM supplemented with 10% FBS, 200 ng/mL puromycin, and 100 μg /mL hygromycin at 37°C in 5% CO2. This protocol can also be applied after transient transfection (see "problem 1").

Purification and fluorescence labeling of LC3 protein

Timing: 4–5 days

1. Express the LC3 protein in E.coli BL21.
   a. Transfect the pET28a-Cys-LC3B plasmid into E.coli BL21 competent cells and spread onto a kanamycin selection plate. Incubate at 37°C for 12 h.
   b. Pick a single clone into 5 mL LB broth media containing 50 μg/mL kanamycin and culture it at 37°C, 220 rpm for 12 h.
   c. Inoculate 500 μL bacteria suspension into 500 mL LB broth medium containing 50 μg/mL kanamycin and culture at 37°C, 220 rpm until the OD600 reach 0.6–0.7.
   d. Add 100 μM IPTG to induce protein expression at 22°C, 160 rpm for 12 h.
   e. Collect bacteria by centrifuging at 5,000 × *g* for 30 min at 4°C and discard the supernatant.
   f. Resuspend the bacteria pellet with 30 mL ice-cold PBS and transfer to a 50 mL tube. Collect bacteria by centrifuging at 5,000 × *g* for 15 min at 4°C and discard the supernatant. The bacteria pellet can be directly used for the following purification or can be frozen at −80°C if desired.
2. Purify the LC3 protein using Nickel Sepharose.
   a. Resuspend the bacteria pellet with 15 mL ice-cold lysis buffer (2×PBS, 10 mM imidazole, 0.3 mM DTT, 150 μL protease inhibitor cocktail). Add a final concentration of 0.5 mg/mL lysozyme and incubate on ice for 30 min.
   b. Add a final concentration of 0.5% Triton X-100 and ultrasonicate the bacteria for 15 s and interval of 30 s, repeat 4–6 times.
   c. Centrifuge the bacterial lysate at 45,000 × *g* for 40 min at 4°C.
   d. Prepare column containing 0.5 mL Nickel Sepharose. Equilibrate column by adding 20 mL ice-cold 2×PBS and allowing the contents to drain.
   e. Add the supernatant after centrifugation into the column, place the caps on the column and incubate for 2 h on a rotor at 4°C.
   f. Remove the caps and drain the contents of the column.
   g. Wash Sepharose with 50 mL ice-cold wash buffer A (2×PBS, 25 mM imidazole, 0.1% Tween-20), followed by 50 mL ice-cold wash buffer B (2×PBS, 25 mM imidazole).
   h. Elute protein with 5 mL ice-cold elution buffer (2×PBS, 250 mM imidazole).
3. Concentrate the LC3 protein and remove the imidazole.
   a. Add the protein eluent into an Amicon Ultra 10k Centrifugal Filter and centrifuge at 4,000 × *g* for about 30 min at 4°C to concentrate the protein eluent to 0.5 mL.
   b. Equilibrate the PD miniTrap G-25 column with 10 mL ice-cold PBS and discard the flow-through by gravity.
   c. Add the 0.5 mL concentrated protein to the column and allow the contents to drain.
   d. Elute protein with 1 mL ice-cold PBS and collect the protein eluent.
4. Determine the concentration of the LC3 protein.
   a. Prepare a range of 0.5–5 μg/μL BSA dissolved in PBS as standard proteins.
   b. Dissolve 1 μL LC3 protein and standard proteins in 10 μL 1× SDS loading buffer respectively.
   c. Load the 11 μL samples into 12.5% PAGE gel and conduct SDS-PAGE as conventional methods (a constant voltage of 80 V for stacking gel and 120 V for separating gel).
   d. Stain the gel with Simply Blue (Invitrogen) as the instruction manual (https://assets.fishersci.com/TFS-Assets/LSG/manuals/simplyblue_man.pdf). Image the gel and measure the band intensities. Make a standard curve based on the BSA band intensities and corresponding loading amount and calculate the concentration of the LC3 protein. About 5 mg LC3 protein can be purified from 500 mL bacterial culture medium.

e. The LC3 protein can be directly used for following fluorescence labeling or split into 1.5 mL tube (100 μL each tube) and flash freeze in liquid nitrogen. The proteins can be stored at −80°C for up to 5 years.

Caution: Take safety precautions when working with liquid nitrogen.

Note: Save some unlabeled LC3 protein for the *in vitro* reconstitution experiment below.

5. Fluorescence labeling of LC3 protein.
   a. Dilute the LC3 protein with PBS to 100 μM (500 μL total volume) and incubate with 1 mM DTT for 10 min at 4°C to reduce the disulfide bonds of proteins.
   b. Remove excessive DTT with a PD miniTrap G-25 column by following steps 3b–d. 1 mL LC3 protein with a concentration of about 50 μM will be obtained after this step.
   c. Dissolve the whole tube of Alexa Fluor 488 C2 maleimide (Invitrogen, 1 mg/tube) in 138.8 μL DMSO to a concentration of 10 mM immediately before use and protect from light.
   d. Add 100 μL maleimide stock solution to the 1 mL LC3 protein solution dropwise while stirring. The final concentration of maleimide is about 1 mM (20 times of the LC3 proteins). The reaction is allowed to proceed for 12 h at 4°C protected from light.
   e. Add 3 mM glutathione (3 times of maleimide) to consume any unreacted thiol reactive reagent.
   f. Concentrate the protein solution into 0.5 mL and remove the free Alexa Fluor dye with a PD miniTrap G-25 column by following steps 3b–d.
   g. Determine the concentration of the labeled LC3 protein by following steps 4a–d.
   h. Split the protein into 1.5 mL tubes (50 μL each tube) and flash freeze in liquid nitrogen. The proteins can be stored at −80°C to avoid light for up to 5 years.

