# Regulation of autophagic flux via dynamic trafficking of the SNARE Stx17: Studies using a chemical biology approach

Thesis submitted for the degree of

**DOCTOR OF PHILOSOPHY**

by

Somya Vats


Molecular Biology and Genetics Unit

Jawaharlal Nehru Centre for Advanced Scientific Research

Jakkur, Bangalore-560064

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# DECLARATION

I hereby declare that the work described in this thesis entitled **'Regulation of autophagic flux via dynamic trafficking of the SNARE Stx17: Studies using a chemical biology approach'** is the result of investigations carried out by myself under the guidance of **Dr. Ravi Manjithaya** at Autophagy Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore-560064, India. This work has not been submitted elsewhere for the award of any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described has been based on findings of other investigators. Any omission, which might have occurred by oversight or misjudgement, is regretted.

Somya Vats

Place: Bangalore

Date:

Jawaharlal Nehru Center for Advanced Scientific Research
Jakkur Campus, Jakkur Post
Bengaluru 560064, INDIA
Office Tel: +91(80)22082924
Office Fax: +91(80)22082766
+91(80)22082767
Email: ravim@jncasr.ac.in

Ravi Manjithaya, PhD
Associate Professor
Molecular Biology and Genetics Unit
JNCASR, Bangalore

# CERTIFICATE

This is to certify that the work described in this thesis entitled **'Regulation of autophagic flux via dynamic trafficking of the SNARE Stx17: Studies using a chemical biology approach'** is the result of investigations carried out by Ms. Somya Vats at Autophagy laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, under my supervision and guidance. The results presented here have not previously formed the basis for the award of any degree or diploma.

November, 2019 Dr. Ravi Manjithaya

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# Abbreviations

| | |
|---|---|
| nM | nanomole |
| µm | micrometre |
| µM | micromole |
| ATP | Adenosine triphosphate |
| BafA1 | Bafilomycin $A_1$ |
| CCCP | Carbonyl cyanide 3-chlorophenylhydrazone |
| CQ | Chloroquine |
| DFCP1 | Double FYVE domain-containing protein 1 |
| EACC | Ethyl (2-(5-nitrothiophene-2-carboxamido) thiophene-3-carbonyl) carbamate |
| EBSS | Earle's balanced salt solution |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| FDA | Food and drug administration |
| GFP | Green fluorescent protein |
| GM | Growth medium |
| HOPS | Homotypic fusion and vacuole protein sorting |
| LC3 | Microtubule-associated proteins 1A/1B light chain 3B |
| Mdivi1 | Mitochondrial division inhibitor 1 |
| mTOR | mammalian target of rapamycin |
| RFP | Red fluorescent protein |
| SNARE receptor | Soluble N- ethylmaleimide sensitive factor attachment protein |

| | |
|---|---|
| Stx17 | Syntaxin17 |
| SEM | Standard error of mean |
| ULK1 | Unc-51 like autophagy activating kinase 1 |
| WIPI2 | WD repeat domain phosphoinositide-interacting protein 2 |

# Thesis Synopsis

# Regulation of autophagic flux via dynamic trafficking of the SNARE Stx17: Studies using a chemical biology approach

Submitted by

**Somya Vats**

Molecular Biology and Genetics Unit

Jawaharlal Nehru Centre for Advanced Scientific Research

Jakkur, Bangalore-560064, India

**Thesis Advisor: Dr. Ravi Manjithaya**

## Introduction

Macroautophagy (herein autophagy) is a major intracellular pathway that is indispensable for maintaining cellular homeostasis. Autophagy has been evolutionarily conserved in organisms ranging from yeast to humans. The word autophagy, which is Latin for self-eating, was first coined by Christian de Duve (Deter *et al.*, 1967). He identified autophagy as a process of self-cannibalization which involves sequestration of cellular components within double membrane structures called autophagosomes, which then fuse with lysosomes to form autolysosomes wherein complex macromolecules are degraded and recycled for cellular use. The rate at which dynamic turnover of cellular components occurs by the process of autophagy is called 'autophagic flux'. Basal level of autophagy occurs in all cells and tissues, and is indispensable for maintaining cellular homeostasis by protein and organelle turnover (Mizushima *et al.*, 2004; Musiwaro *et al.*, 2013). Cellular stresses such as nutrient starvation, pathogen invasion, organellar damage and protein aggregation

can increase levels of autophagy as an adaptive stress response. As autophagy is central to maintaining cellular homeostasis, dysfunctional autophagy has been attributed to a variety of major human disease such as cancer, neurodegeneration and cardiovascular diseases (Jiang and Mizushima, 2013).

**Relevance of the study**

The importance of autophagy in human physiology is well documented. Dysfunctional autophagy has been implicated in neurodegenerative diseases, intracellular infections and cancers. Pharmacological modulation of autophagy to restore its levels is being pursued as an attractive therapeutic approach (Galluzzi L *et al.*, 2017). A big challenge in the application of autophagy modulators in therapy is the lack of specific and potent inhibitors. Several popular autophagy inhibitors such as Bafilomycin A1 and Chloroquine inhibit autophagy as a result of affecting lysosomal pH and hence are not specific autophagy modulators.

In this work, we have characterized a novel small molecule inhibitor of autophagy EACC which blocks autophagic flux by preventing autophagosome-lysosome fusion. Interestingly, EACC acts specifically on autophagy and does not affect other vesicular trafficking pathways such as endocytosis. Further investigations into its mechanism of action showed that EACC affects the translocation of autophagy specific SNARE Stx17 and its partner SNARE SNAP29 on autophagosomes without hindering the completion of autophagosomes. Additionally, EACC mediated block in autophagy is reversible, and upon removing EACC Stx17 can translocate to autophagosomes and mediate autophagosome-lysosome fusion. As Stx17 trafficking is quite dynamic, molecules like EACC can be used as a tool to study Stx17 trafficking as well as to identify molecular players involved in this process.

**Chapter 1: General introduction and scope of the present investigation**

Chapter 1 contains the general introduction about the history of autophagy and the physiological roles of autophagy. Different types of autophagy and the steps involved in process of autophagy have also been covered. The chapter also covers the role of autophagy in health and disease with special emphasis on neurodegeneration, cancer and intracellular infections. Finally, this chapter summarizes the need for modulation of autophagy in therapeutics. It talks in detail about pharmacological modulation of autophagy by the use of autophagy inducers and inhibitors and the need for more specific and potent modulators.

**Chapter 2: Identification of EACC as a novel and selective inhibitor of autophagic flux**

This chapter describe the assays performed to establish EACC as a late stage inhibitor of autophagic flux. We showed that EACC inhibits autophagosome-lysosome fusion in a dose dependent manner and causes accumulation of autophagic cargo, p62. EACC treatment does not affect cell viability within the intended treatment time. We also showed that EACC selectively inhibits autophagic flux but does not affect lysosomes or other vesicular trafficking events culminating at lysosomes.

**Chapter 3: Step-by-step dissection of the effect of EACC on the process of autophagy**

In the previous chapter, we performed several assays to establish EACC as a selective inhibitor of starvation induced autophagic flux. In this chapter, we have tried to understand which step of autophagy is inhibited by EACC. By a step-by-step study of the autophagy process, we conclude that EACC does not affect early steps of autophagy, i.e., autophagy signalling, formation and elongation of the isolation membrane or cargo capture by autophagosomes.

**Chapter 4: EACC reversibly affects translocation of autophagosomal SNARE Stx17 onto autophagosomes**

In this chapter, we perform several assays to obtain a detailed mechanistic insight into the action of EACC. We showed that EACC affects the translocation of autophagy specific SNARE Stx17 and its partner SNARE SNAP29 on autophagosomes. EACC inhibits the interaction of Stx17 with the tethering complex HOPS and the lysosomal SNARE VAMP8. We conclude that EACC renders autophagosomes 'fusion incompetent' but does not affect the fusion competence of the lysosomes. Interesting, the action of EACC is reversible and washing out EACC allows Stx17 translocation onto autophagosomes and rescues autophagic flux.

**Chapter 5: EACC sheds light on the regulation of autophagic flux via Stx17 trafficking**

In chapter 5, we discuss the established role of Stx17 in mitochondrial dynamics in conjunction with mitochondrial fission regulator Drp1. We show that presence of EACC enhanced the interaction between Stx17 and Drp1. Furthermore, inhibiting this interaction between Stx17 and Drp1 by either genetic or chemical means not only allowed Stx17 translocation onto autophagosomes, but promoted autophagosome-lysosome fusion even in presence of EACC.

**Chapter 6: Discussion and future directions**

Here we summarize all the chapters, discuss the implications of our findings and also describe how this work could be taken ahead in future to address some of the major questions in the field of autophagy.

The work described in Chapters 2-4 have been published in Molecular Biology of the Cell

Vats S and Manjithaya R. **A reversible autophagy inhibitor blocks autophagosome-lysosome fusion by preventing Stx17 loading onto autophagosomes.** Mol Biol Cell., 2019 Aug 1; 30(17):2283-2295.

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**Publications and patents from the work are as follows:**

1. Vats S and Manjithaya R. **A reversible autophagy inhibitor blocks autophagosome-lysosome fusion by preventing Stx17 loading onto autophagosomes.** Mol Biol Cell., 2019 Aug 1; 30(17):2283-2295.
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7. Indian Patent Application No. 6596/CHE/2015 Title: Modulator and modulation of autophagy and applications thereof. Inventors: Ravi Manjithaya, Aravinda Chavalmane, Piyush Mishra, Suresh SN, Somya Vats and Veena A. 2015. International patent applied (PCT Application No. PCT/IB2016/057498).

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# Introduction

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# Introduction

## 1.1 Overview

Cells are dynamic entities and hence are constantly exposed to physical and chemical stressors. In order to survive and perform at an optimal level, cells must maintain a constant environment within. This continuous strive to resist change and maintain a steady state is termed as cellular homeostasis. One of the requirements to achieve cellular homeostasis is the proper quality control of cellular components like proteins and organelles. Molecular chaperones aid in proper folding of proteins and in refolding of misfolded proteins but if even after intervention by chaperones a protein remains improperly folded, then it is targeted to the proteolytic system. Ubiquitin-proteasome system and autophagy are the two major proteolytic (quality control) pathways in the cells. Along with transcription and translation, protein degradation helps to maintain a steady state.

Ubiquitin-proteasome system degrades short-lived and soluble unfolded proteins, but long-lived proteins and insoluble protein aggregates are taken care of by autophagy. The degradative potential of autophagy is not limited to proteins. Autophagy is the only mechanism by which cells can get rid of damaged or superfluous organelles. Another unique feature of autophagy is the recycling of simpler biomolecules. Since it culminates at the lysosomes which are the cellular hubs for degradative enzymes, autophagy can degrade macromolecules such as lipids, proteins and nucleotides into their basic constituents which are further recycled back into the cytoplasm for reuse. This feature of autophagy makes it indispensable in adaptive stress response. Mechanistically, autophagy is an evolutionarily conserved intracellular degradation pathway in which cytoplasmic components are captured in double membrane autophagosomes and are transported to lysosomes for degradation and recycling. The upcoming subheadings would aim at

explaining the genesis of this field, its molecular mechanism and its rising impact on human health and disease.

## 1.2 History of macroautophagy

Nobel Prize winning Belgian cytologist and biochemist Christian de Duve coined the term 'autophagy' in 1963 at the Ciba Foundation Symposium on lysosomes. His ingenuity and expertise in biochemical methods had led to the discovery of lysosomes in 1955. Several groups (Ashford and Porter, 1962; Clark, 1957; Novikoff, 1959; Novikoff and Essner, 1962) reported the existence of a mechanism by which cells could isolate their own components in membrane compartments which were later shown to contain lysosomal enzymes (Novikoff and Essner, 1962). A comprehensive review written by Christian de Duve and Robert Wattiaux (De Duve and Wattiaux, 1966) which sought out to assemble all available knowledge involving lysosomes and associated structures at that point of time coined the term 'autophagic vacuole' for membrane-lined vacuoles containing morphologically recognizable cytoplasmic components.

The earliest links for the role of autophagy in catabolism were established when multiple reports suggested that autophagy in rat liver cells was increased by nutrient limitation or glucagon treatment and decreased by insulin (Amherdt et al., 1974; Deter and De Duve, 1967; Pfeifer, 1978). Seglen et al., reported that presence of amino acids particularly leucine can strongly inhibit the rate of autophagy (Seglen and Gordon, 1984). Another paper by the same group identified 3-methyladenine (3-MA) as the first specific inhibitor of autophagy (Seglen and Gordon, 1982).

The knowledge that autophagy is inhibited by the presence of amino acids in rat hepatocytes led to the establishment of reciprocal link between the phosphorylation of ribosomal S6 protein kinase and autophagy. Rapamycin, a molecule which could dephosphorylate and

inhibit ribosomal S6 protein kinase was identified as an inducer of autophagy (Blommaart et al., 1995).

In the thirty years following these discoveries, autophagy was mainly studied using biochemical methods and electron microscopy. However, molecular characterization of proteins involved in autophagy remained unaccomplished. Major breakthrough in this regard came through when Yoshinori Ohsumi performed the first genetic screen in yeast to dissect the autophagic process. Takeshige et al., first characterized the process of autophagy in yeast (Takeshige et al., 1992). Yeast vacuole is easily observable under a phase contrast microscope and when exposed to nitrogen starvation, there is an accumulation of autophagic bodies in the vacuole. Tsukada and Ohsumi identified the first autophagy-defective mutant, *apg1* which did not show any accumulation of autophagic bodies even in nitrogen starvation conditions (Tsukada and Ohsumi, 1993). They also found 15 complementation groups which showed phenotypes similar to the *apg1* mutant. This led to the identification of first 15 autophagy *(apg)* related genes. Baba et al., showed that the process of autophagy in yeast is quite similar to the lysosomal system in mammalian cells (Baba et al., 1994). As mentioned earlier, the very first studies in the field of autophagy were mostly conducted on rat hepatocytes but the genius shift to the yeast system meant relative ease of genetic manipulation which revolutionized the search for autophagy genes. The 2016 Nobel Prize in Physiology or Medicine was awarded to Yoshinori Ohsumi ‘for his discoveries of mechanisms for autophagy’.

Almost at the same time as Ohsumi, independent groups around the globe (Michael Thumm, Daniel J Klionsky, William A Dunn, Suresh Subramani, Yasuyoshi Sakai and Marten Veenhuis) also identified autophagy related genes involved in macroautophagy and selective autophagy using the power of yeast genetics (Harding et al., 1995; Mukaiyama et al., 2002; Sakai et al., 1998; Thumm et al., 1994; Titorenko et al., 1995; Yuan et al., 1997).

These genes also partially overlapped with the list of genes identified by Ohsumi. In 2003, the autophagy community agreed on a unified nomenclature for ***A**u**T**opha**G**y-related* genes *(Atgs)* (Klionsky et al., 2003).

Mizushima et al., showed the importance of ATG5-12 conjugation system in autophagosome biogenesis which was conserved from yeast to mammals (Mizushima et al., 1998a). The second ubiquitin-like system involved in ATG8/LC3 lipidation was almost simultaneously identified in yeast and mammalian cells (Ichimura et al., 2000; Kabeya et al., 2000). LC3 has been studied extensively since and is considered as an autophagosomal marker.

The importance of autophagy in human health and disease came into the picture with the identification of an essential autophagy gene Beclin1 as a tumor suppressor which was mono-allelically deleted in 40-75% of sporadic breast and ovarian cancers (Liang et al., 1999). This was a major finding suggesting the role of autophagy in preventing tumorigenesis. Later studies showed that the role of autophagy in cancer is highly context dependent and varies greatly depending on the type, stage and genetic makeup of the tumor (White, 2012). A study by Ravikumar et al., showed that autophagy is responsible for degradation of huntingtin aggregates (Ravikumar et al., 2002). Studies like these and several others shed light on the neuroprotective role of autophagy. The most notable studies on the role of autophagy in controlling intracellular infections were performed by Tamostu Yoshimori and colleagues who showed intracellular pathogens like *Streptococcus pyrogens* and *Salmonella typhimurium* trapped in autophagosome-like vesicles. Gutierrez et al., reported that autophagy is a defense mechanism that protects macrophages against *Mycobacterium tuberculosis* infection (Gutierrez et al., 2004a).

The last decade saw a surge in research into methods to modulate autophagy including extensive research to identify chemical activators and inhibitors of autophagy which could be of therapeutic interest for a variety of disorders. Hydroxychloroquine (HCQ), a derivative of a well-studied autophagy inhibitor chloroquine (CQ) was the first autophagy modulator to be approved by the Food and Drug Administration (FDA) for use in a variety of cancers in conjunction with chemotherapeutic agents.

The field of autophagy has seen an exponential growth between the 1990s and the present time both in terms of understanding the mechanism as well as its roles in health and disease. However, a lot more work is required to unravel several underlying questions in the field and to link the knowledge to autophagy's role in human physiology, disease and therapeutics.

### 1.3 Autophagy: Process and Function

As mentioned in earlier sections, macroautophagy (herein autophagy) is a lysosomal mediated degradation pathway which is highly evolutionarily conserved from lower to higher eukaryotes. Autophagy genes were first identified in yeast and later homologs of most of them were identified in mammalian cells. The rate at which the dynamic turnover of cellular components takes place via the process of autophagy is called autophagic flux. As autophagy is a multistep process, it is important to study the autophagic flux which accounts for all the steps of autophagy.

Using transgenic mice expressing the fluorescent autophagosome marker GFP-LC3, Mizushima et al. showed that although autophagy is induced in all tissues as a response to nutrient starvation, the rate of autophagic flux varies significantly across tissues. Muscle fibres had the highest autophagic flux whereas significant levels of autophagy were not observed in the brain even 48 hours post starvation. They even found differences in the

basal autophagic flux because several tissues such as thymic epithelial cells, lens epithelial cells and podocytes as well as some exocrine gland cells such as gastric chief cells and seminal vesicle cells showed high basal levels of autophagy even under nutrient rich conditions (Mizushima et al., 2004).

## 1.4 Types of autophagy

Depending on how the cargo is sequestered, autophagy is of three types: Macroautophagy, Microautophagy and Chaperone mediated autophagy (CMA). In Macroautophagy, cargo is sequestered in double membrane autophagosomes and taken to lysosomes. Microautophagy is the direct invagination of cargo into the lysosomal lumen. This can occur in bulk or selectively with the help of the chaperone Hsc-70 (Li et al., 2012). Chaperone mediated autophagy (CMA) is the targeted delivery of proteins harbouring the pentapeptide KFERQ to the lysosomes. The proteins are bound to chaperones such as Hsc-70. This protein-chaperone complex then binds to the lysosomal membrane receptor LAMP-2A which helps in its internalization into lysosomes (Kaushik and Cuervo, 2018). Depending on the selectivity of cargo, autophagy is of two types: general and selective autophagy. The core machinery required for the process is largely same in both general and selective autophagy. The first selective autophagy receptor to be identified in mammalian cells was p62/SQSTM1 (Bjorkoy et al., 2005). p62 can bind to ubiquitinated proteins via the C-terminal Ubiquitin-associated (UBA) domain and to LC3 by the LC3 interacting region (LIR) motif (Bjorkoy et al., 2009). Depending on the cargo captured, several subcategories of selective autophagy have now been characterized.

### 1.4.1 Aggrephagy

Selective degradation of protein aggregates by autophagy is termed as aggrephagy. Accumulation of undegraded protein aggregates due to dysfunctional autophagy is the major hallmark of most age-related neurodegenerative disorders. Aggregate prone proteins

like alpha-synuclein (Winslow et al., 2010), β-Amyloid (Pickford et al., 2008) and huntingtin (Ravikumar et al., 2004) are very well studied autophagy substrates. p62/SQSTM1, Neighbour of BRCA1 (NBR1) and Optineurin (OPTN) are autophagic adaptor proteins implicated in aggrephagy (Kirkin et al., 2009; Korac et al., 2013; Pankiv et al., 2007). A PI3P- binding protein Alfy has been reported as a scaffold in autophagy. Alfy binds to and facilitates complex formation between p62 and autophagy proteins (Filimonenko et al., 2010).

### 1.4.2 Mitophagy

Mitochondria has indispensable roles in maintaining and regulating cellular energetics. Mitophagy is the selective degradation of superfluous or damaged mitochondria. It was first observed by Rodriguez-Enriquez et al. in cultured rat hepatocytes treated with glucagon in absence of serum (Rodriguez-Enriquez et al., 2006). Improper removal of damaged mitochondria can lead to Reactive oxygen species (ROS) accumulation and increased oxidative stress in cells. Mitophagy has important physiological roles. During erythrocyte development, removal of mitochondria in reticulocytes occurs by mitophagy (Sandoval et al., 2008). Decrease in mitophagy is linked to several neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Kurihara et al., 2012; Redmann et al., 2014). Major regulators of mammalian mitophagy are mitochondrial kinase PINK1 and an E3 ubiquitin ligase Parkin. PINK1 accumulates on damaged mitochondria and enhances translocation of Parkin from cytosol to mitochondria. Parkin ubiquitinates mitochondrial proteins and induces mitophagosome formation (Kane et al., 2014; Narendra et al., 2008).

In the absence of Parkin, mitophagy can be regulated by various receptors such as BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), FUN14 domain-containing protein1 (FUNDC1) and NIX. These receptors are located on the outer mitochondrial

membrane and have LIRs through which they can interact with LC3 family proteins (Villa et al., 2018).

### 1.4.3 Xenophagy

Selective degradation of intracellular pathogens is termed as xenophagy. Pathogens like *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Shigella flexneri*, *Streptococcus pyrogenes, Listeria monocytogenes* and several others have been studied as substrates for autophagy. The first evidence for xenophagy was shown with reference to capture of Group A Streptococcus in autophagosome-like vacuole. The requirement of ATG5 for this process provided a solid link with autophagy (Nakagawa et al., 2004). S. typhimurium is also captured in vacuoles decorated with LC3 and taken to lysosomes for degradation. They can replicate significantly faster in ATG5 null MEFs (Birmingham et al., 2006). Nuclear dot protein of 52kDa (NDP52) was later identified as an autophagy receptor for ubiquitinated bacteria (Thurston et al., 2009).

### 1.4.4 Reticulophagy

ER stress results in unfolded protein response and induction of autophagy (Yorimitsu et al., 2006). FAM134B reticulon family protein reside in ER and have been identified as receptors for selective degradation of ER i.e. 'ER-phagy'. Upon induction of ER-phagy, FAM134B interacts with LC3 family proteins with the help of LIR motif and helps in packaging of cargo in autophagosomes (Khaminets et al., 2015).

### 1.4.5 Ferritinophagy

Ferritinophagy is the selective degradation of ferritin, a protein involved in binding to keeping free iron in check. When iron levels in the cell are low, the ferritinophagy receptor NCOA4 binds to ferritin and promotes its degradation via autophagy (Mancias et al., 2015; Mancias et al., 2014).

## 1.5 Molecular mechanisms of Autophagy

The process of autophagy involves a sequential set of events comprising of:

- Induction of autophagy by signalling cues
- Vesicle nucleation by contribution from various membrane sources
- Elongation of phagophore, capture of cargo and closure
- Movement towards lysosomes
- Fusion of autophagosome with the lysosome
- Degradation of cargo inside autolysosome and efflux of recycled products

### 1.5.1 Induction of Autophagy by signalling cues

The most well studied trigger of autophagy is nutrient starvation. Depletion of amino acids and growth factors force a cell to induce autophagy for its survival. Mechanistic target of rapamycin (mTOR) is a well conserved serine/threonine kinase which is the main sensor of nutritional status of a cell. The inverse relationship between Tor (a homolog of mTOR in *Saccharomyces cerevisiae*) activity and induction of autophagy was first reported by Noda et al. They reported that adding rapamycin, a Tor inhibitor can induce autophagy even in yeast growing in nutrient rich conditions (Noda and Ohsumi, 1998). In the presence of amino acid signaling, mTORC1 phosphorylates Unc-51 like autophagy activating kinase 1 (ULK1), the mammalian homolog of yeast Atg1. ULK1 forms the first autophagy-specific protein complex along with ATG13, ATG101 and focal adhesion kinase family interacting protein of 200 kD (FIP200). mTORC1 also phosphorylates ATG13 and prevents its interaction with ULK1 (Ganley et al., 2009; Jung et al., 2009). In nutrient starvation conditions or upon mTOR inhibition, ULK1 gets phosphorylated and activated by 5' AMP-activated protein kinase (AMPK). ULK1 in turn phosphorylates Atg13 and FIP200 and autophagy is induced (Kim et al., 2011).

### 1.5.2 Vesicle nucleation by contribution from various membrane sources

In mammals, the origin of the autophagy initiation site i.e. phagophore is not well defined. Phagophore biogenesis occurs as a result of extension of an ER-subdomain also referred to as 'omegasomes'. Omegasomes are rich in Phosphatidylinositol 3-phosphate (PI3P) and are characterized by presence of PI3P-binding proteins such as WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) and Double FYVE domain containing protein (DFCP1) (Hayashi-Nishino et al., 2009; Polson et al., 2010; Yla-Anttila et al., 2009). Other membrane sources for phagophore biogenesis are mitochondria (Hailey et al., 2010), ER-Golgi intermediate compartment (ERGIC) (Ge et al., 2013) recycling endosomes (Puri et al., 2013), ER-Mitochondria contact sites (Hamasaki et al., 2013) and more recently, ER-plasma membrane contact sites (Nascimbeni et al., 2017).

The second autophagy-specific complex is the class III Phosphoinositide 3-kinase (PI3K) complex. The mammalian class III PI3K complex comprises of the class III PI3Kinase VPS34, Beclin1, p150 and ATG14. Class III PI3K complex is essential for phagophore elongation by recruiting aforementioned PI3P-binding proteins and sequential recruitment of downstream autophagy proteins (Funderburk et al., 2010). Beclin1, is a 60kDa Bcl2-homology (BH)3-domain containing protein which is bound to Bcl2 protein in nutrient rich conditions. Beclin1-Bcl2 association inhibits the autophagic activity of Beclin1 (Pattingre et al., 2005). The active ULK1 complex directly phosphorylates Beclin1 at serine 14 and activates the pro-autophagy VPS34 complex to promote autophagosome biogenesis (Russell et al., 2013).

### 1.5.3 Elongation of phagophore, capture of cargo and closure

Elongation of the nascent isolation membrane requires two ubiquitin-like systems which act at the ATG12-ATG5 conjugation step and the LC3 processing step. In the ATG12-ATG5 conjugation step, ATG12 is activated by E1-like enzyme ATG7 and is transferred

to ATG10. Here, ATG10 which is an E2-like enzyme helps in linkage of ATG12 to ATG5 via covalent bonding. The ATG12-ATG5 conjugate further interacts with ATG16L1 and forms a multimeric complex. This ATG12-ATG5-ATG16L1 complex transiently associates with the phagophore and helps in arching of the elongating phagophore by unequal recruitment of LC3 on the membrane (Mizushima et al., 1998a; Mizushima et al., 1998b).

The second ubiquitin-like system is involved in the processing of LC3. LC3 is present as a full-length protein in the cytoplasm which is cleaved by cysteine protease ATG4 to generate a C-terminal exposed glycine residue, this is the LC3-I form. The carboxy terminus of the exposed glycine is activated by E1-like enzyme ATG7 and is transferred to the E2-like enzyme ATG3. ATG3 attaches phosphatidylethanolamine (PE) to the exposed glycine residue of LC3-I which is now the LC3-II form. LC3-II binds to the inner and outer autophagosomal membranes and is the only known protein present on the autophagosome during and after its formation (Kabeya et al., 2000). Therefore, it is routinely used as an autophagosomal marker. This nascent structure expands, captures cargo and closes to form a double membrane autophagosome.

### 1.5.4 Movement towards lysosomes

Complete autophagosomes need to move towards the perinuclear area in the cell wherein majority of the lysosomes are present. It is well documented that autophagosome-lysosome fusion requires microtubules (Aplin et al., 1992; Kochl et al., 2006; Monastyrska et al., 2009). Dynein-dynactin motor complex is utilized by autophagosomes for minus end transport towards lysosomes (Gross et al., 2007).

### 1.5.5 Fusion of autophagosome with the lysosome

Vesicle trafficking events inside the cell requires coordinated efforts by special classes of proteins involved in trafficking namely Rabs, SNAREs and tethers. Rabs are small GTPases involved in membrane trafficking. Each Rab is distinct in its location and hence provides specificity or 'molecular address' to vesicles. Activity of a Rab protein is regulated by GTP binding. Rab7 is located in late endosomes and lysosomes and is involved in autophagosome-lysosome fusion. Knockdown of Rab7 causes a block in autophagic flux (Gutierrez et al., 2004b; Jager et al., 2004). Thapsigargin, an ER-stress inducer blocks autophagosome-lysosome fusion by preventing recruitment of Rab7 (Ganley et al., 2011). Pleckstrin homology domain containing protein family member 1 (PLEKHM1) is a Rab effector that interacts with Rab7, HOPS-SNARE complex and LC3 to facilitate autophagosome-lysosome fusion (McEwan et al., 2015). HOPS complex comprising of VPS33A, VPS11, VPS16, VPS18, VPS39 and VPS41 is a conserved multisubunit tethering complex (Rieder and Emr, 1997). HOPS complex subunits like VPS33A interact with autophagosomal SNARE Stx17 and help in bridging autophagosomal and lysosomal membranes to enable docking and fusion (Jiang et al., 2014). Ectopic P granules protein 5 homolog (EPG5) is another tethering factor that has been reported to stabilize SNARE complexes involved in fusion (Wang et al., 2016). Elegant studies by Noburo Mizushima's group identified Stx17, the first SNARE protein exclusively involved in autophagosome-lysosome fusion. SNARE proteins are mediators of vesicle fusion and will be described in greater detail in the upcoming parts. Stx17 is a Qa SNARE which forms a complex with Qbc SNARE SNAP29. This SNARE complex formation occurs on complete autophagosomes (Itakura et al., 2012). O-linked β-*N*-acetylglucosamine (O-GlcNAc) transferase (OGT) mediated O-GlcNAcylation of SNAP-29 negatively affects Stx17-SNAP29 complex formation (Guo et al., 2014). Stx17 recruitment on autophagosomes is

aided by a small GTPase IRGM (Kumar et al., 2018). The Stx17-SNAP29 complex on autophagosomes is stabilized by accessory proteins such as ATG14. ATG14 binds to the SNARE domain of Stx17. This function of ATG14 is distinct from its roles in early steps of autophagy (Diao et al., 2015). The Qabc Stx17-SNAP29 complex interacts with the R-SNARE VAMP8/VAMP7 on lysosomes. This interaction is possible after these two vesicles are docked with the help of the HOPS complex (Itakura et al., 2012; Jiang et al., 2014; Takats et al., 2013). Recent studies by Noburo Mizushima's group also identified YKT6 as another autophagosomal SNARE. YKT6 depletion partially abrogated fusion in wild type cells and completely in Stx17 KO cells (Matsui et al., 2018). Following the fusion of autophagosomes with lysosomes, autolysosomes are formed. Figure 1 gives a pictorial overview of the proteins involved in autophagosome-lysosome fusion.

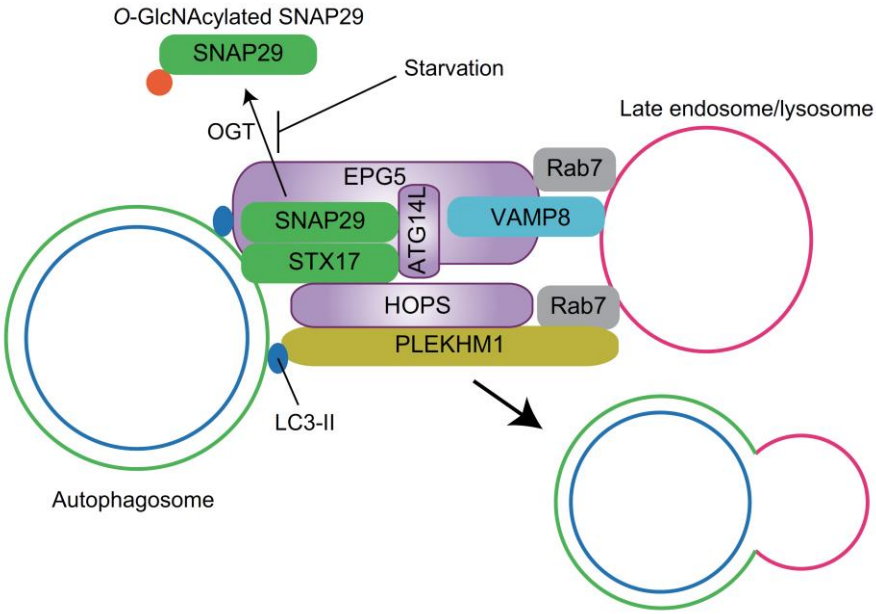


**Figure 1:** A detailed schematic depicting the molecular players involved in autophagosome-lysosome fusion. Reproduced with permission (Nakamura and Yoshimori, 2017).

### 1.5.6 Degradation of cargo inside autolysosome and efflux of recycled products

Completion of the autophagic process requires degradation of autophagic cargo inside autolysosomes. Maintenance of acidic pH inside lysosomes is not only important for autophagosome-lysosome fusion but also for proper action of lysosomal hydrolases. Deficiency of cysteine proteases like cathepsin B and D can block autophagic flux (Tatti et al., 2012). Similar block can also be observed by using protease inhibitors such as E64D and pepstatin. Figure 2 (below) gives a detailed pictorial representation of the molecular players involved in the process of autophagy.

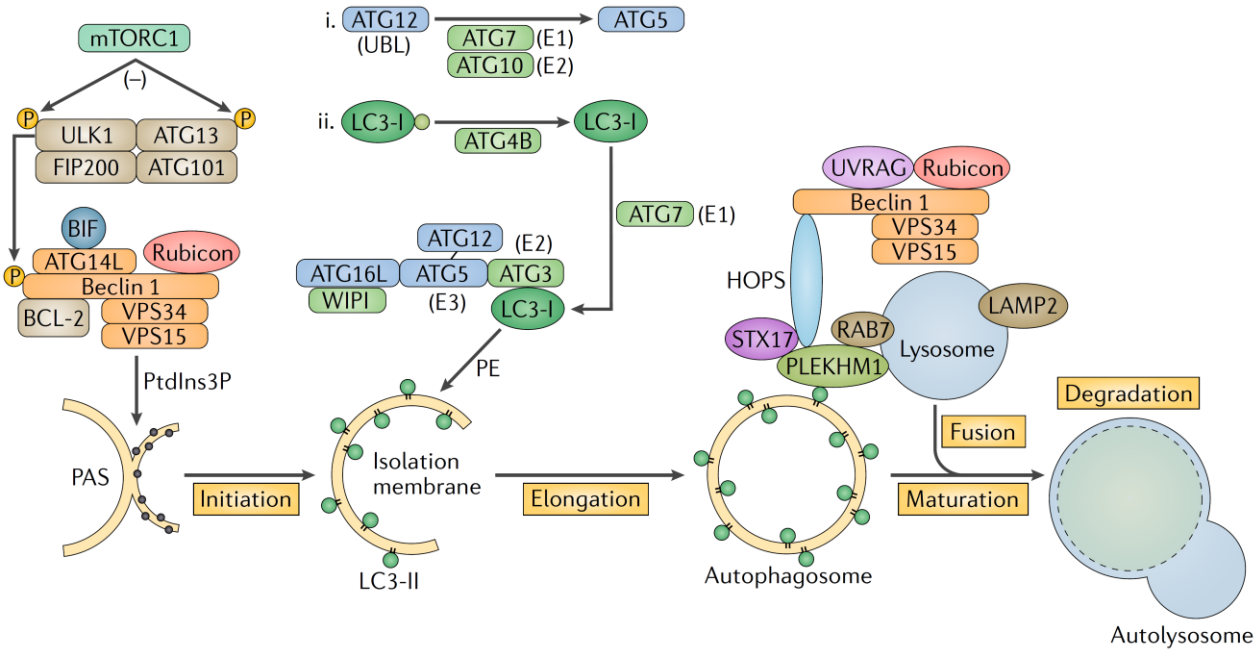


**Figure 2:** Molecular mechanisms of autophagy. The various stages of autophagy and the proteins involved are depicted. Reproduced with permission (Choi et al., 2018).

