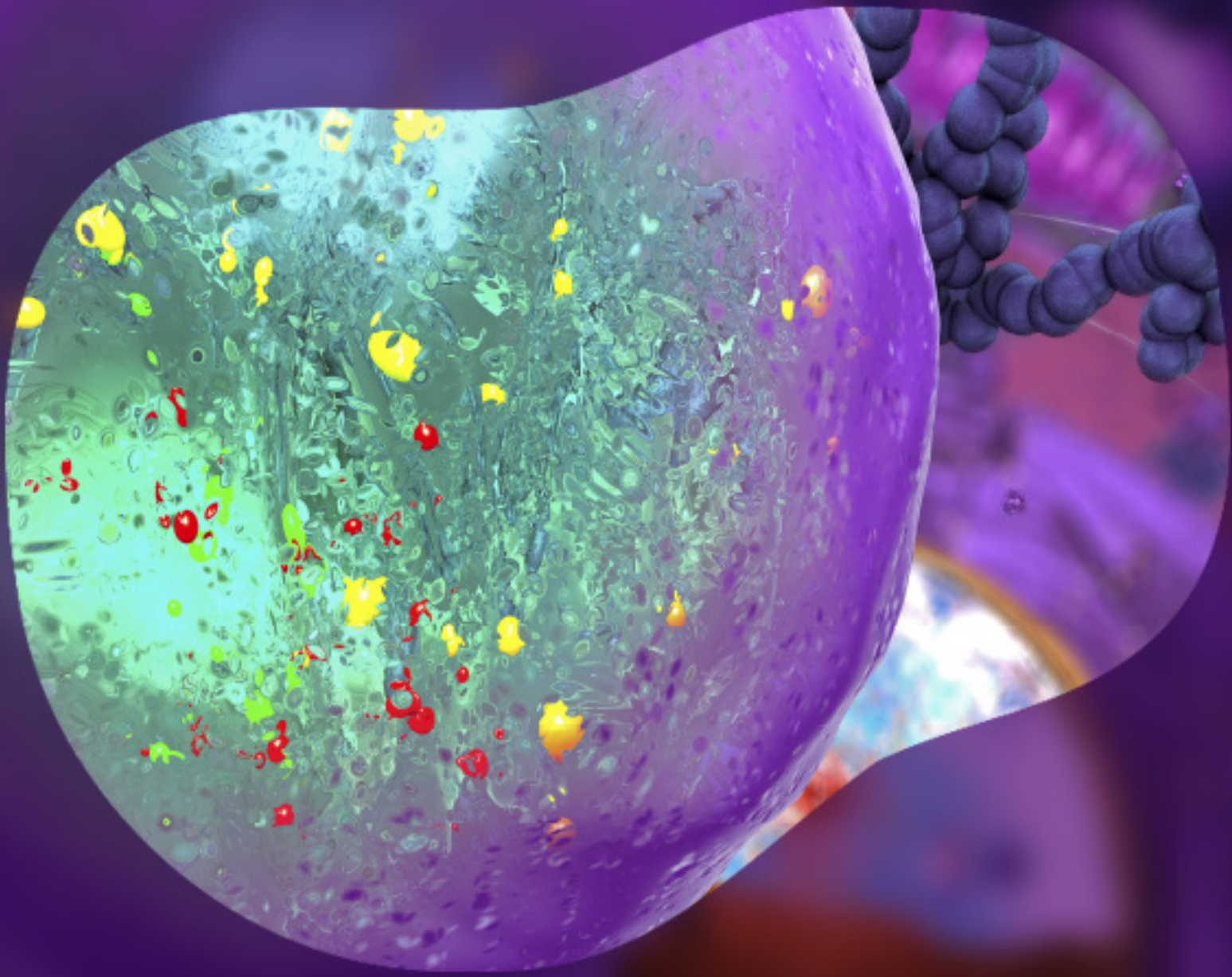
Autophagy regulation.



revvity

Table of contents

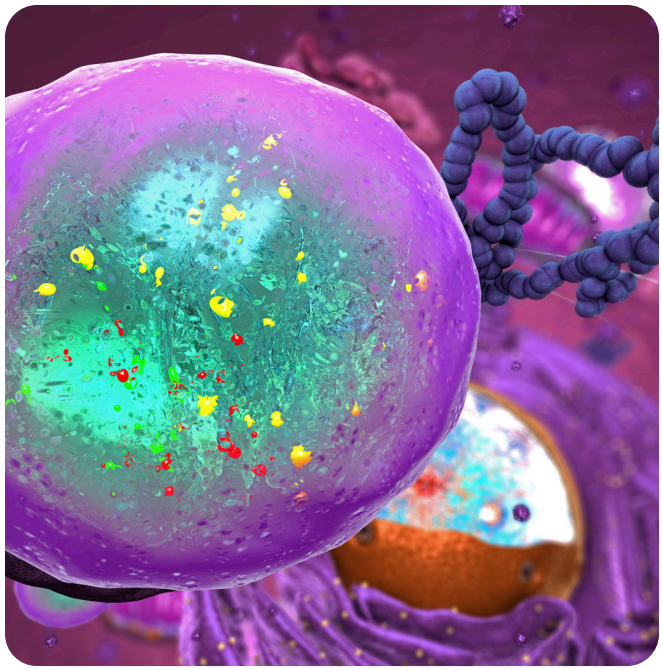
Foreword 2
Infographic: Autophagy 3
Understanding the role of Atg9 vesicles during autophagy 5
Autophagy regulation and mitophagy 8
Infographic: The role of autophagy dysregulation in pathology 11
The impact of autophagy research on drug discovery: Q&A with Eileen Gonzalez 12
Innovative immunoassay solutions for autophagy research 15

Foreword

Autophagy is an essential component of cellular homeostasis, responsible for clearing long-lived proteins and organelles and recycling cellular building blocks (lipids, amino acids, carbohydrates, and nucleotides). This complex cellular mechanism is performed and regulated thanks to the coordinated action of several conserved autophagy-related proteins (Atg).

Since autophagy is involved in numerous physiological processes (e.g., stress responses, cell growth, and aging), its dysregulation is associated with different pathologies such as cancer, neurodegenerative and infectious diseases.

This eBook will 1) introduce the different types of autophagy and some of the main mechanisms underlying its regulation, 2) discuss the present state of autophagy research in health and disease, and 3) highlight the innovative immunoassay solutions that have been developed to further support autophagy research.

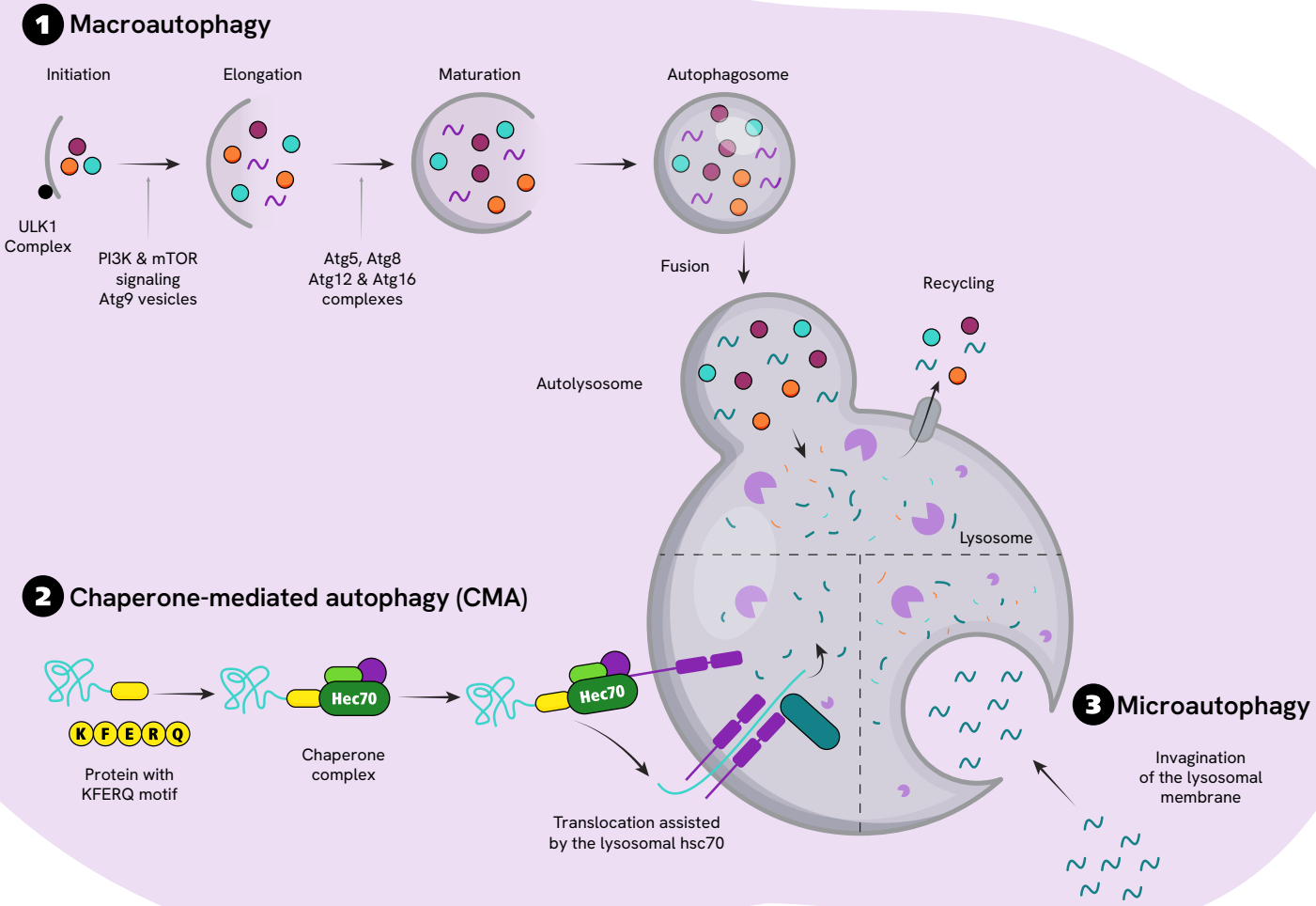


BioLegend®

Autophagy

Autophagy (from the Greek, auto "self" and phagy "eating") is a cellular process whereby a cell eats and digests its own components. It is crucial for the maintenance of homeostasis, and is involved in numerous physiological processes, including stress responses, cell growth, and aging.[1] It is also required for the clearance of long-lived proteins and organelles and in the recycling of building blocks (lipids, amino acids, carbohydrates, and nucleotides).[1]

