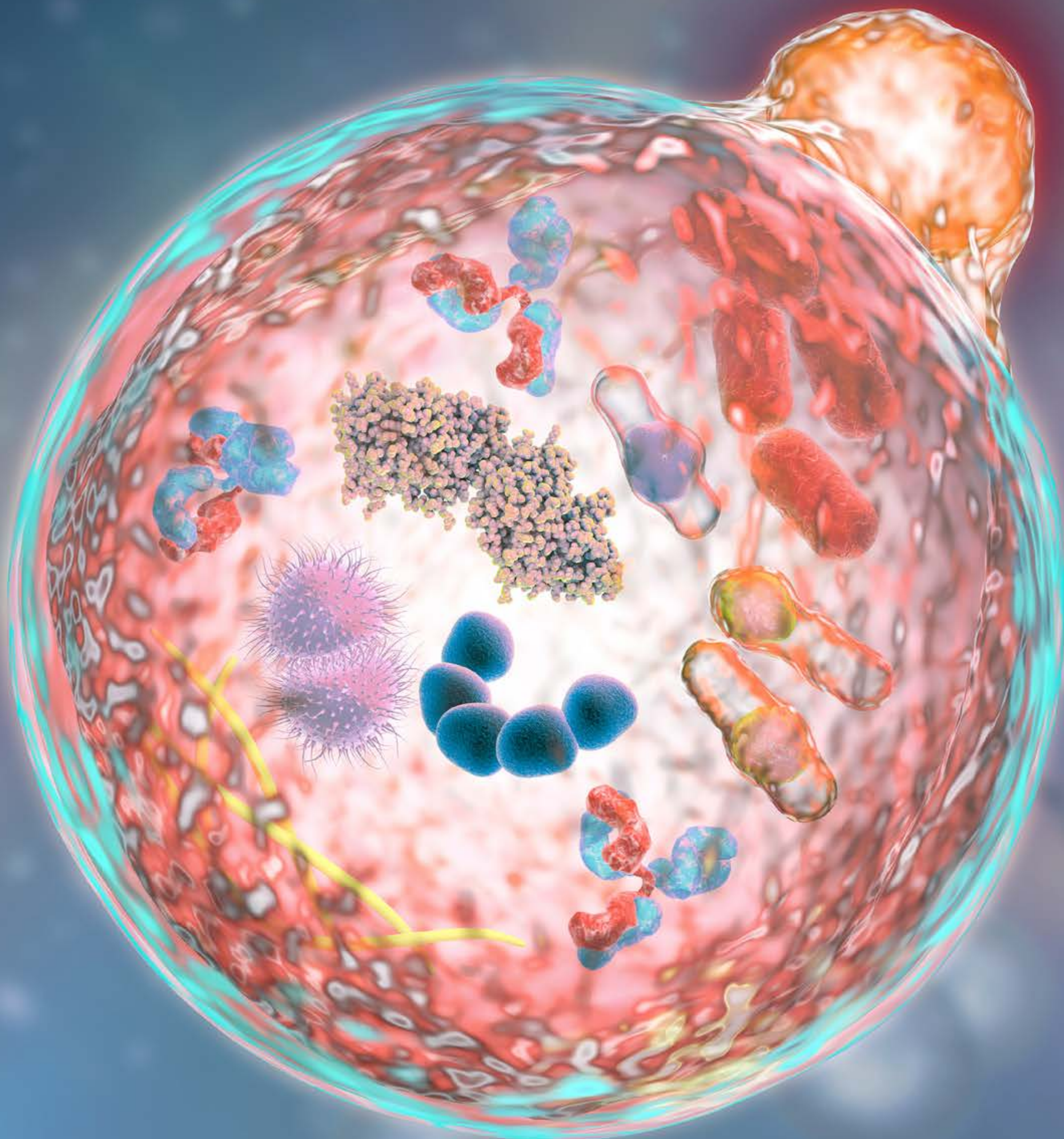
AUTOPHAGY HANDBOOK



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ATG12
ATG10
ATG12
ATG5
ATG16L
ATG12
ATG16L
ATG5
Misfolded Proteins
p62
Ub
Pro-LC3
ATG4
LC3-I
ATG7
PE
VAMP8
SNAP29
STX17
Autolysosome
Lysosome
TLR4
TLR4
Degraded Organelles, Cytosolic Proteins
Macroautophagy Induction
p62
Ub
LC3-II
ATG12
ATG16L
ATG5
LC3-II
ATG4
LC3-II
LC3-II
Elongation
Lamp-2a
Autophagosome Maturation
IFNg TNFa
TRAIL
DR4/5
Type I IFN
TLR3/7/8
CD40L
CD40
Antigen-MHC class II Transport + Presentation
MHC class II
Lamp-2a
TCR
MHC class II
CD4
CD4+ T Cell
Antigen Presentation & Recognition
Cytosolic Protein
Chaperone Mediated Autophagy
Hop
Hip
Hsp70
Bag-1
Hsp40
Hsp90
Hop
Hip
Hsp70
Bag-1
Hsp40
Hsp90
Cytosolic Protein
Lamp-2a
CP
IYS
Hsc70
Cytosolic Protein
ATF6
PERK
IRE1
UPR
XBP1, GRP78
ATF6
ATG16L, ATG12, ATG3, BECN1, p62, ATG7, ATG10, ATG5, etc.
ATF4
sXBP1
BECN1
sXBP1
Nucleus
Cytoplasm
Microautophagy

THE TERM "AUTOPHAGY" IS DERIVED FROM THE GREEK WORD αὐτόφαγος autóphagos THAT MEANS "SELF-DEVOURING"

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INTRODUCTION

Autophagy is a conserved cellular process for the delivery of macromolecules and organelles into lysosomes for degradation and recycling. This process targets excess and defective organelles as well as aggregated and long-lived proteins for elimination. Following hydrolysis, basic molecular components are recycled into the cytosol to serve as building blocks or as energy resources. Therefore, autophagy plays a critical role as a cellular homeostatic mechanism, controlling the balance between available resources and energy expenditure. Several cellular stressors including nutrient deprivation, hypoxia and ER stress may induce autophagy above basal levels. Additionally, deregulated autophagy is implicated in disease states including neurodegeneration and cancer.

CYTOSOLIC PROCESSING

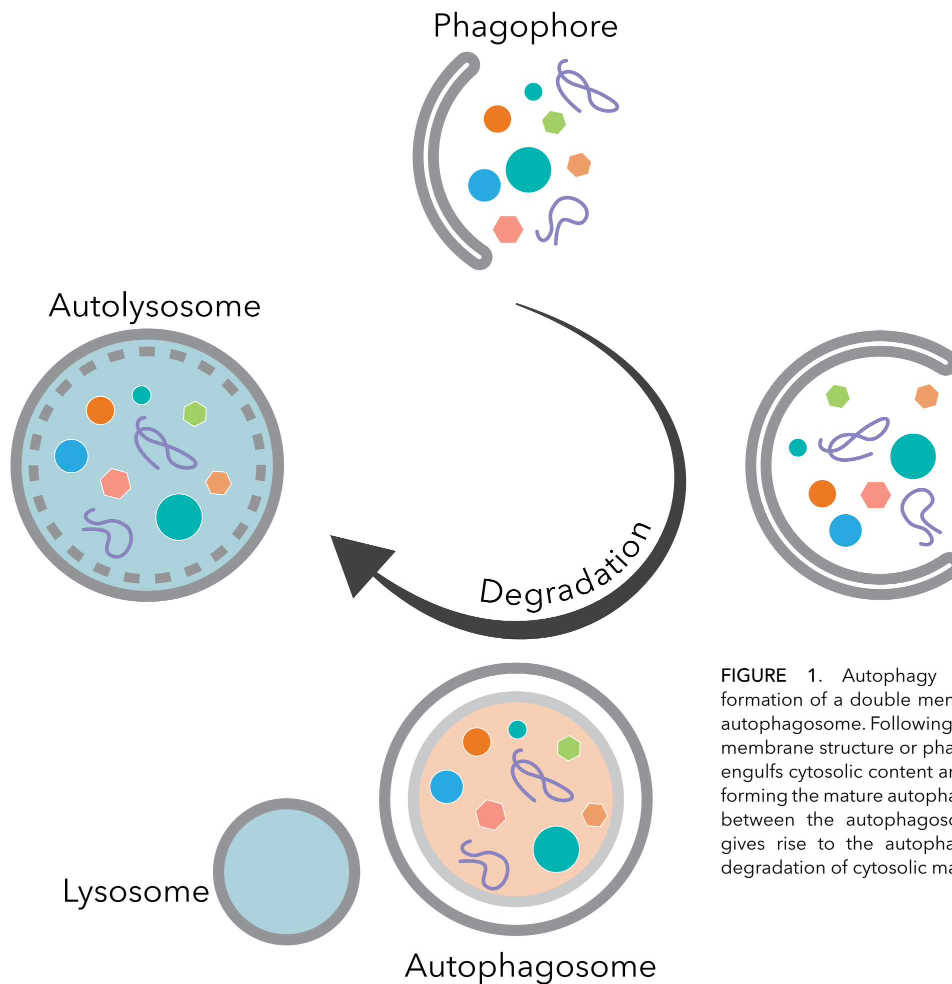


FIGURE 1. Autophagy depends on the formation of a double membrane organelle or autophagosome. Following induction, the initial membrane structure or phagophore elongates, engulfs cytosolic content and closes onto itself, forming the mature autophagosome. The fusion between the autophagosome and lysosome gives rise to the autophagolysosome where degradation of cytosolic material takes place.

This guide provides an overview of the process of autophagy. The main molecular players and regulatory pathways involved in the autophagic process are discussed. An emphasis on validated and standard methods for the quantification of autophagic activity will provide researchers with essential tools for optimizing autophagy measurements in their specific systems.

TYPES OF AUTOPHAGY

Three main types of autophagy are recognized that include **macroautophagy**, **microautophagy** and **chaperone-mediated autophagy**. In all these processes, cytosolic components reach the lysosomes for degradation, however specific differences in delivery mechanisms distinguish these processes.