### 1.6 SNARE proteins- Essential mediators of membrane trafficking

For survival of eukaryotic cells, proper membrane trafficking is of the utmost importance. Molecules often are released from one cellular location and they need to be delivered to another intracellular destinations for proper function. To achieve this, transport vesicles

bud from an intracellular donor organelle and then target, dock and fuse with an acceptor organelle. SNARE proteins mediate vesicle fusion with the target membranes. So far, more than 30 SNAREs have been identified in mammalian cells. SNARE is an abbreviation for Soluble NSF attachment proteins (**SNA**P) **RE**ceptor. The major SNARE superfamilies are Syntaxins (Stx), Synaptosome-associated proteins (SNAP) and Vesicle associated membrane protein (VAMP) which were also known as Synaptobrevins. The first process where the functional role of SNAREs was characterized was neurotransmitter release. VAMP1/synaptobrevin is present on the synaptic vesicle while Stx1 and SNAP-25 are present on the synaptic cleft. These three SNAREs form a macromolecular complex that spans both the membranes and brings them in close apposition. This allows synaptic vesicle fusion with the plasma membrane and neurotransmitter release (Baumert et al., 1989; Bennett et al., 1992; Oyler et al., 1989; Sollner et al., 1993b; Trimble et al., 1988). Several important insights into the functional aspects of SNAREs especially the ones involved in neurotransmitter release have been obtained from studying actions of toxins that block the same. Several botulinum and tetanus toxins cleave the SNAREs VAMP1, Stx1 or SNAP-25 thereby hampering the process of fusion and subsequent release (Montecucco and Schiavo, 1994).

SNAREs proteins have been classified as v-SNAREs (ideally present on the vesicle membrane and includes SNAREs similar to VAMP1) and t-SNARE (ideally present on the target membrane and includes SNAREs similar to Stx1 and SNAP-25). The SNARE hypothesis proposed in 1993 gave the first working model to explain vesicle docking and fusion. It postulated that each vesicle has a distinct v-SNARE that pairs up with a unique cognate t-SNARE which is present at the appropriate target membrane (Sollner et al., 1993a). But with the increasing body of knowledge, this classification based on the location does not always hold true.

Now, SNAREs have been reclassified based on their structure as $Q_a$, $Q_{bc}$ and R-SNAREs. Mechanistically, upon interaction SNAREs form a bundle comprising of four alpha helices. One alpha helix is contributed by $Q_a$ and R-SNARE each and two alpha helices are contributed by $Q_{bc}$ SNAREs. A zero ionic layer is the main site of interaction in the SNARE complex. It is a hydrophilic region in the otherwise hydrophobic SNARE complex and is formed by contribution of three glutamine (Q) residues (One by $Q_a$ SNARE and two by $Q_{bc}$ SNARE) and one arginine (R) residue contributed by the R-SNARE. Almost all the characterized SNARE complexes are of the 3Q:1R type (Fasshauer et al., 1998).

Figure 3 (below) represents a simplified model for SNARE function. Vesicles are brought close to and docked to the target membrane by the help of Rabs and tethers. Following this, the cognate SNARE pairs interact and due to the tight pairing, the membranes are brought into close apposition expelling water molecules at the interface. Lipid molecules between the two interacting leaflets of the membrane flow towards each other leading to hemifusion and formation of a new bilayer. Rupture of this bilayer forms the fusion pore and completes the process of fusion (Chen and Scheller, 2001).

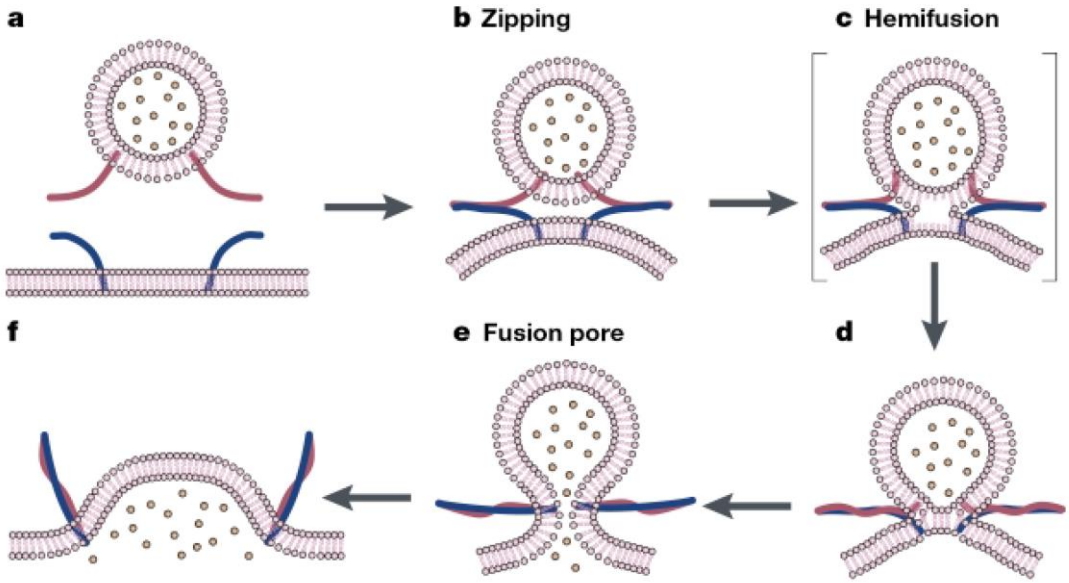

**Figure 3:** A model of SNARE function. Reproduced with permission (Chen and Scheller, 2001).

### 1.7 Syntaxin17: A multifaceted SNARE involved in autophagy and mitochondrial dynamics

Syntaxin17 (Stx17) belongs to the mammalian Syntaxin SNARE family and it has no recognisable homolog in yeast. Early studies showed that it is predominantly present on the smooth ER and is involved in trafficking from the smooth ER (Steegmaier et al., 2000; Steegmaier et al., 1998). Stx17 has a ubiquitous expression in almost all mouse and human tissues.

Stx17 has a long N-terminal cytoplasmic domain followed by a conserved SNARE motif and two adjacent transmembrane domains at the C terminal end which is the unique feature of Stx17 (other Syntaxins have been reported to have only one transmembrane domain). It requires both of its transmembrane domains for membrane anchoring which together form a hairpin-like structure (Itakura et al., 2012; Steegmaier et al., 2000). There are reports suggesting Stx17 cycles between the ER and ERGIC and that it is essential for maintaining the architecture of ERGIC and Golgi (Muppirala et al., 2011).

As mentioned earlier, Noburo Mizushima's group identified Stx17, the first SNARE protein exclusively involved in autophagosome-lysosome fusion. Stx17 is a Qa SNARE which forms a complex with Qbc SNARE SNAP29 and R-SNARE VAMP8 (VAMP7 in *Drosophila melanogaster*). Stx17 has a reticular/tubular pattern in nutrient rich conditions, suggesting ER/Mitochondrial localization. Upon nutrient starvation, Stx17 translocates to complete autophagosomes assuming a punctate localization (Itakura et al., 2012; Takats et al., 2013). Figure 4 shows a simplified model of Stx17 function in autophagosome-lysosome fusion.

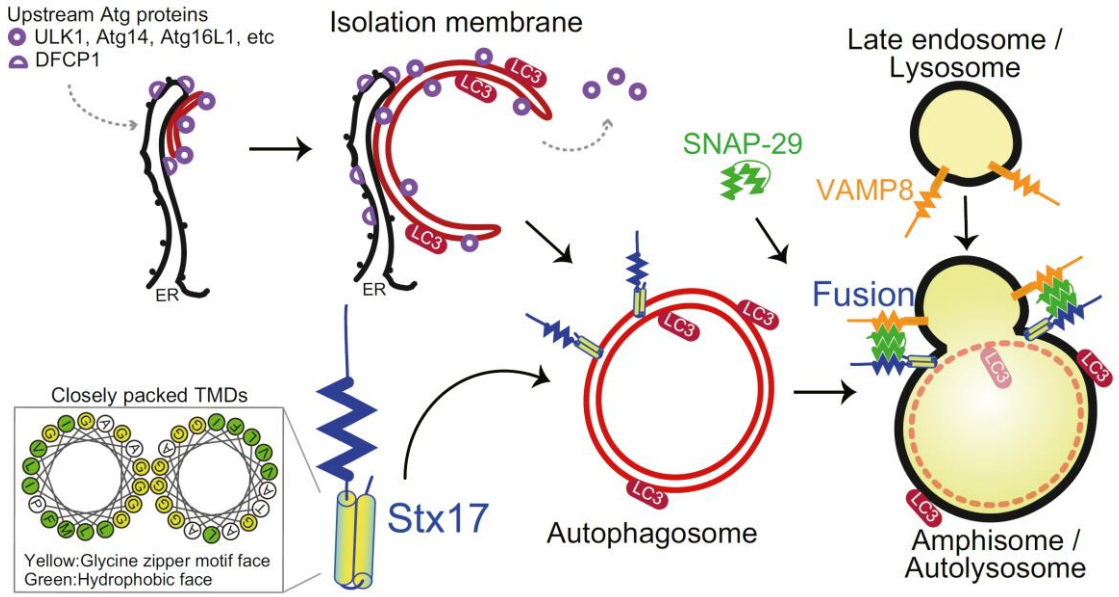


**Figure 4:** A model of Stx17 function in autophagosome-lysosome fusion. Reproduced with permission (Itakura et al., 2012).

In the past few years, additionally roles of Stx17 in processes other than autophagosome-lysosome fusion have emerged. Reports suggest that Stx17 is required for the delivery of stress-induced PINK1/parkin dependent Mitochondrial-derived vesicles (MDVs) to the late endosomes or lysosomes. In this process, Stx17 forms a ternary SNARE complex with SNAP29 and VAMP7 (McLelland et al., 2016). Stx17 also initiates PINK1/parkin independent mitophagy upon depletion of outer mitochondrial membrane protein Fis1. Fis1 loss results in enhanced accumulation of Stx17 on mitochondria. Mitochondrial Stx17 recruits core autophagy proteins thereby initiating mitophagy (Xian et al., 2019).

Another important biological process in which Stx17 has been implicated is mitochondrial dynamics. Mitochondria are highly dynamic organelles and they undergo coordinated cycles of 'fission' and 'fusion' in order to maintain their shape, size and distribution. This is referred to as 'mitochondrial dynamics'. This dynamicity is regulated by two sets of

proteins responsible for fission and fusion respectively. Fission is mediated by Drp1 which is a member of the large GTPase family Dynamin. Drp1 is recruited from the cytosol to form spirals around mitochondria that use energy from GTP hydrolysis to constrict and sever both inner and outer membranes (Frank et al., 2001). Drp1 requires accessory proteins such as Mff and Fis1 to be recruited to the mitochondria (Otera et al., 2010). Fusion between mitochondrial outer membranes is mediated by dynamin family members named Mitofusin1 (Mfn1) and Mitofusin2 (Mfn2) whereas fusion between mitochondrial inner membranes is mediated by Opa1 (Chen et al., 2003; Cipolat et al., 2004). Physiologically, fission generates new mitochondria and maintains quality control while fusion is enhanced in time of high energy demand and stress. It mitigates damage by allowing functional mitochondria to share components with damaged mitochondria. Fission is also required for mitophagy so that the damaged parts of mitochondria can be cut off and packed into mitophagosomes (Youle and van der Bliek, 2012). ER has also been implicated in fission. It has been shown that ER tubules mark the constriction sites for mitochondrial fission (Friedman et al., 2011).

Arasaki et al., showed that Stx17 gets spatially regulated depending on the nutritional status of the cell. In nutrient rich conditions wherein the basal autophagic flux is low, Stx17 is present in raft-like structures on ER-Mitochondria contact sites where it assists in the assembly of GTP-bound Drp1 on mitochondria. Stx17 on mitochondria can also prolong the activity of Drp1 by preventing Protein kinase A (PKA) mediated inhibitory phosphorylation of Drp1 at serine 637.

Starvation redistributes Stx17 from ER-Mitochondria contact sites to autophagosomes wherein it binds to autophagic proteins like LC3 and ATG14. This ensures proper autophagic flux as well as mitochondrial elongation in order to maximize ATP production during nutrient stress. Figure 5 provides a model of Stx17 function in mitochondrial

dynamics and gives a simplified overview of its spatial regulation depending on nutritional status of the cell (Arasaki et al., 2015).

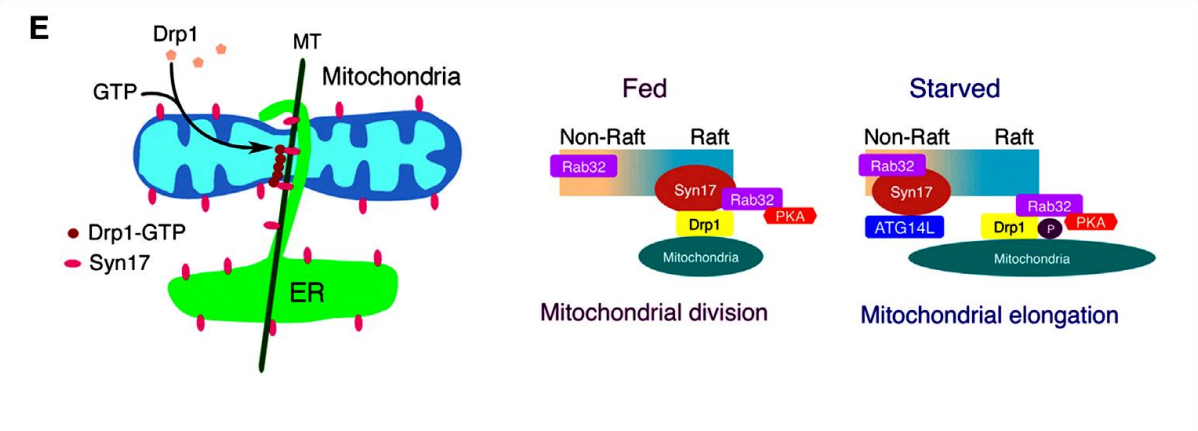


**Figure 5:** A model of Stx17 function in mitochondrial dynamics. Reproduced with permission (Arasaki et al., 2015)

Starvation has been reported to redistribute Stx17 not only to complete autophagosomes but also to mitochondrial associated membranes (MAM). Hamasaki et al., showed that autophagosomes are formed at ER-mitochondria contact. Stx17 along with ATG14 is involved in this process and the knockdown of Stx17 resulted in absence of functional autophagosomes (Hamasaki et al., 2013). Although the role of ER-mitochondria contact sites in autophagosome formation is now well recognized by the autophagy community, the role of Stx17 in autophagosome formation at the MAM in the case of starvation contradicts with other published findings which suggest that Stx17 interacts with Drp1 in nutrient rich conditions and translocates to complete autophagosomes in starvation conditions to ensue autophagosome-lysosome fusion (Arasaki et al., 2015; Itakura et al., 2012).

## 1.8 Autophagy in health and disease

The role of autophagy in normal physiology and some of the major human diseases has been explained under the following subheadings.

### 1.8.1 Physiological roles of autophagy

The physiological importance of autophagy is very well documented. The roles of autophagy are described under the following subheadings.

#### 1.8.1.1 Adaptive stress response to nutrient starvation

Autophagy is indispensable for adaptation to starvation through the generation of amino acids. In both yeast and mammalian cells, autophagy is immediately activated following amino acid withdrawal (Mizushima et al., 2010; Takeshige et al., 1992).

#### 1.8.1.2 Intracellular quality control

Basal autophagy occurs constitutively at low levels in all cells. This is very consequential for maintaining intracellular quality control. Mice with liver-specific ATG7 deletion develop hepatomegaly and hepatic failure (Komatsu et al., 2005) and neuron specific ATG5 and ATG7 deletion results in neurodegeneration and progressive motor deficits (Hara et al., 2006; Komatsu et al., 2006).

#### 1.8.1.3 Development

Autophagy has several roles during mammalian development. Autophagy is important for preimplantation development, survival during neonatal starvation, and erythropoietic and lymphopoietic differentiation and adipogenesis (Mizushima and Levine, 2010).

#### 1.8.1.4 Anti-aging

There is an inverse relationship between autophagy and aging. Caloric restriction which also results in autophagic induction is a very effective method for extending the life spans

of various species from yeast to mammals. Genetic studies also suggest that autophagy is important for extending life span. A *C. elegans* mutant lacking the insulin signaling gene *daf-2* resulting in caloric restriction also shows a life extension phenotype, which is not observed if autophagy-related genes are simultaneously mutated (Hansen et al., 2008; Levine and Klionsky, 2004).

### 1.8.2 Autophagy in pathophysiology

As already explained in the previous sections, autophagy as an intracellular recycling process has indispensable roles in cell growth, development and survival. Deregulation of autophagic flux has been implicated in several human diseases such as cancer, neurodegeneration and infectious diseases and thereby studying and understanding autophagic flux in disease conditions is of crucial importance.

#### 1.8.2.1 Autophagy in cancer

The role of autophagy in cancer has been rightly designated as a ‘double-edged sword’. It depends on the type, stage and genetic context of the tumor. The tumor suppressor role of autophagy in early tumorigenesis is well documented. Beclin1, a core autophagy protein and a member of the PI3K complex has a clearly proven tumor suppressor role and its loss is seen in 40-75% of breast, prostate and ovarian cancers. Mice with monoallelic deletion of Beclin1 are predisposed to liver tumors (Qu et al., 2003). Deletion of essential autophagy genes like ATG5 and ATG7 also results in hepatomas (Takamura et al., 2011).

Hampered quality control due to decreased autophagy function results in decreased protein and organelle quality control and increased ROS production. A constitutive level of basal autophagy is needed for cellular homeostasis and loss of autophagy leads to accumulation of damaged mitochondria, DNA damage response, genomic instability and aneuploidy. Chronic tissue damage can cause inflammation and contribute to tumor progression.

Accumulation of the autophagy substrate p62 due to reduced autophagic flux results in activation of the nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathway. NRF2 is generally bound to and kept in check by Kelch-like ECH-associated protein 1 (KEAP1) but high levels of p62 binds to KEAP1 and frees NRF2 to activate antioxidant-defence genes responsible for cell survival (Jaramillo and Zhang, 2013).

In primary tumors, autophagy prevents metastasis by limiting necrosis and inflammation and managing nutrient stress (Degenhardt et al., 2006). There are few examples of excessive autophagy resulting in cell death especially in cancer cells that are resistant to apoptosis. Tamoxifen induced cell death in MCF7 cells which have mutated caspases are marked by rapid induction of Beclin1 levels (Scarlatti et al., 2004).

The first evidence that autophagy helped in survival of advanced cancers came from the fact that it was highly upregulated in hypoxic tumor regions and promoted tumor cell survival (Degenhardt et al., 2006). The elevated levels of autophagy help tumor cells to survive oxygen stress and enhanced resistance to the lack of blood supply. Due to these effects, autophagy is one of the mechanisms responsible for poor efficacy and acquired resistance in anti-angiogenesis therapy and the combining anti-angiogenesis therapy with autophagy inhibition is seen as a promising alternative (Nishikawa et al., 2010).

The best examples of pro-survival autophagy are the KRAS driven cancers such as non-small cell lung cancers (NSCLC). ATG7 deficiency in KrasG12D-driven lung tumor reduced tumor cell proliferation and tumor burden (Guo et al., 2013)

Autophagy defects in these Ras-driven tumors causes metabolic impairment and reduced mitochondrial quality control leading to reduced tumor growth, survival, and malignancy. Tumor cells obtain energy from aerobic glycolysis rather than oxidative phosphorylation. This phenomenon called ‘Warburg effect’ eliminates the need of oxygen which is a limiting

factor particularly in hypoxic tumor regions (Warburg, 1956). On the other hand, proceeding with aerobic glycolysis results in deficiency of substrates to run the Tri carboxylic acid (TCA) cycle for ATP production. Autophagy comes to the rescue in this scenario by scavenging biomolecules to produce more substrates for the TCA cycle (White, 2013).

Autophagy has a very consequential role during the process of invasion and metastasis. As mentioned earlier that autophagy prevents metastasis during early tumorigenesis but it has pro-survival roles during later stages of metastasis. Once tumor cells detach from the extracellular matrix, a type of apoptotic cell death known as anoikis is initiated. This is to prevent cells from leaving their designated location. Induced autophagy in these cells provides 'anoikis resistance' (Fung et al., 2008). Studies have identified that inducing autophagy by starvation results in increased metastasis and invasion of hepatocellular carcinoma cells which is regulated by TGF-β/Smad3 signaling (Li et al., 2013).

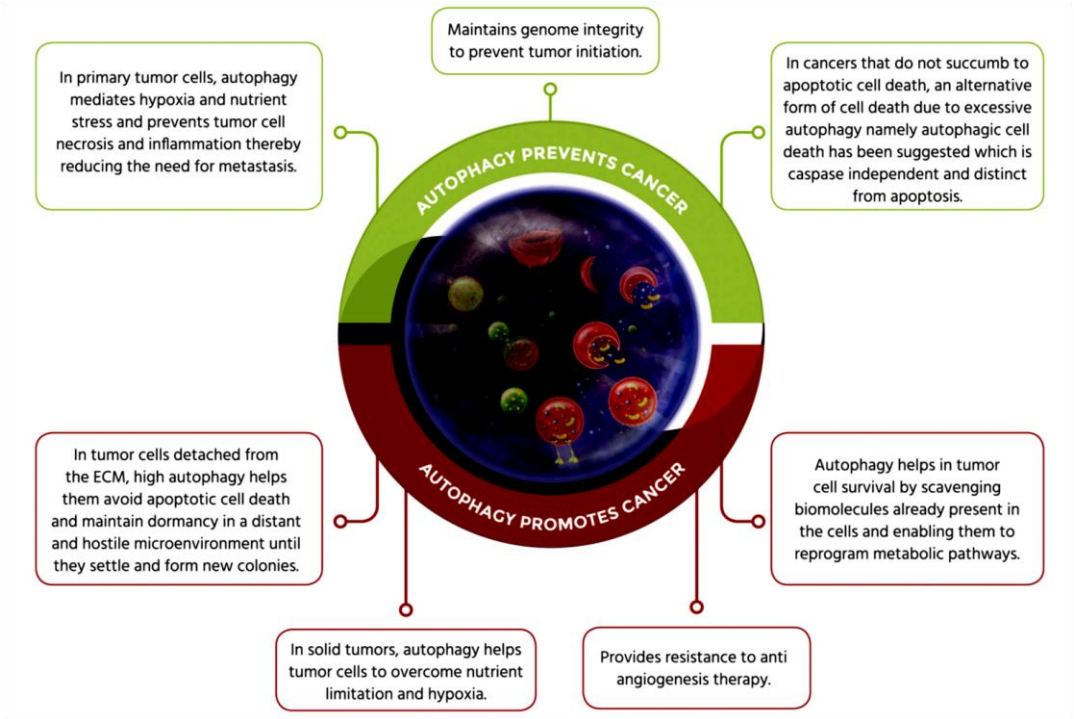

**Figure 6:** The dual nature of autophagy in cancer. The role of autophagy in cancer depends on the type, stage and genetic context of the tumor. Reproduced with permission (Singh et al., 2018).

### 1.8.2.2 Autophagy in neurodegeneration

Autophagy as a cellular homeostasis process is exceedingly important in neurons because they are post-mitotic and rely heavily on autophagy for protein and organellar quality control. Deletion of key autophagy proteins such as ATG5 in mice leads to neurodegeneration (Hara et al., 2006). Hampered autophagic flux due to deletion of ATG5 or ATG7 also affected neurogenesis and synaptic pruning (Kim et al., 2017; Xi et al., 2016). The two hallmarks of neurodegenerative disorders like Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) is the accumulation of aggregated and misfolded proteins such as beta-amyloid, alpha-synuclein and mutant huntingtin as well as damaged mitochondria (Dikic and Elazar, 2018). As shown in Figure 7 autophagy maintains neuronal health by eliminating toxic aggregates and ROS-generating damaged mitochondria. In case of neurodegenerative disorders, autophagic flux decreases either due to increasing age or a defect in autophagy often due to presence of pathogenic proteins. Decreased autophagic flux thereby is not enough to remove the abovementioned toxic species from the cell. Several autophagy proteins and effectors get trapped in aggregates which renders them non-functional (Menzies et al., 2017). Mutant α-synuclein can bind to and prevent the nuclear translocation of transcription factor EB (TFEB), thereby reducing the transcriptional activation downstream autophagic and lysosomal genes (Decressac et al., 2013).

In Huntington's disease, both general autophagy and selective autophagy such as mitophagy and aggrephagy are characterized by presence of empty autophagosomes. Although autophagy induction and autophagosome biogenesis occur, the cargo recognition

machinery fails to efficiently recognize misfolded proteins and damaged organelles (Martinez-Vicente et al., 2010). Presenilin 1 (PS1) mutation which is present in Alzheimer's disease disrupts proper lysosomal acidification resulting to a block in autophagosome-lysosome fusion (Lee et al., 2015). Mutations and post-translational modifications of α-synuclein impairs lysosome-associated membrane protein-2A (LAMP-2A) organization. LAMP-2A is required for translocation of substrate proteins from the cytosol into the lysosomal lumen and its disruption blocks degradation by chaperone-mediated autophagy (Cuervo et al., 2004).

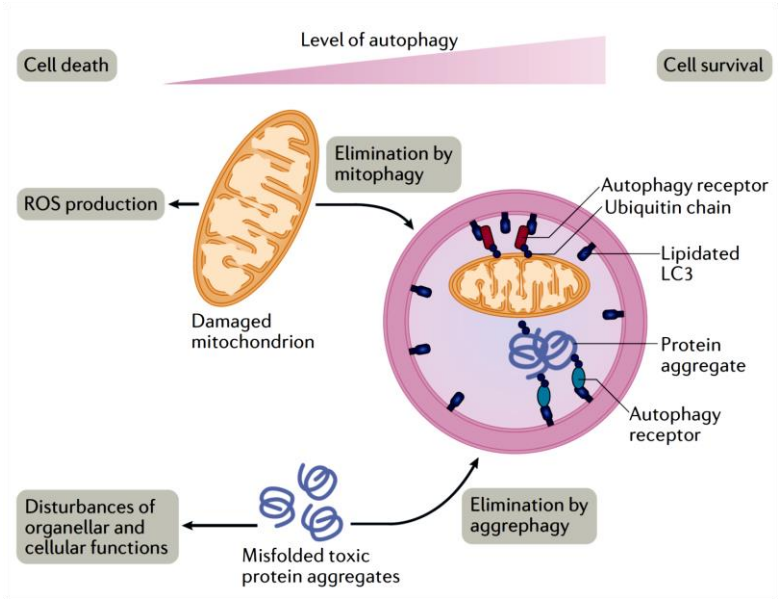


**Figure 7:** Role of autophagy in neurodegeneration. Autophagy maintains neuronal cell health by mediating protein and organellar quality control. Reproduced with permission (Dikic and Elazar, 2018).

### 1.8.2.3 Autophagy in immunity

In addition to the role of autophagy in cellular homeostasis, it also has an intricate role in innate and adaptive immunity. As explained in the previous parts, autophagy can capture and degrade intracellular pathogens such as *Mycobacterium tuberculosis*, *Salmonella*

*typhimurium*, *Shigella flexneri*, *Streptococcus pyrogenes*, *Listeria monocytogenes* and several others.

Cells have surface and cytosolic pattern recognition receptors like Toll-like receptors (TLRs) and NOD-like receptors (NLRs) which recognise pathogen-associated molecular patterns (PAMPs) and recruit autophagic adaptors and proteins for their capture and elimination. During *M. tuberculosis* infection, the innate immunity response is triggered. After detection of bacteria by TLRs, the TLR-MyD88-NFKB signaling is initiated which recruits DNA damage associated autophagy modulator 1 (DRAM1) which in turn induces autophagy (Khan et al., 2016; van der Vaart et al., 2014). Immunity related GTPase IRGM was shown to be involved in assembling core autophagy machinery in response to intracellular infections (Chauhan et al., 2015). Another category of PAMPs that can activate autophagy are the NLRs. NLRs can activate ULK1 and cause increased translocation of ATG16L1 at the infection sites thereby enhancing the rate of autophagosome formation (Irving et al., 2014). The process of autophagy therefore is very consequential for cell-autonomous immunity.

Autophagy controls inflammation which is triggered by multiprotein complexes called inflammasomes. These inflammasomes induce conversion of inactive cytokines into their active forms. ROS and damaged mitochondria are shown to be triggers for NLPR3 inflammasome formation and by removing such harmful entities from the cell, autophagy limits inflammatory response (Zhong et al., 2016). Autophagy also inhibits the production of Type1 Interferon (IFN-1) in response to the presence of viral RNA. Viral RNA can trigger translocation of retinoic acid-inducible gene 1 (RIG-1) and the mitochondrial antiviral signaling adaptor (MAVS) to mitochondria. Mitophagy can degrade the MAVS complex located on mitochondria thereby limiting IFN-1 production (Lei et al., 2012; Zhao et al., 2012).

Autophagy has significant roles in immune cell development and function. It aids in monocyte survival and monocyte-macrophage differentiation (Zhang et al., 2012). Generation of free fatty acids by autophagy mediated lipid degradation supports essential mitochondrial respiration during neutrophil differentiation (Riffelmacher et al., 2017). Autophagy also supports survival of metabolically active B1a cells (Clarke et al., 2018). Conventionally during immunogenic cross-presentation, antigens from the extracellular environment are taken up by antigen presenting cells such as dendritic cells and degraded. The resulting peptides are displayed on the surface by MHC class II antigen-presenting molecules leading to activation of CD4 T lymphocytes. Autophagy has been implicated in the degradation of exogenous antigens therefore aiding in antigen presentation. The autophagy gene ATG5 has been shown to be essential for dendritic cells to process and present antigens for MHC class II presentation (Lee et al., 2010). Figure 8 (below) summarizes the role of autophagy in immunity. Impairment of autophagy can result in exacerbated infection or inflammatory diseases such as Crohn's disease.

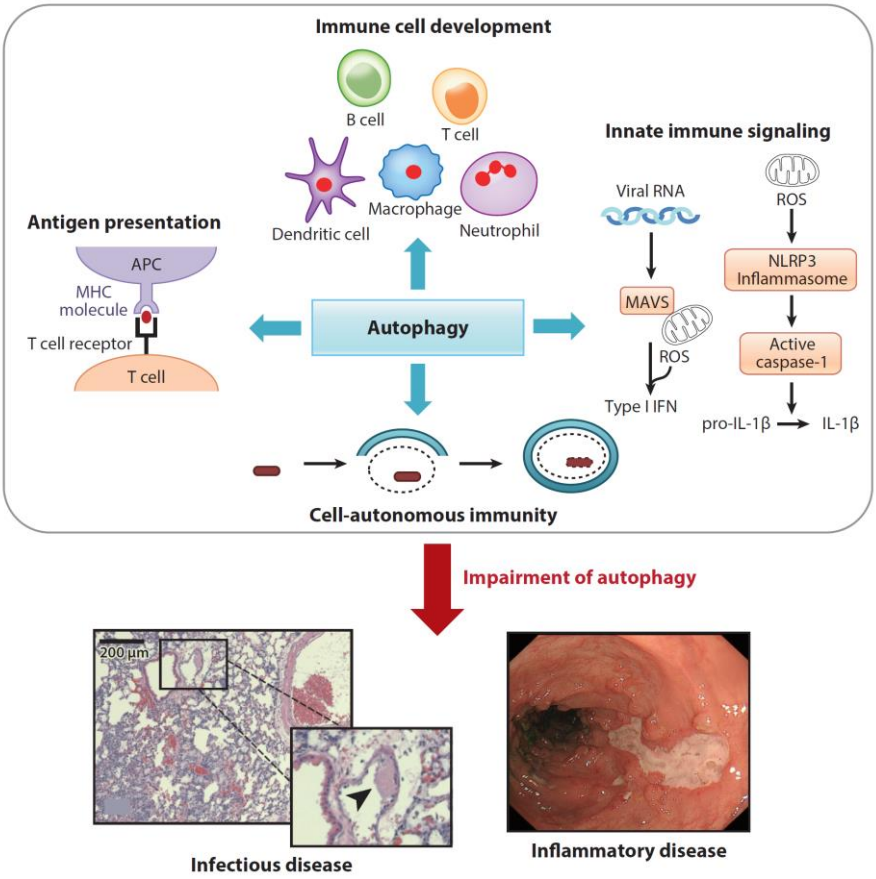


**Figure 8:** Role of autophagy in immunity. Reproduced with permission (Matsuzawa-Ishimoto et al., 2018).

### 1.9 Modulation of autophagy in therapeutics

As the above subheadings have already covered in detail, autophagy is very consequential for maintaining protein and organellar quality control and overall cellular homeostasis and therefore deregulation of autophagic flux is crucial in development of some major human diseases. In cancer, autophagy has a tumor suppressor role in early tumorigenesis and largely cytoprotective function in established tumors. Decreased autophagy leads to accumulation of misfolded protein aggregates and damaged mitochondria resulting in neurodegeneration. Autophagy is also the effector in many adaptive and innate immunity pathways. Owing to these observations, modulating i.e. enhancing or inhibiting autophagy

is being seen as a very promising potential target in therapeutics. Autophagy as a pathway is highly druggable and offers several crucial points to target.

The most important thing to understand before utilization of autophagy modulation in therapy is how autophagy affects aetiology of the disease to be targeted. For example, if in a diseased condition initiation of autophagy is affected, molecules which could act upstream and boost vesicle nucleation will yield more potent effects than the ones which could enhance lysosomal degradation (Galluzzi et al., 2017).

Autophagy modulation can be done by either genetic or pharmacological means. In case of genetic modulation, whole-body knockouts of few autophagy genes like Beclin1 are embryonic lethal so the preferred approach is to generate tissue-specific or inducible knockouts. Results obtained with these genetically altered models have implicated that alterations of autophagy can be useful for wide range of clinically relevant disorders (Galluzzi et al., 2017).

### 1.9.1 Pharmacological modulation of autophagy

Pharmacological modulation is advantageous over genetic manipulations because the phenotype can be observed just on the addition of the compound and the action can potentially be reversed on its withdrawal. The method is less laborious and the putative modulators could be used as leads for pharmacological purposes in certain disease conditions. Pharmacological modulation of autophagy entails usage of drugs, usually small molecules which can induce or inhibit autophagy.