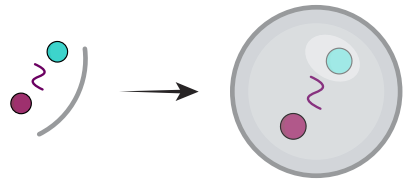
Different types of autophagy



1) Macroautophagy	2) CMA	3) Microautophagy
Sequestering of cytosolic components (cargo) in autophagosomes prior to lysosomal fusion	Sequestering of proteins containing KFERQ-like sequences by chaperone complex and internalization via a lysosomal receptor	Sequestering of cytosolic proteins via lysosomal wrapping mechanism (LWM)
Non-selective or selective	Selective	Selective
Triggered by short periods of fasting (<12h)	Triggered by long periods of fasting (>12h)	
Clearance of various cellular components. Recycles nutrients	Clearance of cytosolic proteins with KFERQ-like sequences (~40% of soluble proteins)	Clearance of cytosolic proteins

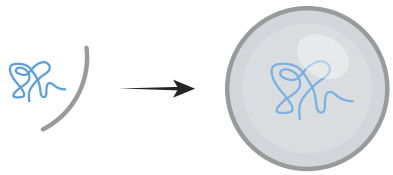
Non-selective ("bulk") and selective macroautophagy[2]

Non-selective

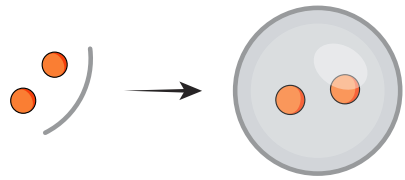


Triggered by starvation
Helps cell survival by recycling building blocks

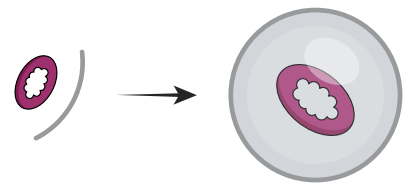
Selective



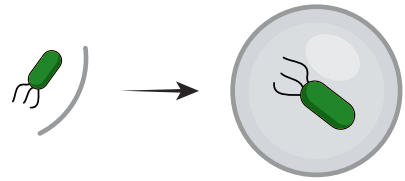
Proteins (proteaphagy), Proteins aggregates (aggregaphagy)



Lipid droplets (lipophagy)



Dysfunctional organelles, e.g., mitochondria (mitophagy), endoplasmic reticulum (reticulophagy)



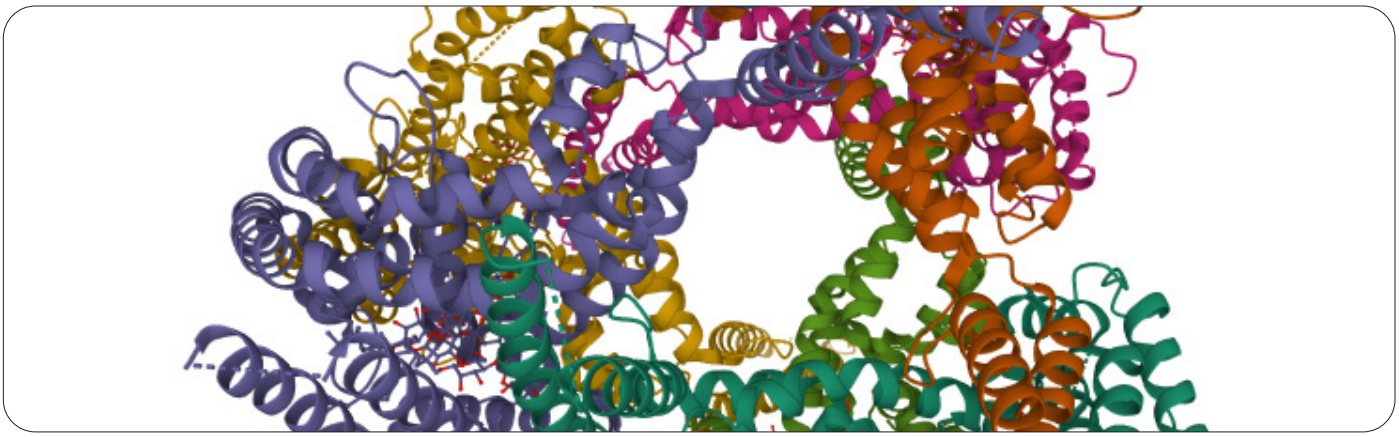
Intracellular pathogens (xenophagy)

Proteins involved in autophagy

The molecular mechanism of autophagy involves several conserved autophagy-related proteins (Atg).[3]

Functional Units	Yeast	Humans
Atg1/ULK1 Complex	Atg1	ULK1/2
	Atg13	Atg13
	Atg17	F1P200 Atg101
	Atg29	
	Atg31	
Atg9 vesicles	Atg9	Atg9A/B
P1K3 Class3-complex1	Vps34	Vps34
	Vps15	Vps15
	Vps30/Atg6	Beclin1
	Atg14	Atg14
	Atg38	NRFB2
Atg2-Atg18 complex	Atg2	Atg2A/B
	Atg18	WIPI 1/2/3/4
Atg16 complex	Atg12	Atg12
	Atg7	Atg7
	Atg10	Atg10
	Atg5	Atg5
	Atg16	Atg16
Atg8 complex	Atg3	Atg3
	Atg4	Atg4 A/B/C/D
	Atg7	Atg7
	Atg8	LC3 A/B/B2/C
		GABARAP

1. *Khandia R, Dadar M, Munjal A, et al. A comprehensive review of autophagy and its various roles in infectious, non-infectious, and lifestyle diseases: current knowledge and prospects for disease prevention, novel drug design, and therapy. Cells. 2019;8(7):674. doi:* ***10.3390/cells8070674.***
2. *Aburto MR, Hurlé JM, Varela-Nieto I, Magariños M. Autophagy during vertebrate development. Cells. 2012;1;428-448. doi:* ***10.3390/cells1030428.***
3. *Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. Curr. Top. Microbiol. Immuno l. 2009;335:1-32. doi:* ***10.1007/978-3-642-00302-8_1.***



Understanding the role of Atg9 vesicles during autophagy

Autophagy is a cellular process mediating the degradation of cytoplasmic material within lysosomes.[1] For example, misfolded proteins or damaged organelles are captured in a double membrane-bound compartment called the autophagosome. The autophagosome then fuses with the lysosome to become an autolysosome which degrades the materials contained within it.[1] This process is triggered by stressful conditions and/or limited resources and serves as a dynamic recycling system that produces new building blocks.[1] Autophagy is essential for cellular homeostasis, thus, its dysregulation is associated with different pathologies such as neurodegeneration, cancer, and infectious diseases.[2] A fundamental question in autophagy research is how the autophagosome forms de novo in the cell. A team led by Dr. Sascha Martens at the Max Perutz labs in Vienna have recently addressed this question by reconstructing the first steps in the formation of autophagosomes in vitro.[3] This article will discuss the results and conclusions of this study.

Autophagosome formation

During autophagy, the cytoplasmic material to be degraded, usually referred as "the cargo", is sequestered within double membrane vesicles made of phospholipids. These vesicles form de novo in the cytoplasm and first appear as small membrane structures called isolation membranes (or phagophores). These gradually grow until the cargo is engulfed within the autophagosome. The formation of the autophagosome depends on a number of highly conserved autophagy-related (Atg) proteins, which work together in a hierarchical manner to nucleate and expand the isolation membranes.[4] During selective autophagy, cargo receptors interact with scaffold proteins to direct this machinery towards specific cargos.[5] Although many of the factors have been described, the mechanisms that mediate autophagosome formation are not well understood. For example, the role of Golgi-derived Atg9 vesicles has remained enigmatic. Atg9 vesicles are required for nucleation of the isolation membrane but do not provide the lipids to form the autophagosomal membrane (Figure 1).[6] Instead, the bulk of the lipids appear to be derived from the endoplasmic reticulum (ER).[7] The flow of lipids from one membrane to the other is mediated by lipid transfer proteins.