AUTOPHAGY MECHANISMS

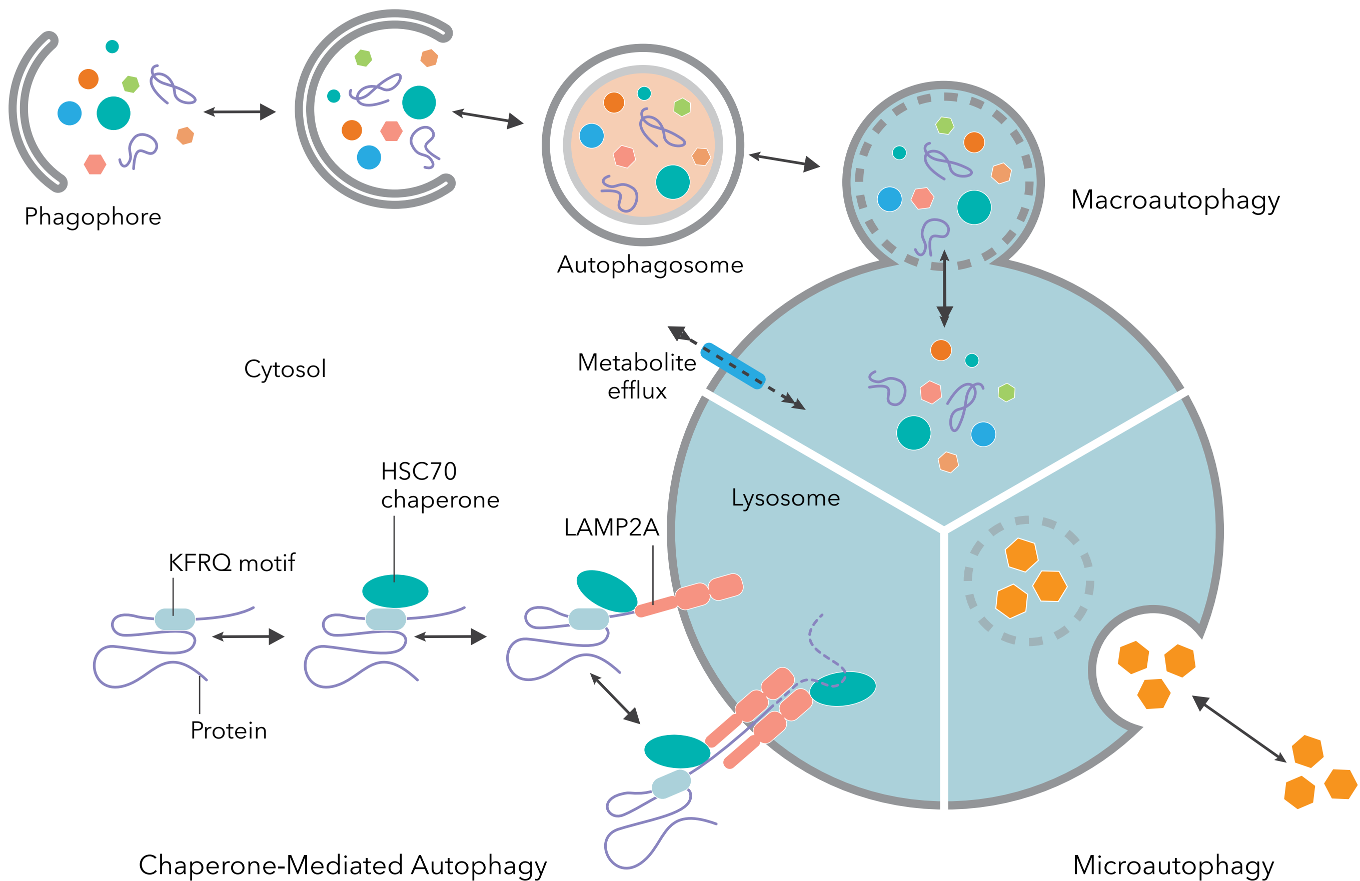


FIGURE 2. Autophagy may be classified under three main categories including macroautophagy, microautophagy and chaperone-mediated autophagy. The autophagosome participates in the degradation of cytosolic components only during macroautophagy.

- **Macroautophagy**: Cytosolic components are sequestered within a double membrane organelle or autophagosome. Fusion of autophagosomes with lysosomes forms the autolysosome, where degradation of cytosolic components occurs. This guide focuses primarily on the process of macroautophagy (hereafter referred to as autophagy).
- **Microautophagy**: Lysosomes directly engulf cytosolic components via lysosomal membrane invagination.
- **Chaperone-Mediated Autophagy**: Chaperone proteins (e.g., heat shock cognate 70 protein; HSC-70) interact with cytosolic proteins destined for degradation. This complex is recognized by a lysosomal-associated membrane protein-2A (LAMP-2A), resulting in the translocation of the unfolded cytosolic protein into the lysosome.

SELECTIVE AUTOPHAGY

Cytosolic components are targeted for degradation in bulk or by selective autophagy. Selective autophagy in mammals depends on two main components: selective-autophagy receptors and lipidated LC3 (microtubule-associated protein light chain 3) proteins. Their interaction ensures specific degradation of various cellular components including mitochondria, endoplasmic reticulum, aggregated proteins and ribosomes, among other components.

TYPES OF SELECTIVE AUTOPHAGY RECEPTORS

Selective autophagy pathways are broadly classified as ubiquitin-independent or -dependent and involve an adaptor protein or selective autophagy receptor that interacts directly or via ubiquitin with cellular targets, respectively.

- **Ubiquitin-Dependent Autophagy**: Selective autophagy receptors interact with ubiquitinated cargo via their ubiquitin binding domain (UBD). This selective autophagy pathway cooperates with the ubiquitin-proteasome system for the elimination of protein aggregates. Many other cellular components are targeted for degradation by these receptors. The factors that determine the specificity of the receptor to cargo interactions have not been completely elucidated. Considerable overlap exists in the selectivity of the receptors.
- **Ubiquitin-Independent Autophagy**: Receptors target directly a variety of molecules and organelles as cargo including proteins, lipids, peroxisomes and lysosomes, among many other cellular components.

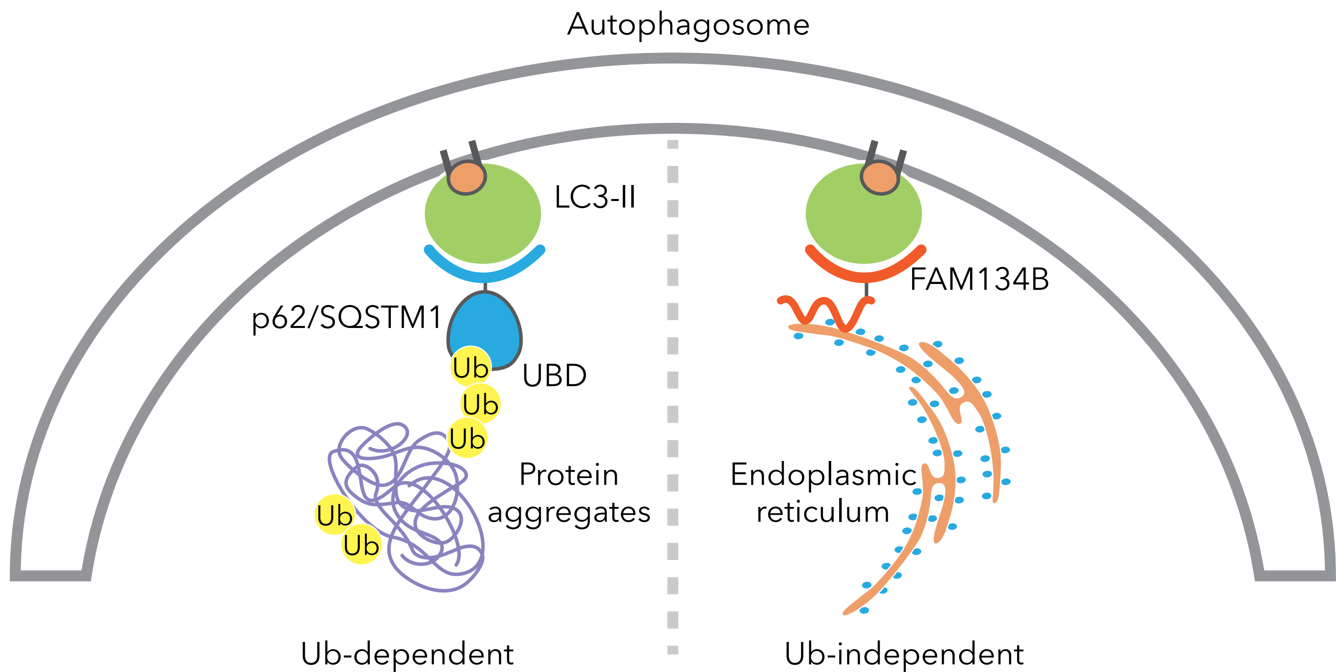


FIGURE 3. Selective autophagy receptors contain an LC3 interacting region (LIR motif) for binding to LC3 family members. They interact with cellular cargo via a second binding motif that is ubiquitin-dependent or -independent.