#### 1.9.1.1 Autophagy inducers

Pharmacological induction of autophagy can be useful in neurodegenerative disorders, infectious diseases, aging and metabolic disorders. By enhancing the autophagic flux, inducers can aid in elimination of misfolded proteins, toxic aggregates, intracellular pathogens. Autophagy inducers can additionally boost immunity, reduce inflammation,

ROS and other harmful species which might cause genomic instability (Levine et al., 2015). There are still significant challenges in the use of autophagy inducers. The first group of autophagy inducers identified were rapamycin (and rapalogs) which are approved for human use by the FDA but due to the inhibitory effect on mTORC, they cause immunosuppression and other issues which make them unsuitable for long term use. There are other FDA approved inducers such as lithium and metformin which have effects on several pathways including autophagy and hence it is difficult to be certain that the effect obtained is primarily due to autophagy induction or not. Additionally, for an inducer to be used in neurodegenerative disorders, the biggest challenge is to ensure that it crosses the blood-brain barrier. Table 1 lists the autophagy inducers identified so far and their targets.

| Compound | Mechanism of autophagy induction |
|---|---|
| **FDA approved drugs** | |
| Carbamazepine | Lowers inositol and Ins(1,4,5)$P_3$ levels |
| Clonidine | Lowers cAMP levels |
| Lithium | Lowers inositol and Ins(1,4,5)$P_3$ levels |
| Metformin | Upregulates AMPK, which phosphorylates ULK1 and beclin 1 |
| Rapamycin (and rapalogs) | Inhibits mTORC1 |
| Rilmenidine | Lowers cAMP levels |
| Sodium valproate | Lowers inositol and Ins(1,4,5)$P_3$ levels |
| Verapamil | Inhibits L-type $Ca^{2+}$ channel, lowering intracytosolic $Ca^{2+}$ |
| Trifluoperazine | Unknown |
| Statins | Depletion of geranylgeranyl diphosphate activates AMPK |
| Tyrosine kinase inhibitors | Inhibit Akt-mTOR signaling and beclin 1 tyrosine phosphorylation, increase beclin 1/Parkin interaction |
| **Investigational drug** | |
| BH3 mimetics | Disrupt binding between beclin 1 and Bcl-2 family members |
| **Nutritional supplements** | |
| Caffeine | Inhibits mTOR signaling |
| Omega-3 polyunsaturated fatty acids | Inhibit Akt-mTOR signaling; disrupt beclin 1 and Bcl-2 binding |
| Resveratrol | Activates sirtuin 1 |
| Spermidine | Acetylase inhibitor |
| Vitamin D | Calcium signaling, hCAP18/LL37-dependent transcription of autophagy genes |
| Trehalose | Unknown |

**Table 1:** Autophagy inducers and their mechanism of action. Adapted with permission (Levine et al., 2015).

### 1.9.1.2 Autophagy inhibitors

Inhibitors of autophagy can be classified as early- or late-stage inhibitors. 3-MA, Wortmannin, and LY294002 target the vesicle nucleation process by inhibiting class III PI3K (VPS34) and are classified as early stage inhibitors. On the other hand, drugs which block autophagosome-lysosome fusion by most likely affecting the lysosomal pH are late stage inhibitors. These include lysosomotropic drugs like monensin and chloroquine/hydroxychloroquine (CQ/HCQ) and vacuolar ATPase inhibitor BafilomycinA1. The most well studied autophagy inhibitor is CQ which was first identified as an anti-malarial drug. Autophagy inhibition using CQ has shown enhanced chemo-sensitivity and tumor regression in xenograft models (Yang et al., 2011).

Autophagy inhibition also enhanced the antitumor effect of chemotherapy drugs and hence CQ/HCQ is used in combinatorial therapy along with chemotherapy drugs. There are currently several ongoing phase I/II clinical trials which are testing the combinatorial approach in different cancers (Rosenfeld et al., 2014; Singh et al., 2018).

However so far, autophagy inhibition has shown limited success as a viable therapeutic option in cancer. There are several reasons for this, the biggest being a lack of specific and potent autophagy inhibitors. CQ/HCQ, the only FDA approved autophagy inhibitor, has a long half-life and micromolar concentrations of it are needed to inhibit autophagy. These factors limit its efficacy in human trials. Additionally, any molecule that affects lysosomal pH will most likely affect a plethora of vesicular trafficking pathways culminating at the lysosomes. Several in the scientific community have challenged chloroquine's classification as an autophagy inhibitor and they feel it should be more aptly characterized as a lysosomal inhibitor. Even the early stage inhibitors of autophagy often act indiscriminately on phosphatidylinositol 3-kinase pathway again causing a plethora of off-target effects. Table 2 summarizes the status of various autophagy inhibitors and the major

limitations in their development. It is very evident that successful application of autophagy inhibitors in cancer therapy requires specific and potent inhibitors. This thesis describes one such autophagy inhibitor which inhibits autophagosome-lysosome fusion but does not affect lysosomal pH or function.

| Agent | Mode of action | Blood–brain barrier permeant | Status | Major limitations |
|---|---|---|---|---|
| ***Inhibitors (cont.)*** | | | | |
| Melatonin* | Altered ROS production | Yes | In clinical trials for treatment of a wide panel of conditions | Potentially interferes with ROS-sensitive processes and has been associated with autophagy activation in some models |
| MRT67307 | ULK1 inhibition | Unknown | In preclinical development | Also inhibits ULK2, IKK and TBK1 |
| MRT68921 | ULK1 inhibition | Unknown | In preclinical development | Also inhibits ULK2 |
| NSC185058 | ATG4B inhibition | Unknown | In preclinical development | Exhibits improved selectivity for the autophagic pathway |
| SAR405 | VPS34 inhibition | Unknown | In preclinical development | Exhibits improved selectivity compared with 3-MA and wortmannin |
| SBI-0206965 | ULK1 inhibition | Unknown | In preclinical development | Exhibits improved selectivity for the autophagic pathway |
| VPS34-IN1 | VPS34 inhibition | Unknown | In preclinical development | Exhibits improved selectivity compared with 3-MA and wortmannin |
| Wortmannin | VPS34 inhibition | No | Experimental agent | Inhibits various class III PI3Ks |
| ***Inhibitors*** | | | | |
| 3-MA | VPS34 inhibition | Yes | Experimental agent | Inhibits various class III PI3Ks |
| Azithromycin | Unknown | Yes | Approved for treatment of multiple bacterial infections | Unclear MOA, blocks autophagic flux |
| Bafilomycin A1 | Lysosomal inhibition | Yes | Experimental agent | Inhibitor of lysosomal functions |
| Cardiac glycosides | $Na^+/K^+$-ATPase inhibition | Yes | Extensively used in the past for treatment of cardiac disorders | Narrow therapeutic window but specific for autosis |
| Chloroquine | Lysosomal inhibition | Yes | Extensively used in the past as an antimalarial agent | Inhibitor of lysosomal functions |
| Compound C (also known as dorsomorphin) | AMPK inhibition | Unknown | In preclinical development | Potentially interferes with AMPK-dependent processes |
| Edavarone | ROS scavenger | Unknown | Experimental agent | Potentially interferes with many ROS-sensitive processes |
| HCQ | Lysosomal inhibition | Yes | Extensively used in the past as an antimalarial agent | Inhibitor of lysosomal functions |
| LY294002 | VPS34 inhibition | Yes | In clinical trials for the treatment of refractory neuroblastoma | Exhibits improved selectivity compared with 3-MA and wortmannin but commonly considered nonspecific |
| Lys05 | Lysosomal inhibition | Yes | In preclinical development | Exhibits increased potency compared with HCQ |
| Mdivi-1 | Mitophagy inhibition | Yes | In preclinical development | Inhibitor of mitochondrial fragmentation |

**Table 2:** Status of various autophagy inhibitors and the major limitations in their development. Adapted with permission (Galluzzi et al., 2017).

### 1.10 Rationale of the present study

The importance of autophagy in human physiology is well documented. Dysfunctional autophagy has been implicated in neurodegenerative diseases, intracellular infections and cancers. Pharmacological modulation of autophagy to restore its levels is being pursued as an attractive therapeutic approach but the field is lagging behind due to the lack of specific and potent modulators. Since autophagy shares several molecular machineries with the endocytic pathway and they both culminate at the lysosomes, hence developing inhibitors specific to autophagy possesses unique challenges.

In this work, we have characterized a novel small molecule inhibitor of autophagy EACC which blocks autophagic flux by preventing autophagosome-lysosome fusion. Interestingly, EACC acts specifically on autophagy and does not affect other vesicular trafficking pathways. Further investigations into its mechanism of action showed that EACC affects the translocation of autophagy specific SNAREs Stx17 and its partner SNARE SNAP29 on autophagosomes without hindering the completion of autophagosomes.

Additionally, using EACC we linked the role of Stx17 in autophagic flux and mitochondrial dynamics. EACC enhanced the interaction between Stx17 and Drp1 even in starvation conditions. Furthermore, inhibiting this interaction between Stx17 and Drp1 by either genetic or chemical means not only allowed Stx17 translocation onto autophagosomes, but also restored autophagosome-lysosome fusion even in presence of EACC. Interestingly, EACC mediated block in autophagy although quite robust can be reversed by washing. We observed that upon removing EACC, Stx17 can translocate to autophagosomes and rescue

autophagic flux. As Stx17 trafficking is quite dynamic, molecules like EACC can be used as a tool to study Stx17 trafficking as well as to identify new molecular players involved in the process.

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# Chapter 2

# Identification of EACC as a novel and selective inhibitor of autophagic flux

# Chapter 2

# Identification of EACC as a novel and selective inhibitor of autophagic flux

## 2.1 Overview

In the introduction, we have described in detail the role of autophagy in maintaining protein and organellar quality control and overall cellular homeostasis and also explained how deregulation of autophagic flux is crucial in the development of some major human diseases. Due to this intricate correlation of autophagy with health and disease, modulating i.e. enhancing or inhibiting autophagy by using novel 'drug-like' molecules is being seen as a very promising potential target in therapeutics. Autophagy being a multistep pathway, is highly druggable and offers several crucial points to target. Additionally, although we have exponentially improved our knowledge about the process of autophagy since the 1990s, there are still a lot of open questions in the field related to autophagosome biogenesis, SNARE mediated vesicular trafficking in early and late steps of autophagy and mechanisms regulating autophagic flux. Small molecule which can modulate autophagy can also provide mechanistic insights into the process of autophagy and depending on their target of action can be helpful in answering some of the big questions in the field.

Our laboratory has standardized a luciferase-based high-throughput screen for identification of novel small molecule modulators of autophagy. The principle of the assay is based on detection of the levels of luciferase activity in order to monitor the rate of autophagic flux in S. cerevisiae. The strength of the assay is that it is not target-driven and it can yield hits across all steps of autophagy. Owing to the conserved nature of autophagy, the hits identified in yeast were later identified as inhibitors of autophagy in both yeast and mammalian systems (Mishra et al., 2017a; Mishra et al., 2017b).

Utilizing this assay, we performed a screening for 1999 compounds of the Microsource Discovery Systems library and identified EACC as one of the hits. EACC stands for Ethyl (2-(5-nitrothiophene-2-carboxamido) thiophene-3-carbonyl) carbamate. To test for its potential to modulate autophagy, EACC was further tested in mammalian system.

## 2.2 Results

### 2.2.1 EACC inhibits autophagic flux

Starvation is a potent inducer of autophagic flux and in order to test if EACC could modulate starvation induced autophagic flux, we treated HeLa cells with an increasing dose of EACC in starvation conditions (2.5-25μM) and performed a LC3 processing assay (Kabeya et al., 2000). An enhanced conversion of LC3 (LC3-I to LC3-II) was seen with increasing dose of EACC (**Fig. 2.1 A, B**). This would mean either increase or a late stage block in autophagic flux. To address this, we performed autophagic flux assay in which accumulation of LC3-II is analyzed in the presence or absence of a known autophagy inhibitor, BafilomycinA1 (BafA1). An autophagy inducer added along with BafA1 will increase LC3-II levels over and above that of BafA1 alone. On the other hand, in case of an inhibitor the LC3-II levels will remain unchanged (Mizushima and Yoshimori, 2007; Mizushima et al., 2010). The accumulation of LC3-II in combinatorial treatment with BafA1 was similar to that of BafA1 alone. This suggests that EACC is an inhibitor rather than an inducer of autophagic flux (**Fig. 2.1 C, D**).

To further validate these observations and dissect the step of autophagic flux affected by EACC, we employed tandem-fluorescent-tagged LC3 reporter, mRFP-GFP-LC3 (Kimura et al., 2007). This reporter is based on the acid-labile nature of GFP. Using this reporter, while autophagosomes appear yellow, autolysosomes (the fusion product of autophagosomes with lysosomes) are seen as red because the green fluorescence of GFP

gets quenched due the acidic nature of lysosomes. HeLa cells were transfected with mRFP-GFP-LC3 construct and treated with increasing concentrations of EACC (2.5-25μM) for 2 hours in starvation conditions. We saw a significant dose dependent increase in the number of autophagosomes ($mRFP^+/GFP^+$) and a concomitant decrease in the number of autolysosomes ($mRFP^+/GFP^-$) (**Fig. 2.1 E, F**) in EACC treated cells.

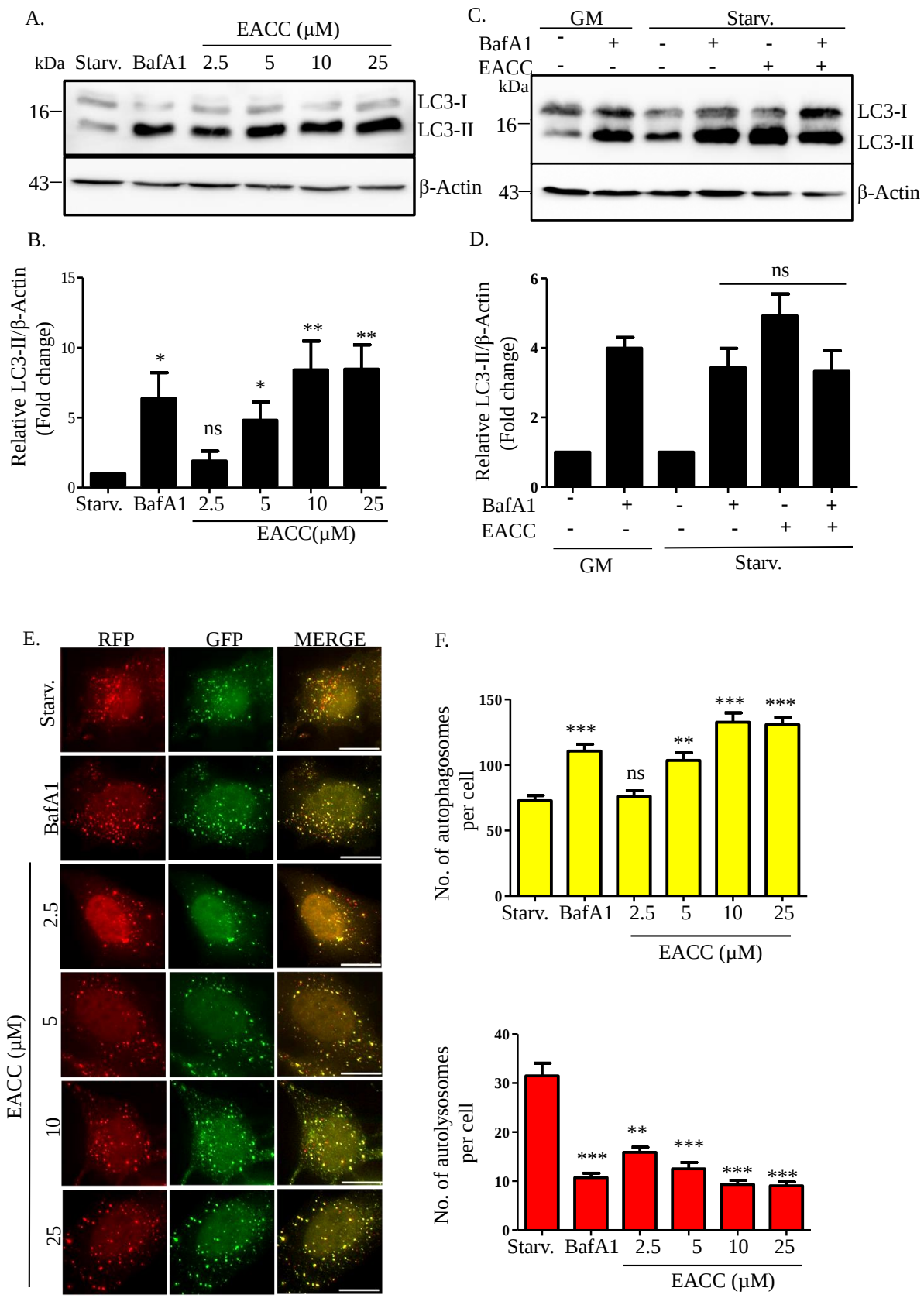
A.
EACC (μM)
kDa Starv. BafA1 2.5 5 10 25
16
43
LC3-I
LC3-II
β-Actin
C.
GM
Starv.
BafA1
EACC
kDa
16
43
LC3-I
LC3-II
β-Actin
B.
Relative LC3-II/β-Actin (Fold change)
Starv. BafA1 2.5 5 10 25
EACC(μM)
ns
D.
Relative LC3-II/β-Actin (Fold change)
ns
BafA1
EACC
GM
Starv.
E.
RFP
GFP
MERGE
Starv.
BafA1
EACC (μM)
2.5
5
10
25
F.
No. of autophagosomes per cell
Starv. BafA1 2.5 5 10 25
EACC (μM)
ns
No. of autolysosomes per cell
Starv. BafA1 2.5 5 10 25
EACC (μM)

**Figure 2.1: EACC inhibits autophagic flux**

**(A)** HeLa cells were either left untreated, treated with BafA1 (100nM) or EACC (2.5-25μM) for 2 hours in starvation conditions. Samples were collected and immunoblotted for anti-LC3 and anti-β-Actin antibodies. **(B)** Relative levels of LC3-II: β-Actin in untreated versus treated samples were quantitated for 3 independent experiments. $**P < 0.01$, $*P < 0.05$, ns= non-significant (Two-way ANOVA, replicate means compared with Bonferroni post-test) (**C)** HeLa cells were either left untreated or pretreated with BafA1 (100nM) in basal or starvation conditions for 1hour in order to block the autophagic flux. This was followed by treatment with EACC (10μM) for 2 hours. Samples were collected and immunoblotted for anti-LC3 and anti-β-Actin antibodies. **(D)** Relative levels of LC3-II: β-Actin in untreated versus treated samples were quantitated for 3 independent experiments. ns= non-significant (Student's unpaired t-test) **(E)** HeLa cells transfected with tandem tagged ptfLC3 (mRFP-GFP-LC3) construct were either left untreated, treated with BafA1 (100nM) or EACC (2.5-25μM) for 2 hours in starvation conditions. Scale=10 μm. **(F)** The number of autophagosomes ($RFP^+/GFP^+$ structures) and autolysosomes ($RFP^+/GFP^-$ structures) per cell were counted using the cell counter plugin of ImageJ software. Data shown represents number of autophagosomes ($RFP^+/GFP^+$) and autolysosomes ($RFP^+/GFP^-$) of a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$, ns= non-significant

### 2.2.2 EACC does not affect cell viability within the intended treatment time

In order to test if EACC significantly affects HeLa cell viability, we performed a cell viability assay. The Cell-Titer Glo assay is based on the principle that production of ATP from live cells can fuel conversion of luciferin to luciferase, the values of which can be recorded in a luminometer. The relative luminescence values are compared to the untreated control and converted into percentage. **Fig. 2.2 A** represents a graph of percent cell viability five hours post treatment in starvation medium. EACC (10μM) did not significantly affect the cell viability even five hours post starvation. All the upcoming experiments were performed with 10μM EACC for a time period of two hours until stated otherwise.

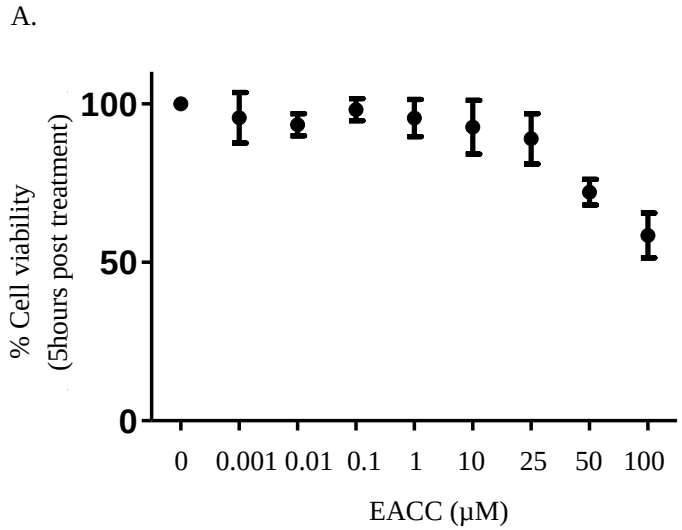


**Figure 2.2: EACC does not affect cell viability within the intended treatment time**

**(A)** HeLa cells were counted and equal numbers (1500 cells/well) were plated in 384 well plate in growth medium. The following day, post washing with D-PBS, different concentrations of EACC ranging from 100nM to 100μM were mixed in starvation media, added onto the cells and incubated for five hours. Post incubation, CellTiter-Glo Reagent was added to each well, and luminescence was measured using Varioskan Flash (Thermo Fisher Scientific). Graph represents percent cell viability five hours post EACC treatment in starvation conditions.

### 2.2.3 EACC causes accumulation of autophagic cargo

Next, we assessed the effect of EACC on autophagic adaptor p62/SQSTM1. p62 binds to ubiquitinated cargo on one end via its UBA domain and to LC3 via its LC3 interacting region (LIR) region on the other. This step helps in sequestration of cargo in autophagosomes. As, p62 is also degraded by autophagy hence an accumulation of p62 hints at decreased autophagic flux. EACC treatment resulted in enhanced colocalization between p62 and LC3 suggesting that EACC while inhibiting autophagic flux, did not affect adaptor loading and LC3 recruitment (**Fig. 2.3 A, B**). This suggests that treatment with EACC results in a late stage block in the autophagic flux.

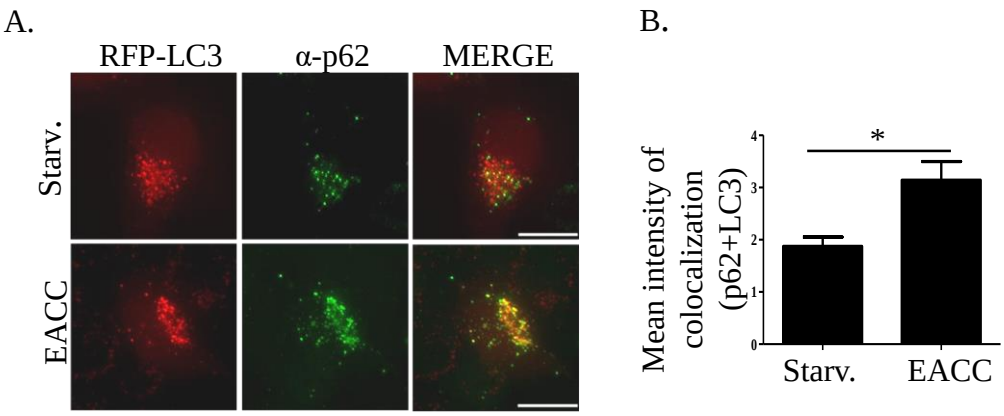


**Figure 2.3: EACC causes accumulation of autophagic substrate p62**

**(A)** Immunostaining with anti-SQSTM1/p62 antibody in RFP-LC3 transfected HeLa cells treated with EACC (10µM) for 2 hours in starvation conditions. Scale=15 µm **(B)** Graph showing the mean intensity of colocalization between p62 and RFP-LC3 in control versus EACC treated group. Mean intensity of colocalization was measured using colocalization and analyse plugins of ImageJ software. Data shown here represents a minimum of 60 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. *$P < 0.05$.

### 2.2.4 EACC reduces LAMP1 and LC3 interaction and autolysosome formation

The process of autophagy proceeds by fusion of autophagosomes with the hydrolase rich lysosomes to form autolysosomes. In order to further understand the inhibitory action of EACC on autophagic flux, we checked the colocalization between the autophagosome marker LC3 and the lysosomal marker, LAMP1. In RFP-LC3 transfected HeLa cells treated with EACC, we saw a decrease in the percentage of autolysosomes (RFP-LC3$^+$/LAMP1$^+$) (**Fig. 2.4 A, B**). A similar decrease in number of autolysosomes was also observed in EACC treated cells immunostained for endogenous LC3 and LAMP1 **(Fig. 2.4 C, D)**. To further understand if EACC directly impedes LC3-LAMP1 interaction endogenous immunoprecipitation (IP) was employed. Control and EACC treated lysates were subjected to IP using LC3 antibody. The levels of LC3-II were significantly high as compared to

untreated control in both LC3 input and LC3 immunoprecipitate but the levels of endogenous LAMP1 in the LC3 IP as detected by immunoblotting remains unchanged indicating that treatment with EACC reduced interaction between LC3 and LAMP1 **(Fig. 2.4 E)**.

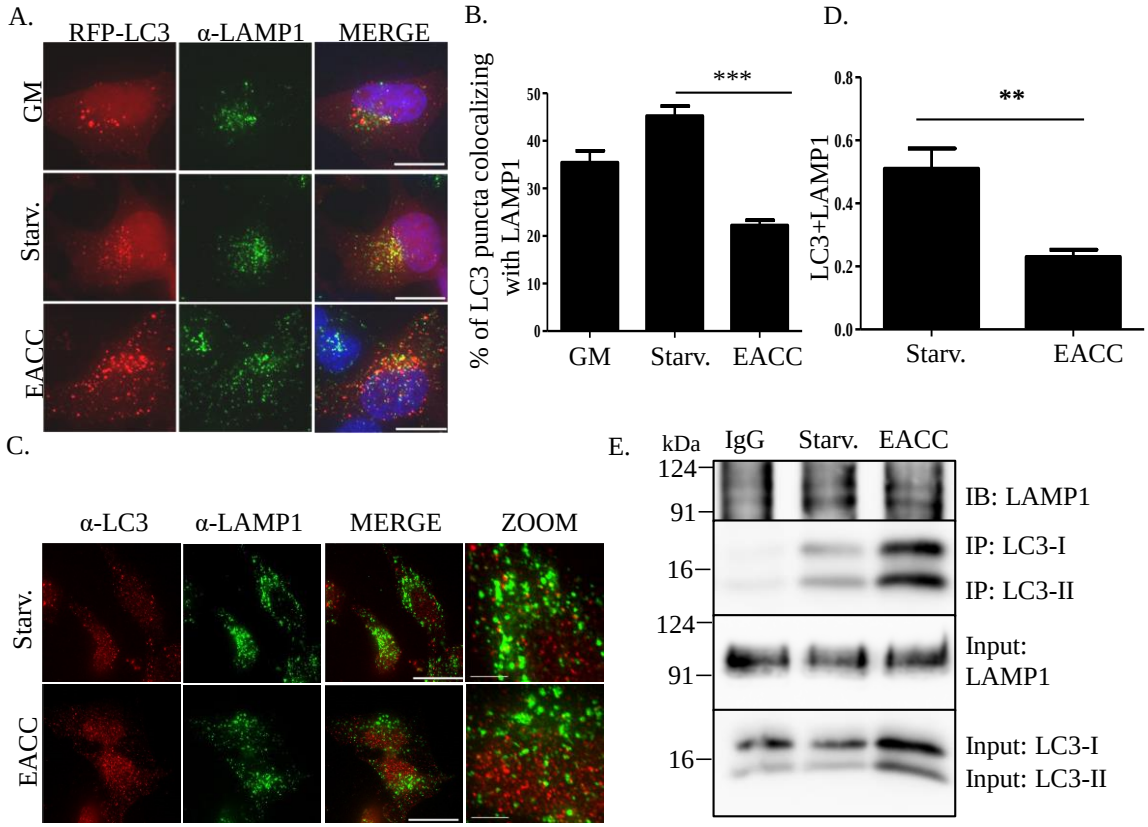


**Figure 2.4: EACC inhibits autolysosome formation**

**(A)** RFP-LC3 transfected HeLa cells were immunostained with anti-LAMP1 antibody and treated with EACC (10μM) for 2 hours in starvation conditions. Scale=10 μm **(B)** Graph showing percent colocalization between LAMP1 and RFP-LC3 (autolysosomes) in starvation conditions and EACC treatment. The number of colocalized dots were counted using colocalization and cell counter plug-ins of ImageJ software and plotted with respect to total number of LC3 puncta. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ***$P < 0.001$. **(C)** HeLa cells were treated with EACC (10μM) for 2 hours in starvation conditions and immunostained with anti-LC3 and anti-LAMP1 antibody. Scale=15 μm. **(D)** Graph showing the mean intensity of colocalization between

LC3 and LAMP1 measured as in **Figure 2.3 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ***$P < 0.001$. **(E)** HeLa cells were treated with EACC (10µM) for 2 hours in starvation conditions and immunoprecipitated with anti-LC3 antibody. Anti-Mouse IgG was used as an isotype control. The immunoprecipitates were immunoblotted with anti-LAMP1 and anti-LC3 antibodies.

### 2.2.5 EACC does not affect lysosomes or other vesicular trafficking culminating at lysosomes

The most common mode of blocking autophagic flux particularly at the late stage is by impeding lysosomal pH or function. Lysosomes harbor variety of hydrolases which require an acidic pH to function. v-ATPases present on the lysosomal membranes pump protons to maintain optimal acidic pH inside lysosomes. Lysosomes also need glycoproteins like LAMP1 to maintain membrane integrity so that the proteolytic enzymes do not leak into the cytoplasm and cause damage. Most widely used late stage autophagy inhibitors such as chloroquine and BafA1 affect the acidic pH of lysosomes. As the autophagy process requires macromolecular degradation in lysosomes and recycling of simpler biomolecules for its completion, protease inhibitors like E64D and pepstatin can also block autophagic flux by causing accumulation of undegraded cargo in lysosomes. As lysosomes are a culminating point for several vesicular trafficking pathways, the major downside of using inhibitors that affect lysosomes is that in addition to inhibiting autophagy they also impede processes like endocytosis. We have already seen that EACC causes a late step block in autophagic flux. We further wanted to investigate if this effect of EACC on autophagic flux is because it affects lysosomal pH or function. To test this, we checked the expression of LAMP1 in presence or absence of EACC. HeLa cells treated with EACC were either immunostained or immunoblotted with LAMP1 antibody. We did not observe any significant change in the LAMP1 expression in control versus treated cells (**Fig. 2.5 A-D**).

According to some reports, lysosomal positioning can also regulate autophagic flux (Korolchuk et al., 2011).

We also did not see any obvious difference in lysosomal positioning in EACC treated cells **(Fig. 2.5 A, B)**.

Although EACC did not affect the overall levels of lysosomes, we still wanted to check if similar to BafA1 and CQ it causes a loss of acidification of lysosomes. To test the effect of EACC on lysosomal acidification, we used Lysotracker Deep Red which is a probe that preferentially accumulates in acidic compartments. The intensity of Lysotracker staining remained unchanged in EACC treated cells but was diminished in v-ATPase inhibitor BafA1 treated cells suggesting that EACC does not affect lysosomal pH (**Fig. 2.5 E, F**).

As mentioned before, lysosomal hydrolases are important for completion of the autophagy pathway. We checked the expression and processing of Cathepsin B (CTSB) which is a lysosomal cysteine protease that gets cleaved inside the lysosomes to release its proteolytically active mature form. EACC treatment did not impede the conversion of pro-cathepsin B to mature-cathepsin B (**Fig. 2.5 G**).

Finally, in order to test the effect of EACC on other vesicular trafficking pathways such as endocytosis, we performed the Epidermal Growth Factor Receptor (EGFR) degradation assay. In order to regulate Epidermal Growth Factor (EGF) signaling when excess EGF is present, EGFR bound to the ligand EGF gets internalised via endocytosis and gets degraded in lysosomes upon EGF treatment. Hence, the temporal decrease in levels of EGFR after EGF pulse is indicative of endocytic trafficking of the receptor to the lysosomes. The rate of EGFR degradation with time followed a comparable trend in EACC treated versus untreated cells thereby showing that EACC does not affect endocytosis (**Fig. 2.5 H, I**).

These results clearly indicate that EACC is a selective inhibitor of autophagosome-lysosome fusion and it does not affect lysosomes or other vesicular trafficking pathways. This is a desirable trait in an autophagy inhibitor because being a selective inhibitor of autophagy avoids a plethora of side effects that arises from lysosomal inhibition.

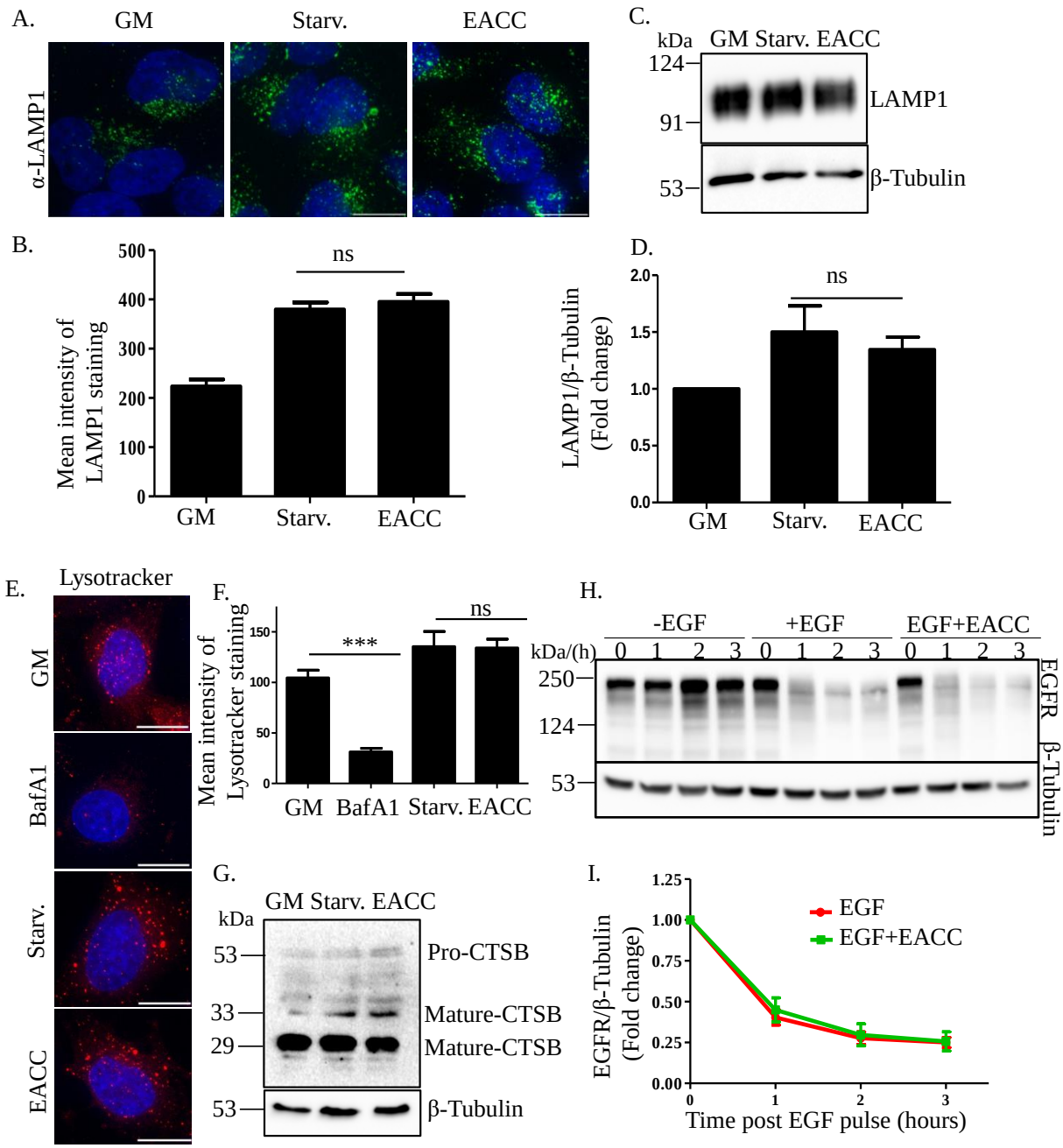

**Figure 2.5: EACC causes accumulation of autophagic substrate p62**

**(A)** HeLa cells were treated with EACC (10μM) for 2 hours in starvation conditions and immunostained with anti-LAMP1 antibody. Scale=10 μm **(B)** Graph represents the mean intensity of LAMP1 staining which was measured using analyse plugin in ImageJ. Data shown here represents a minimum of 60 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ns=non-significant. **(C)** HeLa cells were treated with EACC (10μM) for 2 hours in starvation conditions and immunoblotted with anti-LAMP1 and anti-β-Tubulin antibodies. **(D)** Relative levels of LAMP1: β-Tubulin in untreated versus treated samples were quantitated for 3 independent experiments. Statistical significance was analysed by Student's unpaired t-test. ns= non-significant **(E)** HeLa cells were either treated with BafA1 (100nM) in basal conditions or EACC (10μM) in starvation conditions for 2 hours. Lysotracker Deep Red (100nM) was added in the media in the last 15 minutes of treatment**.** Cells were fixed and imaged**.** Scale=15 μm **(F)** Graph showing the mean intensity of lysotracker staining measured as in **Figure 2.5 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ***$P < 0.001$, ns=non-significant. **(G)** Samples collected after EACC treatment were immunoblotted with anti-cathepsin-B and anti-β-Tubulin antibodies. **(H)** HeLa cells were serum starved for 3 hours and either left untreated or pre-treated with EACC prior to addition of EGF (100ng/ml) for the indicated time periods. Samples were collected and immunoblotted for anti-EGFR and anti-β-Tubulin antibodies. **(I**) Relative levels of EGFR: β-Tubulin in untreated versus treated samples were quantitated for 3 independent experiments.