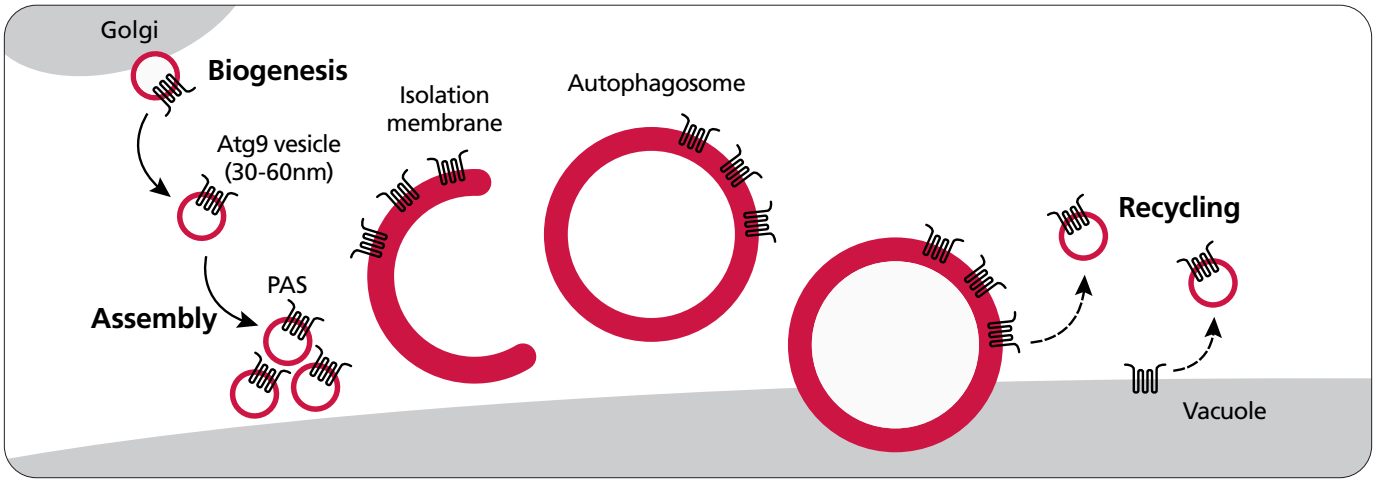


Figure 1: Diagram summarising the role of Atg9 vesicles during autophagosome formation. During starvation, a small number of Atg9 vesicles derived from the Golgi assemble into pre-autophagosomal structures (PAS) to become part of the isolation membrane and ultimately part of the autophagosomal outer membrane. Atg9 vesicles are then recycled back to the cytoplasm (adapted from6).

Atg9 vesicles as seeds for autophagosomes formation

To better understand how Atg9 vesicles work with rest of the autophagy machinery during autophagosome formation, Dr. Martens' team reconstituted a large part of the yeast autophagy machinery in vitro.3 The team developed an elaborate toolkit to isolate and characterize 21 proteins involved in selective autophagy (Figure 2) using recombinant components from yeast. In this way, they were able to reconstitute the early steps of autophagosome biogenesis (nucleation) in a controlled manner in the 'test tube'.

Figure 3 summarises the results of the study. During selective autophagy, Atg9 vesicles are recruited to the prApe1 cargo via the Atg19 receptor and Atg11 scaffold axis. Next, the Atg9 vesicles recruit Atg2-Atg18 and the lipid kinase complex (PI3K). The PI3P binding lipid transfer complex (Atg2-Atg18) recruits Atg21 and the E3-like Atg12–Atg5-Atg16 complex. The membrane-positioned E3-like complex directs Atg8–PE conjugation to the vesicle. Atg8 lipidation is sustained by Atg2-mediated lipid transfer from the ER. The resulting lipid influx expands the isolation membrane until the complete autophagosome is formed.

Therefore, the team concluded that Atg9 vesicles act as seeds which establish membrane contact sites to initiate lipid transfer from compartments such as the endoplasmic reticulum. Importantly, as Atg9 vesicles are abundant in the cell, they can be rapidly recruited when autophagosomes are needed.

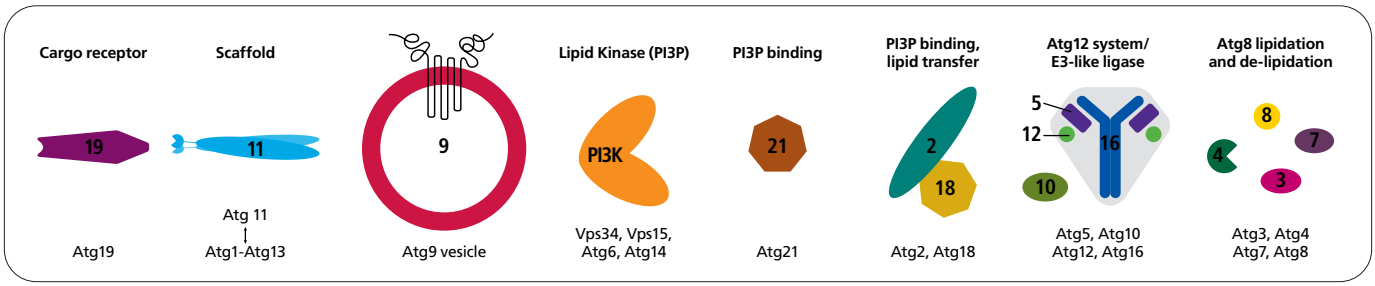


Figure 2: Proteins isolated and characterized in the study by Sawa-Makarska et al. 2020 (adapted from3).

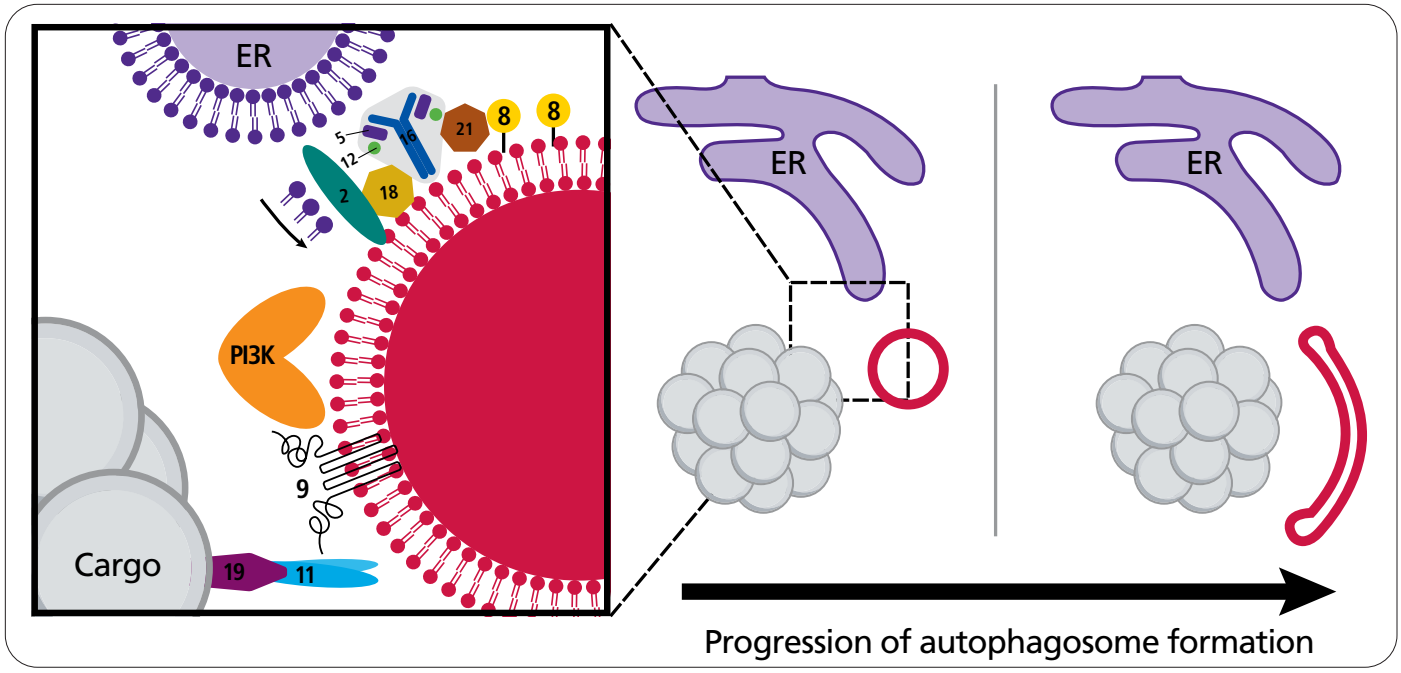


Figure 3: Diagram showing the initial steps of the autophagosome formation during selective autophagy (adapted from3).

Conclusion

In order to understand the functioning of complex machinery, it is often helpful to take it apart and rebuild it. That is what the team of Dr. Martens did to unravel the early steps of autophagosome biogenesis during selective autophagy. Their result showed that Atg9 vesicles recruited other proteins of the autophagy machinery (PI3K, PI3P, Atg21, Atg2-Atg18 and Atg12–Atg5-Atg16 complex). Combining these elements together allows the subsequent incorporation of lipids to the vesicle and the consequent expansion of the membrane for autophagosome formation. Future work will reveal how the recruitment of the autophagy machinery, including the Atg9 vesicles, is sterically and temporally coupled to the formation of membrane contact sites with the ER.

References

1. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell.* 2011;147(4):728-741. doi: 10.1016/j.cell.2011.10.026.

2. Levine B, Kroemer G. Biological functions of autophagy genes: a disease perspective. *Cell.* 2019;176(1-2):11-42. doi: 10.1016/j.cell.2018.09.048.