Caution: Take safety precautions when working with liquid nitrogen.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-LC3. Working dilution: 1:500 for IF.	CST	Cat# M152-3; RRID: AB_1279144
Rabbit monoclonal anti-HA. Working dilution: 1:2000 for WB, 1:500 for IF.	CST	Cat# 3724; RRID: AB_1549585
Rabbit monoclonal anti-GFP. Working dilution: 1:5000 for WB.	CST	Cat# 2956; RRID: AB_1196615
Rabbit polyclonal anti-Ribophorin1(RPN1). Working dilution: 1:5000 for WB.	Dr. Randy Schekman	(Schindler and Schekman, 2009)
Chemicals, peptides, and recombinant proteins		
LB Broth Agar	Sangon Biotech	Cat# A507003-0250
LB Broth	Sangon Biotech	Cat# A507002-0250
Kanamycin	Inalco	Cat# 1758-9316
NaCl	Merck	Cat# 106404
KCl	Merck	Cat# 104936
Na2HPO4	Merck	Cat# 106586
KH2PO4	Merck	Cat# 119898
HEPES	Merck	Cat# 391338
Sorbitol	Merck	Cat# 56755
Potassium acetate	VWR	Cat# VWRC85507.290
Magnesium acetate	VWR	Cat# VWRC0131
SDS	Solarbio	Cat# S8010
Urea	AMRESCO	Cat# 0378

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Glycerol	Macklin	Cat# G810575
2-Mercaptoethanol	GEN-VIEW	Cat# GM195
Bromophenol blue	Solarbio	Cat# B8120
Nickel Sepharose	GE Healthcare	Cat# GE17531802
Amicon Ultra 10k Centrifugal Filters	Millipore	Cat# UFC8010
PD miniTrap G-25 column	GE Healthcare	Cat# GE28918007
Simply Blue	Invitrogen	Cat# LC6060
Alexa Fluor 488 C2 maleimide	Invitrogen	Cat# A10254
DMEM	Gibco	Cat# C11965500BT
FBS	Sunrise	Cat# SR100180.03
Doxycycline	Selleck	Cat# S8608
Protease inhibitors Cocktail	Sigma	Cat# P8340
DTT	GEN-VIEW	Cat# CD116
Imidazole	Sangon	Cat# A600277
Tween-20	Macklin	Cat# T6335
Triton X-100	AMRESCO	Cat# T0694
BSA	VWR	Cat# 97061-420
Glutathione	VWR	Cat# VWRC0399
DNase I	Sigma	Cat# DN25
RNase A	Sigma	Cat# R5125
Ammonium bicarbonate	Sigma	Cat# A6141
Acetonitrile	Thermo Scientific	Cat# 85188
Iodoacetamide	Sigma	Cat# I1149
Formic acid	Thermo Fisher Scientific	Cat# 85178
Trypsin	Promega	Cat# V5280
X-tremeGENE HP DNA Transfection Reagent	Roche	Cat# XTGHP-RO
ProLong Gold Antifade Mountant	Invitrogen	Cat# P36930
Cycloheximide	CST	Cat# 2112S
Bafilomycin A1	Selleck	Cat# S1413
Experimental models: Cell lines		
U2OS Cells	Dr. Randy Schekman	N/A
U2OS Q91-HTT-mCherry Cells	Dr. Kirill Bersuker (Dr. Ron Kopito lab)	(Bersuker et al., 2016)
Recombinant DNA		
pET28a-His-LC3B	Lab stock	N/A
pEGFPN1-Q103-HTT	Lab stock	N/A
pBFPN1-Q103-HTT	Lab stock	N/A
pFUGW-empty	Lab stock	N/A
pFUGW-HA- (candidate receptors)	Lab stock	N/A
Software and algorithms		
Fiji (ImageJ)	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
Prism 8	GraphPad	https://www.graphpad.com
Flowjo	FlowJo	https://www.flowjo.com
Proteome Discoverer Alternative: Maxquant (https://www.maxquant.org/)	Thermo Fisher Scientific	https://www.thermofisher.cn/order/catalog/product/cn/zh/OPTON-30810
Other		
BD LSRFortessa	BD Biosciences	N/A
BD FACSAria SORP	BD Biosciences	N/A
Vacuum centrifuge CV200	Beijing JM Technology	N/A
UltiMate 3000 RSLCnano System	Thermo Scientific	N/A
Thermo Q Exactive mass spectrometer Alternative: LTQ Orbitrap velos, Orbitrap Fusion Tribrid, Orbitrap Fusion LUMOS, Synapt G2, etc	Thermo Scientific	N/A
Olympus FV3000 confocal microscope Alternative: Nikon A1, Leica LSM780, etc	Olympus	N/A

MATERIALS AND EQUIPMENT

2×PBS (pH 7.4) (1×PBS is directly diluted the 2×PBS with ddH_2O)		
Reagent	Final concentration	Amount
NaCl	274 mM	16 g
KCl	5.4 mM	0.4 g
Na2HPO4	20 mM	2.88 g
KH2PO4	3.6 mM	0.49 g
ddH_2O	N/A	N/A
Total	**N/A**	**1 L**
Store at 4°C for up to 1 year after filtration sterilization.		

5 M imidazole: Weigh 17 g imidazole and dissolve in ddH2O to a final volume of 50 mL.

Store at 4°C and avoid light for up to 1 year.

1 M DTT: Weigh 154 mg DTT and dissolve in ddH2O to a final volume of 1 mL.

Store at −20°C for up to 1 year.

The lysis buffer/wash buffer A/wash buffer B/elution buffer for protein purification are prepared before use based on the 2×PBS according to the recipes in the steps.

B88 buffer		
Reagent	Final concentration	Amount
HEPES (pH 7.2)	20 mM	2.38 g
Sorbitol	250 mM	22.77 g
Potassium acetate	150 mM	7.36 g
Magnesium acetate	5 mM	0.54 g
ddH_2O	N/A	N/A
Total	**N/A**	**500 mL**
Store at 4°C for up to 1 year after filtration sterilization.		

Solutions used in the mass spectrometry sample preparations:

Decolorizing buffer: 50 mM Ammonium bicarbonate, 50% Acetonitrile (ACN).

Reduction buffer: 25 mM Dithiothreitol, 50 mM Ammonium bicarbonate.

Alkylation buffer: 55 mM iodoacetamide, 50 mM Ammonium bicarbonate.

Digestion buffer: 10 μg/mL trypsin, 25 mM Ammonium bicarbonate.

Extraction buffer: 0.1% Formic acid, 50% ACN.

> *Note:* Prepare these solutions before use. ACN is toxic to inhale and highly flammable, solutions that contain ACN should be prepared and used in a fume hood.