SELECTIVE AUTOPHAGY RECEPTORS

CYTOSOLIC CARGO	UBIQUITIN-DEPENDENT	UBIQUITIN-INDEPENDENT
Mitochondria	OPTN, NDP52, TAX1BP1, p62	NIX, BNIP3, FUNDC1, ATG32
Protein Aggregates	p62, NBR1, OPTN TOLLIP, Cue5	OPTN
Peroxisomes	NBR1, p62	ATG30, ATG36
Bacteria	p62, OPTN, NDP52, TAX1BP1	Galectin-8/NDP52
RNA granules	NDP52, p62	
Proteasome	RPN10	
Endoplasmic Reticulum		FAM134B, ATG40
Viruses		TRIM5α, SMURF1, p62
Nuclear Envelope		ATG39

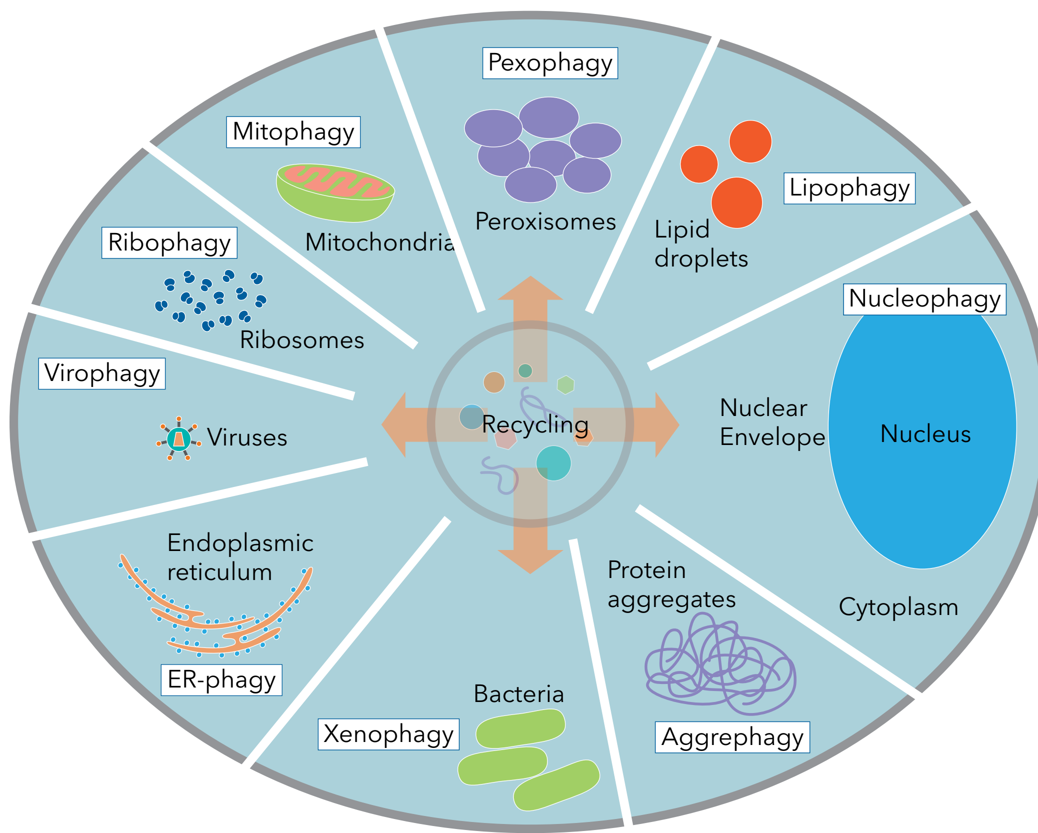


FIGURE 4. Autophagy may occur in bulk or via a selective mechanism. Selective autophagy pathways are named based on the type of cargo targeted for degradation.

LC3 ISOFORMS

Multiple LC3 family members have been identified in mammals that belong to two main sub-families, LC3 and GABARAP. Different isoforms may play specific roles in selective autophagy.

LC3 AND GABARAP FAMILY MEMBERS	
SUB-FAMILY	ISOFORMS
LC3	MAP1LC3A, MAP1LC3B, MAP1LC3B2, MAP1LC3C
GABARAP	GABARAP, GABARAPL1, GABARAPL2, GABARAPL3*

Isoforms share 29-94% sequence identity. *Only subfamily member not involved in formation of autophagosomes.

AUTOPHAGIC MACHINERY AND FLUX

A total of 31 autophagy-related proteins (ATG) regulate this process in yeast, and many ATG proteins are conserved in mammals. Each ATG protein supports specific steps in the dynamic process of autophagy.

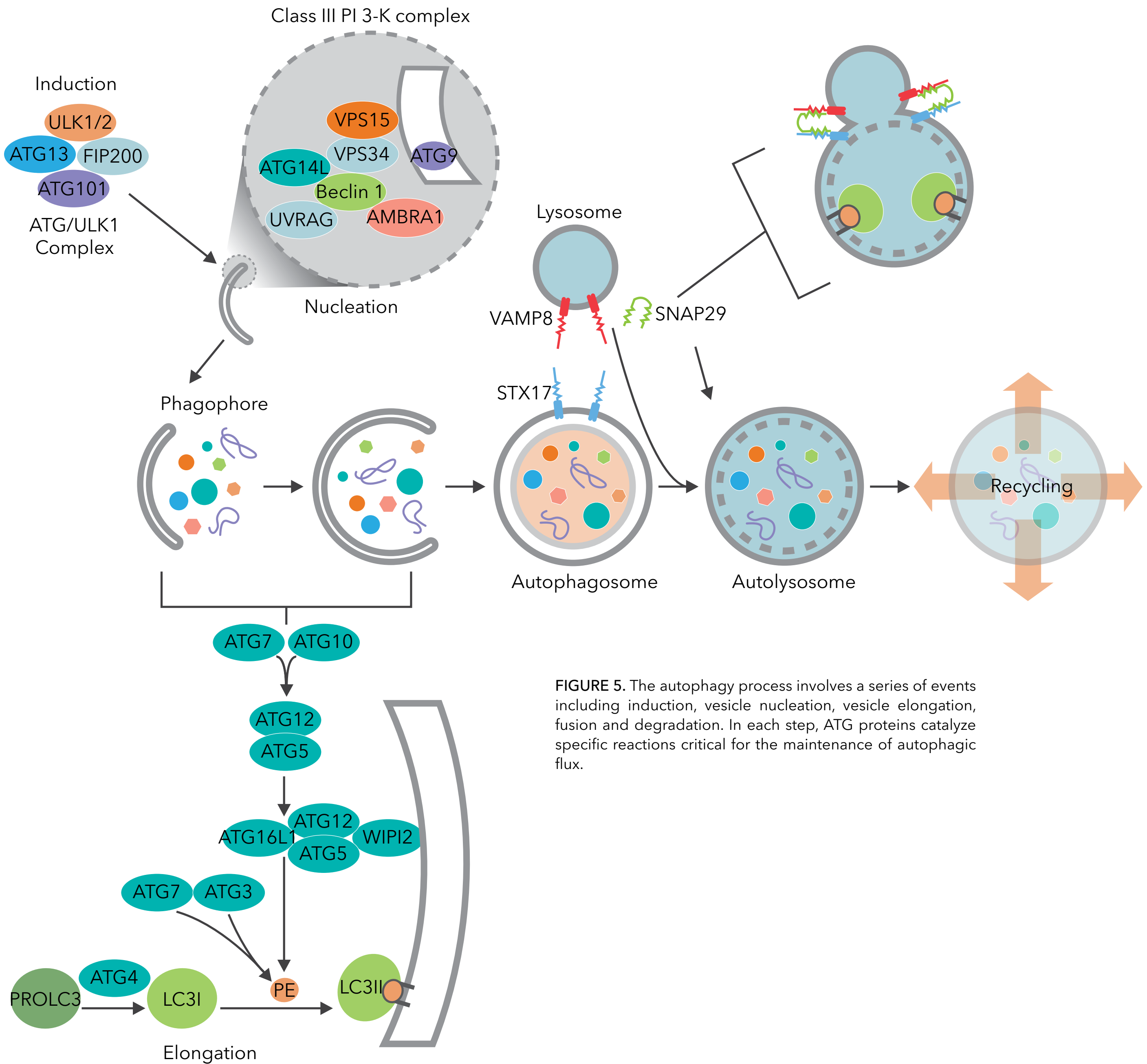


FIGURE 5. The autophagy process involves a series of events including induction, vesicle nucleation, vesicle elongation, fusion and degradation. In each step, ATG proteins catalyze specific reactions critical for the maintenance of autophagic flux.

- **Induction:** Starvation or reduced nutrient availability is one of the most studied triggers in autophagy research. The induction of autophagy in response to starvation is often dependent on the inhibition of mTOR kinase (mammalian target of rapamycin), a key regulator of nutrient signaling. Upon mTOR inhibition, the concomitant decrease in the phosphorylation status of a protein complex including ULK1 (unc-51-like kinase 1), ATG13 and RB1CC1 (RB1-inducible coiled-coil 1), increases the activity of ULK1 and induces autophagy.

AUTOPHAGIC MACHINERY AND FLUX

- **Nucleation and Phagophore Formation**: The membrane that forms the nascent phagophore may originate from the Golgi, endoplasmic reticulum, mitochondria or endosomes. Formation of the initial engulfing organelle or phagophore depends on the release of Beclin1 and AMBRA1 (activating molecule in BECN1 regulated autophagy protein 1) from their inhibitor Bcl-2. Release from inhibition allows for the formation of the PtdIns3K (class III phosphatidylinositol 3-kinase) complex including ATG14, VPS15 and VPS34 (vacuolar sorting proteins). This complex gives rise to phosphatidylinositol-3-phosphate and promotes the recruitment of ATG proteins into the nascent membrane.

- **Elongation, Autophagosome Formation**: Vesicle elongation is facilitated by the activation of two ubiquitin-like conjugation complexes. In the first complex, ATG7 and ATG10 participate in the covalent binding of ATG12 to ATG5. Interaction of the ATG12/ATG5 covalently-bound complex with ATG16L1 (ATG16-like 1) may promote the second ubiquitin-like conjugation complex. In the second complex, ATG4, ATG7 and ATG3 participate in the processing of soluble LC3 to its lipid conjugated form LC3-II. LC3 is lipidated to phosphatidylethanolamine groups associated with the phagophore's membrane. Together, these two ubiquitin-like conjugation complexes participate in the formation of the autophagosome. By the end of the elongation stage, the phagophore's membrane has expanded and closed onto itself completing the sequestration of cytosolic content. All ATG proteins dissociate from the autophagosome and return to the cytosol. Lipidated LC3 is the only ATG protein known to remain associated with the autophagosome's membrane.

- **Fusion and Degradation**: During starvation, the SNARE protein STX17 (syntaxin 17) localizes to completed autophagosomes. Interaction of STX17 with SNAP29 and lysosomal VAMP8 allows fusion of the autophagosome with lysosomes to form the mature hydrolytic organelle. Upon fusion, proton pumps and hydrolases are added to the autophagosome for formation of the autolysosome, enabling cargo acidification and hydrolysis.