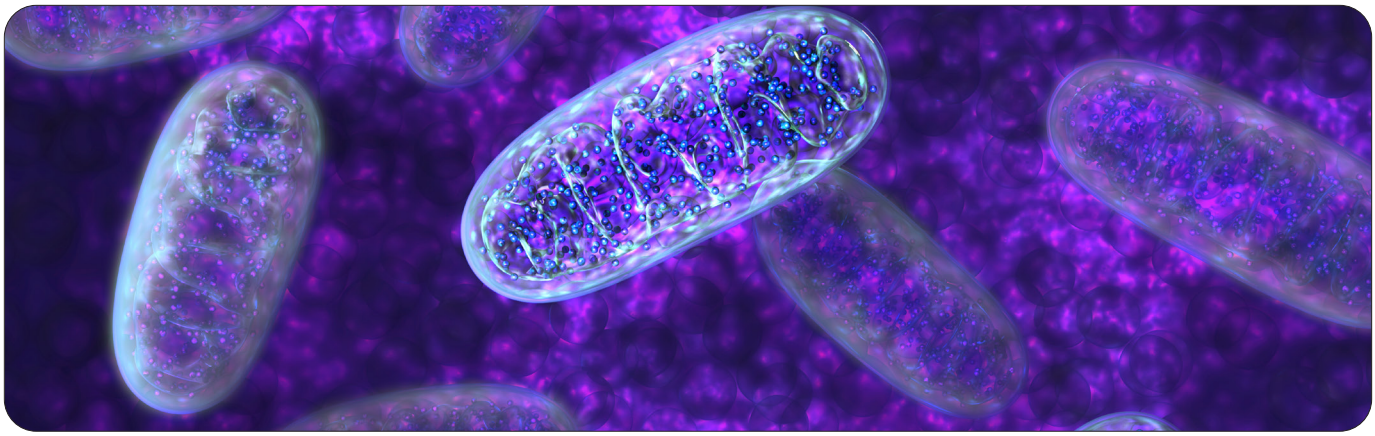
3. Sawa-Makarska J, Baumann V, Coudevylle N, et al. Reconstitution of autophagosome nucleation defines Atg9 vesicles as seeds for membrane formation. *Science.* 2020;369(6508):eaaz7714. doi: 10.1126/science.aaz7714.

4. Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. *Nat. Cell Biol.* 2007;9(10):1102-1109. doi: 10.1038/ncb1007-1102.

5. Turco E, Fracchiolla D, Martens S. Recruitment and activation of the ULK1/Atg1 kinase complex in selective autophagy. *J. Mol. Biol.* 2020;432(1):123-134. doi: 10.1016/j.jmb.2019.07.027.

6. Yamamoto H, Kakuta S, Watanabe TM, et al. Atg9 vesicles are an important membrane source during early steps of autophagosome formation. *J. Cell Biol.* 2012;198(2):219-233. doi: 10.1083/jcb.201202061.

7. Schütter M, Giavalisco P, Brodesser S, Graef M. Local fatty acid channeling into phospholipid synthesis drives phagophore expansion during autophagy. *Cell.* 2020;180(1):135-149.e14. doi: 10.1016/j.cell.2019.12.005.



Autophagy regulation and mitophagy

Under normal conditions, a constitutive basal autophagy ensures intracellular quality control. However, in stress conditions, such as nutrient deprivation, the autophagy process is rapidly induced to maintain the pool of amino acids and ensure cell survival. Multiple regulations in the autophagic pathways occur at both post-translational and transcriptional levels.

Post-translational regulations

The main post-translational modifications involved in autophagy regulation are phosphorylation, ubiquitination, and acetylation. When nutrients are present, the upstream AKT kinase phosphorylates and activates mTORC1, which in turn inhibits autophagy by phosphorylating ULK1 at Ser638 and Ser758, as well as its associated partners ATG13 at Ser389 and AMBRA1 at Ser52. In nutrient deprivation conditions, mTORC1 is downregulated and AMBRA1 is dephosphorylated. This results in ULK1 autophosphorylation at Ser180 and the subsequent phosphorylation of ATG13 (Ser389), ATG101 (Ser11 and Ser203), FIP200 (Ser943, Ser986, Ser1323), and AMBRA1 (Ser465, Ser635). AMP-activated protein kinase (AMPK), which is a nutrient sensor, is a positive regulator of autophagy involved in the activation of ULK1 by phosphorylation at Ser317 and Ser777. In fact, the ULK complex is an upstream hub which integrates and relays the activities of mTORC1 and AMPK.

A complex network of autophagic regulators modulates autophagy either positively (green arrows in Figure 1) or negatively (red arrows in Figure 1). For example, dephosphorylated AMBRA interacting with ULK1 leads to TRAF6 mediated-ULK1 ubiquitination and stabilization; phosphorylation of BCL2 by JNK releases Beclin-1 and promotes autophagy, whereas EGFR and Cdk5 phosphorylate and deactivate Beclin-1 functions. While MAPK15/ERK8 positively regulates autophagy through pro-LC3 phosphorylation, PKA plays the opposite role. Autophagy is inhibited by the acetyltransferase p300, which acetylates ATG7, ATG5, LC3, and ATG12, whereas the deacetylase SIRT1 is a positive autophagy regulator.[1,2,3,4]

Transcriptional regulations

At the transcriptional level, TFEB and FOXO3 are key transcriptional factors which positively regulate both autophagy and lysosomal biogenesis. Transcription Factor EB (TFEB) is considered the master regulator of lysosomal and autophagic function. Inactive TFEB is phosphorylated by mTOR and sequestered in the cytoplasm. Upon dephosphorylation by phosphatases such as Calcineurin, TFEB translocates into the nucleus, where it binds to specific CLEAR DNA sequences and induces the upregulation of proteins involved in lysosome biogenesis and in the autophagy pathway. Whereas the transcription factors HIF1, ATF4, PPARa, or NRF2 also positively regulate autophagy, ZSCAN3, FXR, TCF4, or NFKB down regulate it.[1,5]

Spotlight on mitophagy

Mitochondria are essential organelles that provide cellular energy and contribute to cell death. This organelle is continuously exposed to intra and extra mitochondrial threats. For instance, mitochondrial oxidative phosphorylation produces ATP and by-products such as reactive oxygen species (ROS) that can cause mitochondrial DNA damage.

The removal of damaged mitochondria is critical for maintaining cellular homeostasis, and here mitophagy plays a key role in ensuring a selective control process to maintain mitochondria quality and quantity.

Mitophagy is controlled by two major proteins: PINK1 (PTEN-induced putative kinase 1) which is a serine/threonine-protein kinase, and PARKIN which is an E3-ubiquitin ligase. PINK1 is addressed to heathy polarized mitochondria through a mitochondrial targeting sequence and is processed by matrix processing peptidases (MPP) and the PARL protease in the mitochondrial inner membrane. The 52kD mature form of PINK is then released into the cytosol, where it is ubiquitinated and degraded by the proteasome.

In damaged depolarized mitochondria, PINK1 accumulates on the mitochondrial outer membrane at the TOM complex (Translocase of the Outer Membrane). Following its autophosphorylation, activated PINK1 in turn phosphorylates ubiquitin on serine 65 (Ser65) which promotes Parkin stabilization. In an active conformation, PINK1 directly phosphorylates and fully activates Parkin. Once activated, Parkin ubiquitinates many targets at the Mitochondrial Outer Membrane (MOM), such as mitofusin (MNF), VDAC, or the pro-apoptotic factor BAK, as well as cytosolic proteins. Polyubiquitinated mitochondrial substrates bind to LC3 adapters, such as OPTN or NDP52, which are phosphorylated by TBK1. Finally, damaged mitochondria are trapped in autophagosomes and further degraded by lysosomal enzymes.[6,7,8] Figure 2 showcases the mechanisms in healthy versus damaged mitochondria in more detail.

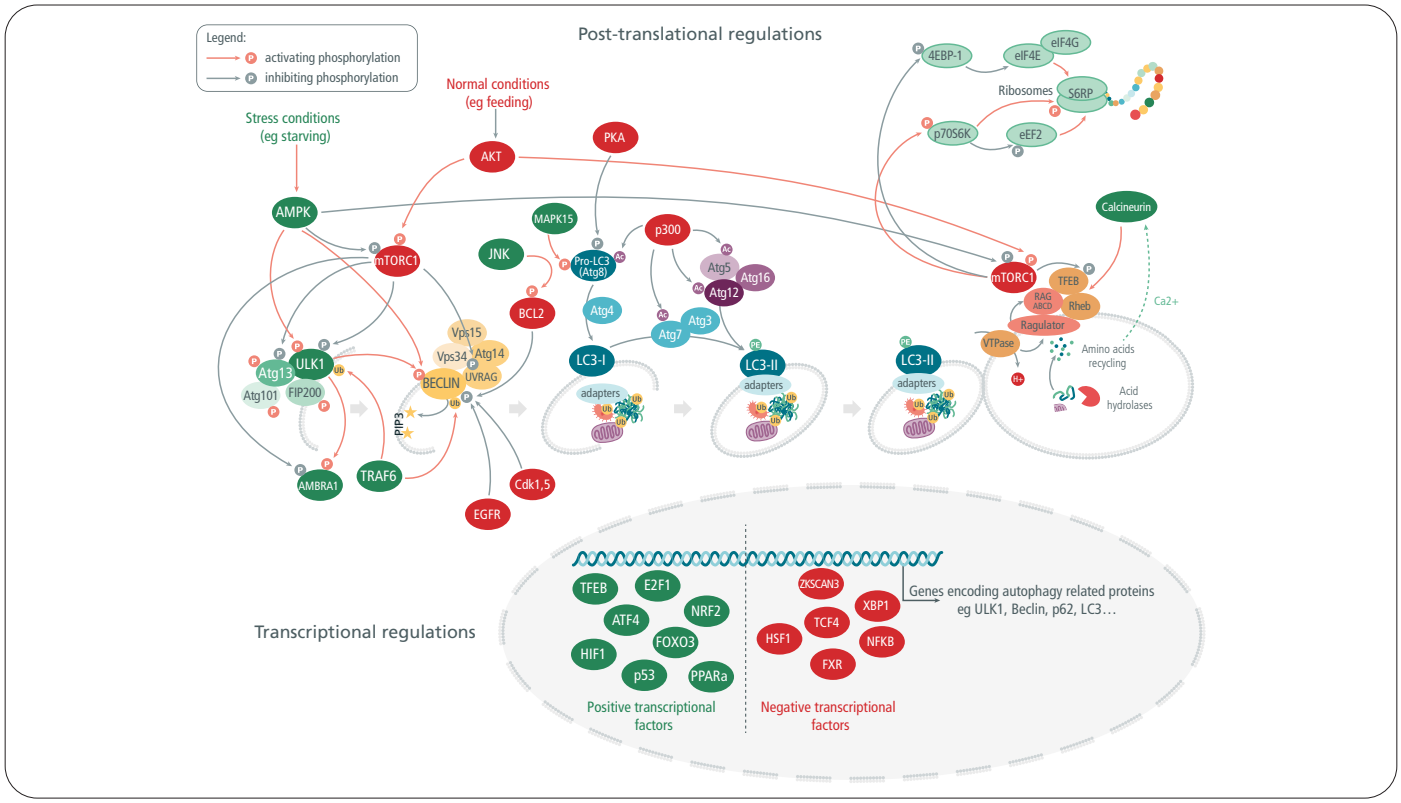


Figure 1: Post-translational regulations vs transcriptional regulations.