Aggregates solubilization buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 6.8)	62.5 mM	0.38 g
SDS	15%	7.5 g
Urea	8 M	24 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Glycerol	10%	5 mL
ddH_2O	N/A	N/A
Total	**N/A**	**50 mL**

Heat to 40°C to dissolve.
Store at −20°C for up to 1 year.

4× Loading buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 6.8)	150 mM	0.91 g
SDS	12%	6 g
Glycerol	30%	15 mL
2-Mercaptoethanol	6%	3 mL
Bromophenol blue	0.2%	0.1 g
ddH_2O	N/A	N/A
Total	**N/A**	**50 mL**

Store at −20°C for up to 1 year.

Aggregates loading buffer		
Reagent	Final concentration	Amount
Aggregation solubilization buffer	N/A	3 mL
4× Loading buffer	N/A	2 mL
ddH_2O	N/A	3 mL
Total	**N/A**	**8 mL**

Store at 25°C for up to 1 month.

Transfer buffer		
Reagent	Final concentration	Amount
Tris	25 mM	6 g
Glycine	200 mM	30 g
Methanol	20%	400 mL
ddH_2O	N/A	N/A
Total	**N/A**	**2 L**

Pre-cold at 4°C and can be re-used 3–4 times.

TBST		
Reagent	Final concentration	Amount
Tris-HCl (pH 7.6)	20 mM	2.42 g
NaCl	137 mM	8 g
Tween-20	0.2% (V/V)	2 mL
ddH_2O	N/A	N/A
Total	**N/A**	**1 L**

Store at 25°C and use within 1 week.

STEP-BY-STEP METHOD DETAILS

In vitro reconstitution system

Timing: 1 day

The *in vitro* reconstitution system is to recapitulate the recruitment of autophagosomes to protein aggregates by adding a fluorescence-labeled LC3 protein to the IBs formed by polyQ-HTT.

1. *In vitro* reconstitution.
   a. Plate about 8 × 10^5 U2OS HTT-Q91-mCherry cells in a 6-cm dish. Maintain the cells in DMEM supplemented with 10% FBS, 200 ng/mL puromycin, and 100 μg /mL hygromycin at 37°C in 5% CO2 for 12 h to allow the cells to adherence.

   Note: We recommend users plate another dish of cell without expression of HTT-Q91-mCherry to prepare a control sample for the following FACS analysis. The control sample is prepared together with experimental group as the following 1c–f.

   b. Incubate the cells with 1 μg/mL doxycycline for 24 h to induce the expression of HTT-Q91-mCherry and IBs formation.
   c. Wash cells once with 5 mL PBS, scrape off cells with a cell scraper in 1 mL PBS, and transfer into a 1.5 mL centrifuge tube. Centrifuge at 600 × *g* for 5 min at 25°C to collect cells.
   d. Add 400 μL B88 buffer plus 1% Triton X-100, 4 μL cocktail protease inhibitors (Sigma, solution), 100 U/mL DNase, and 1 U/mL RNase to the cell pellet and homogenize by passing through a 22 G needle for 10 times. (U refers to Kunitz unit here and below.).
   e. Centrifuge at 300 × *g* for 8 min at 25°C, and carefully remove the supernatant.
   f. Wash the pellet containing IBs with 1 mL B88 buffer plus 0.5% Triton X-100 and centrifuge again at 300 × *g* for 8 min at 25°C.
   g. Resuspend the pellet in 400 μL B88 buffer plus 0.5% Triton X-100, 4 μL cocktail protease inhibitors, 50 U/mL DNase and divide into four equal samples (Figure 1):
      i. No protein added.
      ii. Add 5–10 μg/mL fluorescence-labeled LC3 protein.
      iii. Add 5–10 μg/mL fluorescence-labeled LC3 protein and 2–5 times BSA.
      iv. Add 5–10 μg/mL fluorescence-labeled LC3 protein and 2–5 times non-labeled LC3 protein.

      Incubate the reactions for 1 h at 30°C and protect from light.

   Note: We tried a series of the concentration of fluorescence-labeled LC3 (from 0.5 μg/mL to 10 μg/mL). The different concentration only caused a variable LC3 fluorescence intensity on IBs but didn't change the results (the fluorescent LC3 on IBs can be competed by non-fluorescent LC3 but not BSA).

   h. Centrifuge at 1,000 × *g* for 5 min, wash the pellet with 500 μL PBS plus 0.5% Triton X-100 and centrifuge again.
   i. Resuspend the pellet in 500 μL PBS for FACS analysis.
2. FACS analysis of LC3 specific association with IBs.
   a. Use a pulse shape analysis (PulSA) based on flow cytometry strategy (Ramdzan et al., 2013) to quantify the recruitment of LC3 to the IBs:
      i. Create histograms of mCherry-A and 488-A. Use the control sample and sample ii in step 1g to dynamically modify the range by adjusting the voltage of the PMTs until the sample signal spans the full detector range.

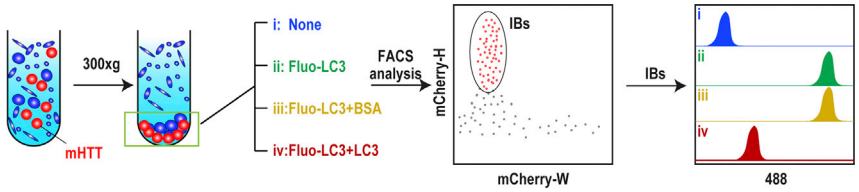


Figure 1. A scheme of major steps for the in vitro constitution system

Cells expressing mHTT IBs are lysed and centrifuged to collect IBs. Incubate the IBs with indicated proteins. Use flow cytometry to analysis IBs and quantify the LC3 (488) on IBs.

ii. Create a dot plot of pulse heights versus pulse widths of the mCherry.

Note: If your flow cytometry cannot collect the pulse width and height of the fluorescence, you can also use the mCherry versus FSC.

iii. By comparing the control sample with the experimental sample, gate the unique population characteristic of IBs (Figure 1).
iv. Collect data for at least 20,000 IBs.
v. Analyze the fluorescence of LC3 (488) on IBs.

b. Compare the LC3 fluorescence on IBs in four different groups from step 1g. A successful *in vitro* reconstitution should show no LC3 fluorescence in 1g (i), higher LC3 fluorescence in 1g (ii & iii), and lower LC3 fluorescence in 1g (iv) because of the competition by non-labeled LC3 (Figure 1).