## 2.3 Discussion

We used a yeast based high-throughput assay already established in the laboratory to screen the Microsource Discovery Systems library and obtained EACC as a hit. Owing to the conserved nature of autophagy, EACC could modulate autophagic flux in mammalian systems as well. By performing LC3 processing assay and the autophagic flux assay, we showed that treatment with EACC caused a dose dependent accumulation of LC3-II over

and above that of the starvation control. Upon performing the mRFP-GFP-LC3 reporter-based assay, we saw an accumulation of yellow autophagosomes and a concomitant decrease in red autolysosomes with an increasing dose of EACC.

We further showed that EACC does not significantly affect cell viability. Treatment with EACC resulted in accumulation of the autophagic cargo p62 and a decrease in LC3-LAMP1 interaction and autolysosome formation. Furthermore, EACC did not affect LAMP1 levels or lysosomal positioning. By using a fluorescent dye that preferentially accumulates in the acidic organelles, we showed that EACC, unlike other widely used autophagy inhibitors BafA1 and CQ does not affect lysosomal pH. EACC did not affect the activity of the lysosomal hydrolase Cathepsin B (CTSB). Interestingly, EACC also did not inhibit the trafficking and processing of endocytic cargo.

These results together establish EACC as a selective late-stage inhibitor of the autophagic flux. Surprisingly, the action of EACC to modulate autophagy was limited to starvation induced autophagic flux. In the later chapters, we will try to get more details about the mechanistic aspects of the autophagy inhibition mediated by EACC.

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# Chapter 3

# Step-by-step dissection of the effect of EACC on the process of autophagy

# Chapter 3

# Step-by-step dissection of the effect of EACC on the process of autophagy

## 3.1 Overview

In the previous chapter, we performed several assays to establish EACC as a selective inhibitor of starvation induced autophagic flux. The next obvious question is which step of autophagy is inhibited by EACC. Three possible steps at which EACC might be acting are:

1. Induction stage prior to isolation membrane formation
2. Isolation membrane formation and elongation to form autophagosomes
3. When mature autophagosomes fuse with lysosomes to form autolysosomes

As we observe plenty of conjugated LC3 in presence of EACC even over and above that of starvation control, the action of EACC is most likely focused at the penultimate step of autophagy i.e. fusion of autophagosomes with lysosomes. We still tested the effect of EACC on all the steps of autophagy process and performed a step-by-step analysis for the same. Autophagy is a multistep process and the hierarchical order in which different autophagy related proteins participate is very well studied.

Autophagy is triggered by nutrient starvation which results in shut down of the major nutrient sensor in the cell i.e. mammalian target of rapamycin and its effectors. Following this, the ULK1 complex comprising of ULK1, ATG13, ATG101 and FIP200 assembles. ULK1 activates several other proteins of the autophagy pathway which leads to assembly of the second complex comprising of VPS34, Beclin1, ATG14 and p150. This complex generates PI3P which is important for recruiting PI3P binding proteins such as DFCP1 and WIPI2 to the phagophore assembly site (PAS) wherein isolation membrane formation

occurs. Elongation of isolation membrane and its conversion into an autophagosome requires two ubiquitin like systems involving the ATG5-ATG12-ATG16L1 complex and LC3 (Itakura and Mizushima, 2010; Mercer et al., 2018; Shibutani and Yoshimori, 2014). Our results so far suggest that EACC selectively affected autophagic flux. So, our next approach was to narrow down to the step of autophagy at which EACC acts.

## 3.2 Results

### 3.2.1 The accumulation of LC3 upon EACC treatment is not dependent on enhanced transcription or translation.

Firstly, we checked whether the accumulation of LC3-II upon EACC treatment is dependent on enhanced transcription or translation. We pretreated HeLa cells with transcription inhibitor Actinomycin D (ActD) or translation inhibitor cycloheximide (CHX) in basal (GM) or starvation conditions (Starv.) for 1 hour. The accumulation of LC3-II in presence of EACC remained unchanged even after pretreating with Actinomycin D or cycloheximide **(Fig. 3.1 A-D)**.

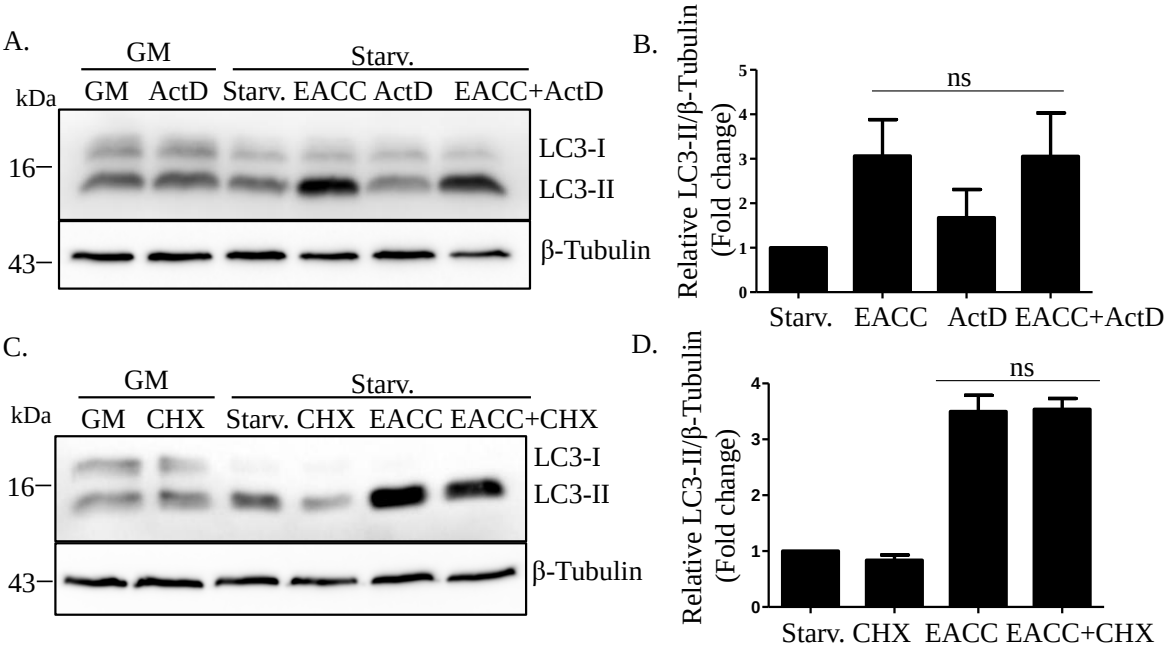


**Figure 3.1: The accumulation of LC3 upon EACC treatment is not dependent on enhanced transcription or translation**

**(A)** HeLa cells were either left untreated or pretreated with Actinomycin D (ActD) in basal (GM) or starvation conditions (Starv.) for 1 hour in order to block transcription. This was followed by treatment with EACC (10μM) for 2 hours in presence of Actinomycin D. Samples were collected and immunoblotted for anti-LC3 and anti-β-Tubulin antibodies. **(B)** Relative levels of LC3-II: β-Tubulin in untreated versus treated samples were quantitated for 3 independent experiments. Statistical significance was analysed by Student's unpaired t-test. ns=non-significant. **(C)** HeLa cells were either left untreated or pretreated with cycloheximide (CHX) in basal or starvation conditions for 1 hour in order to block protein translation. This was followed by treatment with EACC (10μM) for 2 hours in presence of cycloheximide. Samples were collected and immunoblotted for anti-LC3 and anti-β-Tubulin antibodies. **(D)** Relative levels of LC3-II: β-Tubulin in untreated versus treated samples were quantitated for 3 independent experiments. Statistical significance was analysed by Student's unpaired t-test. ns=non-significant.

### 3.2.2 EACC does not affect induction of autophagy

Next, we tested the effect of EACC on mTOR signaling. Nutrient starvation which is the most potent inducer of autophagy, inhibits mTOR complex. Inhibition of mTOR leads to

dephosphorylation of mTOR substrates P70S6 kinase and Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) (Fingar et al., 2002). HeLa cells treated with EACC were immunoblotted for phospho-P70S6 kinase and phospho-4EBP1. We observed loss of phosphorylation of these substrates in EACC treated cells similar to that of control which suggested that mTOR is inhibited (**Fig. 3.2 A**). Upon mTOR inhibition, ULK1 gets dephosphorylated at serine 757 position which allows assembly of ULK1 complex and induction of autophagy (Kim et al., 2011). Unaltered dephosphorylation of mTOR substrates and ULK1 in presence of EACC suggested that the early signaling events that lead to autophagy induction are not perturbed. We also checked the expression of proteins involved in early and middle stages of the autophagy pathway such as ATG14, ATG5 and ATG16L1 and found that they were unchanged upon EACC treatment (**Fig. 3.2 B**).

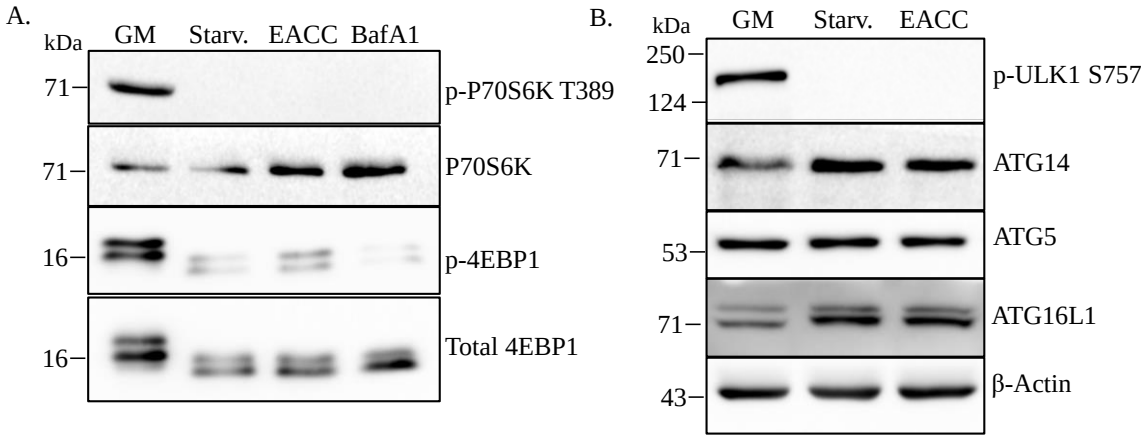


**Figure 3.2: EACC does not affect induction of autophagy**

**(A)** HeLa cells were either left untreated or treated with BafA1 (100nM) or EACC (10μM) for 2 hours in starvation conditions. Samples were collected and immunoblotted with anti-phospho-P70S6K (T389), anti-P70S6K, anti-phospho-4EBP1 and anti-4EBP1 antibodies. **(B)** Samples collected after EACC treatment were immunoblotted with anti-phospho-ULK1 (S757), anti-ATG14, anti-ATG5, anti-ATG16L1 and anti-β-Actin antibodies.

### 3.2.3 EACC does not affect isolation membrane formation

As mTOR mediated control of autophagy was unaltered, we next investigated the effect of EACC on molecular events that lead to autophagosome biogenesis. Autophagosome biogenesis begins with isolation membrane or phagophore formation upon autophagy induction. The phagophore assembly site (PAS) is characterized by the presence of Phosphatidylinositol 3-phosphate (PI3P) generated by Vacuolar protein sorting 34 (VPS34) kinase complex activity. PI3P-binding proteins like Double FYVE-domain-containing protein 1 (DFCP1) and WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) recognize and bind to PI3P and this event is important for isolation membrane formation (Axe et al., 2008; Dooley et al., 2014; Hayashi-Nishino et al., 2009). In addition, this nascent membrane is also marked by ATG14 (Hamasaki et al., 2013; Itakura and Mizushima, 2010). In order to look at autophagosome biogenesis sites, we checked the triple colocalization between ATG14, DFCP1 and LC3 in control and EACC treated cells. The percentage of LC3 puncta with ATG14 and DFCP1 remained unchanged upon EACC treatment **(Fig. 3.3 A, B)**. We also looked at the colocalization between LC3 and WIPI2 and the results again suggest that the number of sites of autophagosome biogenesis are unaffected upon EACC treatment **(Fig. 3.3 C, D)**. These results suggest that EACC does not affect isolation membrane formation.

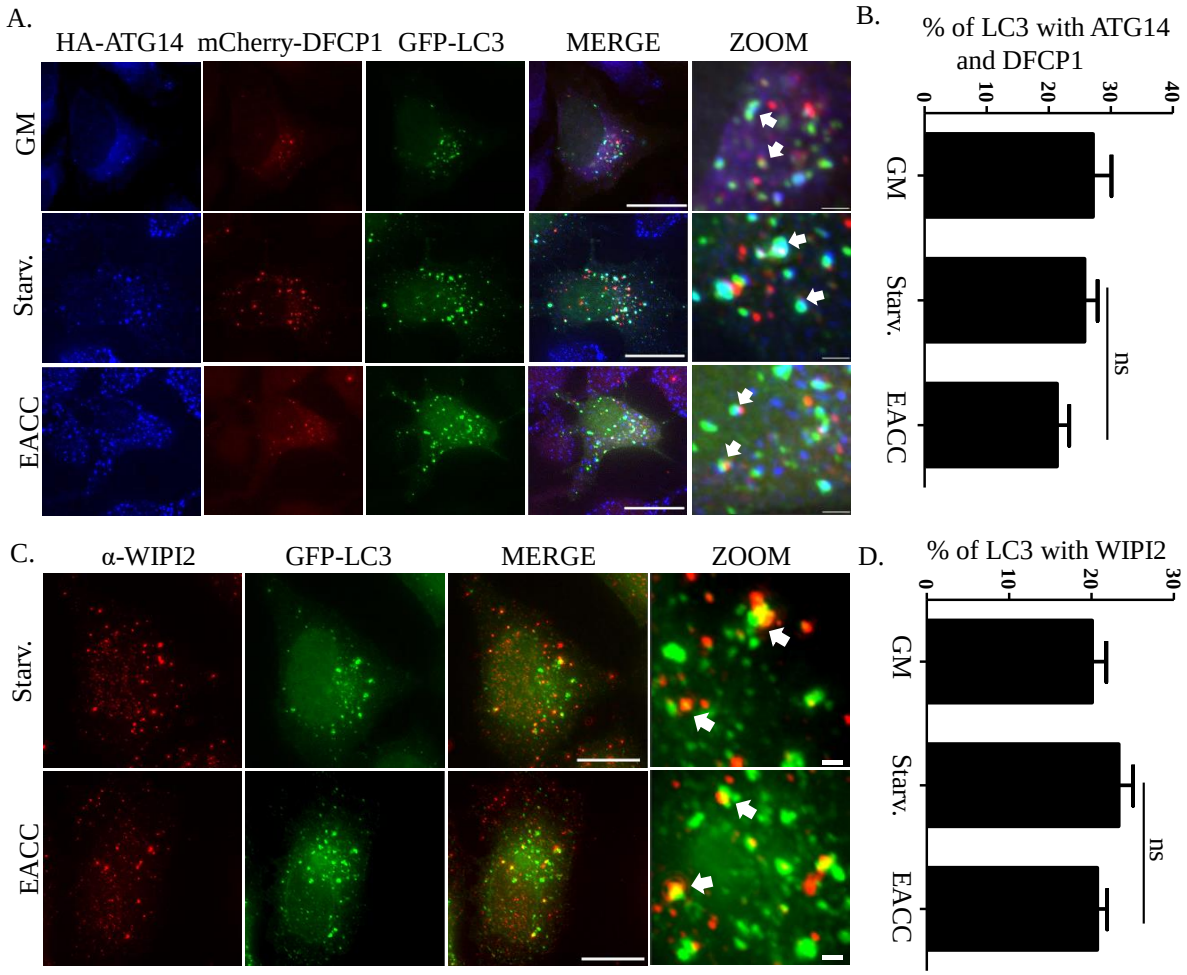


**Figure 3.3: EACC does not affect isolation membrane formation**

**(A)** HeLa cells co-transfected with mCherry-DFCP1, GFP-LC3 and HA-ATG14 were either left untreated or treated with EACC and immunostained with anti-HA antibody. Scale=15 µm, 1µm **(B)** Graph showing the percent of LC3 puncta colocalizing with DFCP1 and ATG14. This population represents immature autophagosomes. The number of colocalized dots were counted and plotted as in **Fig. 2.4 B.** Data shown here represents a minimum of 50 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ns=non-significant. **(C)** GFP-LC3 transfected HeLa cells were treated with EACC (10µM) for 2 hours in starvation conditions and immunostained with anti-WIPI2 antibody. Scale=15 µm **(D)** Graph showing the percent of LC3 puncta colocalizing with WIPI2. This population represents isolation membrane. The analysis was done similarly as in 3D. Data shown here represents a

minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ns=non-significant

### 3.2.4 EACC does not affect expansion of the isolation membrane

Expansion of the isolation membrane and formation of autophagosome takes place by sequential recruitment of ATG5-12/16 complex and LC3. The ATG12-ATG5 conjugate interacts with ATG16L1 and forms a multimeric complex. This ATG12-ATG5-ATG16L1 complex transiently associates with the isolation membrane and helps in recruitment of LC3 on the membrane (Fujita et al., 2008; Mizushima et al., 1998a; Mizushima et al., 1998b).

In HeLa cells transfected with RFP-LC3, the colocalization between ATG5 and LC3 as well as ATG16L1 and LC3 that represents expanding isolation membrane was comparable to that of starvation control (**Fig. 3.4 A-D).**

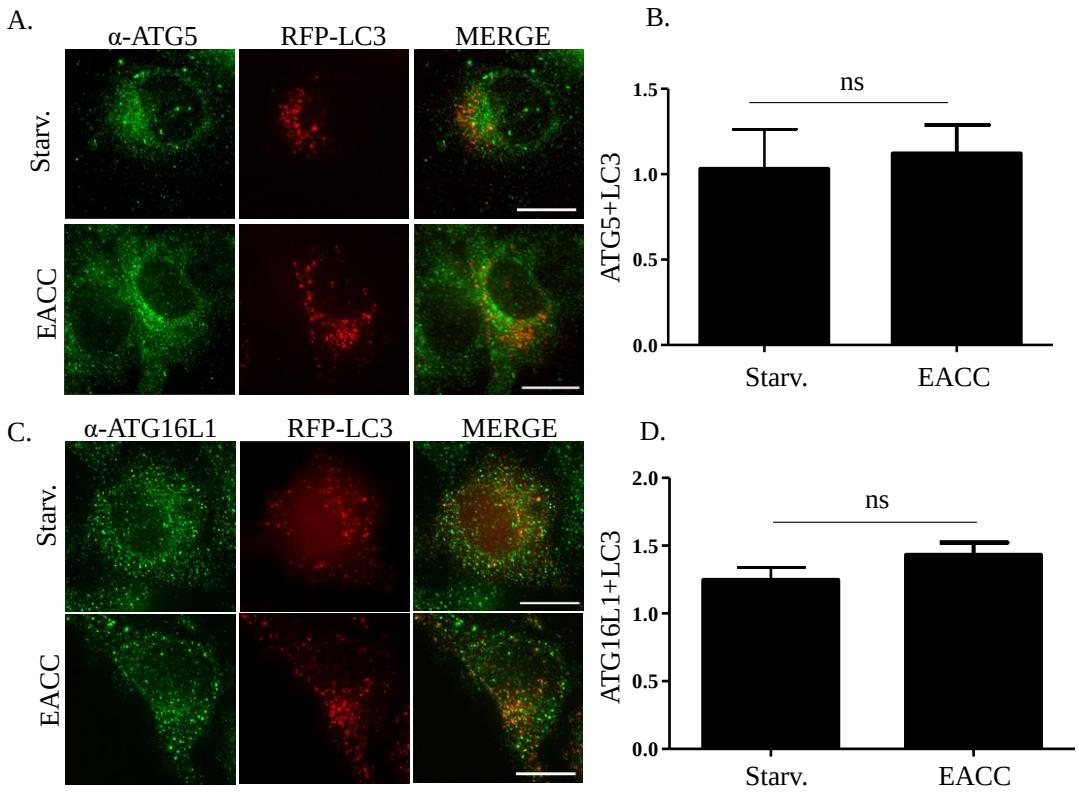


**Figure 3.4: EACC does not affect expansion of the isolation membrane**

**(A)** HeLa cells were transfected with RFP-LC3, treated with EACC and immunostained with anti-ATG5 antibody. Scale=15 µm **(B)** Graph showing the mean intensity of colocalization between ATG5 and RFP-LC3 measured as in **Fig. 2.3 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ns=non-significant. **(C)** RFP-LC3 transfected HeLa cells were treated with EACC (10µM) for 2 hours in starvation conditions and immunostained with anti-ATG16L1 antibody. Scale=15 µm **(D)** Graph showing the mean intensity of colocalization between ATG16L1 and RFP-LC3 measured as in **Fig. 2.3 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ns=non-significant.

In the previous chapter we have checked if cargo recognition was affected by EACC. The colocalization analysis of the autophagy adaptor p62/SQSTM1 with LC3 showed enhanced

colocalization between these proteins (**Fig. 2.3 A, B**). These results indicate that signaling events leading to autophagy induction, the isolation membrane formation and expansion and cargo recognition remains unaltered in presence of EACC. The accumulation of autophagic cargo suggests that EACC is a late-stage inhibitor of autophagic flux.

### 3.3 Discussion

In the previous chapter, we performed detailed analysis to show that EACC is a selective inhibitor of autophagic flux. This chapter focuses on trying to understand the effect of EACC on early steps of autophagy.

Pre-treatment with transcription and translation inhibitors showed that EACC does not enhance transcription or translation of LC3. Nutrient starvation inhibits mTOR and its substrates and induces autophagy. We observed that EACC did not affect the dephosphorylation of mTOR substrates and in turn induction of autophagy. ULK1, the first autophagy protein in the hierarchical order, gets phosphorylated and inhibited by active mTOR. Induction of autophagy by starvation leads to dephosphorylation and activation of ULK1. This dephosphorylation event was unchanged upon EACC treatment. 3-MA and wortmannin, the earliest autophagy inhibitors to be identified (Blommaart et al., 1997; Seglen and Gordon, 1982) act on the autophagy process by inhibiting PI3kinases. Generation of PI3P by the action of PI3kinases is important for binding of PI3P-binding proteins such as WIPI2 and DFCP1 and isolation membrane formation. We looked at two different markers of the isolation membrane and their colocalization with EACC and showed that EACC treatment does not affect isolation membrane formation.

We also checked the effect of EACC on expansion of the isolation membrane. Action of ATG5-12/16 complex is important for recruitment of LC3 and expansion of autophagosomes. We observed that EACC does not affect the expansion of

autophagosomes by quantitating the mean intensity of colocalization between ATG16L1 and ATG5 with LC3. This data is further corroborated with colocalization analysis of the autophagy adaptor p62/SQSTM1 with LC3 which showed enhanced colocalization between these proteins and thereby suggests the cargo loading is unaffected in presence of EACC.

As autophagy is a multi-step process and a block in any of the steps could lead to an overall block in autophagic flux, it is important to do a step-by-step analysis to understand the effect of an autophagy modulator. The results in this chapter suggest that EACC does not affect the early steps of autophagy. They also corroborate the findings of the last chapter that EACC treatment most likely causes an accumulation of mature autophagosomes. It also suggests that EACC might be acting on the penultimate step of autophagy i.e. autophagosome-lysosome fusion. The upcoming chapter focuses on studying the effect of EACC on various molecular players involved in the process of autophagosome-lysosome fusion.

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# Chapter 4

# EACC reversibly affects translocation of autophagosomal SNARE Stx17 onto autophagosomes

# Chapter 4
# EACC reversibly affects translocation of autophagosomal SNARE Stx17 onto autophagosomes

## 4.1 Overview

In the previous chapters, we have performed several assays to show that EACC is a selective inhibitor of autophagic flux. We showed that although EACC blocks autophagosome-lysosome fusion, unlike BafA1 or CQ it does not affect lysosomal pH or function or the other vesicular trafficking pathways culminating at the lysosomes. In Chapter 2, we looked at the early steps of autophagy in order to understand the effect of EACC on the same. We showed that induction of autophagy, isolation membrane formation, its elongation and cargo capture is unaffected in presence of EACC.

In this chapter, we look at the various molecular players involved in autophagosome-lysosome fusion and the effect of EACC on them. Stx17 was identified as an autophagosomal Qa SNARE. Stx17 has a reticular/tubular pattern in nutrient rich conditions, suggesting ER/Mitochondrial localization. Upon nutrient starvation, Stx17 translocates to complete autophagosomes assuming a punctate localization (Itakura et al., 2012).

Firstly, we checked the status of Stx17 upon EACC treatment. By performing various assays, we showed that EACC inhibits the translocation of Stx17 onto autophagosomes and LC3-Stx17 interaction. The autophagosomes in presence of EACC were devoid of not only Stx17 but also SNAP29. We further looked at the interaction of Stx17 with the accessory protein ATG14. Stx17-ATG14 colocalization was reduced upon EACC treatment.

Next, we also checked the effect of EACC on the interaction of Stx17 with the tethering complex HOPS and the lysosomal R-SNARE VAMP8 (Itakura et al., 2012; Jiang et al., 2014). EACC inhibited the interaction of Stx17 with HOPS subunit VPS33A and the lysosomal SNARE VAMP8 but does not affect lysosomal SNARE localization. We concluded that EACC renders autophagosomes 'fusion incompetent' but does not affect the fusion competence of the lysosomes. Interestingly, the action of EACC is reversible and washing out EACC allows Stx17 translocation back onto autophagosomes and rescues autophagic flux.

## 4.2 Results

### 4.2.1 EACC inhibits translocation of SNARE Stx17 onto autophagosomes

Noburu Mizushima's group identified Stx17 as an autophagosomal SNARE that translocates to autophagosomes upon induction of autophagy. Stx17 is $Q_a$ SNARE that partners with $Q_{bc}$ SNARE SNAP29 and endo/lysosomal R-SNARE VAMP8 with the help of multi-subunit tethering complex like HOPS and Rab proteins like Rab7. Depletion of Stx17 blocked autophagic flux by inhibiting fusion of autophagosomes with lysosomes (Itakura et al., 2012).

We co-transfected HeLa cells with FLAG-Stx17 and GFP-LC3 and quantitated the colocalization between Stx17 and LC3. Similar to previous reports, in nutrient rich conditions, Stx17 depicted a reticulate/tubular pattern suggesting an ER/mitochondrial localization. Upon induction of autophagy by nutrient starvation, Stx17 forms more puncta which have significant colocalization with LC3. This colocalization increased even further in the presence of a late-stage inhibitor such as BafA1 which blocks fusion causing accumulation of autophagosomes without affecting the autophagosomal SNAREs (Itakura

et al., 2012). Interestingly, upon EACC treatment the number of colocalized puncta between Stx17 and LC3 reduced significantly (**Fig. 4.1 A, B**).

A recent publication showed that the pathogenic bacterium *Legionella pneumophila* can block autophagic flux in order to escape autophagic capture by degrading Stx17 (Arasaki et al., 2017). However, presence of EACC did not affect significantly Stx17 expression (**Fig. 4.1 C**).

We further performed a Co-Immunoprecipitation (Co-IP) assay in order to further understand the effect of EACC on LC3-Stx17 interaction. We transfected HeLa cells with either FLAG-Stx17 or an empty vector and treated them with EACC, 48 hours after transfection. We probed for the levels of LC3-II in Flag-Stx17 immunoprecipitates. The relative levels of LC3-II in FLAG-Stx17 IP (after normalising it to input LC3) was reduced upon EACC treatment (**Fig. 4.1 D, E**).

In order to corroborate the decrease in LC3-Stx17 interaction upon EACC treatment, we performed a proximity ligation assay to quantitate endogenous protein-protein interactions between LC3 and Stx17. The number of PLA positive dots representing LC3-Stx17 interaction decreased upon EACC treatment (**Fig. 4.1 F, G**). These results together suggest that EACC affects the starvation induced translocation of the autophagosomal SNARE Stx17 onto autophagosomes.

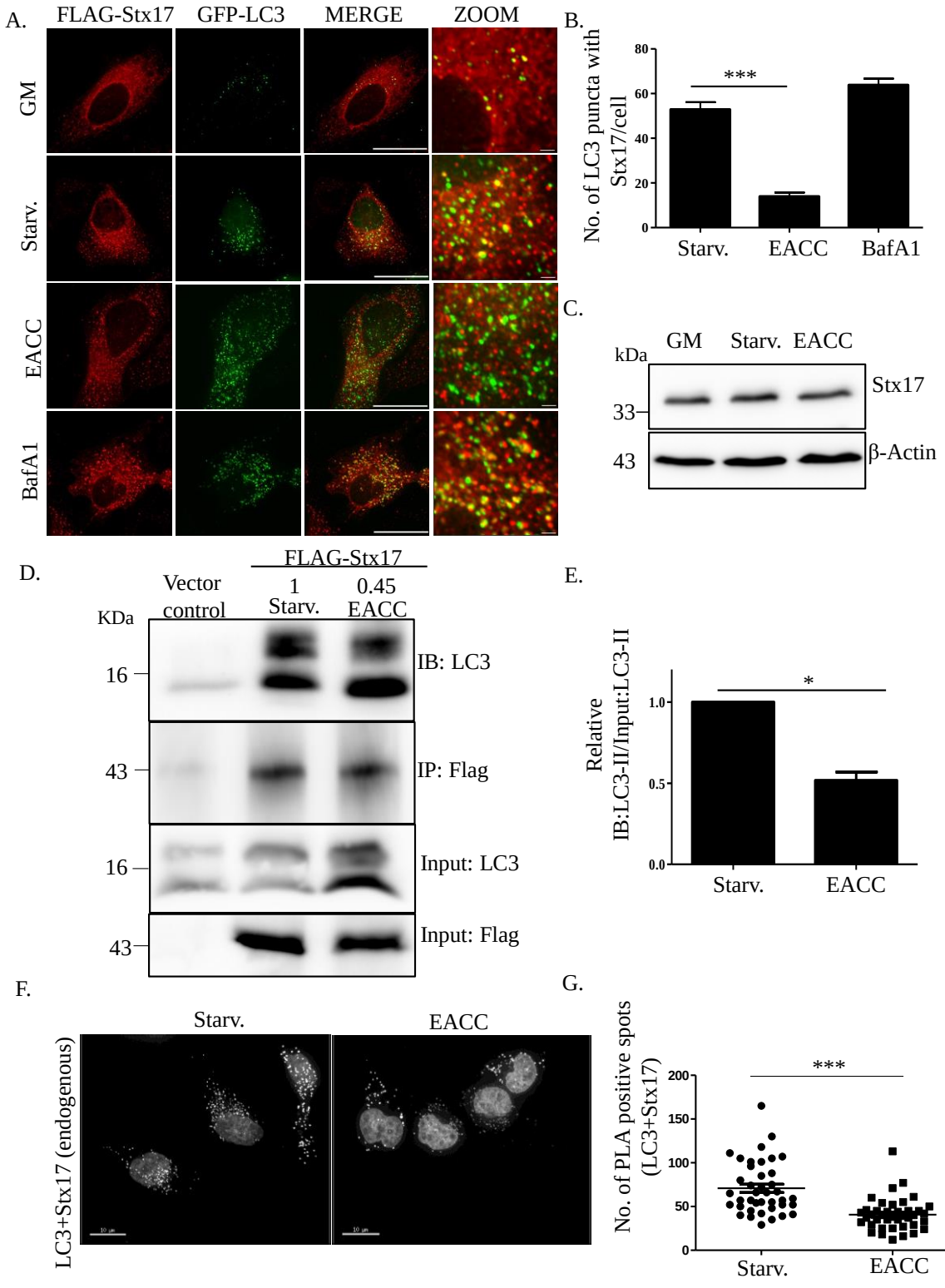
A.
FLAG-Stx17
GFP-LC3
MERGE
ZOOM
GM
Starv.
EACC
BafA1
B.
No. of LC3 puncta with Stx17/cell
***
Starv.
EACC
BafA1
C.
kDa
GM
Starv.
EACC
33
43
Stx17
β-Actin
D.
FLAG-Stx17
Vector control
1 Starv.
0.45 EACC
KDa
16
43
16
43
IB: LC3
IP: Flag
Input: LC3
Input: Flag
E.
Relative IB:LC3-II/Input:LC3-II
*
Starv.
EACC
F.
Starv.
EACC
LC3+Stx17 (endogenous)
G.
No. of PLA positive spots (LC3+Stx17)
***
Starv.
EACC

**Figure 4.1: EACC inhibits the translocation SNARE Stx17 onto autophagosomes**

**(A)** HeLa cells co-transfected with FLAG-Stx17 and GFP-LC3 were treated with BafA1 (100nM) or EACC (10µM) for 2 hours in starvation conditions and immunostained with anti-FLAG antibody. Scale=15 µm, 1µm **(B)** Graph represents the number of colocalized dots of FLAG-Stx17 and GFP-LC3. The number of colocalized dots were counted as mentioned in **Fig. 2.4 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ****P* < 0.001 **(C)** Samples from EACC or BafA1 treated HeLa cells were immunoblotted with anti-Stx17 and anti-β-Actin antibodies. **(D)** Co-IP analysis of interaction between FLAG-Stx17 and endogenous LC3B in HeLa cells either left untreated or treated with EACC. Relative levels of LC3B-II in untreated and EACC treated cells are mentioned. **(E**) Data indicates mean ± SEM of relative levels of LC3B-II in FLAG-Stx17 IP normalized to Input LC3B-II from 3 independent experiments. Statistical significance was analysed by Student's Paired t-test. **P* < 0.05**. (F)** HeLa cells treated with or without EACC were subjected to PLA with LC3-Stx17 antibody pair. Scale=10 µm **(G)** The number of PLA positive dots representing LC3-Stx17 interaction were counted using the cell-counter plugin of ImageJ. Graph represents the number of PLA positive dots representing LC3-Stx17 interaction plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ****P* < 0.001

### 4.2.2 EACC also inhibits the translocation of SNAP29 onto autophagosomes

Stx17 is a $Q_a$ SNARE that partners with $Q_{bc}$ SNARE SNAP29 (Guo et al., 2014; Itakura et al., 2012). In Hela cells, transfected with FLAG-SNAP29, Myc-Stx17 and RFP-LC3, the percentage of autophagosomes having both SNAP-29 and Stx17 was reduced in EACC treated cells as compared to control (**Fig. 4.2 A, B**). Interestingly, the colocalization between the partner SNAREs Stx17 and SNAP29 was largely unaffected post EACC treatment (**Fig 4.2 C**).