PROTEINS PLAYING KEY ROLES IN AUTOPHAGY	
ATG PROTEINS	ROLE IN AUTOPHAGY
ULK1 complex	Decreased phosphorylation activates complex of ULK1, ULK2, ATG13, ATG101 and FIP200 Involved in induction
Class III phosphatidylinositol 3-kinase (PtdIns3K)	Complex of VPS34, VPS15, AMBRA1, UVRAG, BIF1, ATG14 and Beclin 1 Displacement of Bcl-2 activates VPS34 complex Involved in nucleation
ATG9	Transmembrane protein present only in the phagophore Involved in nucleation
Beclin/ATG6	Interacts with Bcl-2 Interacts with GAPR-1, a negative regulator of autophagy
ATG7 and ATG10	E1 and E2-like enzymes Involved in the conjugation of ATG12-ATG5
ATG12-ATG5 complex	Necessary for phagophore elongation
ATG4	Cysteine protease that cleaves LC3
ATG3	E2-like enzyme participates with ATG7 in the conversion of LC3-I to its lipidated form LC3-II
p62/SQSTM1	Cargo receptor that is preferentially degraded by autophagy
LC3/ATG8	Only ATG protein associated to the autophagosome's membrane

Autophagy antibodies available at Novus: novusbio.com

MEASURING AUTOPHAGY

Autophagy is a multistep and dynamic process that presents specific challenges for its quantitative assessment. Traditionally, the process of autophagy has been studied using electron microscopy. The discovery and characterization of the ATG proteins facilitated the development of molecular tools and approaches to identify and quantitate autophagic activity. Ideally, a combination of approaches should be implemented including steady-state and flux measurements for the assessment of autophagic activity in different systems.

STEADY-STATE ASSAY: AUTOPHAGOSOME NUMBER

Assays to determine the amount or number of autophagosomes generally focus on the LC3 protein. LC3 may be found in the cytosolic and nuclear compartments. The lipidated form or LC3-II is the only protein known to associate with the autophagosome's inner membrane. LC3-II levels detected by immunofluorescence (ICC/IHC/IF) and immunoassay (WB) provide a good estimation of autophagosome number. With over 1,000 citations, the Novus LC3B antibody (catalog# NB100-2220) is the most widely trusted and used antibody to monitor LC3 levels.

- **LC3-II Puncta (ICC/IHC/IF):** This method combines the use of antibodies to LC3 and fluorescence microscopy to quantify fluorescent puncta, which correlate with the number of autophagosomes. This assay reports the number of puncta per cell or number of cells with puncta. Nevertheless, in most cells some basal autophagic activity is present and therefore most will have puncta.

PROTOCOL: DETECTION OF AUTOPHAGOSOME NUMBER BY LC3 IMMUNOCYTOCHEMISTRY (ICC/IF)

MATERIALS

- 1X PBS
- 4% paraformaldehyde in 1X PBS (make fresh)
- 1X PBS with 0.5% Triton X-100
- LC3 primary antibody in 1% normal serum or BSA in 1X PBS (~5 µg/mL or ~1:200 for catalog# NB100-2220; 1:5,000 for catalog# NB600-1384)
- Fluorophore-conjugated anti-rabbit secondary antibody in 1% normal serum or BSA in 1X PBS
- 1-5% normal serum or BSA in 1X PBS
- DAPI or Hoechst (1-10 µg/mL)

PROTOCOL: DETECTION OF AUTOPHAGOSOME NUMBER BY LC3 IMMUNOCYTOCHEMISTRY (ICC/IF)

METHODS

01 Harvest cells from a semiconfluent culture and plate them on sterile glass coverslips. For optimal adhesion, some cell types may require coated coverslips (e.g., poly-L-lysine).

Tip: Coverslips may be sterilized with ethanol and flaming or by exposure to UV radiation. To facilitate handling and incubations, place several small circular coverslips in a single culture dish.

02 Grow cells to semi-confluency (70-75%).

Tip: Gently move seeded coverslips to the incubator to ensure sufficient cell adhesion onto the coverslips. Do not let coverslips dry out and avoid adding solutions directly on the cells to reduce detachment.

03 Aspirate the culture medium from the dish, and gently wash with 1X PBS at room temperature.

04 Incubate the coverslips in freshly prepared 4% paraformaldehyde in 1X PBS at room temperature for 10 minutes.

Tip: For more information, see our Immunocytochemistry handbook at novusbio.com/icc-handbook.

05 Rinse coverslips with 1X PBS for 2 minutes.

06 Incubate the coverslips in 1X PBS, 0.5% Triton X-100 at room temperature for 5 minutes.

07 Wash away the permeabilization buffer by incubating in 1X PBS for 5 minutes.

08 Block the coverslips in 1-5% normal serum or BSA (in 1X PBS) for 1 hour at room temperature.

09 Dilute primary antibody in 1% normal serum or BSA (in 1X PBS). Incubate coverslips with ~5 μg/mL rabbit anti-LC3 primary antibody (catalog# NB100-2220) for 1 hour at room temperature (37°C is optional), or 16 hours at 4°C.

Tip: Antibody concentrations may range from 5-20 μg/mL.

PROTOCOL: DETECTION OF AUTOPHAGOSOME NUMBER BY LC3 IMMUNOCYTOCHEMISTRY (ICC/IF)

10. Wash the coverslips in 1X PBS, 3 times for 5 minutes each.

11. Prepare an appropriate dilution of fluorophore-conjugated anti-rabbit secondary antibody in 1% normal serum or BSA (in 1X PBS). Generally, concentrations range from 1-2 µg/mL, however antibody concentrations need to be optimized for every system.

12. Incubate the coverslips in the secondary antibody dilution for 1 hour at room temperature in the dark.

13. Wash the coverslips in 1X PBS, 3 times for 5 minutes each.

14. Once all washing steps have been completed, the coverslips can be counter stained with DAPI or Hoechst (1-10 µg/mL) to stain the nuclei.

15. Invert the coverslip onto a glass slip with mounting media. Use an antifade containing mounting media to reduce photobleaching.

16. Carefully remove any excess mounting media and seal as required with nail polish.

17. Use a fluorescence microscope to examine and image the cells.

    Note: Quantitation of LC3-II positive puncta may be performed manually or automated by the use of appropriate software for image analysis. However, a standardized approach should be applied across samples to prevent the introduction of bias and to ensure reproducibility.

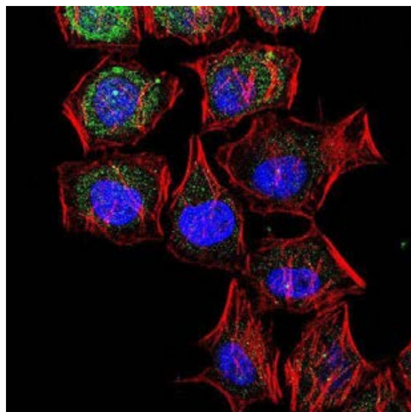


FIGURE 6. Immunocytochemistry/Immunofluorescence: Confocal analysis of HeLa cells using LC3B antibody (catalog# NB600-1384, 1:5,000). An Alexa Fluor 488-conjugated Goat anti-rabbit IgG was used as secondary antibody (Green). Actin filaments were labeled with Alexa Fluor 568 Phalloidin (Red). DAPI was used to stain the cell nuclei (Blue).

MEASURING AUTOPHAGY

- **LC3-Conversion (WB)**: This method measures the conversion of LC3, from unlipidated LC3-I to the lipidated form LC3-II. An increased conversion ratio correlates with increased autophagosome number. Quantification should be based on the comparison of LC3-II levels to the levels of a housekeeping protein (e.g., actin). However, in some systems the levels of housekeeping proteins are affected by autophagy and LC3-I may be used for comparison.

CAVEATS TO THE USE OF LC3 FOR THE ASSESSMENT OF AUTOPHAGIC ACTIVITY	ICC/IHC	WB
1. Tissues and cells differ in the magnitude of LC3 turnover.	√	√
2. LC3-II level alone is not enough evidence of autophagy.	√	√
3. LC3-II may associate with other membranous structures.	√	√
4. Some antibodies may not sufficiently bind to LC3-I.	√	√
5. LC3-I is more sensitive to degradation than LC3-II.		√
6. LC3 protein levels may be heterogeneous in a cell population or tissue.		√
7. Different LC3 family members may be involved in autophagy requiring the use of multiple antibodies.	√	√

MONITORING AUTOPHAGIC FLUX

Autophagic flux refers to the complete processing of cargo, from sequestration to its degradation and recycling of basic components back to the cytosol.

- **LC3 Turnover Assay (WB, ICC/IF)**: This assay monitors the accumulation of autophagosomes to determine autophagic flux. Lysosomotropic agents and protease inhibitors are used to block autophagic flux and prevent LC3-II degradation. The difference in the amount of LC3-II in the presence and absence of these agents provides a measure of autophagic flux. This assay allows researchers to distinguish between true autophagy induction and blockade of autophagic flux.

MEASURING AUTOPHAGY

	REAGENTS FOR AUTOPHAGY TURNOVER ASSAY	INHIBITORY MECHANISM
PHARMACOLOGICAL	Chloroquine Bafilomycin A1 Ammonium chloride Azithromycin	Inhibitors of Lysosome Acidification
PHARMACOLOGICAL	E64d Pepstatin A1	Protease Inhibitors
PHARMACOLOGICAL	Xanthohumol ML 240	Inhibitors of Autophagosome Maturation
PHARMACOLOGICAL	Nocodazole Vinblastine	Inhibitors of Lysosome-Autophagosome Fusion
GENETIC	LAMP2/CD107b RNAi	Inhibitors of Autolysosome Maturation
GENETIC	LAMP2 CRISPR Knockout	Inhibitors of Autolysosome Maturation

Available at Tocris: www.tocris.com

PROTOCOL: LC3-II TURNOVER ASSAY BY WESTERN BLOT

MATERIALS

- Chloroquine diphosphate (CQ) (10 mM) in dH2O
- 1X PBS
- Sample buffer, 2X Laemmli buffer: 4% SDS, 5% 2-mercaptoethanol (BME), 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8
- RIPA buffer: 150 mM NaCl, 1% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, 20 mM Tris-HCl, pH 7.5
- 1X Running Buffer: 25 mM Tris-base, 192 mM glycine, 0.1% SDS. Adjust to pH 8.3
- 1X Transfer buffer (wet): 25 mM Tris-base, 192 mM glycine, 20% methanol pH 8.3
- TBS
- TBST, TBS and 0.1% Tween
- Blocking solution: TBST, 5% non-fat dry milk
- Rabbit anti-LC3 primary antibody (catalog# NB100-2220) in blocking buffer (~2 µg/mL)

PROTOCOL: LC3-II TURNOVER ASSAY BY WESTERN BLOT

METHODS

01 Grow cells (e.g., HeLa or Neuro2A) in vitro to semi-confluency (70-75%).

02 Add CQ to culture dishes to a final concentration of 50 μM and incubate 16 hours. Remember to include an untreated sample as a negative control.