Screen for candidate aggrephagy receptors

Timing: 1 day for step 3; 2–3 days for step 4

After establishing an effective *in vitro* reconstitution system, we divide IBs into two groups according to the LC3 fluorescence and conduct an unlabeled quantitative mass spectrometry to compare protein components enriched in two groups.

3. Sort IBs into two groups (Figure 2).
   a. Plate U2OS HTT-Q91-mCherry cells at 2 × 10^6 cells / dish in four 10-cm dishes. After 12 h, add 1 μg/mL doxycycline for 24 h to induce the expression of HTT-Q91-mCherry and IBs formation.
   b. Wash cells once with 5 mL PBS and scrape off cells with a cell scraper in 3 mL PBS for each dish. Transfer cell suspension into a 15 mL centrifuge tube. Centrifuge at 600 × *g* for 5 min at 25°C to collect cells.
   c. Add 1.6 mL B88 buffer plus 1% Triton X-100, 16 μL cocktail protease inhibitors (Sigma, solution), 100 U/mL DNase, and 1 U/mL RNase to the cell pellet and homogenize by passing through a 22 G needle for 10 times.
   d. Centrifuge at 300 × *g* for 8 min at 25°C, and carefully remove the supernatant.
   e. Wash the pellet containing IBs with 1 mL B88 buffer plus 0.5% Triton X-100 and centrifuge again at 300 × *g* for 8 min at 25°C.

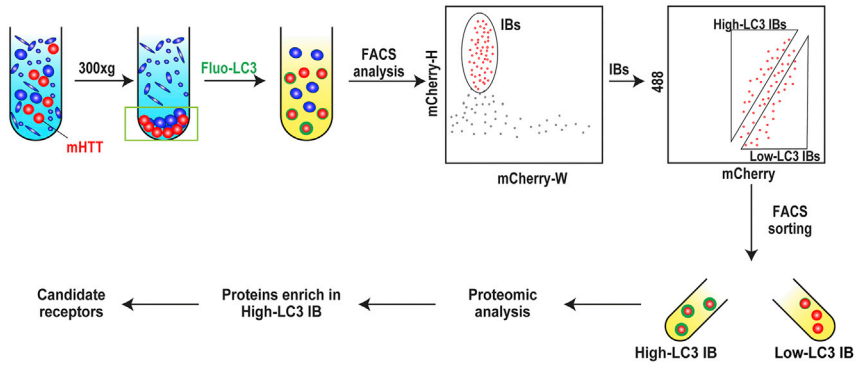


Figure 2. A scheme of major steps to sort IBs

Cells expressing mHTT IBs are lysed and centrifuged to collect IBs. Incubate the IBs with fluorescent LC3. Use flow cytometry to analysis IBs and divide IBs into two groups according to the LC3 fluorescence. Conduct proteomic analysis based on mass spectrometry to identify proteins enrich in High-LC3 IBs as candidate aggrephagy receptors.

f. Resuspend the pellets in 1.6 mL B88 buffer plus 0.5% Triton X-100, 16 μL cocktail protease inhibitors, and 50 U/mL DNase.
g. Add 0.5–1 μg/mL fluorescence-labeled LC3 protein to solution and incubate for 1 h at 30°C in dark.

Note: The sorting steps need a larger reaction system than FACS analysis in step 1. To save protein, we used a lower concentration of fluorescence-labeled LC3 compared to step 1g.

h. Centrifuge at 1,000 × *g* for 8 min, wash the pellet with 500 μL PBS plus 1% Triton X-100 and centrifuge again.
i. Resuspend the pellet in 2 mL PBS for sorting two groups of IBs with flow cytometry:
   i. Gate the population of IBs.
   ii. Divide the IBs into two groups according to the LC3 fluorescence on them: High- LC3 IBs and Low-LC3 IBs.

Note: The "High" and "Low" are the relative fluorescent intensity of LC3 under a specific fluorescent intensity of IBs. You can mark the medians of LC3 intensity (488) under several IBs intensity (mCherry) and join these points into a line. Signals above this line are High-LC3 IBs and vice versa. Gate farther away the line will get more variability but waste more samples.

   iii. Sort 1 million IB particles for each group.
j. The IB solutions were centrifuged at 3,000 × *g* for 30 min.
k. Add 25 μL Aggregates Loading buffer to the IB pellets and boil at 100°C for 10 min.
l. Load all samples into 10% PAGE gel and conduct SDS-PAGE as conventional methods (a constant voltage of 80 V for stacking gel and 120 V for separating gel).

Note: Leave one lane empty between the two samples and the SDS-PAGE can be finished when the bromophenol blue enters 3–4 cm into the separating gel to ease the following processing.

m. Stain the gel with Simply Blue (Invitrogen) as the instruction manual (https://assets.fishersci.com/TFS-Assets/LSG/manuals/simplyblue_man.pdf).

Note: It is good for the flow sorter to be able to collect the pulse width and height of the fluorescence (e.g., BD FACSAria SORP). Otherwise, a BD influx also works in a two-color gate mode.

4. Mass-spectrometry-based label-free quantification.

Note: The reagents in this workflow should use mass spectrometry grade reagents.

a. Excise the whole lanes above the bromophenol blue from the gel, each lane was split into three samples of equal length to increase the number of protein identifications, then cut the gel into small pieces less than 1 cubic millimeter.
b. Incubate the gel particles with 1 mL decolorizing buffer for 1 h, then remove the supernatant (Figure 3A). Repeat decolorizing step twice to fully remove the Simple Blue dye.the

⚠**Caution:** ACN is toxic to inhale and highly flammable, all operations in this step should be done in a fume hood.

c. Add 1 mL 100% ACN and incubate for 10 min to dry the gel particles, then remove the supernatant (Figure 3B). Repeat the drying step twice.