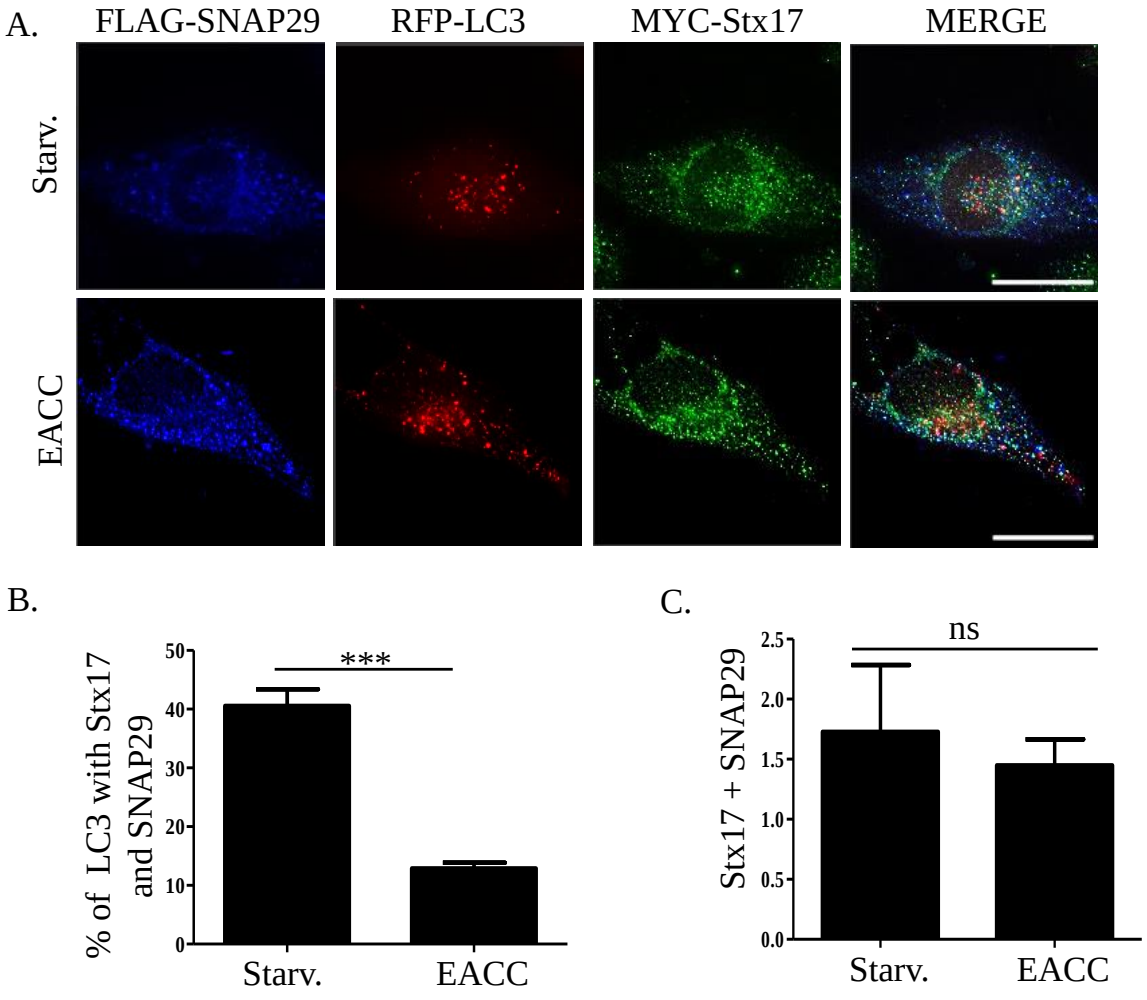


**Figure 4.2: EACC also inhibits the translocation of SNAP29 onto autophagosomes**

**(A)** HeLa cells co-transfected with MYC-Stx17, RFP-LC3 and FLAG-SNAP29 were either left untreated or treated with EACC and immunostained with anti-FLAG and anti-MYC antibodies. Scale=15 µm **(B)** Graph represents the percentage of LC3 puncta colocalizing with Stx17 and SNAP-29. The number of colocalized dots were counted as mentioned in **Fig. 2.4 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ***$P < 0.001$. **(C)** Graph showing the mean intensity of colocalization between FLAG-SNAP29 and MYC-Stx17 measured as explained in **Fig. 2.3 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ns=non-significant.

### 4.2.3 Presence of EACC reduces the interaction between Stx17 and ATG14

In addition to the Qa and Qbc SNAREs, ATG14 is also involved in autophagosome-lysosome fusion. It binds to the SNARE domain of Stx17 and stabilizes the Stx17-SNAP29 complex on autophagosomes. ATG14 binding to Stx17 can enhance the fusion capabilities of the autophagic SNAREs. This function of ATG14 is independent of its role in autophagosome biogenesis (Diao et al., 2015; Hamasaki et al., 2013). EACC treatment reduced the mean intensity of colocalization of ATG14 and Stx17 (**Fig. 4.3 A, B**).

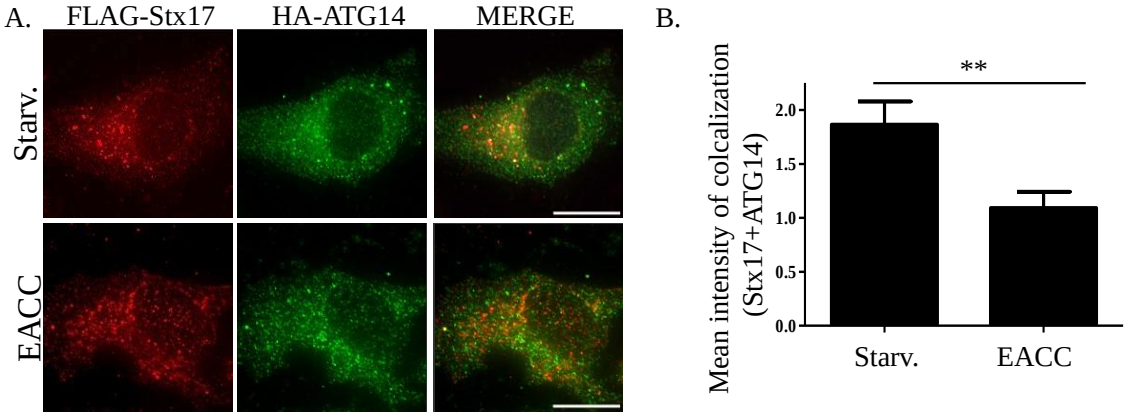


**Figure 4.3: Presence of EACC reduces the interaction between Stx17 and ATG14**

**(A)** HeLa cells co-transfected with FLAG-Stx17 and HA-ATG14 were treated with EACC and immunostained with anti-FLAG and anti-HA antibodies. Scale=15 µm **(B)** Graph showing the mean intensity of colocalization between FLAG-Stx17 and HA-ATG14 measured as explained in **Fig. 2.3 B**. Data shown here represents a minimum of 30 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. $^{**}P < 0.01$.

Overall, all these results suggest that EACC renders autophagosomes 'fusion incompetent' by preventing Stx17 translocation onto autophagosomes. These autophagosomes are also devoid of SNAP29. Finally, EACC treatment reduces the interaction between Stx17 and ATG14.

### 4.2.4 EACC treatment inhibits the interaction of Rab7 with LC3

Apart from SNAREs, autophagosome-lysosome fusion also requires accessory proteins like small GTPase RAB7 and multi-subunit tethering complex HOPS. RAB7 interacts with Stx17 and LC3 and is essential for autolysosome formation (Hyttinen et al., 2013). While control cells showed significant association of RAB7 with LC3, EACC treatment decreased the percentage of LC3 puncta that colocalized with RAB7 reiterating that EACC affects autophagosome-lysosome fusion (**Fig. 4.4 A, B**).

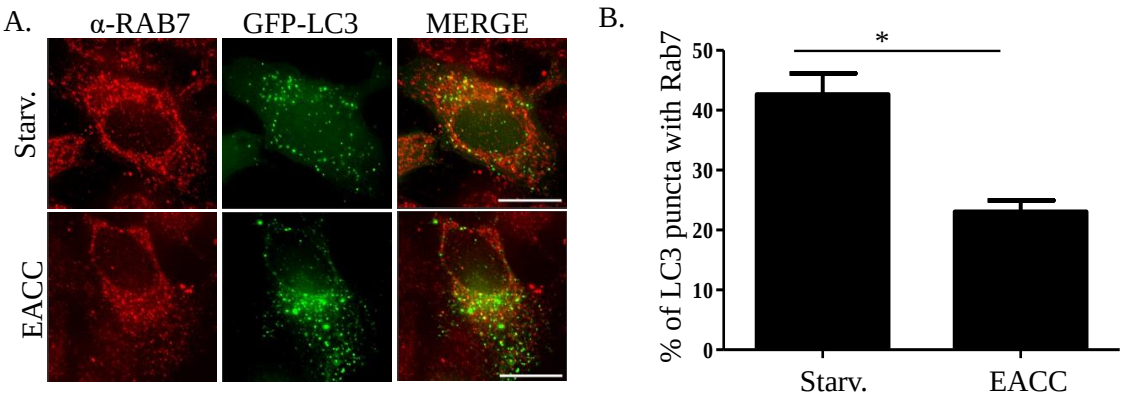


**Figure 4.4: EACC treatment inhibits the interaction of Rab7 with LC3**

**(A)** GFP-LC3 transfected HeLa cells were treated with EACC and immunostained with anti-RAB7 antibody. Scale=10 µm **(B)** Graph represents the number of LC3 puncta colocalizing with RAB7. The number of colocalized dots were counted as in **Fig. 2.4 B.** Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. **P* < 0.05.

### 4.2.5 EACC inhibits the interaction of tether HOPS with Stx17

HOPS is a multi-subunit tethering complex comprising of VPS33A, VPS16, VPS41, VPS18, VPS11, VPS39. HOPS subunits interact with Stx17 and directly promote autophagosome-lysosome fusion. HOPS complex can bridge autophagosomal and lysosomal membranes and facilitate SNARE complex formation (Jiang et al., 2014; Takats et al., 2014). In HeLa cells co-transfected with FLAG-Stx17 and HA-VPS33A, we calculated the Pearson's Correlation Coefficient (PCC) between Stx17 and VPS33A. We observed that the colocalization between HOPS specific subunit VPS33A and Stx17 was decreased in presence of EACC (**Fig. 4.5 A, B**). In HeLa cells co-transfected with HA-VPS33A and FLAG-Stx17, we performed a Co-IP assay using FLAG-tagged magnetic beads. EACC treatment reduced the levels of HA-VPS33A in FLAG-Stx17 immunoprecipitate (**Fig. 4.5 C**). These results together suggest that EACC inhibits the interaction of tether HOPS with Stx17.

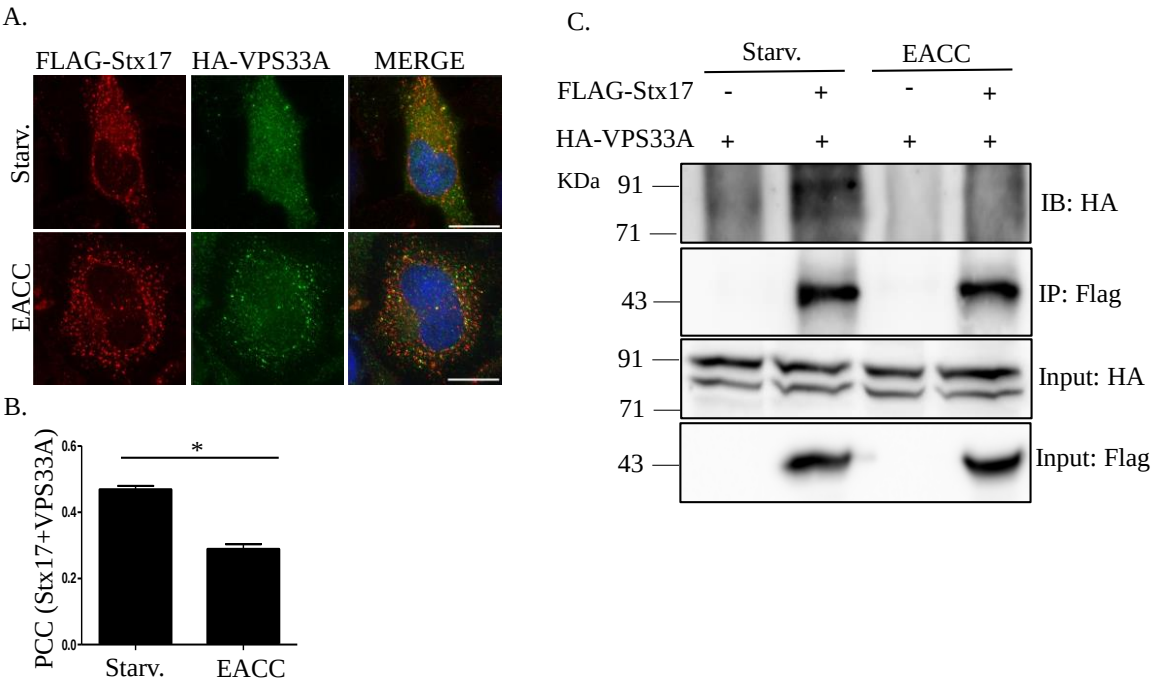

**Figure 4.5: EACC inhibits the interaction of tether HOPS with Stx17 (A)** HeLa cells co-transfected with FLAG-Stx17 and HA-VPS33A were either left untreated or treated with EACC (10µM) for 2 hours. Scale=10 µm **(B)** Graph showing Pearson's Correlation Coefficient (PCC) between Stx17 and VPS33A. PCC was measured using SoftWorx software from DeltaVision. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. *$P < 0.05$. **(C)** HeLa cells transfected with FLAG-Stx17 and HA-VPS33A or only HA-VPS33A were either left untreated or treated with EACC. IP was performed using FLAG-tagged magnetic beads and the levels of HA-VPS33A and FLAG-Stx17 was checked by immunoblotting.

### 4.2.6 EACC inhibits the interaction of lysosomal SNARE VAMP8 with Stx17 but does not affect lysosomal 'fusion competence'

Our experiments so far suggested that EACC treatment resulted in accumulation of 'fusion incompetent' autophagosomes devoid of autophagosomal SNAREs. We further tested if EACC could affect the 'fusion competence' of lysosomes. As shown in chapter 2**,** EACC treatment did not perturb EGFR degradation which hints that lysosomes are competent enough to fuse with the incoming traffic. We further investigated the status of the fusion machinery on lysosomes, in particular the R-SNARE VAMP8 required for autolysosome formation (Itakura et al., 2012). In HeLa cells transfected with GFP-VAMP8 and immunostained for LAMP1, there was no apparent change in VAMP8 and LAMP1 association after EACC treatment as compared to control (**Fig. 4.6 A, B)**. As EACC prevents Stx17 translocation onto autophagosome and blocks autophagosome-lysosome fusion, we checked the colocalization between Stx17 and the lysosomal R-SNARE VAMP8. EACC treatment in cells co-transfected with FLAG-Stx17 and GFP-VAMP8 showed decreased colocalization between Stx17 and VAMP8 (**Fig. 4.6 C, D**).

To further consolidate these findings, we used immunoprecipitation assay to check the effect of EACC on Stx17-VAMP8 interaction. In HeLa cells co-transfected with GFP-VAMP8 and FLAG-Stx17, we performed a Co-IP assay using GFP-Trap beads. EACC treatment reduced the levels of FLAG-Stx17 in GFP-VAMP8 immunoprecipitate **(Fig. 4.6 E)**.

These results suggest that EACC treatment reduces Stx17 interaction with tethering complex HOPS and the lysosomal R-SNARE VAMP8. All these factors collectively prevent autophagosome-lysosome fusion and block autophagic flux.

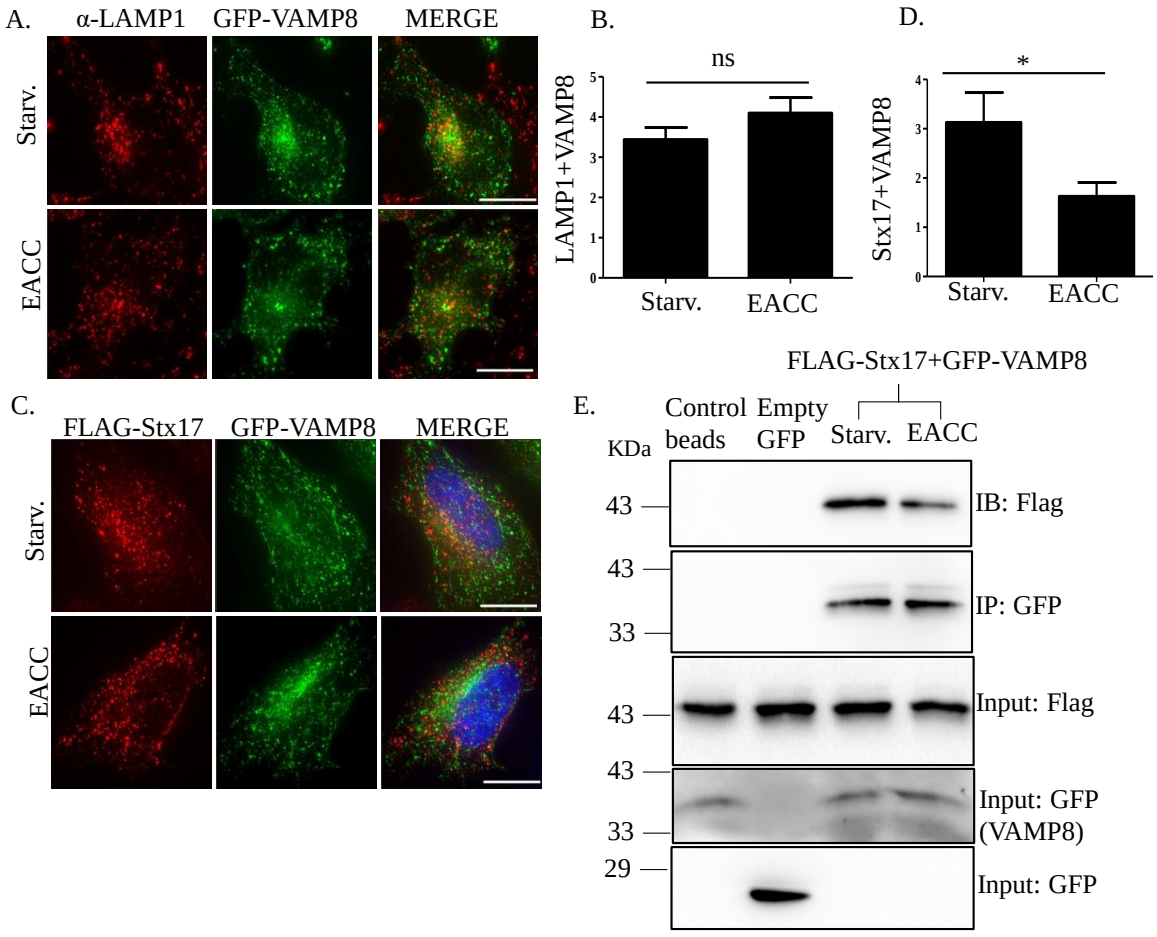


**Fig. 4.6: EACC inhibits the interaction of lysosomal SNARE VAMP8 with Stx17 but does not affect lysosomal 'fusion competence'**

**(A)** GFP-VAMP8 transfected HeLa cells were immunostained with anti-LAMP1 antibody. Scale=10 µm **(B)** Graph representing the mean intensity of colocalization between LAMP1 and VAMP8. The mean intensity of colocalized dots was measured as in **Fig. 2.3 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ns=non-significant. **(C)** HeLa cells co-transfected with FLAG-Stx17 and GFP-VAMP8 were either left untreated or treated with EACC. Scale=10 µm **(D)** Graph representing the mean intensity of colocalization between Stx17 and VAMP8. The mean intensity of colocalization was measured as in. **Fig. 2.3 B** Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. $*P < 0.05$ **(E)** HeLa cells transfected with FLAG-Stx17 and GFP-VAMP8 or FLAG-Stx17 and Empty GFP Vector were either left untreated or treated with EACC. IP was performed using control agarose beads or GFP-Trap beads and the levels of GFP-VAMP8 and FLAG-Stx17 was checked by immunoblotting.

### 4.2.7 The action of EACC is reversible

Thus far, we have shown that EACC inhibits autophagic flux by accumulating fusion incompetent (Stx17 negative) autophagosomes. In order to understand if the effect of EACC is reversible, we carried out EACC wash out experiments and followed autophagic flux and loading of Stx17 onto autophagosomes.

We divided EACC treated cells into three subgroups. In the first group, cells in starvation media were treated with EACC for one hour and lysates were collected. In the second group, after a similar treatment with EACC for one hour, cells were washed with DPBS and kept in starvation medium without EACC for three hours and lysates were collected. In the third group, the treatment with EACC was allowed to go on for four hours and lysates were collected after that. All the lysates were probed for LC3-II expression. The accumulation of LC3-II upon EACC treatment was observed after 1hour treatment.

Washing out EACC, decreased the accumulation of LC3-II similar to the levels of starvation control (**Fig. 4.7 A, B**).

We observed a rescue in autophagic flux by washing out EACC, so in order to further corroborate our immunoblotting based results and to understand if the rescue we observe is due to an increase in autolysosome number, we utilized tandem tagged mRFP-GFP-LC3 construct. HeLa cells transfected with mRFP-GFP-LC3 were treated with EACC in a similar manner as explained above. After one-hour treatment, we saw a significant increase in the number of autophagosomes ($mRFP^+/GFP^+$) and a concomitant decrease in the number of autolysosomes ($mRFP^+/GFP^-$) as compared to control. After washing out EACC, the autophagosome and autolysosome numbers became comparable to that of control (**Fig. 4.7 C-E**). These results suggest that washing out EACC can reverse the block in autophagosome-lysosome fusion.

Previous experiments have shown that EACC inhibits translocation of Stx17 to autophagosomes. So, next we tested if the rescue in autolysosome number upon washing out EACC was due to restoration of the SNARE Stx17 onto autophagosomes. In HeLa cells transfected with FLAG-Stx17 and RFP-LC3, we counted the number of $Stx17^+$ autophagosomes before and after EACC wash-out. There were very few $LC3^+/Stx17^+$ puncta in cells treated with EACC for four hours. On the other hand, the number of $LC3^+/Stx17^+$ puncta in cells in which EACC was washed out after an hour was similar to that of control. (**Fig. 4.7 F-H**).

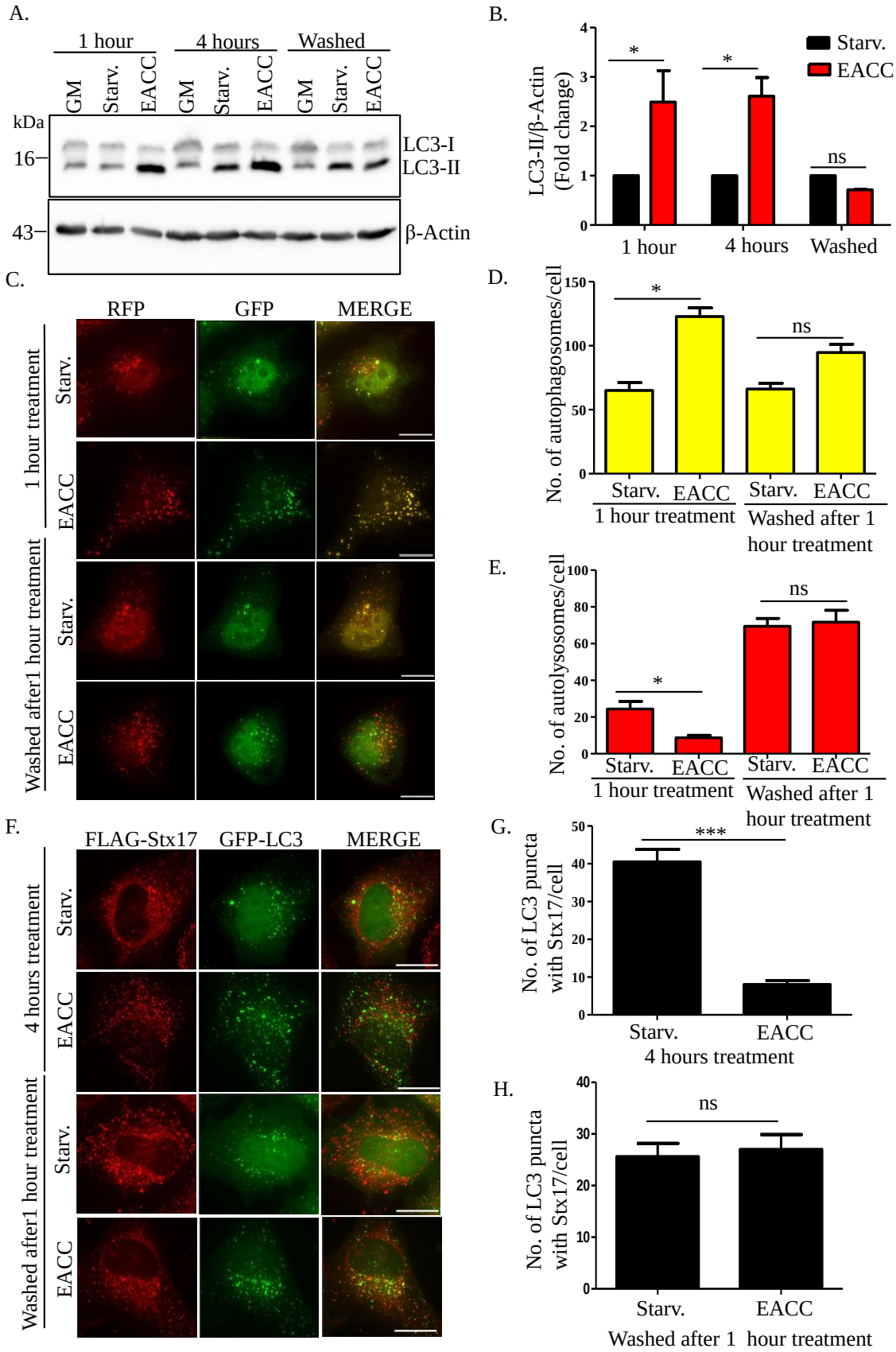
A.
1 hour
4 hours
Washed
GM
Starv.
EACC
kDa
16
43
LC3-I
LC3-II
β-Actin
B.
LC3-II/β-Actin (Fold change)
Starv.
EACC
*
ns
1 hour
4 hours
Washed
C.
RFP
GFP
MERGE
1 hour treatment
Washed after1 hour treatment
D.
No. of autophagosomes/cell
Starv.
EACC
1 hour treatment
Washed after 1 hour treatment
E.
No. of autolysosomes/cell
F.
FLAG-Stx17
GFP-LC3
MERGE
4 hours treatment
G.
No. of LC3 puncta with Stx17/cell
***
4 hours treatment
H.
Washed after 1 hour treatment

**Fig. 4.7: The action of EACC is reversible**

**(A)** We divided EACC treated cells into three subgroups. In the first group, cells in starvation media were treated with EACC for one hour and lysates were collected. In the second group, after a similar treatment with EACC for one hour, cells were washed with DPBS and kept in starvation medium without EACC for three hours and lysates were collected. In the third group, the treatment with EACC was allowed to go on for four hours and lysates were collected after that. All the lysates were probed for LC3B-II expression. **(B)** Relative levels of LC3-II: β-Actin in untreated versus treated samples were quantitated for 3 independent experiments. $*P < 0.05$, ns= non-significant (Two-way ANOVA, replicate means compared with Bonferroni post-test) **(C)** HeLa cells were transfected with tandem tagged mRFP-GFP-LC3 construct for 48 hours and treatment was carried out as explained above. Scale: 15 µm **(D, E)** The number of autophagosomes ($RFP^+/GFP^+$ structures) and autolysosomes ($RFP^+/GFP^-$ structures) per cell in various treatment conditions were counted using the cell counter plugin in ImageJ. Data shown represents number of autophagosomes ($RFP^+/GFP^+$) and autolysosomes ($RFP^+/GFP^-$) for a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. $*P < 0.05$, ns= non-significant. **(F)** HeLa cells transfected with FLAG-Stx17 and GFP-LC3 were treated with EACC (10µM) as explained above and immunostained with anti-FLAG antibody. Scale: 10 µm **(G, H)** Graph represents the number of LC3 puncta colocalizing with Stx17. The number of colocalized dots was counted as mentioned in **Fig. 2.4 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. $***P < 0.001$, ns=non-significant.

## 4.3 Discussion

In this chapter, we show that EACC inhibits the translocation of autophagosome specific SNARE Stx17 thereby blocking autophagosome-lysosome fusion.

We had previously shown that EACC causes accumulation of LC3-II over and above that of starvation induced autophagy and that the increase in LC3-II is due to a block in autophagic flux rather than autophagy induction. Additionally, upon probing each step in the process of autophagy, we narrow down the action of EACC to the penultimate step of

autophagic flux, i.e. fusion of autophagosomes with lysosomes resulting in accumulation of autophagosomes., We further concluded that EACC does not affect the localization of R-SNARE VAMP8 present on lysosomes and thereby the ability of lysosomes to fuse with other incoming traffic.

Previous published literature has shown that during autophagosome-lysosome fusion, first Stx17 gets loaded on autophagosomes followed by SNAP29 recruitment. This $Q_{abc}$ SNARE complex is stabilized by ATG14. Subsequently, successful fusion ensues when SNARE pairing ($Q_a$ Stx17, $Q_{bc}$ SNAP29 and the lysosomal R-SNARE VAMP8) is promoted by small GTPase RAB7 and tethering complex HOPS (Diao et al., 2015; Guo et al., 2014; Itakura et al., 2012; Jiang et al., 2014; Takats et al., 2014).

The striking feature of EACC is that it blocks autophagosome-lysosome fusion by impairing Stx17 loading onto autophagosomes. As per our knowledge, we have not come across any other report suggesting a chemical modulator of autophagy that can selectively prevent Stx17 translocation thereby rendering autophagosomes 'fusion incompetent'. The exact mechanism by which Stx17 is translocated onto complete autophagosomes is not very clear. A recent report suggested that Stx17 recruitment to autophagosomes occurs via interaction with a small GTPase IRGM and mammalian ATG8 proteins (Kumar et al., 2018). We propose that identification of Stx17 binding partners in presence or absence of EACC could give a clue regarding the target of EACC as well as help in identification of any other accessory factors that might be involved in Stx17 recruitment on autophagosomes.

Furthermore, we also showed that the action of EACC is reversible. Washing out EACC can rescue the block in autophagic flux because Stx17 is now able to translocate to autophagosomes and participate in further fusion events. Hence, we propose that due to

the reversible nature of its action, EACC can be used as a useful tool to study the dynamic Stx17 trafficking.

## 4.4 References

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# Chapter 5

# EACC sheds light on the regulation of autophagic flux via Stx17 trafficking

# Chapter 5
# EACC sheds light on the regulation of autophagic flux via Stx17 trafficking

## 5.1 Overview

In the past chapters, we have identified and characterized EACC as an inhibitor of autophagosome-lysosome fusion. We further showed that the effect of EACC is selective to autophagy and it does not affect the lysosomal pH or function or other vesicular trafficking pathways such as endocytosis that culminate at the lysosomes. In order to further understand the mechanism of action of EACC, we did a step-by-step analysis for all the steps of autophagy.

EACC treatment did not affect induction of autophagy or the steps that lead to autophagosome biogenesis such as isolation membrane formation or its expansion. We further showed that EACC blocks autophagic flux by inhibiting translocation of autophagosomal SNAREs onto autophagosomes without impeding the lysosomal 'fusion competence'. It also decreases the interaction between the autophagosomal SNARE Stx17 and the tethering complex HOPS as well the lysosomal SNARE VAMP8. These factors are collectively responsible for the block in autophagic flux mediated by EACC.

Next, we were trying to understand the reasons for inhibition of Stx17 translocation mediated by EACC. According to published reports, Stx17 in nutrient rich conditions is present in ER, Mitochondria and ER-Mitochondria contact sites. Here, it interacts with the mitochondrial fission regulator protein Drp1, assists in its assembly and ensures mitochondrial fission. Whereas upon nutrient starvation, Stx17 translocates onto autophagosomes and regulates autophagosome-lysosome fusion (Arasaki et al., 2015; Itakura et al., 2012). In presence of EACC, as the starvation mediated translocation of Stx17

was blocked, we wondered if the role of Stx17 in mitochondrial dynamics and its role in autophagic flux was connected.

In this chapter, we showed that EACC treatment led to enhanced mitochondrial fission which hinted at it having a role in mitochondria. We further showed that EACC enhances Stx17-Drp1 interaction even in starvation conditions. Furthermore, inhibiting this interaction between Stx17 and Drp1 by either genetic or chemical means not only allowed Stx17 translocation onto autophagosomes, but promoted autophagosome-lysosome fusion even in presence of EACC.

## 5.2 Results

### 5.2.1 EACC enhances mitochondrial fragmentation

In the presence of EACC, Stx17 is not present on the autophagosomes even in starvation conditions. Next, we decided to observe the effect of EACC on other organelles where Stx17 is located such as mitochondria.

HeLa cells transfected with DsRed-Mito7, were treated with a well-studied mitochondrial uncoupler Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (10µM) in GM or EACC (10µM) for 2 hours in starvation conditions and imaged **(Fig. 5.1 A)**. CCCP increases the proton permeability across the mitochondrial inner membrane, and dissipates the mitochondrial membrane potential. This results in enhanced mitochondrial fission (Lim et al., 2001). Starvation on the other hand, results in mitochondrial elongation which maximizes ATP production (Arasaki et al., 2015). With the help of Mitochondrial Network Analysis (MiNa), an Image J plugin, we measured the length of mitochondrial branches in all treatment conditions which was then divided by the number of independent skeletons or fragments. This value is termed as the summed branch length mean (Valente et al., 2017). An increase in fragmentation, leads to decrease in the summed branch length mean values.

EACC treatment decreased the summed branch length mean as compared to starvation control. Interestingly, the mitochondrial fragmentation in presence of EACC was not as drastic as that of CCCP treatment. Secondly, treatment with EACC did not result in accumulation of swollen mitochondria which is a hallmark of CCCP treatment **(Fig. 5.1 A, B)**. Hence, we concluded that EACC enhances mitochondrial fragmentation as compared to starvation control but the enhanced fragmentation was less dramatic as compared to that of the mitochondrial uncoupler CCCP.