Note: Validated autophagy inducers should be included as positive controls.

03 Rinse cells with ice-cold 1X PBS and lyse cells with sample buffer.

Note: LC3-I and LC3-II are sensitive to degradation, although LC3-I is more labile. These proteins are sensitive to freeze-thaw cycles and SDS sample buffers. Fresh samples should be analyzed quickly to prevent protein degradation.

04 Sonicate and incubate cells for 5 minutes at 95°C.

Tip: Cells are lysed directly in sample buffer or may be lysed in RIPA buffer.

05 Load samples of Chloroquine-treated and -untreated cell lysates 10-40 μg/lane on a 4-20% polyacrylamide gradient gel (SDS-PAGE).

Tip: For detection of LC3, it is particularly important to monitor the progress of the gel run as this protein is relatively small (~14kDa).

06 Transfer proteins to a 0.2 μm PVDF membrane for 30 minutes at 100V.

Tip: For more information on Western Blotting, see our Western Blot handbook at novusbio.com/wb-handbook

07 After transfer, rinse the membrane with dH_2O and stain with Ponceau S for 1-2 minutes to confirm efficiency of protein transfer.

08 Rinse the membrane in dH_2O to remove excess stain and mark the loaded lanes and molecular weight markers using a pencil.

PROTOCOL: LC3-II TURNOVER ASSAY BY WESTERN BLOT

09 Block the membrane using blocking buffer solution (5% non-fat dry milk in TBST) for 1 hour at room temperature.

10 Rinse the membrane with TBST for 5 minutes.

11 Dilute the rabbit anti-LC3 primary antibody (catalog# NB100-2220) in blocking buffer (~2 μg/mL) and incubate the membrane for 1 hour at room temperature.

12 Rinse the membrane with dH2O.

13 Rinse the membrane with TBST, 3 times for 10 minutes each.

14 Incubate the membrane with diluted secondary antibody (e.g., anti-rabbit-IgG HRP-conjugated) in blocking buffer for 1 hour at room temperature.

Note: Tween-20 may be added to the blocking or antibody dilution buffer at a final concentration of 0.05-0.2%, provided it does not interfere with antibody-antigen binding.

15 Rinse the membrane with TBST, 3 times for 10 minutes each.

16 Apply the detection reagent of choice (e.g., NovusLume Pico Chemilunescent Substrate, Catalog #NBP2-61915) in accordance with the manufacturer's instructions.

17 Image the blot.

Tip: LC3-I and its lipidated form LC3-II have different electrophoretic mobility properties, with the lipidated form moving faster in an SDS-PAGE gel, albeit its larger molecular weight. LC3-II runs at 14-16kDa while LC3-I runs at 16-18kDa.

Note: This assay measures the difference in the LC3-II signal in the presence and absence of inhibitors (e.g., lysosomotropic agents). When autophagic flux is activated or induced in a system an increase in the LC3-II signal should be observed with the inhibitor.

MEASURING AUTOPHAGY

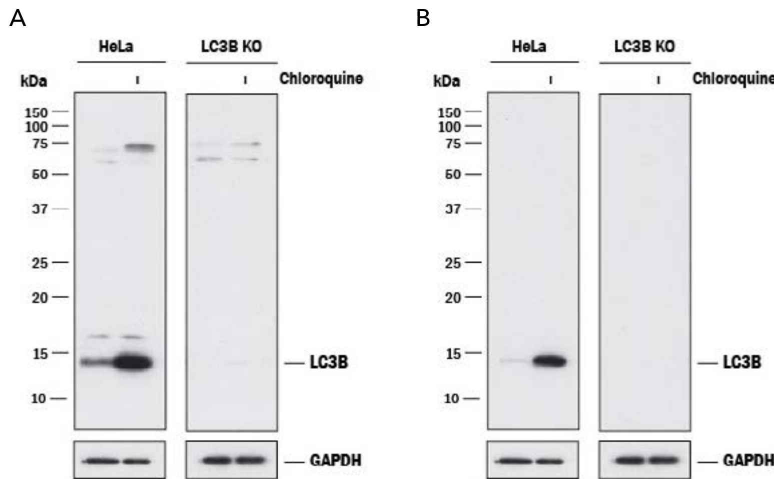


FIGURE 7. HeLa human cervical epithelial carcinoma parental cell line and LC3B knockout HeLa cell line (KO) were untreated (-) or treated (+) with 50 μM Chloroquine for 18 hours. Whole cell protein lysates were prepared in 1x Laemmli sample buffer and approximately 10 μg of each lysate was separated on a 4-15% gel by SDS-PAGE, transferred to 0.2 μm PVDF membrane and blocked in 5% non-fat milk in TBST. PVDF membranes were probed with (A) rabbit anti-LC3B polyclonal (catalog# NB100-2220) and (B) rabbit anti-LC3B monoclonal (catalog# NBP2-46892) followed by HRP-conjugated anti-rabbit IgG secondary antibody. A specific band was detected for LC3B at approximately 15 kDa (as indicated) in the parental HeLa cell line, but is not detectable in the knockout HeLa cell line. GAPDH is shown as a loading control. This experiment was conducted under reducing conditions.

CONSIDERATIONS FOR LC3 TURNOVER ASSAYS	ICC/IHC	WB
1. In cells or tissues with high basal autophagy flux it may be difficult to detect additional autophagy induction.	√	√
2. To avoid signal saturation, treatment with inhibitors should be limited to 1–2 hours.	√	√
3. Incubation periods with inhibitors and their concentrations should be closely monitored to prevent triggering cell death.	√	√
4. The use of fusion blockers (autophagosome-lysosome) may lead to the formation of larger autophagosomes and complicate analysis of autophagic activity.	√	

ALTERNATE TARGETS TO STUDY AUTOPHAGY

p62/SQSTM1 Degradation Assay (WB, FC, ELISA, ICC/IF): Removal of cytosolic p62/SQSTM1 occurs primarily by autophagy. Therefore, the level of p62/SQSTM1 is inversely correlated to autophagic activity. Similar to LC3-II, blocking degradation of cytosolic components with Chloroquine, inhibits autophagic flux and leads to the accumulation of p62/SQSTM1. The levels of p62/SQSTM1 may be quantitated by flow cytometry, immunoblot and ELISA and serve as a biomarker for the analysis of autophagic flux.

FIGURE 8. p62/SQSTM1 is targeted to the autophagosome by interacting with LC3-II via its LIR domain. The UBD domain allows p62/SQSTM1 to bind ubiquitinated cytosolic components (e.g., protein aggregates), targeting them for degradation. Through the PB1 domain p62/SQSTM1 self-oligomerizes or dimerizes with NBR1.

ALTERNATE TARGETS TO STUDY AUTOPHAGY

P62/SQSTM1- STRUCTURAL DOMAINS RELEVANT IN AUTOPHAGY	
DOMAINS	FUNCTION
PB1 (Phox and Bem1 Domain)	Self-oligomerization or heterodimerization with NBR1
UBD (Ubiquitin Binding Domain)	Interaction with ubiquitinated proteins
LIR (LC3 Interacting Region)	Human LIR motif: 335-DDDWTHLS-342

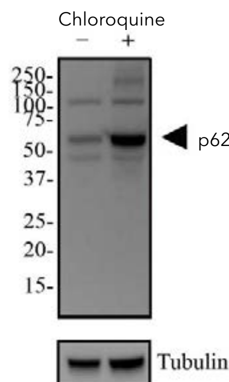


FIGURE 9. Cultured HeLa cells were treated with vehicle (H2O) or with 50 µM Chloroquine for 24 hours as indicated. Cell lysates were prepared and separated on a 12% gel by SDS-PAGE. Protein was transferred to PVDF membrane and blocked in 5% non-fat milk. The membrane was then probed with 1 µg/mL anti-p62/SQSTM1 [catalog# NBP1-48320] in 1% milk and detected with an anti-rabbit HRP secondary antibody using chemiluminescence. Note the upregulation of p62 (arrowhead) in response to Chloroquine treatment which indicates the blockage of autophagy.

CONSIDERATIONS FOR P62/SQSTM1 QUANTITATION

For western blot analysis of p62/SQSTM1, care must be taken with the specific lysis and sample buffers used. p62/SQSTM1 oligomerizes upon inhibition of autophagy (e.g., lysosomotropic agents) and in the presence of protein aggregates leading to its accumulation in the Triton X-100 insoluble fraction. Therefore, a lysis buffer containing 1% SDS is more effective, allowing researchers to evaluate the entire p62/SQSTM1 cellular pool.

In comparison to LC3-II, turnover of p62/SQSTM1 occurs at a slower rate, therefore evaluation of degradation (e.g., after autophagy induction) should include time points beyond 24 hours. To evaluate autophagic flux via p62/SQSTM1 degradation, a combination of western blot and immunostaining in the presence and absence of autophagy inhibitors and inducers is recommended.

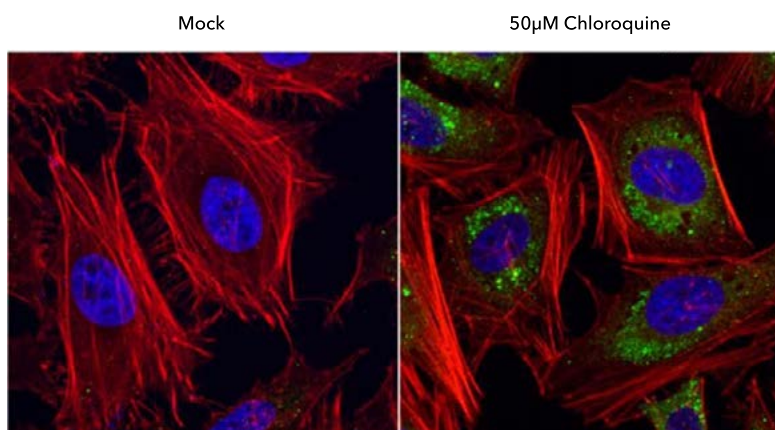


FIGURE 10: HeLa cells mock (left) and treated with 50 µM Chloroquine for 24 hours (right) were fixed in 4% paraformaldehyde at room temperature for 15 minutes. (Green): SQSTM1 protein stained by SQSTM1 antibody (1478) [catalog# NBP2-43663] diluted at 1:1000. (Red): Actin stained with Phalloidin diluted at 1:200. (Blue): Hoechst 33342 staining.