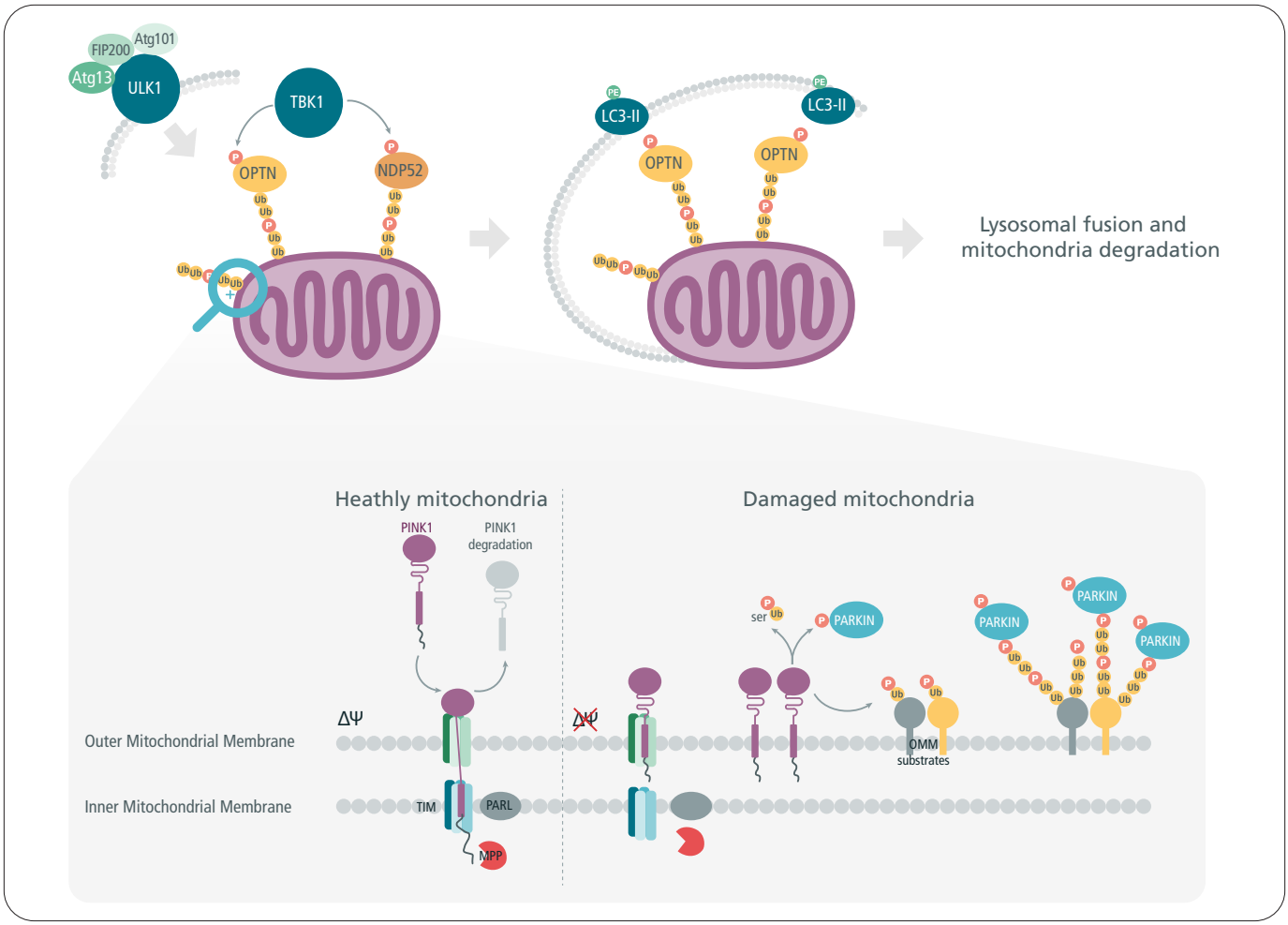


Figure 2: Mechanisms of mitophagy in healthy and damaged mitochondria.

References

1. Botti-Millet A, Nascimbeni C, Dupont N, Morel E, Codogno P. Fine-tuning autophagy: from transcriptional to posttranslational regulation. *Am. J. Physiol. Cell Physiol.* 2016;311:C351–C362. doi: 10.1152/ajpcell.00129.2016.

2. Bach M, Larance M, James DE, Ramm G. The serine/threonine kinase ULK1 is a target of multiple phosphorylation events. *Biochem. J.* 2011;440:283-291. doi: 10.1042/BJ20101894.

3. Chauhan S, Goodwin JG, Chauhan S, et al. ZKSCAN3 is a master transcriptional repressor of autophagy. *Mol. Cell.* 2013;50(1):16–28. doi: 10.1016/j.molcel.2013.01.024.

4. Kim YM, Jung CH, Seo M, et al. mTORC1 phosphorylates UVRAG to negatively regulate autophagosome and endosome maturation. *Mol. Cell.* 2015;57:207-218. doi: 10.1016/j.molcel.2014.11.013.

5. Füllgrabe J, Ghislat G, Cho D-H, Rubinsztein DC. Transcriptional regulation of mammalian autophagy at a glance. *J. Cell Sci.* 2016;129(16):3059-3066. doi: 10.1242/jcs.188920.

6. Seung-Min Yoo, Yong-Keun Jung. A molecular approach to mitophagy and mitochondrial dynamics. *Mol. Cells.* 2018;41(1): 18-26. doi: 10.14348/molcells.2018.2277.

7. Jin SM, Youle RJ. PINK1- and Parkin-mediated mitophagy at a glance. *J. Cell Sci.* 2012;125(4):795-799. doi: 10.1242/jcs.093849.

8. Ashrafi G, Schwarz TL. The pathways of mitophagy for quality control and clearance of mitochondria. *Cell Death Differ.* 2013;20(1):31-42. doi: 10.1038/cdd.2012.81.

The role of autophagy dysregulation in pathology

Autophagy is critical for a range of physiological processes, including the maintenance of cellular homeostasis. Accordingly, defective autophagy mechanisms are linked with a growing list of diseases.[1] This infographic will explore the impact of autophagy dysregulation in various pathologies.

Neurodegenerative diseases

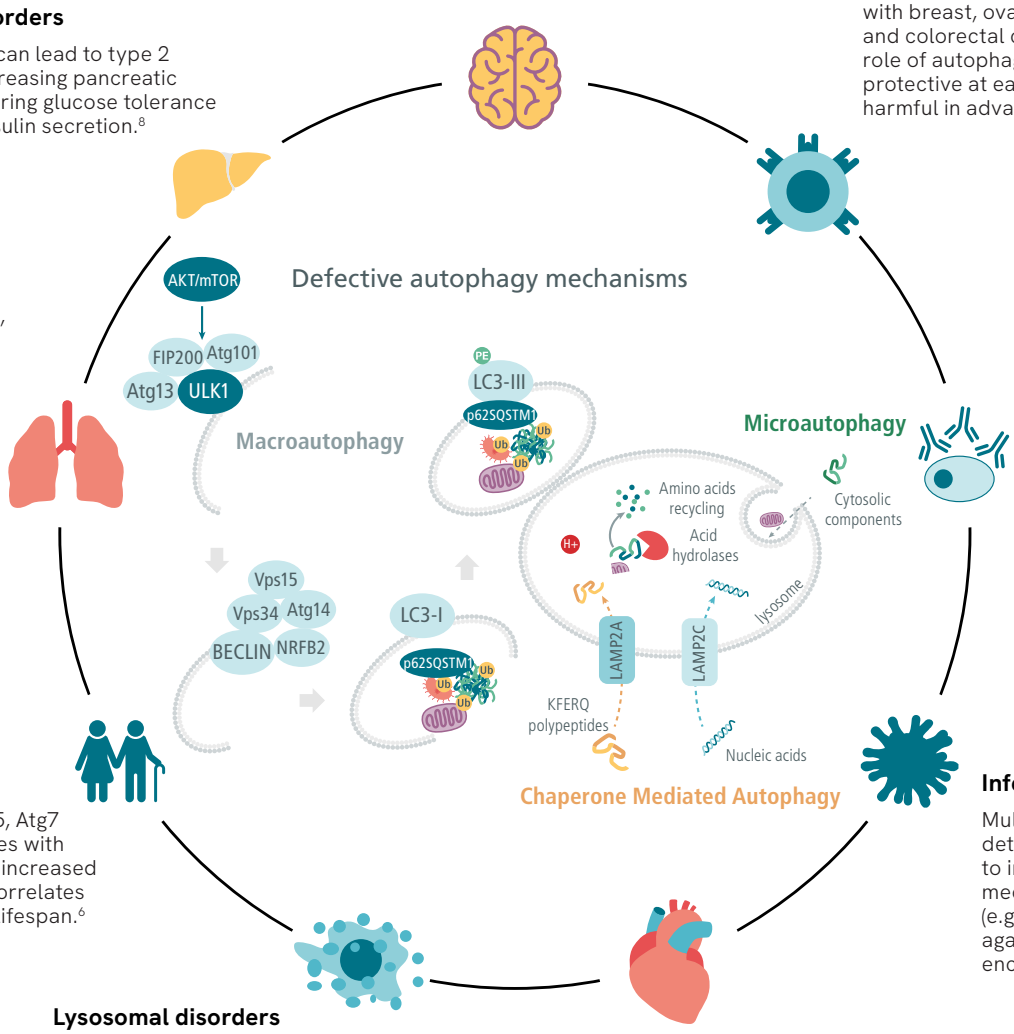
Mutations in the protein a-synuclein that impair its degradation by autophagy are correlated with Parkinson's disease pathogenesis.[2]