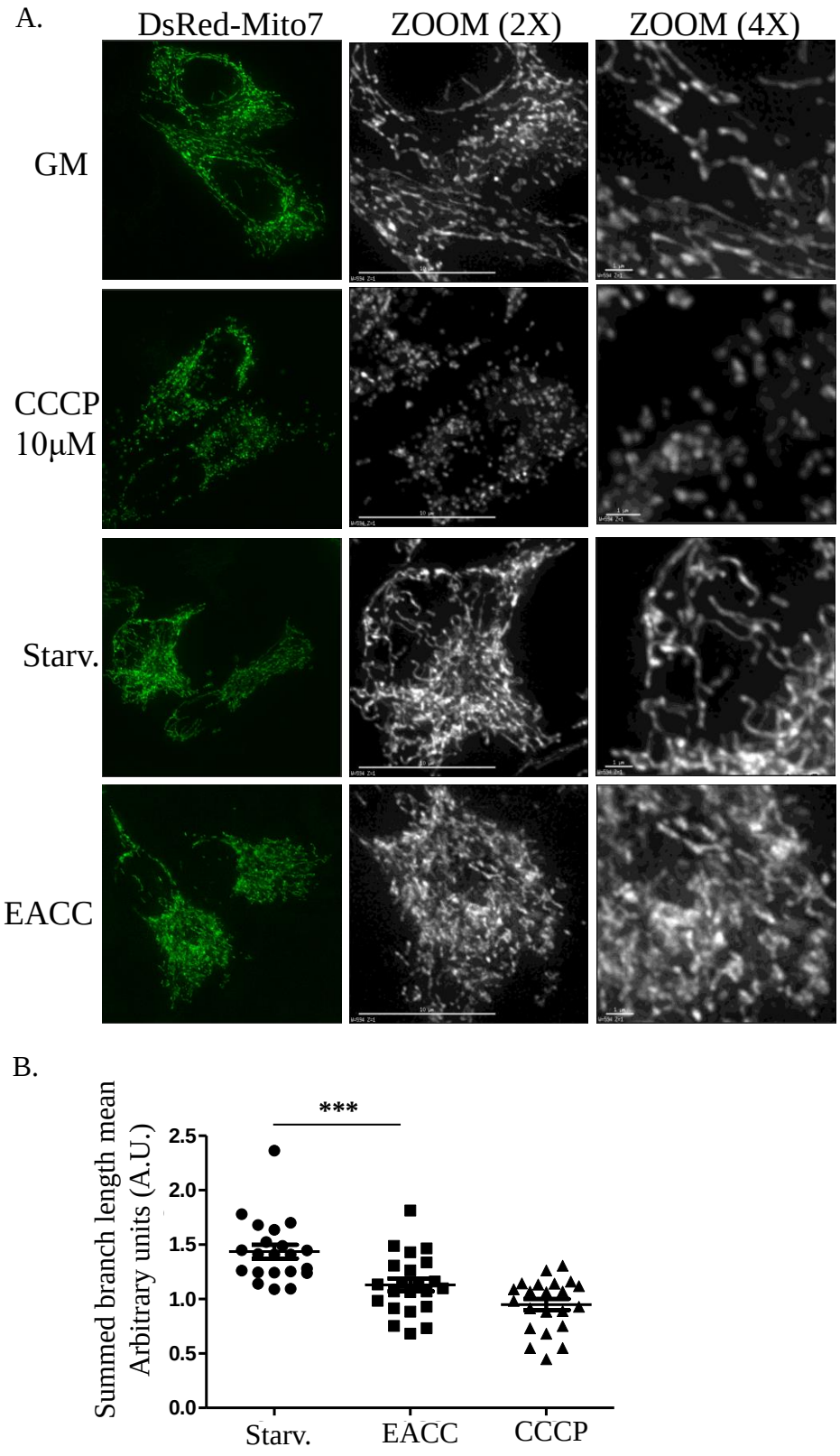


**Figure 5.1: EACC enhances mitochondrial fragmentation**

**(A)** HeLa cells transfected with DsRed-Mito7 were treated with CCCP (10µM) in GM or EACC (10µM) for 2 hours in starvation conditions. Scale=10 µm, 1µm **(B)** Graph

represents the summed branch length mean calculated in Image J using the Mitochondrial Network Analyzer (MiNa) plugin. Data shown here represents 20 panels (~7 cells per panel) for each treatment condition across 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ***$P < 0.001$

### 5.2.2 EACC treatment enhances Stx17-Drp1 interaction

So far, we had two major observations upon EACC treatment. First was the block in autophagic flux due to inhibition of Stx17 translocation from ER, mitochondria and ER-mitochondria contact sites onto autophagosomes. Secondly, we observed enhanced mitochondrial fragmentation upon EACC treatment. Based on these two observations, our hypothesis was to test whether the increase in mitochondrial fragmentation and block in autophagic flux seen upon EACC treatment is due to enhanced Stx17-Drp1 interaction even in starvation conditions.

In order to test this hypothesis, we transfected HeLa cells with GFP-LC3, FLAG-Stx17 and mCherry-Drp1. These cells were were either left untreated, treated with EACC, or pre-treated with Mdivi1 (50µM for 1 hour) followed by EACC treatment for 2hours and immunostained with anti-FLAG antibody **(Fig. 5.2 A)**. Mitochondrial division inhibitor 1 (Mdivi1) is a quinazonilone derivative that inhibits Drp1-dependent mitochondrial fission. Mdivi1 inhibits Drp1 self-assembly and GTPase activity (Cassidy-Stone et al., 2008; Ruiz et al., 2018; Tanaka and Youle, 2008).

As Stx17 interacts with Drp1 in a GTP-dependent manner and helps in Drp1 self-assembly (Arasaki et al., 2015), we used Mdivi1 to disrupt Stx17-Drp1 interaction.

In HeLa cells transfected with GFP-LC3, FLAG-Stx17 and mCherry-Drp1, EACC treatment resulted in decreased colocalization between Stx17 and LC3 and enhanced colocalization between Stx17 and Drp1 as compared to the starvation control. On the other hand, pre-treatment with Mdivi1 (50µM for 1 hour) to disrupt Stx17-Drp1 interaction followed by EACC treatment for 2hours resulted in the levels of mean intensity of

colocalization for Stx17-LC3 and Stx17-Drp1 almost comparable to the starvation control (**Fig. 5.2 B, C**).

In order to corroborate the increase in Drp1-Stx17 interaction upon EACC treatment, we performed a proximity ligation assay to quantitate endogenous protein-protein interactions between Drp1 and Stx17. HeLa cells transfected with HA-Drp1 were either left untreated or treated with EACC. The number of PLA positive dots representing Drp1-Stx17 interaction increased upon EACC treatment (**Fig. 5.2 D, E**). These results together suggest that EACC treatment decreases the interaction between LC3-Stx17 and increases the interaction between Drp1-Stx17 and the use of Drp1 inhibitor such as Mdivi1 can overcome this effect of EACC.

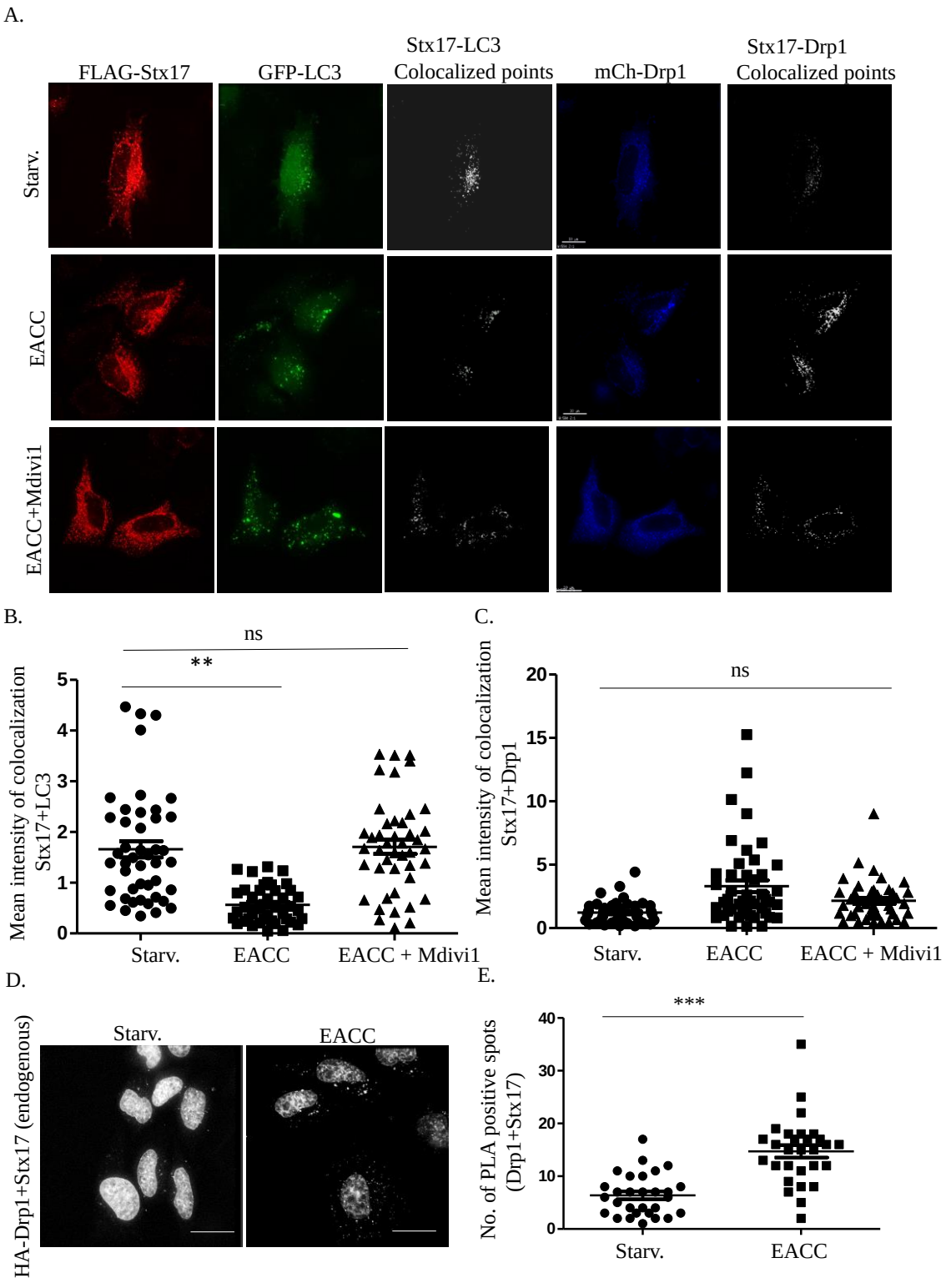


**Figure 5.2: EACC treatment enhances Stx17-Drp1 interaction**

**(A)** HeLa cells co-transfected with FLAG-Stx17, GFP-LC3 and mCherry-DRP1 were either left untreated, treated with EACC, or pre-treated with Mdivi1 (50µM for 1 hour) followed by EACC treatment for 2hours and immunostained with anti-FLAG antibody.

Scale=10 µm **(B)** Graph showing the mean intensity of colocalization between FLAG-Stx17 and GFP-LC3 measured as explained in **Fig. 2.3 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ***$P < 0.001$**,** ns=non-significant. **(C)** Graph showing the mean intensity of colocalization between FLAG-Stx17 and mCherry-Drp1 measured as explained in **Fig. 2.3 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ns=non-significant. **(D)** HeLa cells transfected with HA-Drp1 and treated with or without EACC were subjected to PLA with HA and Stx17 antibody pair. Scale=15 µm **(E)** The number of PLA positive dots representing HA-Drp1 and Stx17 interaction were counted using the cell-counter plugin of ImageJ. Graph represents the number of PLA positive dots representing HA-Drp1 and Stx17 interaction plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ***$P < 0.001$

### 5.2.3 Pretreatment with Drp1 inhibitor Mdivi1 rescues autophagic flux

In the last part, we showed that EACC treatment decreases the interaction between LC3-Stx17 and increases the interaction between Drp1-Stx17 and the use of Drp1 inhibitor such as Mdivi1 can overcome this effect of EACC. We further wanted to test if inhibiting Drp1-Stx17 interaction by genetic and chemical methods can rescue the block in autophagosome-lysosome fusion mediated by EACC.

HeLa cells transfected with tandem tagged mRFP-GFP-LC3 construct for 48 hours were either left untreated, treated with EACC, or pre-treated with Mdivi1 (50µM for 1 hour) followed by EACC treatment for 2hours. In EACC treatment as previously reported, we saw an accumulation of autophagosomes and a concomitant decrease in autolysosomes. On the other hand, in HeLa cells pre-treated with Mdivi1 the number of autolysosomes was comparable to that of the starvation control (**Fig. 5.3 A-C**). Hence, Mdivi1 mediated Drp1 inhibition could rescue autophagic flux even in presence of EACC.

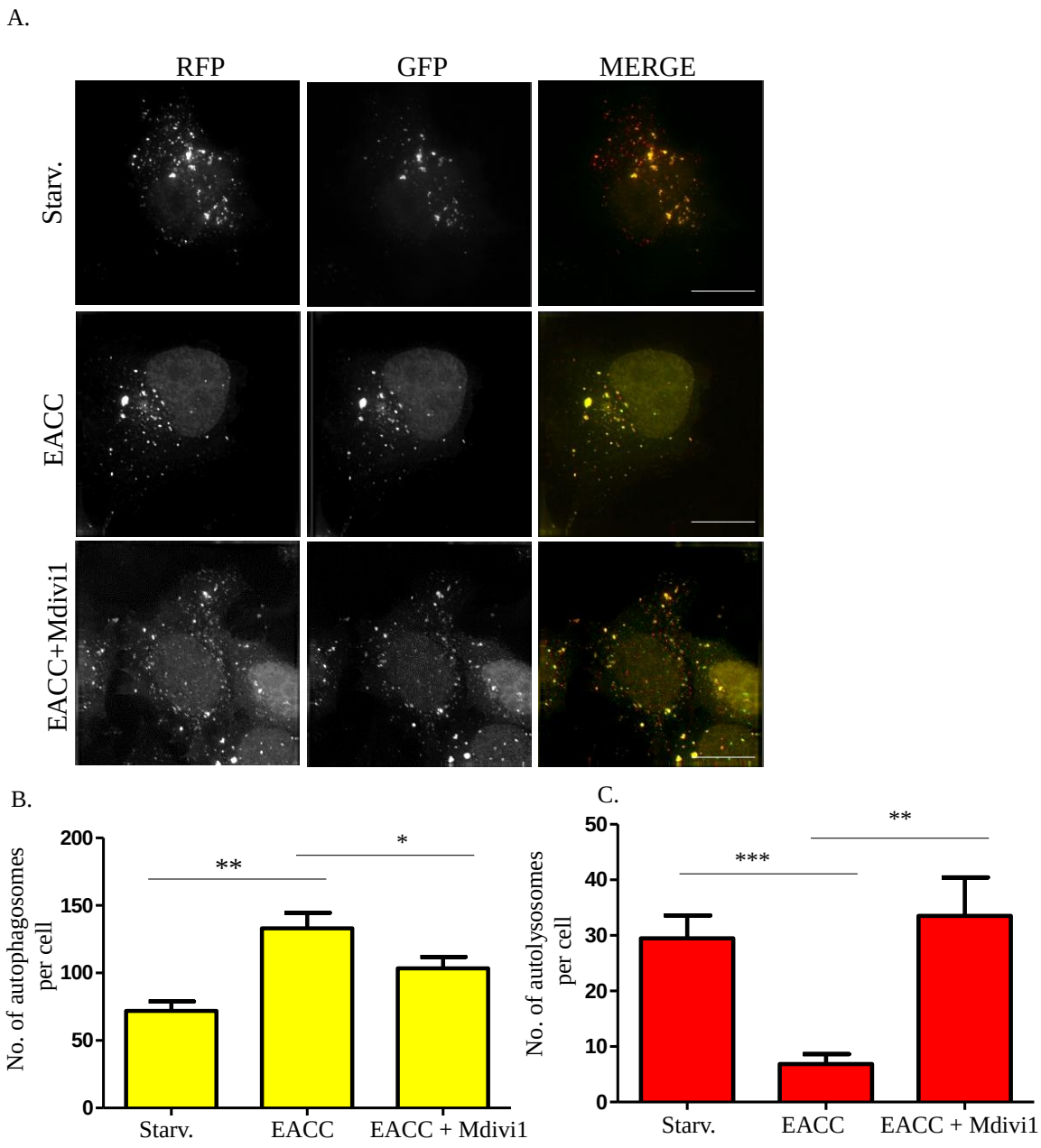


**Figure 5.3: Pretreatment with Drp1 inhibitor Mdivi1 rescues autophagic flux**

**(A)** HeLa cells transfected with tandem tagged mRFP-GFP-LC3 construct for 48 hours were either left untreated, treated with EACC, or pre-treated with Mdivi1 (50µM for 1 hour) followed by EACC treatment for 2hours. Scale: 15 µm **(B, C)** The number of autophagosomes ($RFP^+/GFP^+$ structures) and autolysosomes ($RFP^+/GFP^-$ structures) per cell in various treatment conditions were counted using the cell counter plugin in ImageJ. Data shown represents number of autophagosomes ($RFP^+/GFP^+$) and autolysosomes ($RFP^+/GFP^-$) for a minimum of 45 cells from 3 independent experiments plotted as mean ±

SEM. Statistical significance was analysed by Student's unpaired t-test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns= non-significant.

### 5.2.4 Overexpression of the dominant negative Drp1 rescues autophagic flux

Next, we overexpressed the dominant negative (K38A) form of Drp1. In this mutant, a critical lysine is converted to alanine which reduces the GTP-binding ability of Drp1 (Smirnova et al., 2001; Ugarte-Uribe et al., 2014). Since, the Drp1-Stx17 interaction is GTP-dependent (Arasaki et al., 2015), overexpression of $Drp1^{K38A}$ can inhibit Drp1-Stx17 interaction.

HeLa cells transfected with tandem tagged mRFP-GFP-LC3 and HA-Drp1 or HA-$Drp1^{K38A}$ for 48 hours were either left untreated or treated with EACC. In HeLa cells transfected with mRFP-GFP-LC3 and HA-Drp1, the number of autolysosomes in EACC treated cells was significantly lower than the starvation control whereas in HeLa cells transfected with mRFP-GFP-LC3 and HA-$Drp1^{K38A}$, the number of autolysosomes was increased significantly even in EACC treated cells (**Fig. 5.4 A-C**).

Hence, inhibiting the enhanced Drp1-Stx17 interaction by overexpression of $Drp1^{K38A}$ could rescue autophagic flux even in the presence of EACC.

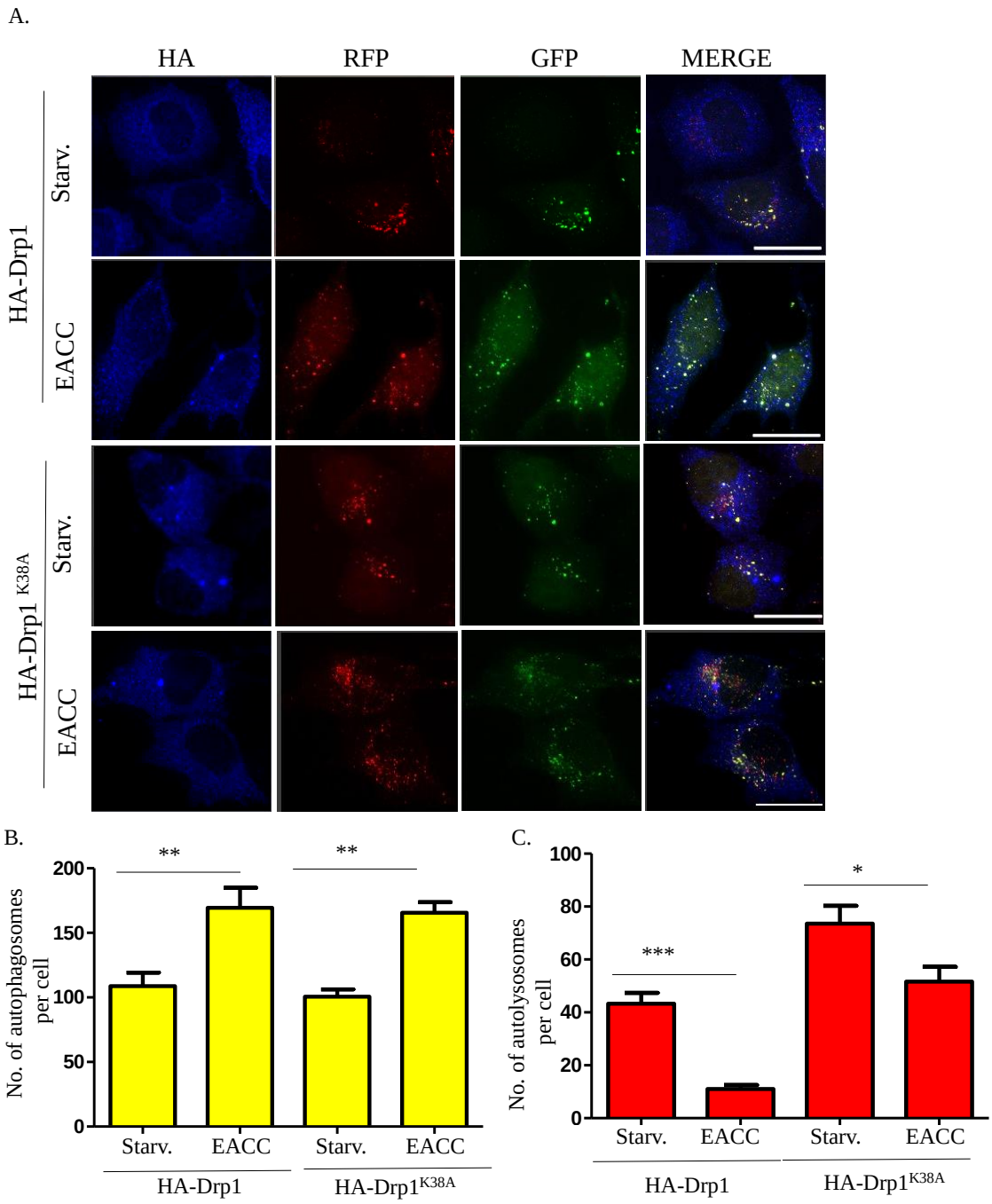


**Figure 5.4: Overexpression of the dominant negative Drp1 rescues autophagic flux**

**(A)** HeLa cells transfected with tandem tagged mRFP-GFP-LC3 and HA-Drp1 or HA-Drp1$^{K38A}$ for 48 hours were either left untreated or treated with EACC. Scale: 25 µm **(B, C)** The number of autophagosomes ($RFP^+/GFP^+$ structures) and autolysosomes ($RFP^+/GFP^-$ structures) per cell in various treatment conditions were counted using the cell counter plugin in ImageJ. Data shown represents number of autophagosomes ($RFP^+/GFP^+$) and autolysosomes ($RFP^+/GFP^-$) for a minimum of 45 cells from 3 independent

experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns= non-significant.

### 5.2.5 Silencing of Drp1 rescues autophagic flux

Finally, we performed siRNA mediated silencing of Drp1 to see if this could rescue the autophagic flux even in the presence of EACC. First to check the efficiency of Drp1 siRNA, HeLa cells were either left untransfected or transfected with scrambled siRNA or siRNA against Drp1 and immunoblotted for Drp1 and β-Actin. The levels of Drp1 were decreased significantly post silencing **(Fig. 5.5 A).** Next, HeLa cells transfected with siRNA against Drp1 (1.5μg) and tandem tagged mRFP-GFP-LC3 construct for 48 hours were either left untreated or treated with EACC. The number of autophagosomes and autolysosomes in Drp1 silenced HeLa cells were now similar to the starvation control even in presence of EACC **(Fig. 5.5 B-D)**. Hence, disrupting the Drp1-Stx17 interaction by silencing Drp1 could rescue autophagic flux even in presence of EACC.

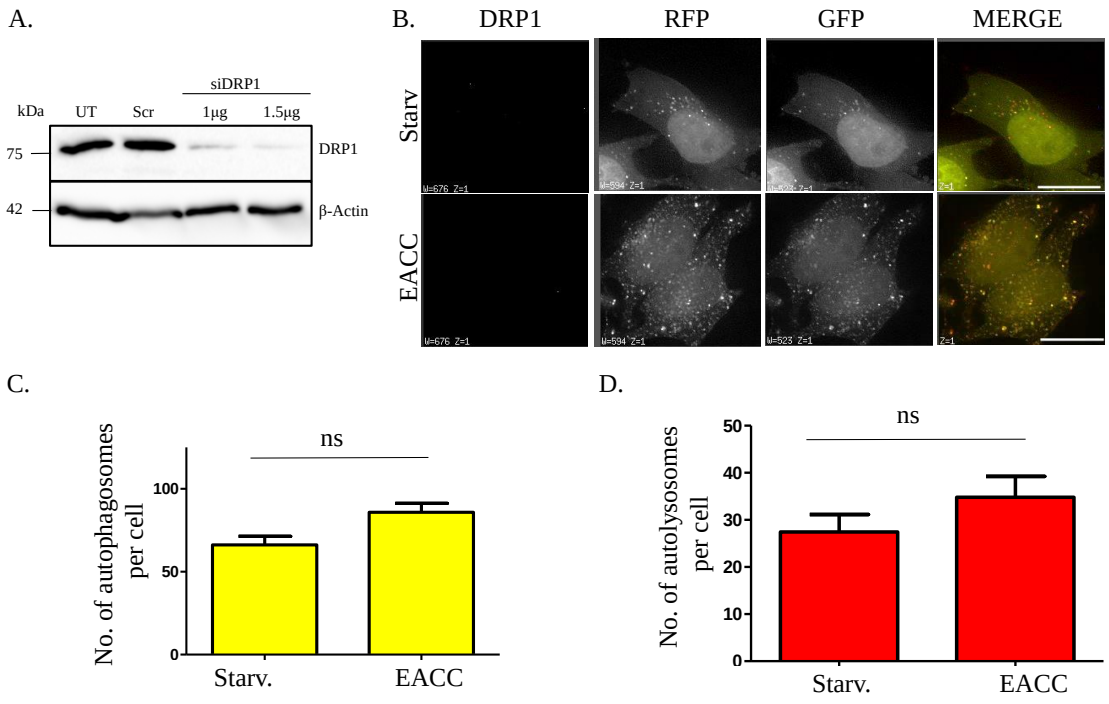


**Figure 5.5: Silencing of Drp1 rescues autophagic flux**

**(A)** HeLa cells were either left untransfected or transfected with scrambled siRNA or siRNA against Drp1 were immunoblotted for Drp1 and β-Actin. **(B)** HeLa cells transfected with siRNA against Drp1 (1.5µg) and tandem tagged mRFP-GFP-LC3 construct for 48 hours were either left untreated or treated with EACC. The same experiment was performed with scrambled siRNA control but it has not been shown here. Scale: 10 µm **(C, D)** The number of autophagosomes ($RFP^+/GFP^+$ structures) and autolysosomes ($RFP^+/GFP^-$ structures) per cell in various treatment conditions were counted using the cell counter plugin in ImageJ. Data shown represents number of autophagosomes ($RFP^+/GFP^+$) and autolysosomes ($RFP^+/GFP^-$) for a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ns= non-significant.

### 5.2.6 Overexpression of Drp1 dominant negative allows Stx17 translocation onto autophagosomes even in presence of EACC

Previously, we showed that inhibiting the enhanced Drp1-Stx17 interaction by overexpression of $Drp1^{K38A}$ could rescue autophagic flux even in the presence of EACC.

Finally, we wanted to understand if this rescue in autophagic flux is because the autophagosomal SNARE Stx17 can now translocate to autophagosomes which we do not observe in presence of EACC (as has been shown in the previous chapter).

HeLa cells transfected with FLAG-Stx17, GFP-LC3 and HA-Drp1 or HA-Drp1$^{K38A}$ for 48 hours were either left untreated or treated with EACC and immunostained with anti-FLAG or anti-HA antibodies. EACC treatment significantly reduced the mean intensity of colocalization of LC3 and Stx17 in HeLa cells overexpressing HA-Drp1. On the other hand, the mean intensity of colocalization of LC3 and Stx17 was rescued and was similar to that of starvation control even in presence of EACC in HeLa cells overexpressing HA-Drp1$^{K38A}$ **(Fig. 5.6 A, B)**. Hence, we conclude that inhibiting the enhanced Drp1-Stx17 interaction by overexpression of Drp1$^{K38A}$ could rescue autophagic flux by allowing translocation of Stx17 onto autophagosomes even in EACC treated cells.

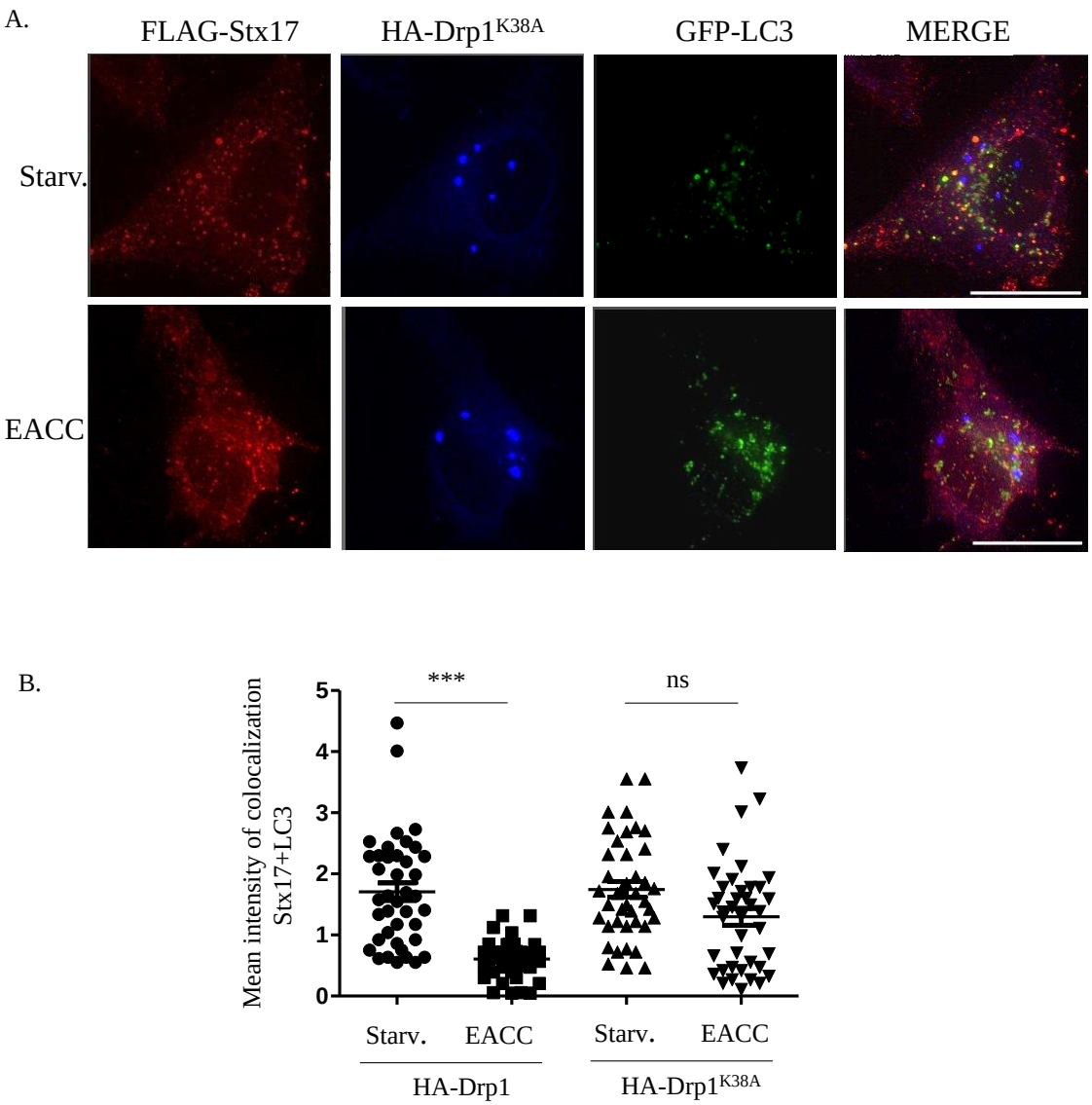


**Fig. 5.6: Overexpression of Drp1 dominant negative allows Stx17 translocation onto autophagosomes even in presence of EACC**

**(A)** HeLa cells transfected with FLAG-Stx17, GFP-LC3 and HA-Drp1 or HA-Drp1$^{K38A}$ for 48 hours were either left untreated or treated with EACC and immunostained with anti-FLAG or anti-HA antibodies. **(B)** Graph showing the mean intensity of colocalization between Flag-Stx17 and GFP-LC3 in HA-Drp1 or HA-Drp1$^{K38A}$ overexpressing cells were measured as explained in **Fig. 2.3 B**. Data shown here represents a minimum of 45 cells from 3 independent experiments plotted as mean ± SEM. Statistical significance was analysed by Student's unpaired t-test. ***$P < 0.001$, ns=non-significant.

## 5.3 Discussion

In this chapter, we looked at non-autophagic roles of Stx17 particularly in regulating mitochondrial dynamics. According to published reports, in nutrient rich conditions Stx17 is present in ER, Mitochondria and ER-Mitochondria contact sites (Arasaki et al., 2015; Itakura et al., 2012). Arasaki *et al.*, also showed that there it interacts with the mitochondrial fission regulator protein Drp1, assists in its self- assembly and in mitochondrial fission. Whereas upon nutrient starvation, Stx17 translocates onto autophagosomes and regulates autophagosome-lysosome fusion which also results in mitochondrial elongation (Arasaki et al., 2015; Itakura et al., 2012).

We had previously shown that in presence of EACC, the starvation mediated translocation of Stx17 was blocked which resulted in inhibition of autophagosome-lysosome fusion and caused accumulation of autophagosomes. Additionally, we observed that EACC enhanced mitochondrial fragmentation. Based on these two observations, we tested our hypothesis whether the increase in mitochondrial fragmentation and block in autophagic flux seen upon EACC treatment is due to enhanced Stx17-Drp1 interaction even in starvation conditions.

We observed that the Stx17-Drp1 interaction is enhanced in EACC treatment which was reciprocal to the effect of EACC on Stx17-LC3 interaction which decreases significantly in the presence of EACC. Furthermore, pre-treatment of cells with a chemical inhibitor of Drp1 such as Mdivi1 resulted in the colocalization for Stx17-LC3 and Stx17-Drp1 being similar to that of starvation control.

This suggested that EACC not only inhibited Stx17 translocation onto autophagosomes but also enhanced the interaction between Drp1 and Stx17. Additionally, disrupting the GTP-dependent Stx17-Drp1 interaction by the use of genetic or chemical inhibitors of Drp1 can potentially rescue the autophagic flux.

Finally, we showed that inhibiting the enhanced Drp1-Stx17 interaction by use of Mdivi1 (Cassidy-Stone et al., 2008; Ruiz et al., 2018; Tanaka and Youle, 2008), overexpression of $Drp1^{K38A}$ (Smirnova et al., 2001; Ugarte-Uribe et al., 2014) or by Drp1 silencing could rescue autophagic flux even in the presence of EACC and this rescue was due to restored translocation of Stx17 onto autophagosomes.

## 5.4 References

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# Chapter 6
# Discussion and Future Directions

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## 6.1 Discussion

The past decade has seen a deluge of studies involving genetic or pharmacological interventions to restore autophagic flux in pathophysiological conditions such as neurodegeneration, cancer and infections with considerable success. This has led to an escalated interest in identifying new small molecule modulators of autophagy that could potentially be of therapeutic value. Several groups have performed luminescence or fluorescence based chemical biology screens for the same (Chauhan et al., 2015; Jo et al., 2011; Min et al., 2018). Our laboratory has standardized a luminescence based high throughput assay to screen for small molecule modulators of autophagy in yeast. The principle of the assay is based on the detection of levels of luciferase activity in order to monitor the rate of autophagic flux in *S. cerevisiae* (Mishra et al., 2017b). The screen identified two novel inhibitors of yeast and mammalian autophagy, Bay11 and ZPCK (Mishra et al., 2017a). The strength of the assay is that unlike target-driven screens which focus on a particular step in the pathway, this assay focuses on the entire pathway and therefore it can yield hits across all steps of autophagy.

Using this assay, we screened ~200,000 molecules across different libraries and obtained several hits. We tested one of the hits, EACC for its potential to modulate autophagy in mammalian cells. We showed that EACC caused an accumulation of LC3-II over and above that of starvation induced autophagy. Furthermore, by using some of the staple assays of the field, we deduced that the increase in LC3-II is most likely due to a block in autophagosome-lysosome fusion rather than autophagy induction. As discussed previously in the introduction, the most commonly used late-stage inhibitors of autophagy

Bafilomycin A1 and chloroquine are not autophagy inhibitors in the truest sense because they primarily affect lysosomal pH and the block in autophagic flux that is observed upon their addition is a consequence of their effects on the lysosome. Therefore, we wanted to test if the late-stage block caused by EACC was due to an effect on lysosomal pH or function. We tested the effect of EACC on lysosomal number, pH and function and ruled out that EACC is affecting the lysosomes. Another issue that arises while working with a lysosomal inhibitor rather than a true autophagy inhibitor is that lysosomes are the final destination for several vesicular trafficking pathways including autophagy and endocytosis. Therefore, a lysosomal inhibitor will not only block the autophagic flux but will have secondary effects on other vesicular trafficking pathways such as endocytosis. In order to further potentiate the finding that the inhibitory nature of EACC is specific to autophagy, we followed the degradation of an endocytic cargo EGFR and found the degradation pattern of EGFR remained unchanged upon EACC treatment.