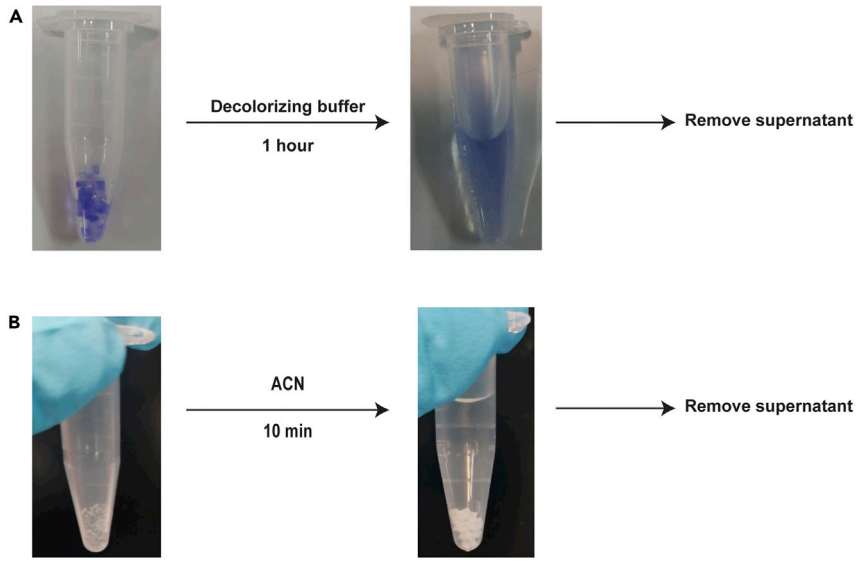


Figure 3. Gel particles destaining and drying

(A) The destaining step to remove the Simple Blue dye in gel particles with destaining buffer.

(B) Drying step to dry the gel particles with ACN.

d. Add reduction buffer to submerge the gel particles and incubated at 55°C for 30 min for cysteine reduction, then remove the supernatant and dry the gel particles with 1 mL 100% ACN (repeat drying step twice) as previously described in step 4c.

e. Add alkylation buffer to submerge the gel particles and incubate in dark for 30 min for cysteine alkylation. Then, remove the supernatant and dry the gel particles with 1 mL 100% ACN (repeat drying step twice) as previously described in step 4c.

f. Add 100 μL digestion buffer to each sample and replenish 25 mM ammonium bicarbonate solution to submerge the gel particles. Incubate at 37°C for 12 h. After incubation, transfer the supernatant to a new EP tube.

g. Recover the digested peptides from the gel using 200 μL extraction buffer nd incubate for 1 h at 37°C. After incubation, transfer the supernatant to merge with the supernatant from step 4f. Repeat recovering step twice.

h. Evaporate the solvent of the recovered solution by vacuum centrifugation.

i. Reconstitute the recovered peptides in 25 μL 0.1% formic acid for LC/MS analysis.

j. Online-desalt and analyze the recovered peptides using an UltiMate 3000 RSLCnano System (Thermo Scientific, USA) which was directly interfaced with a Thermo Q Exactive mass spectrometer (Thermo Fisher Scientific, USA).

Note: Other Liquid Chromatograph Mass Spectrometers (e.g., LTQ Orbitrap velos, Orbitrap Fusion Tribrid, Orbitrap Fusion LUMOS, Synapt G2, etc) can also be used.

i. Separate peptide with UltiMate 3000 RSLCnano System using a 120-min gradient with 0.30 μL/min flow rate.

Note: Mobile phase A is consisted of 5% acetonitrile, 95% water, and 0.1% formic acid. Mobile phase B is consisted of 80% acetonitrile, 20% water, and 0.1% formic acid. The analytical column is a home-made fused silica capillary column (75 μm ID, 150 mm length; Upchurch) packed with C18 resin (300 Å, 5 μm; Varian).

ii. Acquire the MS/MS spectra with the Thermo Q Exactive mass spectrometer operated in the data-dependent acquisition mode using Xcalibur 2.8 software.

Note: Use a single full-scan mass spectrum in the orbitrap (300–1,800 m/z, 70,000 resolution) followed by 20 loop count cycle of data-dependent MS/MS scans at 27% normalized collision energy (HCD).

k. Search the MS/MS spectra from each LC-MS/MS run against the Human Proteome (Uniprot) database using an in-house Proteome Discoverer 1.4 software (Thermo Fisher Scientific).

Note: Set the search criteria as follows: Enzyme: Trypsin (Specific), carbamidomethylation (C): fixed modifications, the oxidation (M): variable modification, precursor ion mass tolerances: 20 ppm for all MS acquired in an orbitrap mass analyzer, fragment ion mass tolerance: 0.02 Da for all MS2 spectra acquired, false discovery rate (FDR) was calculated using percolator and the cut-of was set as 1%.

l. Perform Relative protein quantification with Proteome Discoverer software according to manufacturer's instructions on the reporter ion peak area per peptide (https://files.mtstatic.com/site_13984/6869/0?Expires=1659082727&Signature=qF2MFsBJLzTj7dbHtSdvLowrRljjUiLLpoRwsxil~7wDLwVlMePyhYfMiU4OPDowP4rHDNRB5AUacLiw7bPZnfqQr6lZti7j3v5PzT5FswwDHTvOTEwmNbA72nyka5FOelaFqUozrf4dHbNnGee-Hc~FdNawMgg88Sbc3VHPss8_&Key-Pair-Id=APKAJ5Y6AV4GI7A555NA).

Note: Other software such as Maxquant can also be used.

m. Use the peak area for protein abundance comparison between the High-LC3 IBs and Low-LC3 IBs. We recommend to choose the proteins whose ratio in High-LC3 IBs/Low-LC3 IBs > 2 and the p-value < 0.05 (two-tailed t test from three independent experiments) as candidate receptors for further study.

Generate the candidate receptor plasmid library

Timing: 1–2 weeks

Once finding out new candidate receptors enriched in High-LC3 IBs, prepare plasmids expressing these proteins for further study. The gene can be cloned from cDNA or directly purchased from plasmid suppliers (e.g., Addgene and Sino Biological). We prefer to tag the candidate receptors with HA and insert them into the FUGW backbone. Other tags and vectors can also be used. In the following steps, we take the HA-tagged candidate receptors as examples.

Autophagosome recruitment assay

Timing: 3 days

5. Immunofluorescence assay.
   a. Plate U2OS cells on coverslips in 24-well plate and culture for 12 h to 60%–70% confluency.
   b. Transfect cells with Q103-HTT-BFP together with an empty plasmid or plasmids expressing candidate receptors.
      i. Dilute 0.2 μg Q103-HTT-BFP and 0.3 μg empty / candidate receptors plasmids into 50 μL DMEM and gently pipette up and down to mix.
      ii. Add 1 μL X-tremeGENE DNA transfection reagent to the diluted DNA and gently mix.
      iii. Incubate for 20 min at 25°C.
      iv. Add transfection complex to the cells in a dropwise manner.