Cancer

Beclin deficiency is associated with breast, ovarian, prostate, and colorectal cancers. The role of autophagy seems to be protective at early stages, but harmful in advanced stages.[2]

Metabolic disorders

Atg7 deficiency can lead to type 2 diabetes by decreasing pancreatic beta cells, impairing glucose tolerance and reducing insulin secretion.[8]



Lung diseases

High levels of LC3B-II, Atg4, Atg5-Atg12 complex and Atg7 correlate with chronic obstructive pulmonary disease. Additionally, enhancing autophagy activity is effective against *Mycobacterium tuberculosis infection*.[7]

Autoimmune disease

Mutations in Atg5 genes are associated with autoimmune disease susceptibility such as systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, and multiple myeloma.[2]

Aging

Expression of Atg5, Atg7 and BECN1 declines with age.[5] Additionally, increased levels of Beclin1 correlates with an extended lifespan.[6]

Infectious diseases

Multiple host pathways detect viral infection to initiate autophagy-mediated innate defenses (e.g., Beclin 1 protects against SINV-mediated encephalitis).[3]

Lysosomal disorders

Lysosomal storage disorders (LSDs) are caused by mutations that impair lysosomal function, leading to an accumulation of ubiquitinated proteins and damage to organelles and autophagy proteins such as SQSTM1/p62.[2]

Cardiovascular diseases

Basal levels of cardiomyocyte autophagy are required for cellular proteostasis, e.g., inactivation of Atg5 triggers heart failure. Interestingly, blocking autophagy altogether can also accelerate heart failure.[4]

References

1. Jing K, Lim K Why is autophagy important in human diseases? *Exp. Mol. Med.* 2012;44:69-72. doi: 10.3858/emm.2012.44.2.028.
2. Protein degradation - Guide to the proteasome and autophagy systems. www.revvity.com. Accessed: March 22, 2022.
3. Choi Y, Bowman JW, Jung JU. Autophagy during viral infection — a double-edged sword. *Nat. Rev. Microbiol.* 2018;16:341-354. doi: 10.1038/s41579-018-0003-6.
4. Gatica D, Chiong M, Lavandero S, Klionsky DJ. Molecular mechanisms of autophagy in the cardiovascular system. *Circ. Res.* 2015;116(3):456-467. doi: 10.1161/CIRCRESAHA.114.303788.
5. Aman Y, Schmauck-Medina T, Hansen M. et al. Autophagy in healthy aging and disease. *Nat. Aging.* 2021;1:634-650. doi: 10.1038/s43587-021-00098-4.
6. Barbosa MC, Grosso RA, Fader CM. Hallmarks of aging: an autophagic perspective. *Front. Endocrinol.* (Lausanne). 2019;9:790. doi: 10.3389/fendo.2018.00790.
7. Mizumura K, Cloonan SM, Haspel JA, Choi AMK. The emerging importance of autophagy in pulmonary diseases. *Chest.* 2012;142(5):1289-1299. doi: 10.1378/chest.12-0809.
8. Yang JS, Lu CC, Kuo SC, et al. Autophagy and its link to type II diabetes mellitus. *Biomedicine (Taipei).* 2017;7(2):8. doi: 10.1051/bmdcn/2017070201.

The impact of autophagy research on drug discovery: Q&A with Eileen Gonzalez

Autophagy has been known for over 50 years, but it was only after the brilliant experiments performed in the early 1990's that its fundamental importance in physiology and medicine was recognized.[1,2,3] These studies identified fundamental genes and mechanisms involved in autophagy and the discoveries led to a new paradigm in the understanding of how cells recycle their content. This pioneer research helped to uncover the role of autophagic process in several pathologies including cancer and neurological disease. For these discoveries, Dr. Ohsumi was awarded the Nobel Prize in Physiology or Medicine in 2016.

Eileen Gonzalez completed her PhD at the University of California San Diego (UCSD) where she worked with Dr. Gustafsson, studying the roles of Beclin1 and Beclin2 in autophagy and mitophagy. Using her extensive background in western blotting and microscopy, she is the Associate Product Manager for non-flow antibody applications at BioLegend. In this interview, she discusses the present state of autophagy research in health and disease and the benefits of Revvity's assays in this field.

Q What is autophagy and what role does it play in health and disease?

A Autophagy is a critical cellular process that, at baseline, aids in the turnover of cellular components. In response to starvation, autophagy is increased to recycle amino acids and fatty acids to help cells maintain energy levels. In response to cellular stress, autophagy acts as a quality control mechanism responsible for the removal of damaged protein aggregates and organelles. This process involves the engulfment of cargo by a double membrane structure known as the autophagosome. Autophagosomes then fuse with lysosomes that introduce hydrolytic enzymes to degrade and recycle the enclosed components. Similar to other physiological processes, deregulated autophagy is associated with a number of pathological conditions. Autophagy-associated genes and pathways have been linked to a variety of diseases including neurodegenerative diseases, inflammation, and cancer.

Q How can understanding autophagy help us develop therapies for neurodegenerative disease?

A Since its discovery in 1992, autophagy has been widely studied in the context of both health and disease. Impaired autophagy has been implicated in an assortment of late-onset neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), to name a few. Understanding the mechanism by which autophagy contributes to the development of these conditions can help identify potential therapeutic targets and treatments. For example, alpha-synuclein (SNCA) aggregates, the primary component of Lewy bodies, is the main pathological hallmark of PD. Alpha-synuclein is a substrate of chaperone-mediated autophagy. Analysis of PD patients shows that the expression of LAMP2A and HSPA8 chaperons are decreased, establishing the involvement of compromised autophagy in early PD development. Similarly, in AD mouse models, accumulation of amyloid-beta is accompanied by upregulation of mRNA levels of mitophagy-related genes, such as *Sqstm1, Prkn, Dnm1l, Becn1, Bnip3, Pink1, and Map1lc3*. Although progress has been made, investing in research that expands our understanding of autophagy lends not only to understanding how it directly relates to disease but also offers hope for new therapeutic targets.

Q Can you comment on the switch between protective and non-protective autophagy and how it can impact treatment strategy?

A Within an individual cell, the balance of autophagy is very delicate. When targeting autophagy for therapeutic purposes in the context of disease, in cancer for example, the goal is to stimulate increased rates of autophagy as a protective mechanism. Sometimes however, the elevated rates of autophagy fail to sensitize or protect tumor cells from external treatments and is referred to as non-protective autophagy. To date, there are no distinguishing features of non-protective autophagy to help predict the sensitivity tumor cells will have to either chemotherapy or radiation. This poses a challenge to creating effective treatment solutions when the effectiveness is unpredictable from patient to patient. Thus far, non-protective autophagy has been primarily explored in the context of cancer, however it is highly likely this arises more often as many other disease therapeutics continue to target this pathway.

Q What are the most prominent drug targets currently pursued by Pharmas and Biotechs?

A Several direct or indirect autophagy-enhancing drugs are currently used in patients, including rapamycin, lithium, carbamazepine, and sodium valproate. Therapies that inhibit autophagy have mostly been applied to cancer treatments and include chloroquine and hydroxychloroquine. In general, candidates for drug targeting within the autophagy machinery fall under three types of proteins: kinases, proteases, and ubiquitin conjugating enzymes. Current clinical trials using inhibitors of autophagosome and lysosome fusion show promising results in combinatorial cancer treatment. One of the most exciting findings comes from a recent study in which a candidate drug named CA increases lysosome-associated membrane protein type 2A (LAMP2A) activity, boosting chaperone-mediated autophagy.[4] Using an AD mouse model, the drug significantly reduced levels of tau and amyloid-beta, as well as plaques in the brains of the animals. The treatment also normalized the animals' walking ability and improved visual memory, anxiety- and depression-like behaviors, and neuromuscular strength. Additional efforts to increase treatment options for a variety of diseases in which autophagy may play a role are a major focus in research and are being explored in mouse models.

Q How can Revvity maximize the effectiveness of assays and solutions to help researchers and ultimately patients?