Following this, we set out to understand the process of autophagosome-lysosome fusion in greater detail. As it was clear that the action of EACC was specific to the autophagy pathway, we wanted to focus on the candidate proteins which are involved in autophagosome-lysosome fusion but not in fusion of other vesicles with the lysosomes. Itakura et al., identified Stx17 as the first specific SNARE involved in autophagosome-lysosome fusion. Stx17 is a $Q_a$ SNARE which forms a complex with $Q_{bc}$ SNARE SNAP29. This SNARE complex formation occurs on complete autophagosomes (Itakura et al., 2012). Stx17 has been reported to be present in ER, mitochondria and ER-mitochondria contact sites in nutrient rich conditions (Arasaki et al., 2015; Itakura et al., 2012).

According to already published reports, fusion step proceeds temporally by first translocation/loading of Stx17 on autophagosomes followed by SNAP29 recruitment. This $Q_{abc}$ SNARE complex is stabilized by ATG14. Autophagosome-lysosome docking is

mediated by small GTPase RAB7 and tethering complex HOPS. Subsequently, successful fusion occurs when membranes of the two vesicles are brought in close apposition and a *trans*-SNARE complex comprising of $Q_a$ Stx17, $Q_{bc}$ SNAP29 and the lysosomal R-SNARE VAMP8 is formed (Diao et al., 2015; Guo et al., 2014; Itakura et al., 2012; Jiang et al., 2014; Takats et al., 2014).

We observed that EACC consistently impaired Stx17 loading onto autophagosomes. This was very intriguing because as per our knowledge, there were no reports suggesting any chemical modulator of autophagy that could selectively prevent Stx17 translocation thereby rendering autophagosomes 'fusion incompetent'. There are reports which suggest that in order to prevent being captured and degraded by autophagy some pathogens like *Legionella pneumophila* can degrade Stx17 to block the autophagic flux (Arasaki et al., 2017). However, EACC did not affect Stx17 expression. Although EACC affected the translocation of Stx17 on autophagosomes and therefore the interaction of LC3 with Stx17 and its partner SNARE SNAP29, it did not significantly affect the interaction of Stx17 with SNAP29. This could mean that although EACC is affecting the function of Stx17 and SNAP29 in autophagosome-lysosome fusion, the other trafficking processes mediated by Stx17-SNAP29 interaction such as fusion of MDVs with lysosomes (McLelland et al., 2016) most likely remains unaffected.

We further showed by analysing the presence of lysosomal R-SNARE VAMP8 that the lysosomal 'fusion competence' remained unaltered in the presence of EACC but as expected, there is decreased interaction between Stx17 and HOPS subunit VPS33A as well Stx17 and VAMP8. So, we concluded that the block in autophagosome-lysosome fusion that we see upon EACC treatment is due to impaired translocation of the SNARE Stx17 onto autophagosomes and reduced interaction of Stx17 with the tethering complex HOPS and the cognate lysosomal SNARE VAMP8.

The next major question was to address how Stx17 translocation is regulated. As mentioned before, Stx17 gets spatially regulated depending on the nutritional status of the cell. Since, Stx17 was not getting translocated to autophagosomes in starvation, we sought out to understand the reasons behind it.

We decided to observe the effect of EACC on other organelles such as mitochondria where Stx17 is located. Interestingly, EACC treatment caused enhanced mitochondrial fragmentation. As already explained in the introduction, Stx17 gets spatially regulated depending on the nutritional status of the cell. In nutrient rich conditions wherein the basal autophagic flux is low, Stx17 is present on ER-Mitochondria contact sites where it assists in the assembly of GTP-bound Drp1 on mitochondria and mitochondrial fragmentation. Starvation redistributes Stx17 from ER-Mitochondria contact sites to autophagosomes wherein it binds to autophagic proteins like LC3 and ATG14. This ensures proper autophagic flux as well as mitochondrial elongation in order to maximize ATP production during nutrient stress (Arasaki et al., 2015).

Upon EACC treatment, our two main observation were that Stx17 translocation on autophagosomes is impaired even in starvation conditions and we also observed enhanced mitochondrial fragmentation. Linking the two observations, we wanted to check if the excessive mitochondrial fragmentation seen upon EACC treatment was due to enhanced Stx17-Drp1 interaction and could this be the reason for decreased LC3-Stx17 interaction and the concomitant block in autophagic flux. As the interaction of Stx17 and Drp1 on mitochondria is GTP dependent (Arasaki et al., 2015), we used a chemical inhibitor Mdivi1 which impairs GTP binding as well as a non-hydrolysable GTP mutant of Drp1 ($Drp1^{K38A}$) to disrupt Stx17-Drp1 interaction. We observed that EACC enhanced association of Stx17 with Drp1 even in starvation conditions. This enhanced interaction was attenuated upon pre-treatment with Mdivi1.

Additionally, by performing traffic light assay in conjunction with multiple genetic and chemical methods to inhibit Drp1, we observed a rescue in autophagosome-lysosome fusion even in presence of EACC. Hence, by inhibiting the interaction between Stx17 and Drp1 by either genetic or chemical means not only allowed Stx17 translocation onto autophagosomes, but also restored the autophagic flux even in presence of EACC.

This suggests that there is a critical balance between mitochondrial dynamics and autophagy mediated by Stx17. EACC enhances Stx17-Drp1 interaction even in starvation thereby impairing the intended function of Stx17 in starvation i.e. to mediate autophagosome-lysosome fusion.

Furthermore, we also showed that the action of EACC is reversible. The block in autophagic flux is rescued after washing out EACC because Stx17 is now able to translocate to autophagosomes and participate in further fusion events. As Stx17 trafficking is a dynamic event, molecules like EACC which reversibly impair this translocation can be a useful tool to study Stx17 trafficking.

In conclusion, molecules like EACC can fill the lacuna that exists in the field due to the lack of specific autophagy inhibitors and provide further mechanistic insights into the process of autophagy and its regulation.

### 6.2 Future directions

As mentioned previously, EACC is a reversible inhibitor of Stx17 translocation onto autophagosomes. Molecules like EACC which reversibly impair this translocation can be used as a useful tool to study Stx17 trafficking. The exact mechanism by which Stx17 translocates to mature autophagosomes is still unclear. A recent report suggested that Stx17 recruitment to autophagosomes occurs via interaction with a small GTPase IRGM and mammalian ATG8 proteins (Kumar et al., 2018). Stx17 has been suggested to have various

cellular locations including ER, mitochondria, ER-mitochondria contact sites and even cytosol (Arasaki et al., 2015; Itakura et al., 2012). The recruitment of Stx17 onto autophagosomes could either be vesicle-mediated or by direct connections between autophagosomes and ER/mitochondria or ER-mitochondria contact sites. The latter is more unlikely because it is known that mature autophagosomes do not retain connections with the above-mentioned organelles. In case the translocation of Stx17 is indeed vesicle-mediated, we propose that EACC could be useful in identification of such vesicles. Vesicles from cells either left untreated or treated with EACC can be isolated and purified followed by immunoprecipitation of Stx17 and mass spectrometry to identify the protein components of the vesicle.

We also cannot rule out the involvement of other accessory proteins which decide and regulate the location of Stx17 in response to nutrient status of the cell. Immunoprecipitation of Stx17 in starvation with or without EACC followed by mass spectrometry can potentially give us a list of interacting partners of Stx17 in presence and absence of EACC and bring us closer towards identification of other accessory proteins regulating this process.

Furthermore, we would like to understand which site in the cell is the major source for Stx17 trafficking onto autophagosomes. As EACC affects not only the autophagic flux but also mitochondrial dynamics, we would like to understand if ER-mitochondria contact sites are an important source for Stx17 trafficking. First, we would like to study the effect of EACC on ER-mitochondria by employing high-resolution microscopy and other methods (Csordas et al., 2018; Lopez-Crisosto et al., 2015; Wu et al., 2018). An important point to note here is that ER-mitochondria sites are also implicated as membrane sources for autophagosomes biogenesis (Hamasaki et al., 2013). We do see an accumulation of autophagosomes upon EACC treatment but as there are several other membrane sources known which contribute to autophagosome biogenesis (Tooze, 2013), we cannot

completely rule out the effect of EACC on ER-mitochondria contact sites. Keeping in mind the reported role of Stx17 in autophagosome biogenesis at the MAM (Arasaki et al., 2017; Hamasaki et al., 2013), it will worth probing the effect of Stx17-Drp1 interaction on the type and the composition of autophagosomes that accumulate upon EACC treatment. Finally, we would like to disrupt the ER-mitochondria contact sites either by RNA interference of tethering proteins such as Mfn2, IP3 receptor (IP3R) or Phosphofurin acidic cluster sorting protein 2 (PACS2) or by pharmacological inhibition of the ER-to-mitochondria Ca2+ signaling by targeting the IP3R and Voltage dependent anion channel (VDAC) (Lopez-Crisosto et al., 2015) and see if that affects the translocation of Stx17 onto autophagosomes after washing out EACC.

We would also like to do some chemical studies with EACC to identify which molecular structures are important for its biological activity. Finally, the most challenging future work will involve identifying the target of EACC by affinity-based target identification methods (Futamura et al., 2013) with Biotin-tagged versions of EACC. We believe that further studies with EACC will provide some interesting mechanistic insights into the process of autophagosome-lysosome fusion and its regulation.

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# Chapter 7

# Materials and Methods

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# Materials and Methods

## Materials and methods

### 7.1 Cell culture

HeLa cells were maintained in growth medium comprising of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, D5648) supplemented with 3.7 g/L sodium bicarbonate (Sigma-Aldrich, S5761) plus 10% Fetal Bovine Serum (FBS) (Gibco, 10270-106) and 100 units/ml of Penicillin and Streptomycin (Gibco, 15140-122) at 5% $CO_2$ and 37°C. Autophagy was induced by washing cells with Dulbecco's Phosphate Buffered Saline (D-PBS) (Sigma-Aldrich, D5773) and incubating them in starvation media/Earle's Balanced Salt Solution (Sigma-Aldrich, E7510) made to 1X and supplemented with 2.2 g/L sodium bicarbonate for 2 hours until otherwise stated.

### 7.2 Plasmids used in the study

Plasmids used in the study were as follows: ptfLC3 (mRFP-GFP-LC3) (Addgene plasmid #21074) and pmRFP-LC3 (Addgene plasmid #21075) were gifts from Tamotsu Yoshimori. FLAG-Stx17 (Addgene plasmid #45911) and FLAG-SNAP29 (Addgene plasmid#45915) were gifts from Noburu Mizushima. GFP-VAMP8 was a gift from Thierry Galli (Addgene plasmid #42311), mCherry-DFCP1was a gift from Do-Hyung Kim (Addgene plasmid #86746), HA-hATG14 was a gift from Noburu Mizushima (Addgene plasmid #24294), mCh-Drp1 was a gift from Gia Voeltz (Addgene plasmid #49152), DsRed2-Mito7 was a gift from Michael Davidson (Addgene plasmid #55838), pcDNA3-Drp1$^{K38A}$ was a gift from Richard Youle and Alexander Van der Bliek (Addgene plasmid #45161). Plasmid containing HA-VPS33A was a kind gift from Dr. Mahak Sharma, IISER Mohali. Plasmid containing HA-Drp1 was a kind gift from Dr. Patrick D'Silva, IISc Bangalore. Myc-Stx17

plasmid was a kind gift from Dr. Viktor Korolchuk, Newcastle University. GFP-LC3 plasmid was generated in lab by excising out mRFP fragment from mRFP-GFP-LC3 plasmid.

### 7.3 Antibodies and reagents used in the study

The following primary antibodies were used: LC3 (MBL, M152-3), LC3B (Sigma-Aldrich, L7543), β-Actin (CST, 4970), LAMP1 (CST, 9091), p62/SQSTM1 (Abcam, ab56416), β-Tubulin (DHSB, E7), cathepsin-B (Cloud Clone Corp., PAC964Hu01), EGFR (Santa Cruz Biotechnology, sc-03), phospho-P70S6K (T389) (CST, 9234), Total P70S6K (CST, 9202), phospho-4EBP1 (CST, 2855), Total 4EBP1 (CST, 9452), phospho-ULK1 (S757) (CST,6888), ATG14 (CST, 5504), ATG5 (CST, 12994), ATG16L1 (CST, 8089), WIPI2 (Abcam, ab105459), Stx17 (Sigma-Aldrich, HPA001204), FLAG (Sigma-Aldrich, F3165), FLAG (Sigma-Aldrich, F1804), HA (CST, 3724), Myc (Abcam, ab9106), RAB7 (CST, 9367), GFP (Roche, 11814460001), Drp1 (CST, 5391), Mouse IgG (Genei, IGP3).

Secondary antibodies used were Goat Anti-mouse IgG (H+L) HRP conjugate (Biorad, 1721011), Goat Anti-rabbit IgG (H+L) HRP conjugate (Biorad, 1706515). Fluorescent secondary antibodies used were Atto 633 (goat anti-rabbit IgG, Sigma-Aldrich, 41176), Atto 488 (goat anti-rabbit IgG, Sigma-Aldrich, 41057), Alexa 647 (goat anti-mouse IgG, Invitrogen, A21236), Alexa 405 (goat anti-mouse IgG, Invitrogen, A31556).

Chemicals used in this study were EACC (Life Chemicals, F1358-0554), Bafilomycin A1 (Sigma-Aldrich, B1793), Actinomycin D (Sigma-Aldrich, A1410), Cycloheximide (Sigma-Aldrich, C7698), EGF (Thermo Fisher Scientific, PHG0311L), Mdivi-1 (Sigma-Aldrich, M0199). Lysotracker Deep Red (L12492) was purchased from Thermo Fisher Scientific. Proximity Ligation Assay kit (DUO92101) was purchased from Sigma-Aldrich.

Scrambled siRNA (SIC001) and siRNA against human Drp1 (EHU081751) were purchased from Sigma-Aldrich.

## 7.4 Protocols used in the study

### 7.4.1 Immunofluorescence

Approximately 1 million cellsplated on top of coverslips placed in 60 mm cell culture dishes for transfection. The following day, transfection was done on a 60 mm dish with HeLa cells at 60-70% confluency. Cells were transfected using 5µl of Lipofectamine 2000 (Invitrogen, 11668-019) and 2.5µg of DNA (2:1 ratio) diluted in 100µl of OPTI-MEM (Invitrogen, 31985-070) separately. 48 hours after transfection, cells were either left untreated or treatment with EACC was done for 2 hours. Starvation was induced by treating cells with Earle's balanced salt solution (EBSS). After treatment, cells were fixed in 4% paraformaldehyde and permeabilized using 0.25% Triton X-100. Overnight incubation with primary antibody was done at 4ºC. Excess antibody was washed with PBS and coverslips were incubated with appropriate fluorescent secondary antibody. The coverslips were mounted with Vectashield antifade reagent (H-1000/ H-1200, Vector laboratories).

Imaging for HeLa cells was carried out using DeltaVision microscope, GE (Olympus 60X/1.42, Plan ApoN, excitation and emission filter Cy5, FITC, DAPI and TRITC, polychroic Quad).

### 7.4.2 Immunoprecipitation

For immunoprecipitation assays, approximately 8 million cells from a confluent 100mm dish were lysed in lysis buffer (20 mM Tris-HCl pH 7.2, 2 mM $MgCl_2$, 150 mM NaCl, 0.5% NP-40) supplemented with protease inhibitor/phosphatase inhibitor cocktails for 30 minutes at 4°C and centrifuged at 13000 *g* for 15 minutes. Five hundred micrograms to one milligram protein was incubated with specific primary antibody at 4°C (2 hours to

overnight) on tube rotator followed by incubation with Protein G Dynabeads (Invitrogen, #10004D) for 2 hours at 4°C. The beads were washed thrice with ice-cold PBS and the proteins were eluted from washed beads by boiling for 5 minutes in 2× Laemmli sample buffer and proceeded for Immunoblot analysis.

For immunoprecipitation with GFP-Trap beads (Chromotek), a similar number of cells were lysed in lysis buffer recommended by the manufacturer (10 mM Tris-HCl pH 7.2, 0.5mM EDTA, 150 mM NaCl, 0.5% NP-40) supplemented with protease inhibitor/phosphatase inhibitor cocktails for 30 minutes at 4°C and centrifuged at 13000 *g* for 15 minutes. One milligram protein from the supernatant was used and immunoprecipitation was performed by following manufacturer's instructions.

### 7.4.3 Immunoblotting

Following appropriate treatments, approximately 0.8 million cells from each well of a 6-well dish were washed with ice cold PBS. Cells were then lysed in 100µl of sample buffer (10% w/v SDS, 10 mM DTT, 20% v/v glycerol, 0.2 M Tris-HCl pH 6.8, 0.05% w/v bromophenol blue) and then collected using a rubber cell scraper. The lysates were boiled at 99°C for 15 minutes and stored at −20°C. Immunoblotting was performed using standard methods.

Blots were incubated overnight with above mentioned primary antibodies. Secondary antibody used at 1:10,000 was goat anti-mouse (Bio-Rad # 172-1011) or goat anti- rabbit antibody (Bio-Rad # 172-1019) conjugated to HRP. Blots were developed by using ECL substrate (Bio-Rad #170-5061) and images captured using auto capture or series capture program in Gel documentation system (Syngene G-Box, UK). ImageJ software (NIH) was used for quantitation of band intensities.

### 7.4.4 CellTiter-Glo cell viability assay

Toxicity of the compound was monitored by CellTiter-Glo cell viability assay (Promega, G7570). HeLa cells were counted and equal numbers (1500 cells/well) were plated in 384 well plate in growth medium. The following day, different concentrations of EACC ranging from 100nM to 100μM were mixed in starvation media, added onto the cells and incubated for five hours. After five hours, 15μl CellTiter-Glo Reagent was added to each well, and luminescence measured using Varioskan Flash (Thermo Fisher Scientific).

### 7.4.5 EGFR trafficking

HeLa cells were plated on 6 well plates and allowed to attach. The following day, approximately 0.8 million cells from each well of a 6-well dish were washed with PBS and then starved in DMEM (serum free media) for 3 hours. Pre-treatment with EACC was carried out for 1 hour, following which cells were pulsed with 100ng/ml of EGF and samples were collected at 0, 1, 2 and 3-hour intervals.

### 7.4.6 Lysotracker staining

Lysotracker staining was performed in HeLa cells according to manufacturer's protocols.

### 7.4.7 Proximity Ligation Assay

Proximity ligation assay was performed in HeLa cells according to manufacturer's protocols.

## 7.5 Analysis parameters used in the study

### 7.5.1 Colocalization analysis and mean intensity calculation

ImageJ software (NIH) was used to calculate the mean intensity of staining or mean intensity of colocalization. Images were opened using the split channel plugin. In case of colocalization, Colocalization plugin in the analysis tool was used to obtain the colocalized area between two channels as a separate window. The intensity was measured using the

analyse measure plugin in analysis tools. Cell counter plugin was used to count the number of colocalized structures.

### 7.5.2 Statistical analysis and image preparation

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Statistical analyses were performed by comparing the means using the Paired/Unpaired Student t-test or Two-way ANOVA followed by the Bonferroni post-test to compare replicate means by row. Images were prepared using Softworx software (GE healthcare). Some fluorescent MIP images had their brightness and contrast modified equally in control and treatment conditions just for the purpose of visualization.

# Publications

# A reversible autophagy inhibitor blocks autophagosome–lysosome fusion by preventing Stx17 loading onto autophagosomes

Somya Vats and Ravi Manjithaya*
Autophagy Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore-560064, India

**ABSTRACT** **Autophagy is an evolutionarily conserved intracellular lysosomal degradation pathway. It is a multistep process involving de novo formation of double membrane autophagosomes that capture cytosolic constituents (cargo) and eventually fuse with lysosomes wherein the cargo gets degraded and resulting simpler biomolecules get recycled. In addition to their autophagy function, several of the autophagy-related proteins work at the interface of other vesicular trafficking pathways. Hence, development of specific autophagy modulators that do not perturb general endo-lysosomal traffic possesses unique challenges. In this article, we report a novel small molecule EACC that inhibits autophagic flux by blocking autophagosome–lysosome fusion. Strikingly, unlike other late stage inhibitors, EACC does not have any effect on lysosomal properties or on endocytosis-mediated degradation of EGF receptor. EACC affects the translocation of SNAREs Stx17 and SNAP29 on autophagosomes without impeding the completion of autophagosomes. EACC treatment also reduces the interaction of Stx17 with the HOPS subunit VPS33A and the cognate lysosomal R-SNARE VAMP8. Interestingly, this effect of EACC although quite robust is reversible and hence EACC can be used as a tool to study autophagosomal SNARE trafficking. Our results put forward a novel method to block autophagic flux by impeding the action of the autophagosomal SNAREs.**

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Suresh Subramani
University of California, San Diego

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## INTRODUCTION

Autophagy is an intracellular catabolic pathway in which double membrane autophagosomes containing cytoplasmic cargo are transported to lysosomes to form a single membrane degradative compartment called autolysosomes. Inside autolysosomes, by the action of lysosomal hydrolases, simpler biomolecules are generated that are recycled back to the cytoplasm for reuse. The rate at which this multistep dynamic process occurs inside cells is referred to as autophagic flux. All these steps are tightly regulated and are constantly occurring inside a cell at a basal rate; however, this basal autophagic flux varies according to cell type and environmental cues. Basal autophagic flux and its appropriate responsiveness to external perturbations are critical to maintain cellular homeostasis. On the other hand, external stress stimuli such as nutrient limitation or starvation lead to an increase in autophagic flux.

Dysfunctional autophagic flux has been associated with several human diseases. Impaired autophagic flux has been associated with neurodegenerative and infectious diseases while excessive autophagy sustains survival of several types of solid tumors. Therefore, pharmacological modulation of autophagy and its application in various disease scenarios has garnered a lot of interest (Mizushima, 2007; Glick *et al.*, 2010; Rubinsztein *et al.*, 2012; Deretic *et al.*, 2013; Nixon, 2013; Singh *et al.*, 2018).

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R.M. performed the primary screening at the Molecular Shared Screening Resource, University of California, Los Angeles. R.M. and S.V. designed the experiments, and S.V. performed the experiments. S.V. and R.M. analyzed the data and wrote the manuscript.

The authors declare no potential conflict of interest.

*Address correspondence to: Ravi Manjithaya (ravim@jncasr.ac.in).

Abbreviations used: BafA1, bafilomycin A1; Co-IP, coimmunoprecipitation; DPBS, Dulbecco phosphate buffered saline; EACC, ethyl (2-(5-nitrothiophene-2-carboxamido) thiophene-3-carbonyl) carbamate; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GM, growth medium; HRP, horseradish peroxidase; IB, immunoblotting; IP, Immunoprecipitation; IRGM, immunity related GTPase M; MIP, maximum intensity projection; PCC, Pearson's correlation coefficient; Starv., starvation medium.

Induction of autophagy is tightly regulated inside cells. The mammalian target of rapamycin (mTOR) senses cellular nutrient status and regulates cell growth. In the case of nutrient limitation, mTOR is deactivated, which leads to dephosphorylation of Unc-51–like autophagy activating kinase1 (ULK1) and allows assimilation of the ULK1 complex. This complex translocates to the phagophore or isolation membrane where it promotes assembly of the second complex comprising BECLIN1, ATG14, p150, and VPS34. Expansion of this isolation membrane requires conjugation of the ATG5-12/ATG16L1 complex that in turn brings LC3-II to autophagosomal membrane. Double membrane autophagosomes capture cytoplasmic cargo by binding to ubiquitinated cargo via the LC3 interacting region (LIR) present in adaptor proteins like SQSTM1/p62. Finally, these autophagosomes should fuse with lysosomes so that the captured cargo can be degraded by action of lysosomal enzymes (Bento *et al.*, 2016).

Autophagosome–lysosome fusion, similar to all vesicle fusion events, involves the action of soluble NSF (*N*-ethylmaleimide–sensitive factor) attachment protein receptors (SNAREs; Bonifacino and Glick, 2004; Cai *et al.*, 2007). In yeast, autophagosome–vacuole fusion requires SNAREs Vam3 (Qa), Vti1 (Qb), Vam7 (Qc), and R-SNARE YKT6 (Darsow *et al.*, 1997; Sato *et al.*, 1998; Ishihara *et al.*, 2001; Surpin *et al.*, 2003). In mammalian cells, autophagosome–lysosome fusion is orchestrated by the concerted action of autophagosomal Qa-SNARE Syntaxin17 (Stx17), Qbc-SNARE SNAP29, lysosomal R-SNARE VAMP8, homotypic fusion and protein sorting (HOPS) tethering complex, small GTPase RAB7, and accessory proteins like ATG14 (Itakura *et al.*, 2012; Hyttinen *et al.*, 2013; Jiang *et al.*, 2014; Diao *et al.*, 2015).

Stx17 is abundantly present in endoplasmic reticulum and is involved in smooth endoplasmic reticulum membrane trafficking dynamics (Steegmaier *et al.*, 1998, 2000). Stx17 is unique among the Syntaxin family because it possesses a unique C-terminal hairpin structure that is important for its localization to autophagosomes. Interestingly, the translocation of Stx17 occurs only on complete autophagosomes and not on partially formed autophagosomes. This functions as a regulatory step that prevents fusion of incompletely formed autophagosomes with lysosomes (Itakura *et al.*, 2012). Upon its translocation, Stx17 along with its partner SNARE SNAP29 interact with VAMP8 resulting in the formation of a parallel four-helix bundle consisting of Qa, Qbc, and R-SNAREs (Itakura *et al.*, 2012; Guo *et al.*, 2014). This SNARE bundle is stabilized by ATG14 whose role at this step is largely independent of its role in early steps of autophagy (Hamasaki *et al.*, 2013; Diao *et al.*, 2015).

In this article, we report a novel small molecule inhibitor of autophagy EACC that blocks autophagosomal–lysosomal fusion. EACC inhibits autophagic flux by selectively affecting the translocation of Stx17 on autophagosomes. The autophagic pathway and the endocytic pathway both culminate at the lysosomes and share some components of the fusion machinery such as RAB7 and the HOPS complex (Hyttinen *et al.*, 2013; Jiang *et al.*, 2014; Takats *et al.*, 2014). Owing to this, selectively modulating autophagy without perturbing the endo-lysosomal system is difficult. Our investigations into the mechanism of EACC revealed that its action is largely specific to the process of autophagy. Most importantly, the action of EACC is reversible and hence can be used as a tool to study the dynamic recruitment of autophagy-specific SNAREs.

## RESULTS

### EACC inhibits autophagic flux

Recent reports from our lab described a luciferase-based high-throughput screen for identification of novel small molecule modulators of autophagy (Mishra *et al.*, 2017a,b). Utilizing this assay, we screened 1999 compounds of the Microsource Discovery Systems library and identified EACC as one of the hits. EACC stands for ethyl (2-(5-nitrothiophene-2-carboxamido) thiophene-3-carbonyl) carbamate. To test for its potential to modulate autophagy, EACC was further tested in mammalian systems.

Starvation is a potent physiological inducer of autophagic flux and we wanted to test whether EACC could modulate starvation-induced autophagic flux. We treated HeLa cells with an increasing dose of EACC in starvation conditions (2.5–25 μM) and probed for LC3 expression. An enhanced conversion of LC3 (LC3-I to LC3-II) was seen with increasing dose (Figure 1, A and B). This would indicate either induction or a block in autophagic flux. To address this, we analyzed the accumulation of LC3-II in the presence or absence of a known autophagy inhibitor, bafilomycin A1 (BafA1). An autophagy inducer added along with BafA1 will increase LC3-II levels over and above that of BafA1 alone. On the other hand, in the case of an inhibitor the LC3-II levels will remain unchanged (Mizushima and Yoshimori, 2007; Mizushima *et al.*, 2010). EACC caused an accumulation of LC3-II that was similar to that of BafA1. The combined treatment of BafA1 and EACC did not cause further accumulation of LC3-II, suggesting that EACC is an inhibitor rather than an inducer of autophagic flux (Figure 1, C and D).

To validate these observations and further dissect the step of autophagic flux affected by EACC, we employed tandem-fluorescent-tagged LC3 reporter, mRFP-GFP-LC3 (Kimura *et al.*, 2007). Using this reporter, while autophagosomes appear yellow, autolysosomes (the fusion product of autophagosomes with lysosomes) are seen as red because the green fluorescence of GFP gets quenched due the acidic nature of lysosomes. HeLa cells transfected with mRFP-GFP-LC3 construct were treated with increasing concentrations of EACC (2.5–25 μM) for 2 h. We saw a significant dose-dependent increase in the number of autophagosomes ($mRFP^+/GFP^+$) and a concomitant decrease in the number of autolysosomes ($mRFP^+/GFP^-$) (Figure 1, E and F) in EACC-treated cells. Subsequent experiments were carried out at 10 μM concentration for a period of 2 h and at this concentration, cell viability was unaffected even up to a period of 5 h (Supplemental Figure S1A). Next, we assessed the effect of EACC on autophagic adaptor p62/SQSTM1. p62 binds to ubiquitinated cargo via the UBA domain and LC3 via its LC3 interacting region (LIR) region. This step helps in sequestration of cargo in autophagosomes. p62 is degraded by autophagy and hence decreased autophagic flux leads to accumulation of this protein. EACC treatment resulted in increased colocalization between p62 and LC3 suggesting that EACC, while inhibiting autophagic flux, did not affect adaptor loading and LC3 recruitment (Figure 1, G and H). These results together suggest that EACC inhibits autophagic flux most likely at the later stages.

### EACC blocks autophagosome–lysosome fusion but does not affect endo-lysosomal function

To further understand the inhibitory action of EACC on autophagic flux, we checked the colocalization between the autophagosome marker LC3 and the lysosomal marker, LAMP1. In line with our previous observations, we saw a decrease in the percentage of autolysosomes ($RFP\text{-}LC3^+/LAMP1^+$) in RFP-LC3 transfected HeLa cells treated with EACC (Figure 2, A and B). A similar decrease in number of autolysosomes was also observed in EACC-treated cells immunostained with LC3 and LAMP1 (Supplemental Figure S2, A and B).

To dissect the effect of EACC on LC3-LAMP1 interaction endogenous immunoprecipitation (IP) was employed. Control and EACC-treated lysates were subjected to IP using LC3 antibody. We observed that in EACC-treated lysates, the levels of LC3-II were

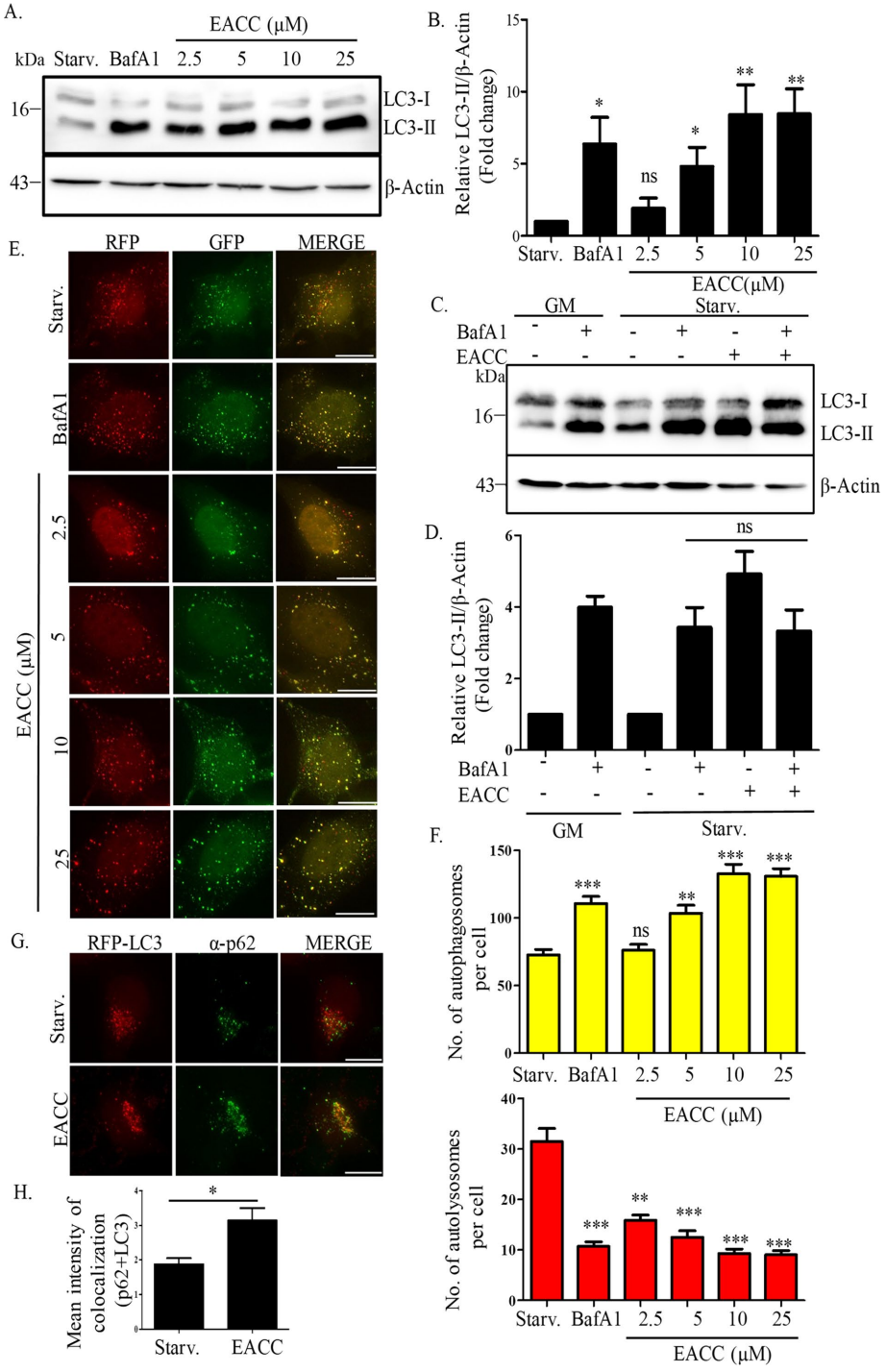


**FIGURE 1:** EACC inhibits autophagic flux. (A) HeLa cells were either left untreated or treated with BafA1 (100 nM) or EACC (2.5–25 μM) for 2 h in starvation conditions. Samples were collected and immunoblotted for anti-LC3 and anti–β-actin antibodies. (B) Relative levels of LC3-II:β-actin in untreated vs. treated samples were quantitated for three independent experiments. **, $P < 0.01$; *, $P < 0.05$; ns = nonsignificant (two-way ANOVA, replicate means compared with Bonferroni posttest). (C) HeLa cells were either left untreated or pretreated with BafA1 (100 nM) in basal or starvation conditions for 1 h in order to block the autophagic flux. This was followed by treatment with EACC (10 μM) for 2 h. Samples were collected and immunoblotted for anti-LC3 and anti–β-actin antibodies. (D) Relative levels of LC3-II:β-actin in untreated vs. treated samples were quantitated for three independent experiments. ns = nonsignificant. Statistical significance was analyzed by Student's unpaired *t* test. (E) HeLa cells transfected with tandem-tagged ptfLC3 (mRFP-GFP-LC3) construct were either left untreated or treated with BafA1 (100 nM) or EACC (2.5–25 μM) for 2 h in starvation conditions. Scale = 10 μm. (F) The autophagosomes ($RFP^+/GFP^+$ structures) and autolysosomes ($RFP^+/GFP^-$ structures) per cell were counted using the cell counter plug-in of ImageJ software. Data shown represent the number of autophagosomes ($RFP^+/GFP^+$) and autolysosomes ($RFP^+/GFP^-$) as compared with control of a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns = nonsignificant. (G) Immunostaining with anti-SQSTM1/p62 antibody in RFP-LC3 transfected HeLa cells treated with EACC (10 μM) for 2 h in starvation conditions. Scale = 15 μm. (H) Graph showing the mean intensity of colocalization between p62 and RFP-LC3 in control vs. EACC-treated group. Mean intensity of colocalization was measured using colocalization and analyze plug-ins of ImageJ software. Data shown here represents a minimum of 60 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. *, $P < 0.05$.

significantly high as compared with control in both LC3 input as well as immunoprecipitates, but the levels of LAMP1 in the LC3 IP as detected by immunoblotting remains unchanged indicating decreased interaction between LC3 and LAMP1 (Figure 2C).