Note: Other transfection reagents such as Lipofectamine 2000 Reagent (Invitrogen), and Neofect Transfectoion Reagent (GenePharma) can also be used.

c. 24 h after transfection, treat cells with 0.5 μg/mL Bafilomycin A1 for 2 h to accumulate the autophagosomes in cells.
d. Dissolve digitonin in ddH2O to prepare 50 mg/mL stock solution. The stock solution can be stored at −20°C for 1 month.
e. Wash the cells with PBS and permeabilize cells with 40 μg/mL digitonin diluted in PBS on ice for 5 min.

Note: The purpose of permeabilizing cells before fixation is to remove soluble proteins for clearer observation of IBs.

f. Wash the cells with cold PBS and immediately incubated them with 4% PFA for 20 min at 25°C.
g. Permeabilize the cells again with 50 μg/mL digitonin diluted in PBS at 25°C for 10 min.
h. Block the cells with 10% FBS diluted in PBS at 25°C for 1 h.
i. Incubate with primary antibodies diluted in 10% FBS at 25°C for 1 h.

Note: Antibody dilution depends on the affinity of your antibody. In our experiment, dilution for LC3 antibody is 1:500, and for HA antibody recognizing HA-tagged candidate receptors is 1:500.

j. Wash the cells three times in PBS for 5 min each.
k. Incubate with secondary antibodies diluted in 10% FBS at 25°C for 1 h in dark.

Note: We used the Alexa Fluor 568 (1:500) for HA and Alexa Fluor 647 (1:500) for LC3. Other fluorescent secondary antibodies can also be used.

l. Wash the cells three times in PBS for 5 min each in dark.
m. Mount samples with a drop of ProLong Gold Antifade Mountant and dry at 25°C for 6–12 h in dark.

6. Confocal microscopy imaging.
   a. Place the sample on the platform of a confocal microscope with a 60× objective (40×∼100× objectives are suitable). We used the Olympus FV3000 for imaging, other confocal microscopes (Nikon A1, Leica LSM780, etc) can also be used.
   b. Find the cells under the eyepiece and switch to imaging mode.
   c. Choose suitable channels: Alexa Fluor 405 for Q103-HTT-BFP, Alexa Fluor 568 for candidate receptors, and Alexa Fluor 647 for LC3.
   d. Preview the image and adjust focal plane, laser intensity, HV (High Voltage), etc.

Note: The optimum layer for observing IBs may be different from cells. Try to find the focal plane that displays the clearest IBs.

   e. Acquire fluorescence images for more than 50 IB-containing cells in each group.
7. Quantify the LC3 area around IBs following the steps in Figure 4.
8. Determine potential receptors for further study.
   a. After obtaining the LC3 area around IBs from at least 50 IBs in each group, enter the results into GraphPad Prism.
   b. Use unpaired T-test to analyze the difference between candidate receptor and control group.
   c. Choose the proteins which can associate with IBs (Figure 6) and recruit more LC3 around IBs compared to the control group ($p<0.05$) as potential receptors for further study.

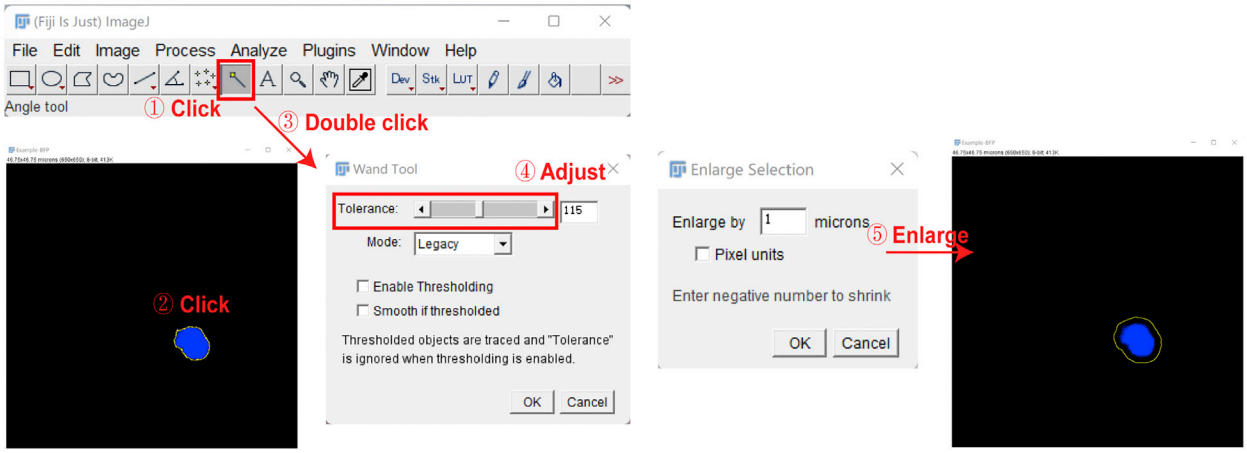
Protein aggregates degradation

Timing: 3 days

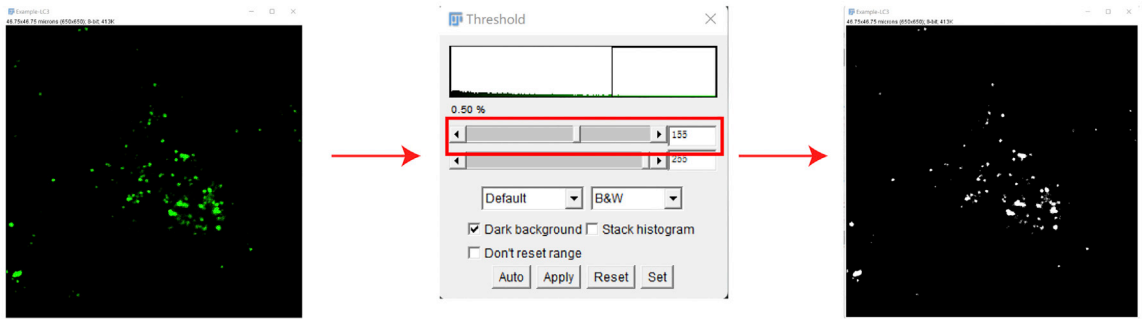
a. Open the picture in image J.