ALTERNATE TARGETS TO STUDY AUTOPHAGY

ADDITIONAL RECOMMENDED TARGETS TO MONITOR AUTOPHAGY		
TARGET	METHOD	CONSIDERATIONS
mTOR, AMPK and ATG1/ULK1*	WB, IP, Kinase Assay	Levels of ULK1 in some systems are too low to detect phosphorylated forms. TOR activity inversely correlates with autophagic activity, although TOR independent pathways also control autophagy.
ATG12, ATG5 and ATG16L1	ICC/IF	Conjugation of ATG12-ATG5 may reflect autophagic activity, but in some cells these proteins mainly exist in the conjugated form.
WIPI (endogenous WIPI1 or WIPI2)	ICC/IF	WIPI positive puncta may not be present in all cells.

*ULK1 serves as a switch for autophagy regulation, mTOR and AMPK directly phosphorylate ULK1 inhibiting or activating its function, respectively.

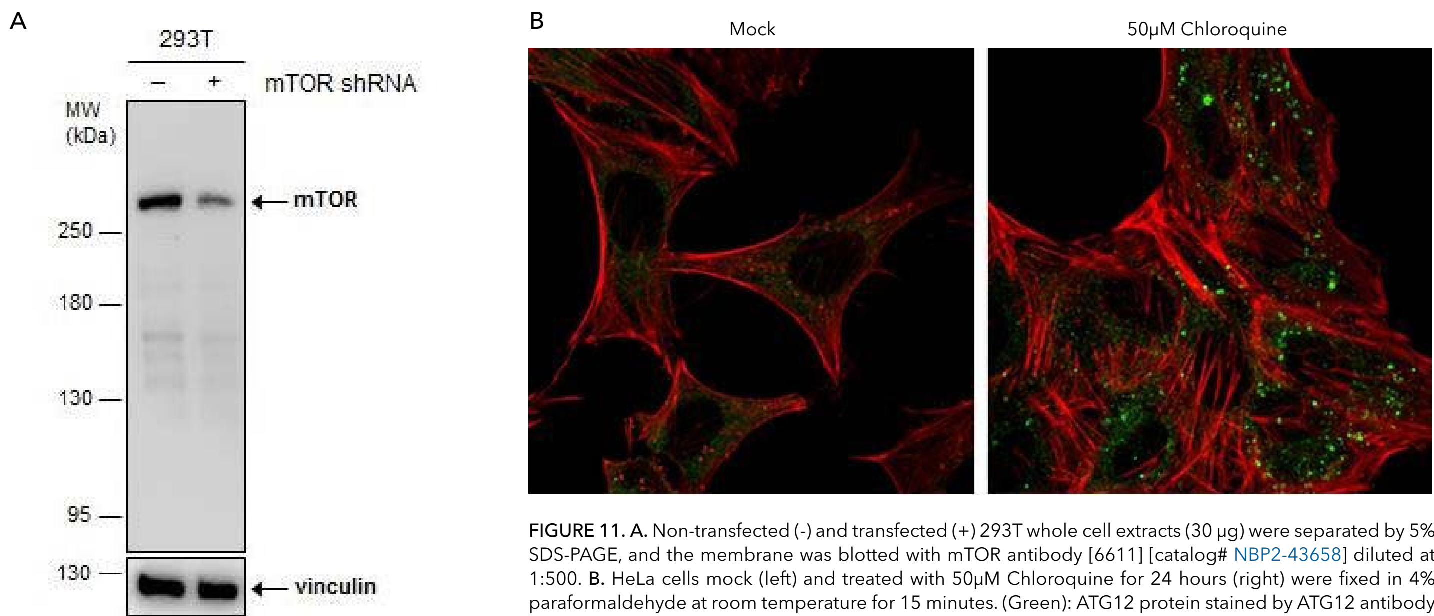


FIGURE 11. A. Non-transfected (-) and transfected (+) 293T whole cell extracts (30 μg) were separated by 5% SDS-PAGE, and the membrane was blotted with mTOR antibody [6611] [catalog# NBP2-43658] diluted at 1:500. **B.** HeLa cells mock (left) and treated with 50μM Chloroquine for 24 hours (right) were fixed in 4% paraformaldehyde at room temperature for 15 minutes. (Green): ATG12 protein stained by ATG12 antibody [166] (catalog# NBP2-43781) diluted at 1:1000. (Red): F-actin stained with Phalloidin.

ADDITIONAL METHODS TO MONITOR AUTOPHAGY	
ASSAY	METHOD
Morphological identification and quantification of autophagosomes and autolysosomes.	TEM
GFP-LC3, monitors number of puncta as a measure of autophagic flux.	FM
Tandem mRFP/mCherry-GFP-LC3, monitors the change in fluorescence (yellow to red) as a measure of autophagic flux.	FM
GFP-LC3 Lysosomal delivery and proteolysis, monitors free GFP as a measure of autophagic flux.	WB

SIMPLE WESTERN FOR AUTOPHAGY TARGETS

Simple Western is a new alternative to conventional western blotting. It provides an automated capillary-based platform for reproducible detection and quantitation of protein targets. Protein separation may be based on size or charge properties, and a wide range of molecular sizes (2-440 kDa) may be resolved in a multiplexed platform.

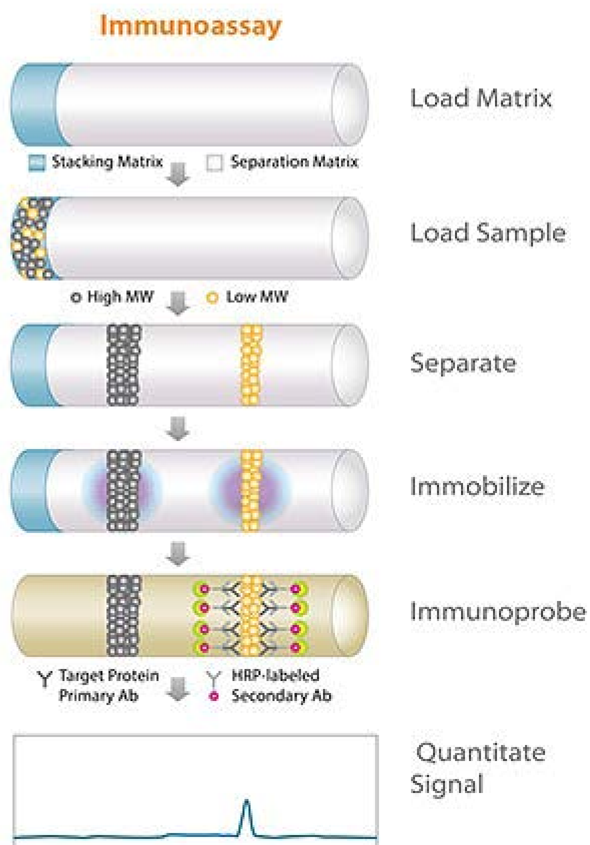


FIGURE 12. In the Simple Western System, separation of proteins and immunoprobing are performed within a capillary cartridge.

Samples are first loaded onto a stacking matrix and proteins are separated according to size or charge as they move through a separation matrix.

Following protein immobilization, immunoprobing with primary and secondary antibodies is performed automatically.

Detection is based on the use of a chemiluminescent reagent.

Simple western automatically provides quantitative information for detected proteins including molecular weight, signal intensity and background or noise measurements.

Sample separation is presented as a virtual image similar to a conventional blot.

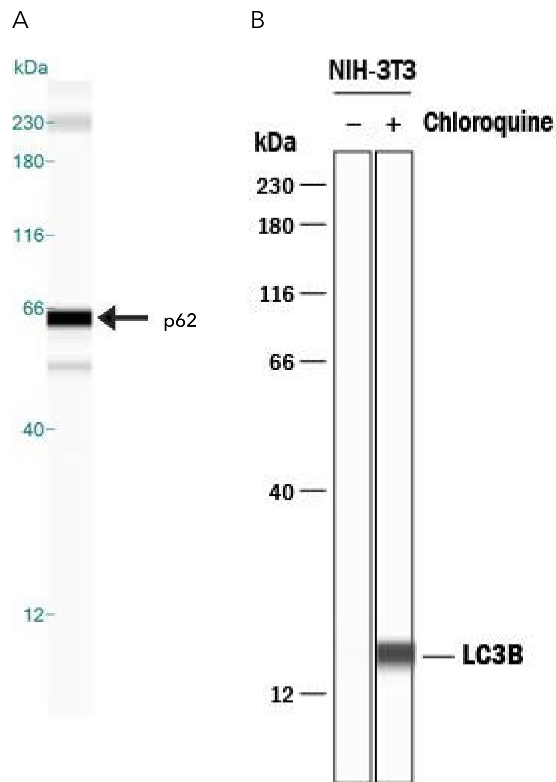


FIGURE 13. A. p62/SQSTM1 Antibody [catalog# NBP1-48320] - Simple Western lane view shows a specific band for p62/SQSTM1 in 1.0 mg/mL of HeLa lysate. B. Simple Western lane view shows lysates of NIH-3T3 mouse embryonic fibroblast cell line treated with vehicle (H2O) (-) or treated (+) with 50 µM Chloroquine for 18 hours, loaded at 0.2 mg/mL. A specific band was detected for LC3B at approximately 17 kDa (as indicated) using 5 µg/mL of Rabbit Anti-Human LC3B Monoclonal Antibody (catalog# NBP2-59800). These experiments were conducted under reducing conditions and using the 12-230 kDa separation system.