A Revvity can contribute by supporting research that aims to expand our working knowledge of autophagy in health and disease. To that end for example, Revvity's solutions and reagents can provide excellent support to identify individual targets as well as protein-protein interactions within compromised pathways. Understanding tissue structure and microenvironment is crucial in AD, PD, cancer, and many other pathologies linked to autophagy. Additionally, companies that are now part of the Revvity extended family, such as BioLegend and Horizon, are well-positioned to help in that effort. Reagents that support this type of research, such as conjugated antibodies or gene editing tools, provide essential support to deepen our understanding of autophagy dynamics within individual disease states.

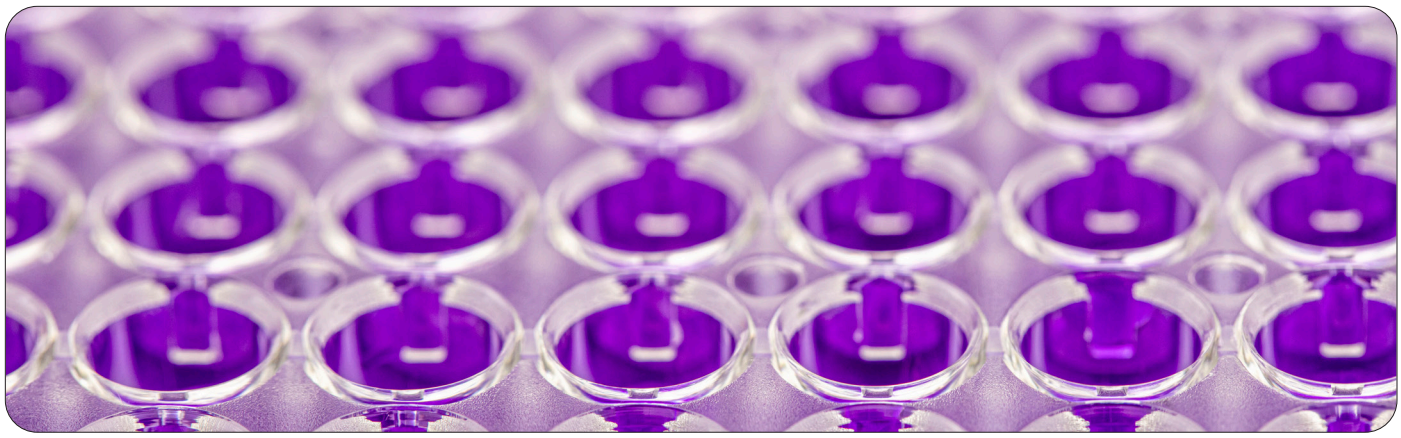
References

1. Takeshige K, Baba M, Tsuboi S, Noda T, Ohsumi Y. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J. Cell Biol.* 1992;119:301-311. doi: 10.1083/jcb.119.2.301.

2. Tsukada M, Ohsumi Y. Isolation and characterization of autophagy-defective mutants of Saccharomyces cervisiae. *FEBS Let.* 1993;333,169-174. doi: 10.1016/0014-5793(93)80398-E.

3. Mizushima N, Noda T, Yoshimori T, et al. A protein conjugation system essential for autophagy. *Nature.* 1998;395:395-398. doi: 10.1038/26506.

4. Bourdenx M, Martín-Segura A, Scrivo A, et al. Chaperone-mediated autophagy prevents collapse of the neuronal metastable proteome. *Cell.* 2021;184(10):2696-2714.e25. doi: 10.1016/j.cell.2021.03.048.



Innovative immunoassay solutions for autophagy research

Immunoassays are used to detect and quantify analytes from various complex samples containing an abundance of proteins and other molecules (e.g., blood, urine or human serum). They have become a fundamental part of scientific research and drug discovery since their introduction in the 1950's. Although traditional immunoassay platforms such as western blots and enzyme-linked immunosorbent assays (ELISAs) are still commonplace throughout research, they are often time- and resource-intensive, involve complex protocols and suffer from limited sensitivity, throughput and detectable range.

Revvity offers a range of ELISA alternative immunoassay technologies, pre-configured kits, and customizable reagents for superior results. From sample compatibility and sample volume, to throughput, reproducibility, automation compatibility, and desired speed of results, these solutions ensure optimal performance of your immunoassay platforms (Figure 1). This article will explore the features of the AlphaLISA and Homogenous Time Resolved Technology (HTRF) immunoassays and their role in autophagy research.

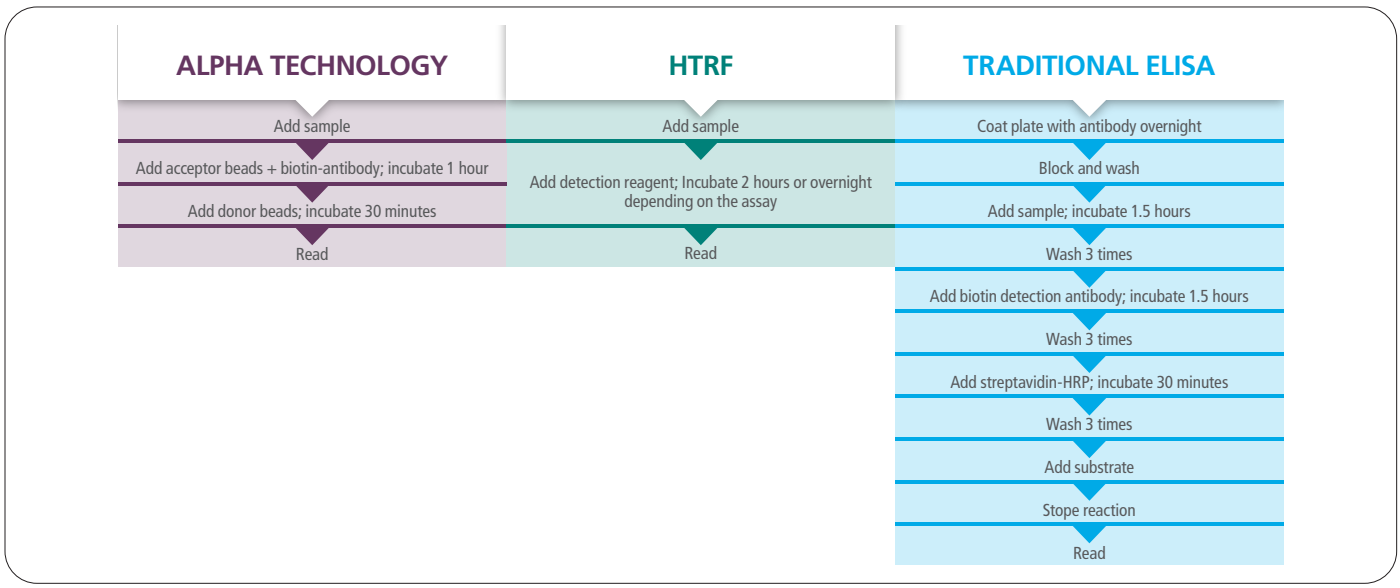


Figure 1: Alpha and HTRF technology vs traditional ELISAs.

Alpha technology

AlphaLISA™ assays are homogenous, no-wash alternatives to ELISAs which enable a simple, streamlined workflow for the detection and quantification of biomolecules. They are bead-based luminescent amplification assays; therefore, they offer greater sensitivity, a wider dynamic range and require smaller sample sizes (~5 µL) compared to traditional ELISAs. Their versatility enables them to detect intracellular and/or membrane-bound analytes, as well as those that are secreted.

As shown in Figure 2A, a biotinylated antibody and an antibody-conjugated AlphaLISA acceptor bead are used to capture the target analyte. After one-hour, the Alpha streptavidin-coated donor bead is added, and the sample is incubated for a further 30-60 minutes. When the analyte is present in the sample, the donor and acceptor beads are brought together. Upon excitation, a photosensitizer inside the donor bead converts ambient oxygen to an excited singlet state. Singlet oxygen diffuses up to 200 nm to produce a chemiluminescent reaction in the acceptor bead, resulting in the emission of light – which is proportional to the amount of analyte present in the sample.

In the AlphaLISA assay, the acceptor beads, anthracene, and rubrene are substituted with an Europium (Eu) chelate. This is directly excited by the 340 nm light resulting from the conversion of thioxene to a di-ketone derivative following its reaction with singlet oxygen. Excited Eu chelate generates an intense light detectable within a narrow wavelength bandwidth centered around 615 nm. The AlphaLISA emission is therefore less susceptible to interference by either artificial or natural compounds, such as hemoglobin, which absorb light between 500-600 nm.

Homogenous Time Resolved Fluorescence technology

AlphaLISA™ assays are homogenous, no-wash alternatives to ELISAs which enable a simple, streamlined workflow for the detection and quantification of biomolecules. They are bead-based luminescent amplification assays; therefore, they offer greater sensitivity, a wider dynamic range and require smaller sample sizes (~5 µL) compared to traditional ELISAs. Their versatility enables them to detect intracellular and/or membrane-bound analytes, as well as those that are secreted.