b. Choose the BFP channel and change the image to 8-bit. (Image -> Type -> 8-bit)

c. Choose the Wand Tool and click the IB to circle it.
Double click the Wand Tool to display setting interface and adjust the Tolerance for best selectoin of IB.
Enlarge the selection by 1 μm. (Edit -> Enlarge -> Enlarge by 1 microns)



d. Choose the LC3 channel and change the image to 8-bit. (Image -> Type -> 8-bit)
Create a suitable threshold to cover all the LC3 puncta signal. (Image -> Adjust -> Threshold)



e. Show the IB selection in the LC3 channel. (Edit -> Selection -> Restore selection)
Quantify the LC3 area inside the IB selection. (Analyze -> Analyze particles)

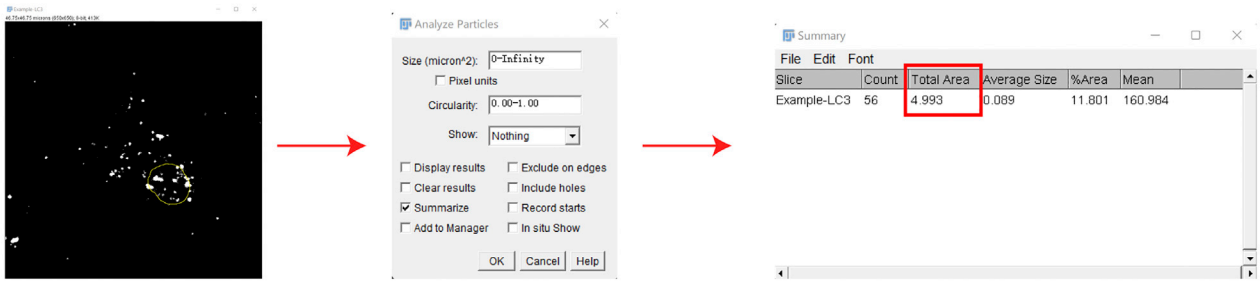


Figure 4. Steps for quantifying LC3 area around IB with image J

Detailed steps to quantify LC3 area around IB with image J after obtaining images. Step-by-step instructions are listed in the figure.

After finding out potential receptors that associate with IBs and recruit more autophagosomes, we use a chase assay to explore the potential receptors' effects on Q103-HTT degradation with cycloheximide (CHX) inhibition of protein synthesis. Caution that when co-expressing with other proteins,

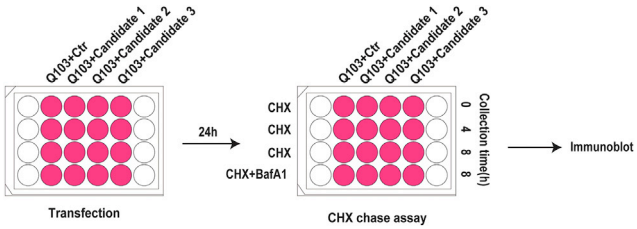


Figure 5. A scheme of CHX chase assay

Transfect cells with Q103-HTT-GFP together with an empty plasmid or plasmids expressing candidate receptors. 24 h after transfection, add CHX and collect cells at indicated time. Perform immunoblot to analyze the amount of Q103 and candidate receptors.

the expression level of Q103-HTT may change. In order to unambiguously compare the degradation level, adjust the amounts of Q103-HTT plasmids when transfecting cells to achieve similar initial expression level of Q103-HTT in each group.

9. CHX chase assay (Figure 5).
   a. Equally plate U2OS cells in 24-well plate at 10^5 cells / well. Each group needs four wells.
   b. Transfect cells with Q103-HTT-GFP together with an empty plasmid or plasmids expressing potential receptors.
   c. 24 h after transfection, treat cells with 50 μg/mL CHX, with or without 0.5 μg/mL Bafilomycin A1 as indicated.
   d. Collect cells at indicated time points, lyse in 200 μL Aggregates loading buffer and boil at 100°C for 10 min.
10. Immunoblot and analyzing.
   a. Load 10 μL/sample into 10% PAGE gel and conduct SDS-PAGE as conventional methods (a constant voltage of 80 V for stacking gel and 120 V for separating gel).
   b. Transfer proteins to 0.45 μm PVDF membranes. We recommend wet transfer method with a constant current of 300 mA for 90 min. The transfer buffer should be pre-cold at 4°C, and the transfer tank should be placed in ice bath.
   c. Block membrane with 5% non-fat milk in TBST for 30 min at 25°C.
   d. Wash membranes three times with TBST.
   e. Incubate with primary antibodies diluted in TBST at 4°C for 12 h.

   Note: Antibody dilution depends on the affinity of your antibody. In our experiment, dilution for GFP antibody recognizing Q103-HTT-GFP is 1:5,000, for HA antibody recognizing HA-tagged candidate receptors is 1:2,000, for RPN1 antibody is 1:5,000. RPN1 is used as internal control. Other controls such as β-actin and GAPDH can also be used.

   f. Wash membranes three times with TBST for 5 min each.
   g. Incubate with secondary antibodies diluted in TBST for 1 h at 25°C.
   h. Wash membranes three times with TBST for 5 min each.
   i. Detect immunoreactivity with Western Blot ECL chemiluminescent substrates and capture images by ChemiDoc imaging system (Bio-Rad).
   j. Quantify each band under the same threshold in ImageJ.
   k. Calculate the ratio of GFP over RPN1 for each sample and normalize to the initial value (time point 0) within each group. The decreased values reflect the degradation of Q103-HTT.

EXPECTED OUTCOMES

The label-free quantification will give the values of peak area for hundreds of proteins in each group. After comparing the protein components in High-LC3 IBs and Low-LC3 IBs, the known

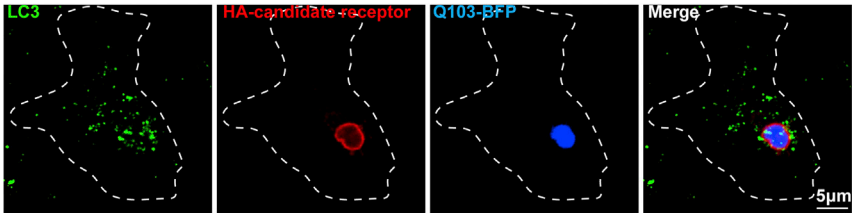


Figure 6. An example image for the autophagosome recruitment assay
Immunofluorescence of U2OS co-expressing Q103-HTT-BFP and HA-tagged candidate receptor with anti-HA and LC3 antibodies.

aggrephagy receptors such as P62, NBR1, TAX1BP1, and CCT2 will enrich in the High-LC3 IBs group (Kirkin et al., 2009; Ma et al., 2022; Pankiv et al., 2007; Sarraf et al., 2020). Other proteins enriched in High-LC3 IBs can be chosen as candidates for new aggrephagy receptors.