BIO-TECHNE: SELECT AUTOPHAGY ANTIBODIES VALIDATED FOR SIMPLE WESTERN	
TARGET	
LC3A	HIF1 α
LC3B	ATG5
p62/SQSTM1	ATG7
PINK1	Beclin1

To learn more go to: www.proteinsimple.com/simple_western_overview

MODULATORS OF AUTOPHAGY

Modulation of autophagy is attractive as this process is implicated in various disease states including cancer, neurodegeneration and cardiomyopathies. Small molecules able to inhibit or induce autophagic activity provide a mechanism to modulate and study autophagy at the single cell and organismal level. In contrast to genetic models, pharmacological agents allow temporal and dose control. Moreover, often the effects of autophagy modulating agents is reversible. For animal model systems, targeted delivery of autophagy modulators to specific organs and tissues may represent a significant advantage.

	SMALL MOLECULE MODULATORS	
	PHARMACOLOGICAL COMPOUND	MODE OF ACTION
AUTOPHAGY INHIBITORS	3-Methyladenine	AMPK inhibitor
	(±)-Bay K 8644	L-type Ca^{2+} channel activator
	Spautin 1	USP10 and USP13 inhibitor
	LY 294002 [*,a] Wortmannin [a]	VPS34 inhibitor
	MRT 67307 MRT 68921	ULK inhibitor
AUTOPHAGY ACTIVATORS	Simvastatin [a] A 769662 [a]	AMPK activator
	Rapamycin [a] Torins (1 and 2) Everolimus PI 103	mTOR inhibitor
	Dexamethasone	Anti-inflammatory glucocorticoid; may act via mTOR pathway
	L-690,330	Inositol monophosphatase inhibitor; independent of mTOR inhibition
	Dorsomorphin	AMPK inhibition-independent
	A23187, free acid [a] Brefeldin A [a] Thapsigargin [a] Tunicamycin [a]	Causes ER stress
	Amiodarone	Causes mitochondrial fragmentation and cell death
	GF 109203X	Protein kinase C inhibitor
	NF 449 [a]	Highly selective P2X1 antagonist; Gsα-selective antagonist
	Perifosine	PKB/AKT inhibitor
	Trichostatin A	Histone deacetylase inhibitor
	Nimodipine Verapamil	Ca^{2+} channel blocker (L-type)
	3-Nitropropionic acid	Irreversible mitochondrial respiratory complex II inhibitor
	Rilmenidine	α2 agonist and I1 ligand; thought to enhance autophagy
	Valproic acid	Reduces inositol levels

[a] Commonly used modulators. *VPS34 inhibitor more selective in comparison to 3-Methyladenine and Wortmannin.

Autophagy modulators available at Tocris: www.tocris.com/autophagy

MODULATORS OF AUTOPHAGY

SPECIFIC INDUCTION OF AUTOPHAGY: TAT BECLIN PEPTIDES

Tat-Beclin peptides provide a very specific approach for the induction of autophagy, preventing the activation of other biologically significant pathways. Tat-Beclin peptides are designed based on the Beclin 1/Nef interacting sequence and coupled to the HIV-1 Tat protein transduction domain (PTD) sequence. The resulting peptide traverses the cell membrane and gains access to its key interacting partner, GAPR-1 (Golgi-associated plant pathogenesis-related protein 1), resulting in the induction of autophagy. The new Tat Beclin peptides (Tat-D11, Tat-L11) are shorter than the original peptide and induce autophagy more efficiently.

To learn more go to: www.novusbio.com/autophagy-inducing-peptides

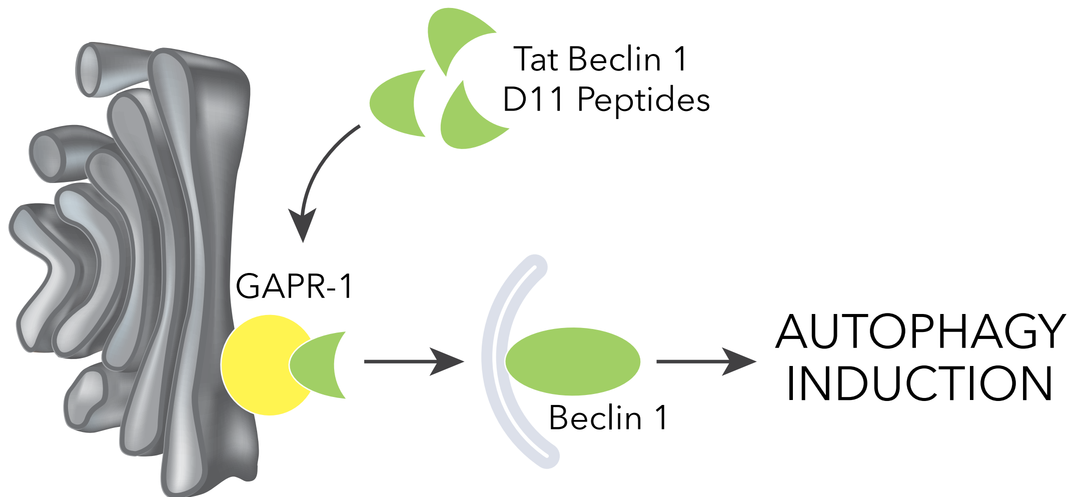


FIGURE 14. GAPR-1/GLIPR2 is a negative regulator of autophagy and binds Beclin 1 to inhibit autophagy. In the presence of Tat-D11 peptides, Beclin 1 bound to GAPR-1 is released allowing Beclin 1 to mediate autophagosome formation and autophagy induction.

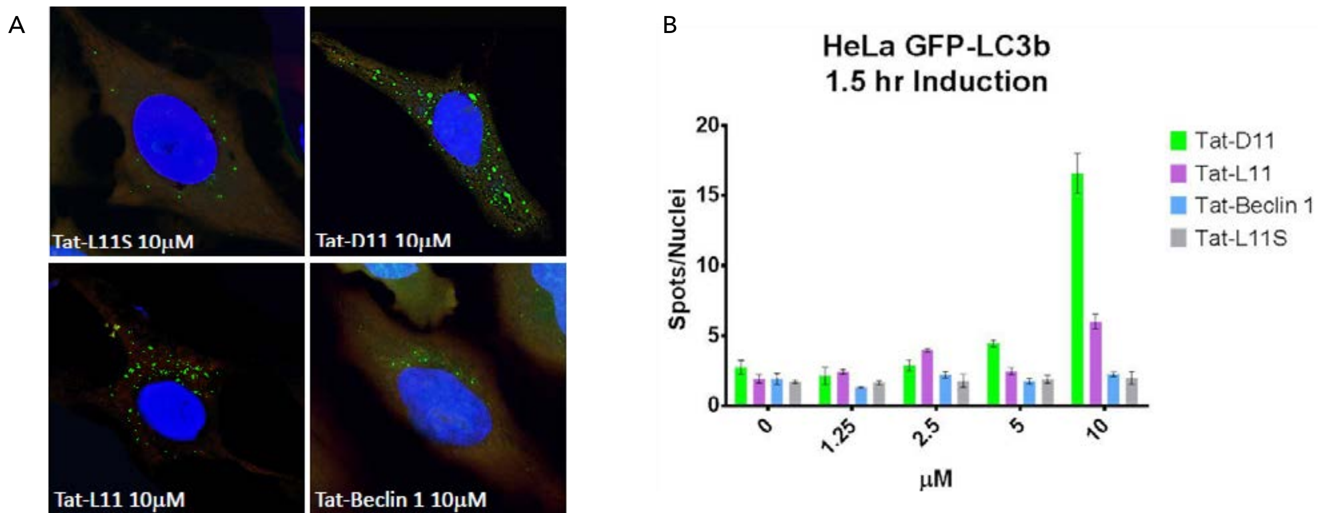


FIGURE 15. Tat-D11 and Tat-L11 are more potent autophagy inducers than Tat-Beclin 1. HeLa GFP-LC3B were treated with Tat-D11, Tat-L11, Tat-L11S or Tat-Beclin for 1.5 hours, and (A) the number of autophagosomes were assessed by fluorescent microscopy and (B) the number of GFP+/ LC3B+ spots were quantified. Cells treated with Tat-D11 had larger numbers of GFP+/ LC3B+ puncta.

NUCLEAR CONTROL OF AUTOPHAGY

Many transcription factors are known to regulate the expression of key autophagy related genes. Transcriptional regulation has rapid outcomes on autophagic activity. Often, translocation of transcription factors between the cytoplasm and the nuclei determines their specific function as modulators of autophagy. Nevertheless, each regulatory axis seems to influence autophagy via unique mechanisms.

TRANSCRIPTIONAL MECHANISMS REGULATING AUTOPHAGY				
TRANSCRIPTION FACTOR	AUTOPHAGY OUTCOME	AUTOPHAGY RELATED GENES TRANSCRIPTIONALLY REGULATED		
FOXO1 (Novus catalog# NBP2-31376)	+	ATG5 ATG12 ATG14	BECN1 BNIP3 LC3	VSP34
FOXO3 (R&D Systems catalog# AF6165)	+/-	ATG4 ATG12 BECN1	BNIP3 LC3 ULK1	ULK2 VSP34
E2F1 (R&D Systems catalog# AF4825)	+	ATG5 BNIP3	LC3 ULK1	
NF-κB (Novus catalog# NBP1-77395)	+/-	BCL2 BECN1	BNIP3 SQSTM1	
TFEB (R&D Systems catalog# MAB9170)	+	ATG4 ATG9 BCL2	LC3 SQSTM1 UVRAG	WIPI
ZKSCAN3 (Novus catalog# NBP2-47570, NBP1-31566)	-	LC3 ULK1 WIPI		

Autophagy outcome: + (Enhanced), - (Decreased).