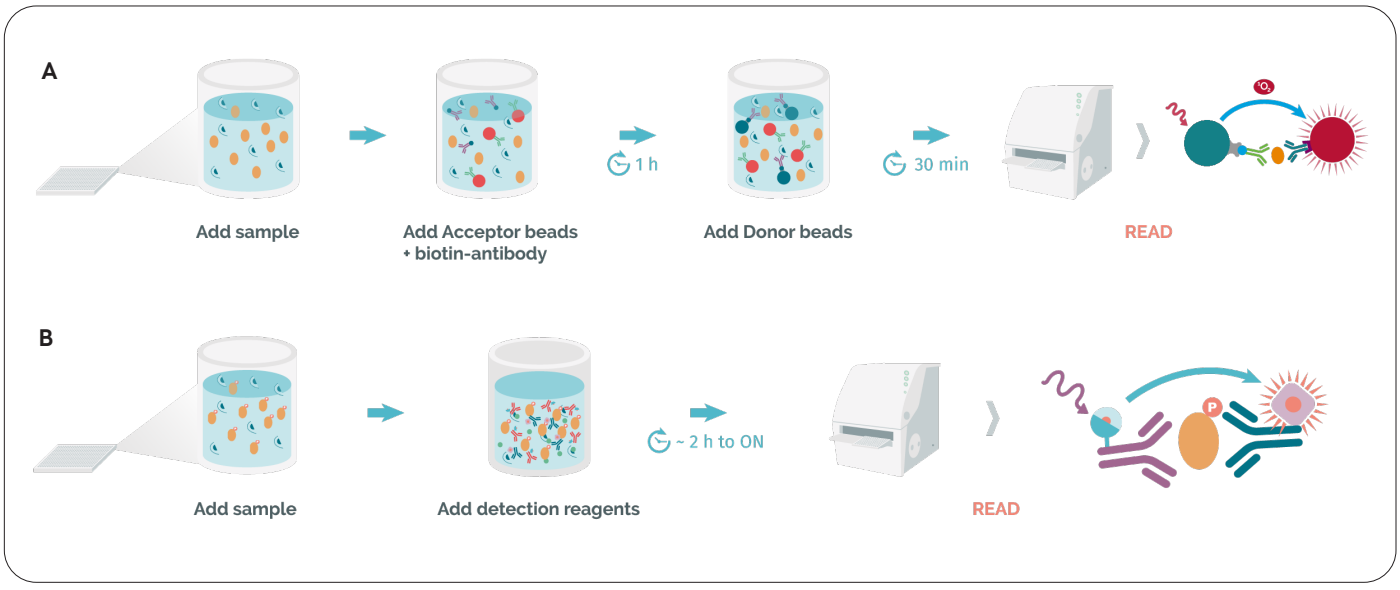


Figure 2: A) The AlphaLISA workflow. B) The HTRF workflow.

Immunoassays for every need

Revvity's immunoassay solutions are expanding research possibilities for a growing number of applications. This section will explore their role in autophagy research in more detail (Table 1).

Table 1: A summary of the autophagy related products offered by Revvity.

Immunoassay			Target
HTRF™	AlphaLISA	AlphaLISA™ *SureFire*® *Ultra*™	
HTRF phospho-mTOR (Ser2448)		AlphaLISA *SureFire Ultra* phospho-mTOR (Ser2448)	phosphorylated mTOR
HTRF Phospho-Ubiquitin (Ser65)		AlphaLISA *SureFire Ultra* Human Phospho-Ubiquitin (Ser65)	phosphorylated Ubiquitin at Ser65
HTRF Phospho-p62/SQSTM1 (Ser403)		AlphaLISA *SureFire Ultra* Human and Mouse Phospho-SQSTM1 p62 (Ser403)	phosphorylated p62/ STSQM1
HTRF phospho-ULK1 (Ser757/Ser758)		AlphaLISA *SureFire Ultra* Human Phospho-ULK1 (Ser556) AlphaLISA ***SureFire Ultra*** Human Phospho-ULK1 (Ser758)	phosphorylated ULK1
HTRF phospho-AMPK (Thr172)		AlphaLISA *SureFire Ultra* Phospho-AMPKα 1/2 (Thr172)	phosphorylated AMPK at Threonine 172
HTRF phospho-TBK1 (Ser172)		AlphaLISA *SureFire Ultra* Phospho-TBK1 (Ser172)	phosphorylated TBK1 at Ser172
HTRF Phospho-BAD (Ser112)			phosphorylated BAD at Ser112
HTRF Cleaved PARP (Asp214)			Cleaved PARP
HTRF TOM20			TOM20
	AlphaLISA Total Beclin-1		Beclin-1
	AlphaLISA Total ATG16L1	AlphaLISA *SureFire Ultra* phospho-ATG16L1 (Ser278)	Atg16L1
	AlphaLISA human LC3B		LC3B
HTRF Phospho ATG14 (Ser29)			phosphorylated Atg14 at Ser29
HTRF Phospho ATG16			phosphorylated Atg16 at Ser278

Detecting general mechanisms of autophagy

Lysosomal dysregulation is implicated in many neurodegenerative diseases, including aging. Tracking proteins associated with the lysosome therefore allows researchers to investigate the mechanisms of autophagy. Sequestosome-1, also known as p62, is incorporated into autophagosomes before degradation in the final steps of autophagy. Visualizing p62 puncta helps to determine a block or increased flux in autophagy, however traditional methods are time-intensive, have a low throughput and require specialized equipment. The homogeneous no-wash AlphaLISA™p62 assay accurately quantify p62 expression levels during autophagy with and without treatment with chloroquine, a common inhibitor of autophagy. The AlphaLISA p62 assay has a large dynamic range, is adaptable to various cell types and can provide quantitative p62 data faster than immunofluorescent staining.

Huntington's Disease

The HTT gene contains a repeat of CAG codon coding for glutamine. When the gene contains 40 or more repeats, the resulting mutation (mHTT) produces an altered protein that leads to neuronal death. High levels of mHTT correlate with the development of Huntington's Disease (HD), therefore, lowering soluble mHTT may provide an effective approach to treat HD by ameliorating downstream toxicity. In this context, several drug discovery strategies have been investigated to treat HD by lowering the levels of mHTT. For example, Li et al. investigated if compounds targeting both the mHTT and the autophagosome protein microtubule-associated protein 1A/1B light chain 3 (LC3) can reduce the levels of the mutant protein. Using HTRF, they found four compounds that were able to bind both proteins and reduce mHTT levels by inducing autophagy clearance of mHTT.1 These results showed that modulation of the autophagy pathway may represent a new paradigm for treating HD. Moreover, the therapeutic effect of the active compounds found in this study could also be extended to treat other poly-Q diseases.

BioLegend offers products that assist in both the detection and visualization of autophagy in cells and tissue. As a trusted source of antibodies, BioLegend's autophagy specific antibodies are applicable for western blotting, immunoprecipitation and microscopy applications (Table 2). Expanding on imaging approaches, BioLegend also carries various chemical probes that can aid in the observation of the cellular environment when autophagy is upregulated as well as some of the target cargo this process can degrade (Table 2).

Table 2: A summary of the autophagy related antibodies and chemical probes offered by BioLegend

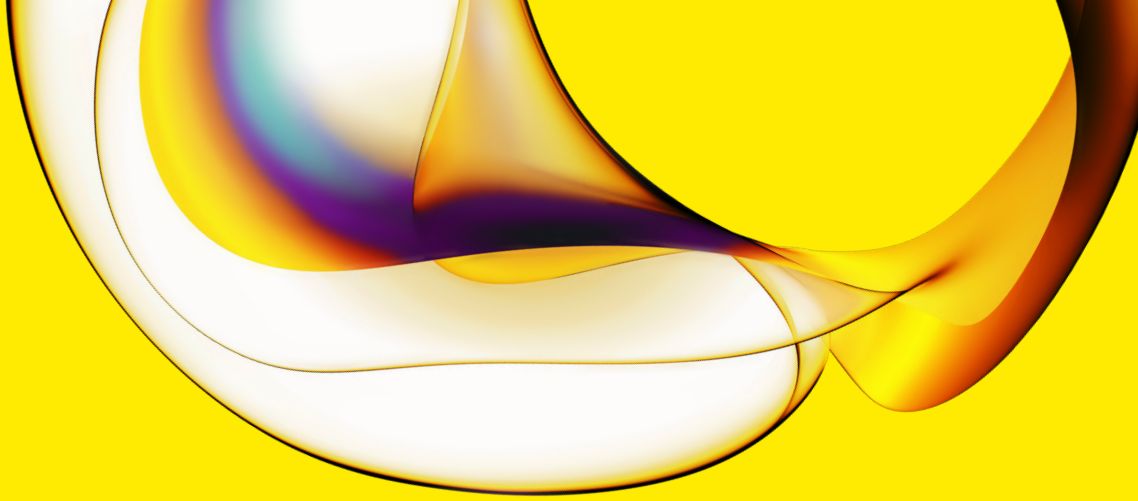
Antibodies	Chemical probes and other products
mTOR	Apotracker
mTOR Phospho (Ser2448)	MitoSpy
Beclin-1	Zombie dye
ATG5	MojoSort
ATG17	
ATG3	
ATG1	
Autophagy antibody sampler kit	
LC3	
Ubiquitin	
Rab7A	
LAMP1	
LAMP2	
p62	
Rab8	
TFEB	
Optineurin	
FBX07	
Tollip	
VPS35	
Parkin	
PINK1	

Conclusion

Western blots and ELISAs have traditionally been the preferred assays for research and drug discovery workflows. However, the solutions offered by Revvity provide several advantages over these technologies, including ease of use and scalability, without compromising on sensitivity and specificity. HTRF technology can be used in competitive and non-competitive formats or performed as cellular or biochemical assays. As a result, it can be used in a variety of applications including GPCRs, kinases, epigenetics, and quantification of a range of biomarkers including cytokines. Similarly, Alpha technology is compatible with a wide range of sample types ranging from simple buffers, cell supernatants, and cell lysates to complex tissue, serum, plasma, and other biological samples.

References

1. Li Z, Wang C, Wang Z, et al. Allele-selective lowering of mutant HTT protein by HTT–LC3 linker compounds. *Nature.* 2019;575:203–209. doi: 10.1038/s41586-019-1722-1.



Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com

1129668 (893117)

www.revvity.com