The autophagosome recruitment assay will primarily assess the ability of candidate receptors to recruit autophagosomes. In most cases, the Q103-HTT-BFP will assemble into a regular IB about 5 μm in diameter. The proteins on the surface of IB are accessible for antibodies so that the candidate receptors should form a shield around IBs in the confocal image. The autophagosomes staining with LC3 antibody are usually 0.5–1.5 μm in diameter and distribute throughout the cell (Figure 6). The potential receptors will recruit more autophagosomes to IBs.

The CHX chase assay will provide direct evidence to judge the function of the potential receptors. Determine the aggrephagy receptors for further study referring to the following criteria: 1) Accelerate the degradation of Q103-HTT. 2) The degradation can be blocked by Bafilomycin A1. 3) The receptors themself are degraded which can be blocked by Bafilomycin A1 (Figure 7).

LIMITATIONS

The common methods to find new autophagy receptors were detecting proximity interactome of LC3 or known receptors (Zellner et al., 2021). However, because the composition of protein aggregates is complex, it's hard to distinguish potential receptors for aggrephagy from the LC3-interactome. In order to solve this problem, we developed this method to bridge the aggregates-interactome and LC3-interactome. In this protocol, we use a pulse shape analysis based on flow cytometry to analyze the large IBs formed by polyQ-HTT. The approach is only applicable for analyzing large aggregates instead of small oligomers due to the limit of conventional flow cytometry. For BD FACS Aria SORP, the minimum resolution of particle size is 0.2 μm, so, we will miss some proteins that act specifically on small oligomers that are less than 0.2 μm.

TROUBLESHOOTING

Problem 1

The U2OS Q91-HTT-mCherry cell line is not available (step 1: *In vitro* reconstitution).

Potential solution

You can also transfect a plasmid expressing fluorescent polyQ-HTT into U2OS or other cells to form IBs if the cell line is not available. Ensure you can get enough IBs (20,000 for analysis in steps 1 and 2 and 1 million for mass spectrometry in steps 3 and 4) for the follow-up experiment.

Problem 2

No unique population of IBs during FACS analysis (step 2: FACS analysis of LC3 specific association with IBs).

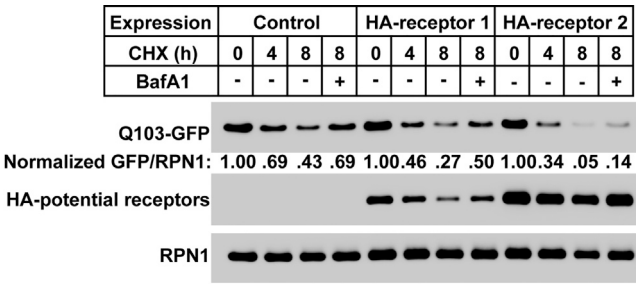


Figure 7. An example result for the CHX chase assay

The turnover of Q103-HTT in a CHX chase assay with or without receptor proteins expression in U2OS.

Potential solution

Confirm the fluorescent polyQ-HTT proteins have formed IBs in cells using a fluorescence microscope. Induce with a higher concentration of doxycycline or extend expression time if the IBs are not formed. You can use control samples without IBs to identify the unique population.

Problem 3

Only few proteins are identified after comparing the mass spectrometry results of High-LC3 IBs and Low-LC3 IBs (step 4: Mass spectrometry-based label-free quantification).

Potential solution

You can try to split each lane into 12 samples of equal length. It will reduce the complexity of each sample and may find more low abundance proteins.

Problem 4

The fluorescence signals are weak (step 5: Immunofluorescence assay).

Potential solution

Increase the concentration of primary antibodies or incubate at 4°C for 12 h in a humidity chamber.

Problem 5

The fluorescence signals of IBs are so strong that interfere with other channels (step 6: Confocal microscopy imaging).

Potential solution

We recommend BFP or mCherry-tagged polyQ-HTT for their less interference. If you use a GFP-tagged polyQ-HTT, avoid bright light excitation before imaging and take images of other channels first.

Problem 6

The protein doesn't decrease during treatment with CHX (step 9: CHX chase assay).

Potential solution

The CHX concentration and treating time depend on your cell types and states, so you may need to explore a suitable condition for your cells. To make sure the CHX works, you can detect the degradation of P62(SQSTM1) which is a typical cargo for autophagy. If the P62 performs well, you can try to increase the chase time in case the candidate proteins work later.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Liang Ge (liangge@mail.tsinghua.edu.cn).

Materials availability

Plasmids and cell lines generated in this study will be made available upon request. We may require a payment and/or a completed Materials Transfer Agreement in case there is potential for commercial application.

Data and code availability

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. This paper does not report original code.

ACKNOWLEDGMENTS

We thank Dr. Kirill Bersuker (previously in Dr. Ron Kopito lab at Stanford and now in Calico, USA) and Dr. Randy Schekman (University of California, Berkeley, USA) for reagents. The work is funded by National Natural Science Foundation of China (32061143009, 91854114, 32130023, 31872826, 31872832), Ministry of Science and Technology of the People's Republic of China (2019YFA0508602; 2021YFA0804802), and Beijing Natural Science Foundation (JQ20028).

AUTHOR CONTRIBUTIONS

M.X. performed the experiments and wrote the manuscript. Z.W. wrote the mass-spectrometry-based label-free quantification section. Z.M., D.H., and G.L. gave suggestions and guidance for writing. All the authors participated in generating the scheme.

DECLARATION OF INTERESTS

L.G., M.Z., and X.M. are inventors on a patent application that covers the use of the chaperones reported in the original manuscript (Ma et al., 2022) as potential therapeutic strategies against human diseases caused by protein aggregation.

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