NUCLEAR CONTROL OF AUTOPHAGY

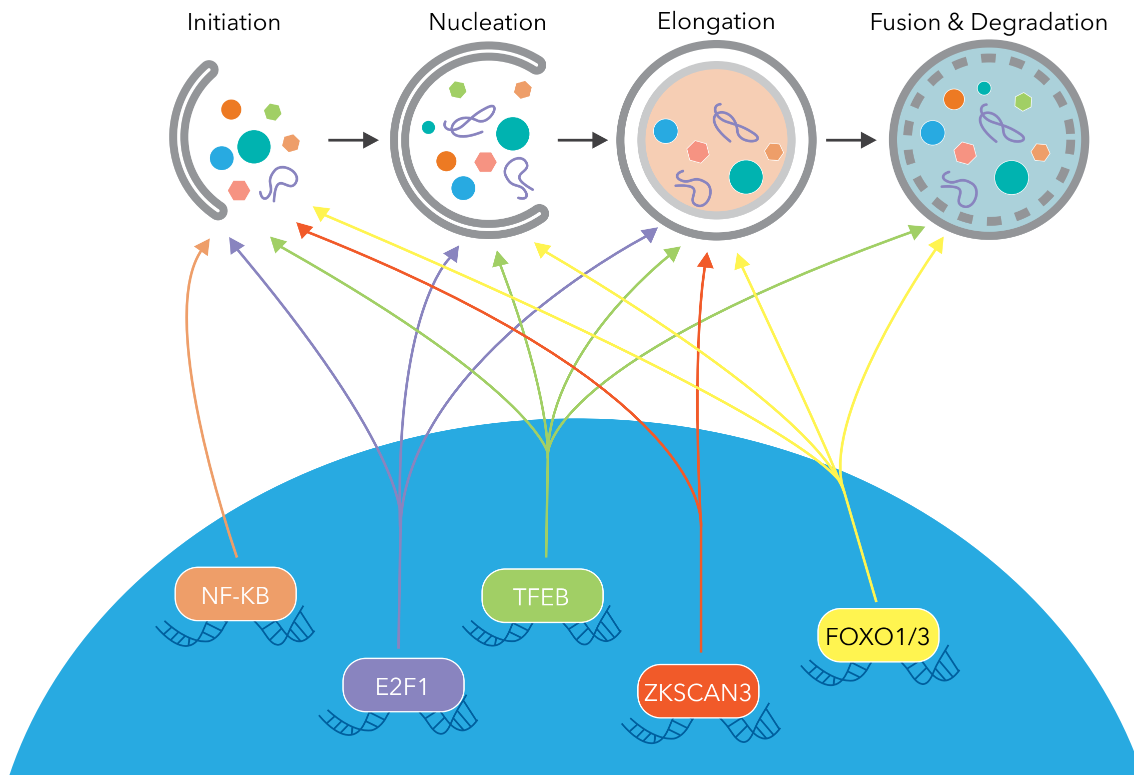


FIGURE 16. Transcriptional regulation of autophagy. The core autophagy machinery comprising all steps of the pathway can be regulated by nuclear transcription factors.

EPIGENETIC CONTROL OF AUTOPHAGY

The role of various epigenetic changes including chromatin and histone modifications on the modulation of autophagy has prompted intense scrutiny. Regulatory pathways involving DNA and histone modifying enzymes dynamically fine-tune the process of autophagy. Several significant modifying enzymes have been identified that induce or inhibit autophagic activity.

IDENTIFIED EPIGENETIC CHANGES INFLUENCING AUTOPHAGY		
ENZYMATIC MECHANISM	AUTOPHAGY RELATED GENES MODULATED	ASSOCIATED AUTOPHAGIC STATE
DNMT2 Hypermethylation	ATG5 and LC3 downregulated	Reduced autophagy
ESA1/RPD3 axis Acetylation/Deacetylation of H4	Ribosomal Protein upregulated/downregulated LC3 expression regulation	Reduced/Increased autophagy
G9A Methylation of H3K9	LC3, WIPI1 and DOR downregulated	Reduced autophagy
hMOF/SIRT1 axis Acetylation/Deacetylation of H4K16	Autophagy related genes induced/ inhibited	Balance between cell death and survival
USP44 deubiquitination of H2B	Downregulated genes: regulation of NF-κB and biosynthetic process Upregulated genes: innate immunity and polyubiquitination	Increased autophagy

ADVANCED CELL DIAGNOSTICS (ACD) RNASCOPE®

The RNAscope® assay is a proprietary RNA (*in situ*) hybridization (ISH) assay based on ACD's patented signal amplification and background suppression technology, which advances RNA biomarker analysis in tissues and cells. Unique to this technology, the RNAscope® assay delivers quantitative, sensitive, and specific molecular detection of RNA targets on a cell-by-cell basis with morphological context. An assay can be designed for any gene in any species and delivered in 2 weeks.

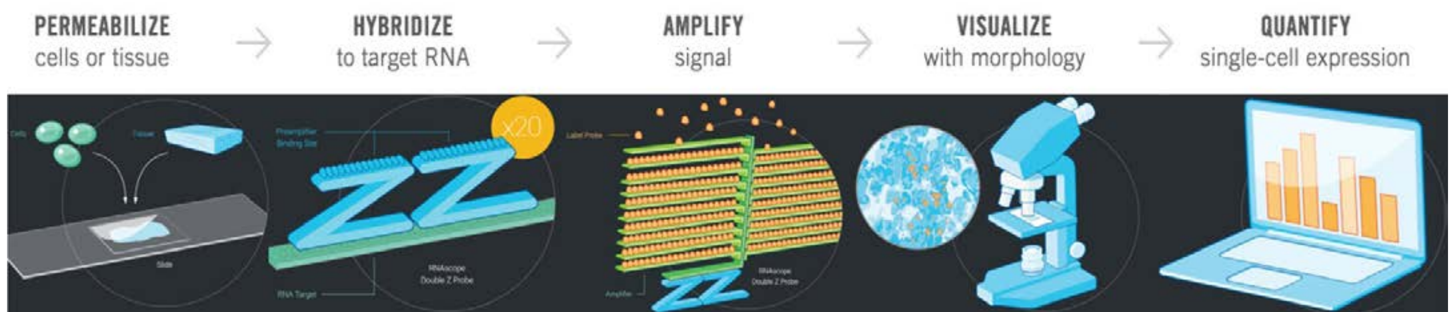
SIGNAL AMPLIFICATION IS ACHIEVED BY A CASCADE OF HYBRIDIZATION EVENTS

Step 1: ~ 20 Double Z target probes hybridize to the RNA target (targeting ~1kb region)

Step 2: Pre-amplifiers hybridize to the 28-base binding site formed by each double Z probe. Hybridization of only one Z probe will inhibit binding of the pre-amplifier, thereby providing high specificity.

Step 3: Amplifiers recognize multiple binding sites on each preamplifier.

Step 4: Labeled probes, containing a fluorescent molecule or chromogenic enzyme, bind to the numerous binding sites on each amplifier. This signal amplification system increases assay sensitivity.



CATALOG NUMBER	PRODUCT NAME	SPECIES	GENE ALIAS
469441	RNAscope® Probe - Mm-Pnpla2	Mouse	Atgl
469711	RNAscope® Probe - Mm-Atg5	Mouse	Atg5
434111	RNAscope® Probe - Hs-BECN1	Human	ATG6
477991	RNAscope® Probe - Mm-Map1lc3b	Mouse	Atg8f
492821	RNAscope® Probe - Hs-MAP1LC3B	Human	ATG8f
445401	RNAscope® Probe - Hs-MAP1LC3A	Human	ATG8e
496561	RNAscope® Probe - Hs-WIPI1	Human	ATG18
434121	RNAscope® Probe - Hs-BECN1P1	Human	BECN2
415881	RNAscope® Probe - Hs-SQSTM1	Human	p62
444221	RNAscope® Probe - Mm-Sqstm1	Mouse	p62
477531	RNAscope® Probe - Hs-ANPEP	Human	p150
439731	RNAscope® Probe - Mm-Ulk1	Mouse	Ulk1

For more information go to: https://acdbio.com/science/applications

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ACRONYMS AND ABBREVIATIONS

AMBRA1	Activating molecule BECN1 regulated autophagy protein 1
AMPK	AMP-activated protein kinase
ATG	Autophagy related proteins
BME	2-mercaptoethanol
BNIP3	BCL-2/adenovirus E1B 19 kDa interacting protein 3
CD40	Cluster of differentiation 40
Cue5	Coupling of ubiquitin conjugation to ER degradation
DNMT	DNA methyltransferase
DOR	Diabetes- and obesity-regulated
ELISA	Enzyme linked immunosorbent assay
ESA	Essential SAS2-related acetyltransferase
FAM134B	Family with sequence similarity 134 member B
FC	Flow cytometry
FIP200	FAK family-interacting protein of 200 kDa
FM	Fluorescent microscopy
FOXO	Forkhead box O
FUNDC1	FUN-domain containing protein
G9A	Euchromatic histone-lysine N-methyltransferase 2
GAPR-1	Golgi-associated plant pathogenesis-related protein 1
HSC-70	Heat shock cognate 70 protein
Hsp	Heat shock protein
ICC	Immunocytochemistry
IF	Immunofluorescence
IHC	Immunohistochemistry
ISH	in situ hybridization
KO	Knockout
LAMP-2A	Lysosomal associated membrane protein-2A
LC3	Microtubule-associated protein light chain 3
LIR	LC3 interacting region
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
NBR1	neighbor of BRCA1 gene 1
NDP52	Nuclear dot protein 52 kDa
NIX	NIP3-like protein X
OPTN	Optineurin
PB1	Phox and Bem1 domain
PE	Phosphatidylethanolamine
PKB	Protein kinase B
PTD	Protein transduction domain
PtdIns3K	Class III phosphatidylinositol 3-kinase
RB1CC1	RB1-inducible coiled-coil 1
RPD3	Reduced Potassium Deficiency 3/Histone Deacetylase
RPN10	Regulatory particle non-ATPase
SIRT1	Silent Mating Type Information Regulation 2, S. Cerevisiae, Homolog 1
SQSTM1	Sequestosome 1
SMURF1	SMAD ubiquitylation regulatory factor
SNAP29	Synaptosome associated protein 29
STX17	Syntaxin 17
TAX1BP1	Human T-Cell Leukemia Virus Type I) Binding Protein 1
TCR	T-cell receptor
TEM	Transmission electron microscopy
TFEB	Transcription factor EB
TLR4	Toll-like receptor 4
TOLLIP	Toll-interacting protein
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRIM5a	Tripartite motif
UBD	Ubiquitin binding domain
ULK1	unc-51-like kinase 1
USP	Ubiquitin Specific Peptidase
UVRAG	UV resistance-associated gene
VAMP8	Vesicle associated membrane protein 8
VPS	Vacuolar sorting protein
WB	Western Blot
WIPI	WD-repeat protein interacting with phosphoinositides
ZKSCAN3	zinc finger with KRAB and SCAN domains 3

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