Autophagic flux inhibition can also be achieved by affecting lysosomal function. As other commonly used late stage autophagy inhibitors (chloroquine and BafA1) affect lysosomal function, we investigated whether the effect of EACC on autophagic flux impinged on lysosomes and related pathways. To test this, we checked the expression of LAMP1 in the presence or absence of EACC. HeLa cells treated with EACC were immunoblotted with LAMP1 antibody. There was no significant change in the LAMP1 expression in control versus treated cells (Figure 2, D and E). We also did not see any obvious difference in lysosomal positioning or LAMP1 signal intensity in EACC-treated cells (Figure 2, F and G). Although the overall levels of lysosomes remain unchanged, we wondered whether there was loss of acidification of lysosomes that stalls all fusion events as seen in chloroquine and BafA1 treatments. To test the effect of EACC on lysosomal acidification, we used LysoTracker Deep Red, which preferably accumulates in acidic compartments. The intensity of LysoTracker staining was diminished in BafA1-treated cells but remained unchanged in EACC-treated cells suggesting that EACC does not affect lysosomal pH (Figure 2, H and I).

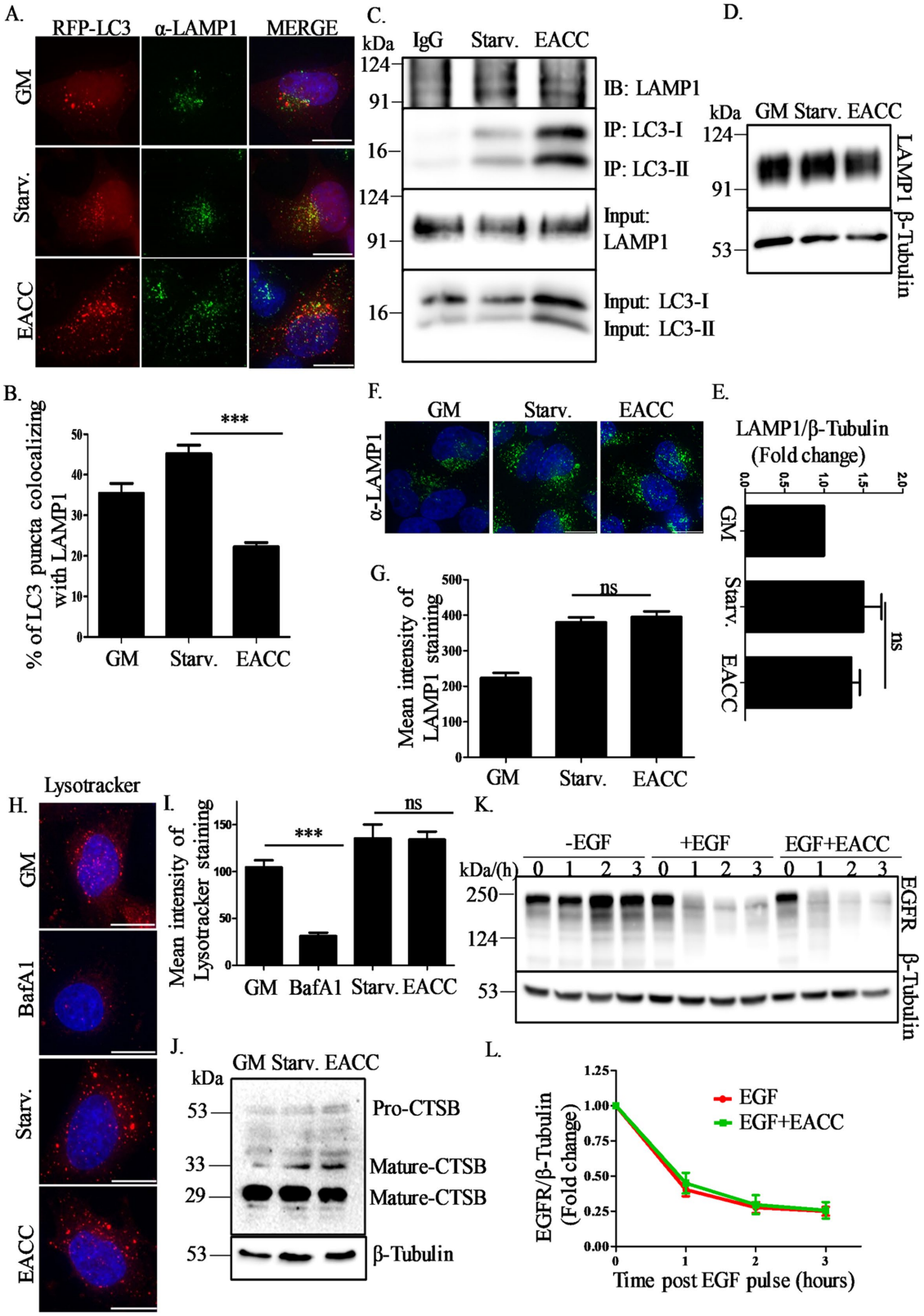


FIGURE 2: EACC blocks autophagosome–lysosome fusion but does not affect endo-lysosomal function. (A) RFP-LC3 transfected HeLa cells were immunostained with anti-LAMP1 antibody and treated with EACC (10 μM) for 2 h in starvation conditions. Scale = 10 μm. (B) Graph showing percent colocalization between LAMP1 and RFP-LC3 (autolysosomes) in starvation conditions and EACC treatment. The colocalized dots were counted using colocalization and cell counter plug-ins of ImageJ software and plotted with respect to the total number of LC3 puncta. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ***, $P < 0.001$. (C) HeLa cells were treated with EACC (10 μM) for

Next, we investigated whether the EACC-treated lysosomes harbored functional hydrolases. We checked the expression and processing of cathepsin B (CTSB), a lysosomal cysteine protease that is cleaved inside the lysosomes to release a proteolytically active mature form. EACC treatment did not impede the conversion of procathepsin B to mature cathepsin B (Figure 2J).

Finally, we tested whether these lysosomes received and processed endocytic pathway cargo upon EACC treatment by performing epidermal growth factor receptor (EGFR) degradation assay.

Upon EGF treatment, EGF bound to EGFR gets internalized via endocytosis and gets degraded in lysosomes. Hence, the temporal decrease in levels of EGFR after EGF pulse is indicative of endocytic trafficking of the receptor to the lysosomes. We found that the rate of EGFR degradation with time followed a comparable trend in treated versus untreated cells (Figure 2, K and L).

These results clearly indicate that EACC prevents autophagosome–lysosome fusion without affecting lysosomes and other vesicular trafficking pathways in general.

### EACC does not affect early autophagic events

Our results so far suggest that EACC selectively affected autophagic flux. So, our next approach was to narrow down to the step of autophagy at which EACC acts.

First, we tested the effect of EACC on mTOR signaling. In nutrient starvation conditions, mTOR is inhibited, which allows induction of autophagy. The status of mTOR can be predicted by the phosphorylation status of its substrates P70S6 kinase and eukaryotic translation initiation factor 4E (eIF4E)–binding protein 1 (4EBP1). HeLa cells treated with EACC were immunoblotted for phospho-P70S6 kinase and phospho-4EBP1. Loss of phosphorylation of these substrates suggested that mTOR is inhibited in EACC-treated cells similar to that of control (Figure 3A). Active mTOR phosphorylates ULK1 at serine 757 and shuts down autophagy, whereas in starvation, inhibition of mTOR activity leads to dephosphorylation of ULK1 at 757 position and induction of autophagy. Unaltered dephosphorylation events of mTOR substrates and ULK1 in the presence of EACC suggested that the early signaling events that lead to starvation-mediated induction of autophagy is not perturbed (Figure 3, A and B). We also checked whether the massive accumulation of LC3-II upon EACC treatment is dependent on enhanced transcription or translation (Supplemental Figure S3, A–D).

Furthermore, relative levels of proteins involved in early and middle stages of the autophagy pathway such as ATG14, ATG5, and ATG16L1 were not changed upon EACC treatment (Figure 3B).

As mTOR-mediated control of autophagy was unaltered, we next investigated the effect of EACC on molecular events that lead to autophagosome biogenesis. Isolation membrane or phagophore formation upon autophagy induction is characterized by the presence of phosphatidylinositol-3-phosphate (PI3P) generated by vacuolar protein sorting 34 (VPS34) kinase complex activity. This local increase in PI3P is recognized by PI3P-binding proteins like double FYVE-domain–containing protein 1 (DFCP1) and WD repeat domain phosphoinositide-interacting protein 2 (WIPI2). In addition, this nascent membrane is also marked by ATG14 (Itakura and Mizushima, 2010; Hamasaki *et al.*, 2013). Triple colocalization results between ATG14, DFCP1, and LC3 in control and EACC-treated cells suggested that there was no decrease in number of autophagosome biogenesis sites (Figure 3, C and D).

As mentioned above, WIPI2 is an effector of mammalian PI3Ps that is recruited to omegasomes and marks the sites of autophagosome biogenesis. We looked at the colocalization between LC3 and WIPI2 and the results again suggest that the number of sites of autophagosome biogenesis (omegasomes) is unaffected upon EACC treatment (Figure 3, E and F). A similar trend was also observed in EACC-treated cells immunostained with LC3 and WIPI antibodies (Supplemental Figure S3E).

Developing autophagosomes undergo expansion of the phagophore and are characterized by localization of the ATG5-12/16 complex. In HeLa cells transfected with RFP-LC3, the colocalization between ATG5 and LC3 as well as ATG16L1 and LC3 that represents expanding phagophores also was comparable to that of control (Supplemental Figure S3, F–I).

As shown earlier, we also tested whether cargo recognition was affected by EACC. The colocalization analysis of the autophagy adaptor p62/SQSTM1 with the autophagosome membrane marker LC3 showed increased association between these proteins (Figure 1, G and H). Taken together, these results indicate that signaling events leading to autophagy induction, the number of autophagosome biogenesis sites, expansion of the phagophore, and cargo recognition remain unaltered in the presence of EACC. Thus, it is likely that the autophagic flux inhibition due to EACC may be affecting some downstream steps.

### EACC inhibits autophagy by preventing SNARE Stx17 loading on autophagosomes

As all our previous observations suggested that autophagosome formation is unaffected upon EACC treatment, we next tested whether these accumulated autophagosomes have the molecular

2 h in starvation conditions and immunoprecipitated with anti-LC3 antibody. Anti-mouse IgG was used as an isotype control. The immunoprecipitates were immunoblotted with anti-LAMP1 and anti-LC3 antibodies. (D) HeLa cells were treated with EACC (10 µM) for 2 h in starvation conditions and immunoblotted with anti-LAMP1 and anti–β-tubulin antibodies. (E) Relative levels of LAMP1:β-tubulin in untreated vs. treated samples were quantitated for three independent experiments. Statistical significance was analyzed by Student's unpaired *t* test. ns = nonsignificant. (F) HeLa cells were treated with EACC (10 µM) for 2 h in starvation conditions and immunostained with anti-LAMP1 antibody. Scale = 10 µm. (G) Graph represents the mean intensity of LAMP1 staining that was measured using the analyze plug-in in ImageJ. Data shown here represent a minimum of 60 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ns = nonsignificant. (H) HeLa cells were either treated with BafA1 (100 nM) in basal conditions or EACC (10 µM) in starvation conditions for 2 h. LysoTracker Deep Red (100 nM) was added in the media in the last 15 min of treatment. Cells were fixed and imaged. Scale = 15 µm. (I) Graph showing the mean intensity of LysoTracker staining measured as in D. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ***, $P < 0.001$; ns = nonsignificant. (J) Samples collected after EACC treatment were immunoblotted with anti–cathepsin B and anti–β-tubulin antibodies. (K) HeLa cells were serum starved for 3 h and either left untreated or pretreated with EACC before addition of EGF (100 ng/ml) for the indicated time periods. Samples were collected and immunoblotted for anti-EGFR and anti–β-tubulin antibodies. (L) Relative levels of EGFR:β-tubulin in untreated vs. treated samples were quantitated for three independent experiments.

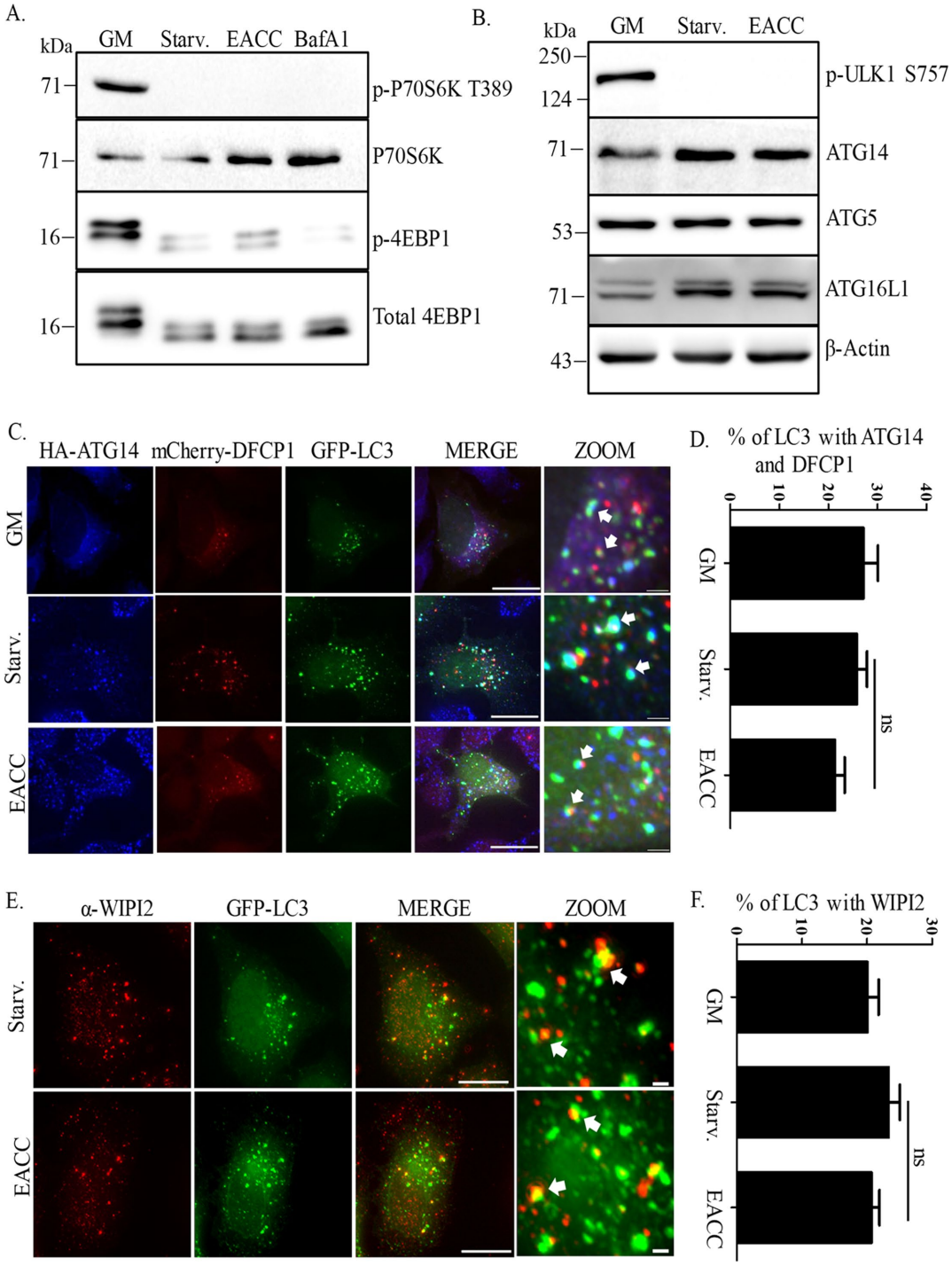


FIGURE 3: EACC does not affect early autophagic events. (A) HeLa cells were either left untreated or treated with BafA1 (100 nM) or EACC (10 μM) for 2 h in starvation conditions. Samples were collected and immunoblotted with anti–phospho-P70S6K (T389), anti-P70S6K, anti–phospho-4EBP1, and anti-4EBP1 antibodies. (B) Samples collected after EACC treatment were immunoblotted with anti–phospho-ULK1 (S757), anti-ATG14, anti-ATG5, anti-ATG16L1, and anti–β-actin antibodies. (C) HeLa cells cotransfected with mCherry-DFCP1, GFP-LC3, and HA-ATG14 were either left untreated or treated with EACC and immunostained with anti-HA antibody. Scale = 15 μm, 1 μm. (D) Graph showing the percent of LC3 puncta colocalizing with DFCP1 and ATG14. This population represents immature autophagosomes. The colocalized dots were counted and plotted as in Figure 2B. Data shown here represent a minimum of 50 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ns = nonsignificant. (E) GFP-LC3 transfected HeLa cells were treated with EACC (10 μM) for 2 h in starvation conditions and immunostained with anti-WIPI2 antibody. Scale = 15 μm. (F) Graph showing the percent of LC3 puncta colocalizing with WIPI2. This population represents omegasomes. The analysis was done similarly as in D. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ns = nonsignificant.

machinery required for fusion with lysosomes. Elegant studies by Noburu Mizushima's group identified Stx17 as an autophagosomal SNARE that translocates to autophagosomes and interacts with SNAP29 and endo/lysosomal SNARE VAMP8 with the help of a multisubunit tethering complex like HOPS. Depletion of Stx17 blocked autophagic flux by inhibiting fusion of autophagosomes with lysosomes (Itakura *et al.*, 2012).

In HeLa cells cotransfected with FLAG-Stx17 and GFP-LC3, we quantitated the colocalization between Stx17 and LC3. Similar to previous reports, in basal conditions, Stx17 depicted a reticulate pattern suggesting ER/mitochondrial localization. Upon induction of autophagy, the Stx17 staining pattern became punctate and showed significantly increased colocalization with LC3. This colocalization increased further in the presence of BafA1 because this treatment blocks fusion by affecting lysosomal pH but does not affect autophagosomal SNARE assembly (Itakura *et al.*, 2012). Interestingly, upon EACC treatment the colocalization between Stx17 and LC3 reduced significantly (Figure 4, A and B).

A recent report showed that the pathogenic bacterium *Legionella pneumophila* can block autophagy by degrading Stx17 (Arasaki *et al.*, 2017). However, the presence of EACC did not affect levels of Stx17 expression (Figure 4C).

To further dissect the effect of EACC on LC3-Stx17 interaction we performed coimmunoprecipitation (Co-IP) analysis. In HeLa cells, transfected with either FLAG-Stx17 or an empty vector and either left untreated or treated with EACC, we probed for the levels of LC3-II. The relative levels of LC3-II in FLAG-Stx17 IP (after normalizing it to input LC3) were reduced upon EACC treatment (Figure 4, D and E).

Stx17 is a Qa SNARE that partners with Qbc-SNARE SNAP29 (Itakura *et al.*, 2012; Guo *et al.*, 2014). The autophagosomes having both SNAP-29 and Stx17 were fewer in EACC-treated cells as compared with control (Figure 4, F and G). Interestingly, the colocalization of the partners SNARE Stx17 and SNAP29 was largely unaffected post EACC treatment (Figure 4H). In addition to these SNAREs, ATG14 also participates in autophagosome–lysosome fusion, where it binds to the SNARE domain of Stx17 and stabilizes the Stx17-SNAP29 complex on autophagosomes. This function of ATG14 is independent of its role in autophagosome biogenesis (Hamasaki *et al.*, 2013; Diao *et al.*, 2015). EACC treatment reduced ATG14 and Stx17 colocalization (Figure 4, I and J).

Overall, all these results suggest that EACC renders autophagosomes fusion incompetent by preventing Stx17 translocation onto autophagosomes.

### EACC does not affect RABs, tethers, and lysosomal SNARE but prevents their interaction with LC3 and Stx17

Apart from SNAREs, autophagosome–lysosome fusion also requires accessory proteins like small GTPase RAB7 and multisubunit tethering complex HOPS.

Through its interaction with Stx17 and LC3, RAB7 is required for autolysosome formation (Hyttinen *et al.*, 2013). Although control cells showed significant association of RAB7 with LC3, EACC treatment revealed decreased RAB7 and LC3 colocalization, reiterating that EACC renders autophagosomes fusion incompetent (Figure 5, A and B).

Multisubunit tethering complex HOPS through its interaction with Stx17 promotes autophagosome–lysosome fusion (Jiang *et al.*, 2014; Takats *et al.*, 2014). We addressed whether this interaction was altered in the presence of EACC by colocalization analysis between HOPS-specific subunit VPS33A and Stx17. In HeLa cells cotransfected with FLAG-Stx17 and HA-VPS33A, we calculated Pearson's correlation coefficient (PCC) between Stx17 and VPS33A. Colocalization between Stx17 and VPS33A decreased in EACC treatment (Figure 5, C and D).

Although EACC treatment resulted in accumulation of fusion-incompetent autophagosomes, we further tested whether the lysosomes were competent to receive incoming vesicles for fusion. As shown in Figure 2, K and L, unperturbed EGFR degradation hinted at unaltered lysosomal competence in the presence of EACC. We further investigated the status of the fusion machinery on lysosomes, in particular the v-SNARE VAMP8 required for autolysosome formation (Itakura *et al.*, 2012). In HeLa cells transfected with GFP-VAMP8 and immunostained for LAMP1, there was no apparent change in VAMP8 and LAMP1 association after EACC treatment as compared with control (Figure 5, E and F). Because EACC prevents Stx17 translocation onto autophagosome and blocks autophagosome–lysosome fusion, as expected, treatment with EACC in cells cotransfected with FLAG-Stx17 and GFP-VAMP8 showed decreased colocalization between Stx17 and VAMP8 (Figure 5, G and H).

To further consolidate these findings, using immunoprecipitation assays we checked the effect of EACC on Stx17-HOPS and Stx17-VAMP8 interactions. Cells cotransfected with HA-VPS33A and FLAG-Stx17 were either left untreated or treated with EACC and subjected to IP using FLAG-tagged magnetic beads. We observed that in EACC-treated lysates, the levels of HA-VPS33A were reduced in FLAG-Stx17 immunoprecipitates (Figure 5I).

We also performed a Co-IP using GFP-Trap beads in cells cotransfected with GFP-VAMP8 and FLAG-Stx17. EACC treatment reduced the levels of FLAG-Stx17 in GFP-VAMP8 Co-IP (Figure 5J).

Taken together, these results suggest that EACC treatment renders autophagosomes "fusion incompetent" by preventing Stx17 translocation onto them. It also reduces Stx17 interaction with tethers (VPS33A) and the lysosomal R-SNARE VAMP8. All these factors collectively prevent autophagosome–lysosome fusion and block autophagic flux.

### The action of EACC is reversible

Thus far, EACC appears to inhibit autophagic flux by accumulating fusion-incompetent (Stx17-negative) autophagosomes. We wondered whether this effect of EACC is reversible. Toward this, we carried out EACC washout experiments and followed autophagic flux and loading of Stx17 onto autophagosomes.

We divided EACC-treated cells into three subgroups. In the first group, cells in starvation media were treated with EACC for 1 h and lysates were collected. In the second group, after a similar treatment with EACC for 1 h, cells were washed with Dulbecco phosphate buffered saline (DPBS) and kept in starvation medium without EACC for 3 h and lysates were collected. In the third group, the treatment with EACC was allowed to go on for 4 h and lysates were collected after that. All the lysates were probed for LC3-II expression. The robust accumulation of LC3-II in EACC treatment was seen as early as 1 h. Interestingly, this accumulation of LC3-II was abrogated after washing out EACC (Figure 6, A and B).

We next corroborated our immunoblotting-based results by utilizing the tandem-tagged mRFP-GFP-LC3 construct. HeLa cells transfected with mRFP-GFP-LC3 were treated with EACC in a similar manner as explained above. After 1-h treatment, we saw a significant increase in the number of autophagosomes ($mRFP^+/GFP^+$) and a concomitant decrease in the number of autolysosomes ($mRFP^+/GFP^-$) as compared with control. After washing out EACC, the autophagosome and autolysosome numbers became

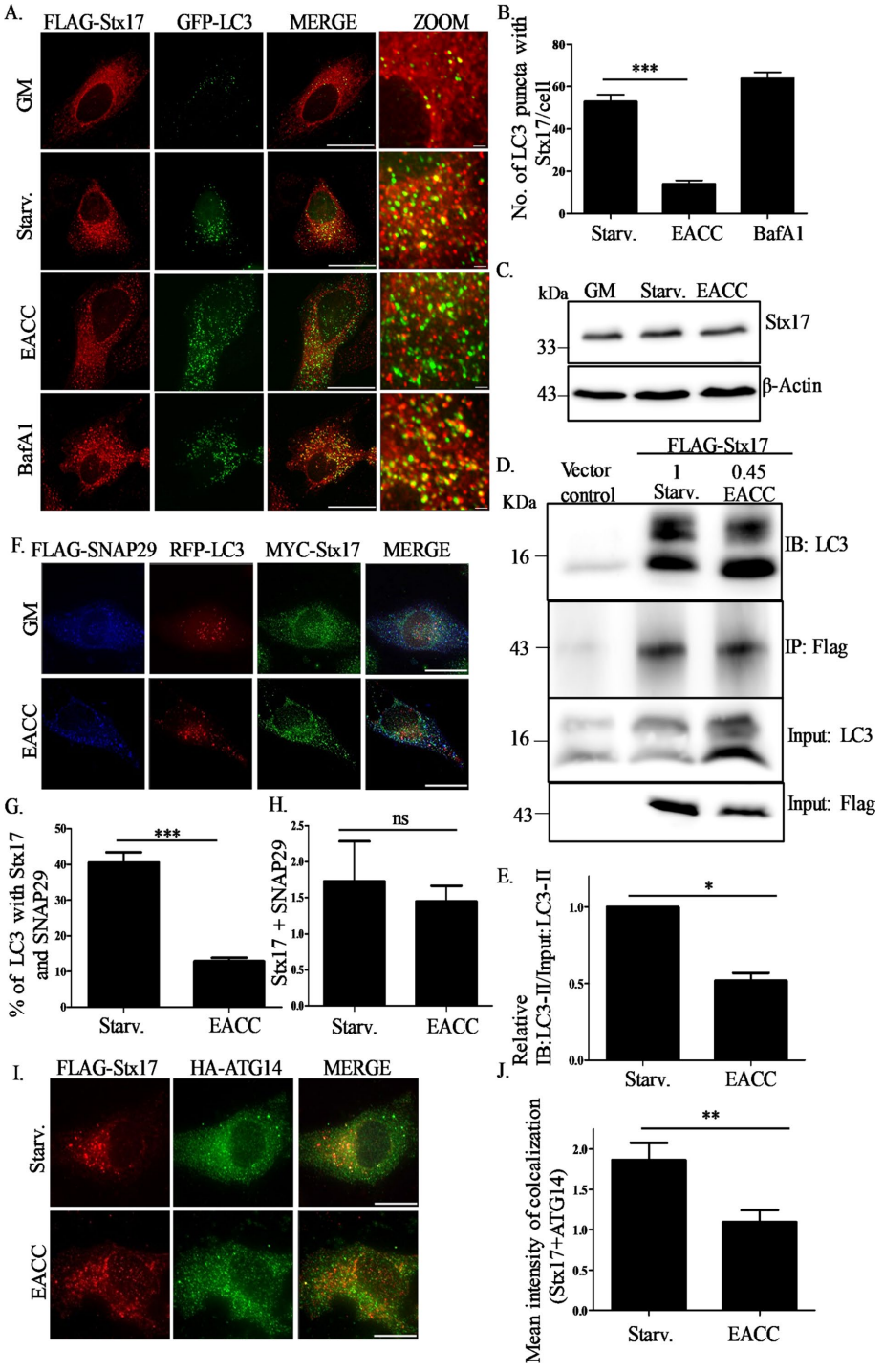


FIGURE 4: EACC inhibits autophagy by preventing SNARE Stx17 loading on autophagosomes. (A) HeLa cells cotransfected with FLAG-Stx17 and GFP-LC3 were treated with BafA1 (100 nM) or EACC (10 μM) for 2 h in starvation conditions and immunostained with anti-FLAG antibody. Scale = 15 μm, 1 μm. (B) Graph represents the number of colocalized dots of FLAG-Stx17 and GFP-LC3. The colocalized dots were counted as mentioned in Figure 2B. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ***, $P < 0.001$. (C) Samples from EACC- or BafA1-treated HeLa cells were immunoblotted with anti-Stx17 and anti–β-actin antibodies. (D) Co-IP analysis of interaction between FLAG-Stx17 and endogenous LC3B in HeLa cells either left untreated or treated with EACC. Relative levels of LC3B-II in untreated and EACC-treated cells are mentioned. (E) Data indicate mean ± SEM of relative levels of LC3B-II in FLAG-Stx17 IP normalized to input LC3B-II from three independent experiments. Statistical significance was analyzed by Student's paired *t* test. *, $P < 0.05$. (F) HeLa cells cotransfected with MYC-Stx17, RFP-LC3, and FLAG-SNAP29 were either left untreated or treated with EACC and immunostained with anti-FLAG and anti-MYC antibodies. Scale = 15 μm. (G) Graph represents the percentage of LC3 puncta colocalizing with Stx17 and SNAP-29. The colocalized dots were counted as mentioned in Figure 2B. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ***, $P < 0.001$. (H) Graph showing the mean intensity of colocalization between FLAG-SNAP29 and MYC-Stx17 measured as explained in Figure 1H. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ns = nonsignificant. (I) HeLa cells cotransfected with FLAG-Stx17 and HA-ATG14 were treated with EACC and immunostained with anti-FLAG and anti-HA antibodies. Scale = 15 μm. (J) Graph showing the mean intensity of colocalization between FLAG-Stx17 and HA-ATG14 measured as explained in Figure 1H. Data shown here represent a minimum of 30 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. **, $P < 0.01$.

comparable to that of control (Figure 6, C–E). Taken together these results suggest that the block in autophagosome–lysosome fusion can be reversed by washing out EACC.

We have shown that EACC inhibits translocation of Stx17 to autophagosomes. So, next we tested whether the localization of SNARE Stx17 can be restored after washing out EACC. In HeLa cells transfected with FLAG-Stx17 and RFP-LC3, we quantitated the number of $Stx17^+$ autophagosomes before and after EACC washout. There were very few $LC3^+/Stx17^+$ puncta in cells treated with EACC for 4 h. On the other hand, the number of $LC3^+/Stx17^+$ puncta was higher in cells in which EACC was washed out after 1 h (Figure 6, F–H).

In summary, EACC is a reversible inhibitor of autophagosome–lysosome fusion and mechanistically, it acts by preventing translocation of Stx17 onto autophagosomes and decreasing its interaction with the HOPS subunit VPS33A and the lysosomal R-SNARE VAMP8.

## DISCUSSION

In this article, we report a novel small molecule EACC that can block autophagic flux in a previously unreported manner. EACC inhibits the translocation of autophagosome-specific SNARE Stx17 thereby blocking autophagosome–lysosome fusion.

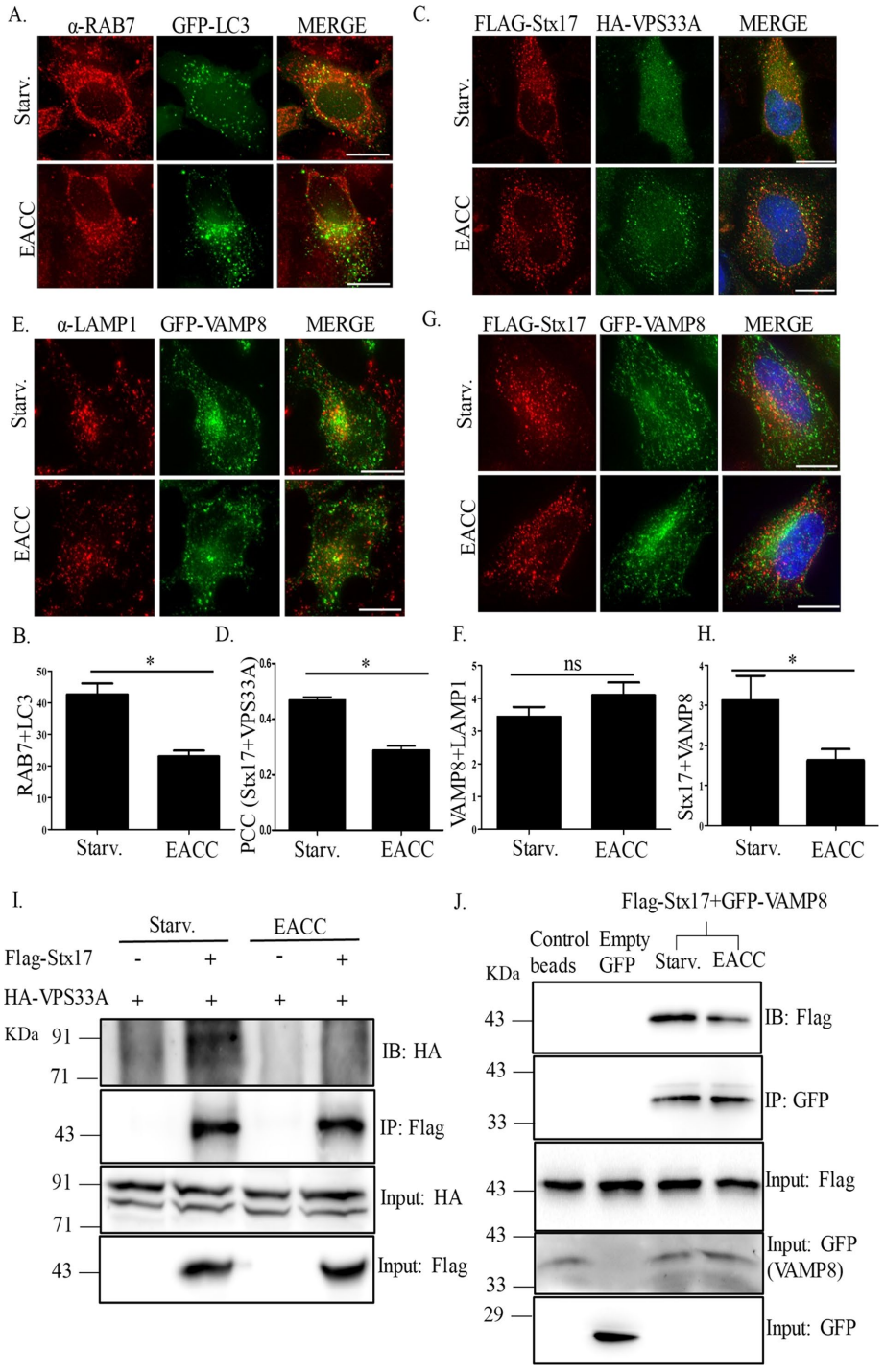


**FIGURE 5:** EACC does not affect RABs, tethers, and lysosomal SNARE but prevents their interaction with LC3 and Stx17. (A) GFP-LC3 transfected HeLa cells were treated with EACC and immunostained with anti-RAB7 antibody. Scale = 10 µm. (B) Graph represents the number of LC3 puncta colocalizing with RAB7. The colocalized dots were counted as in Figure 2B. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. *, $P < 0.05$. (C) HeLa cells cotransfected with FLAG-Stx17 and HA-VPS33A were either left untreated or treated with EACC (10 µM) for 2 h. Scale = 10 µm. (D) Graph showing Pearson's correlation coefficient (PCC) between Stx17 and VPS33A. PCC was measured using SoftWoRx software from DeltaVision. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. *, $P < 0.05$. (E) GFP-VAMP8 transfected HeLa cells were immunostained with anti-LAMP1 antibody. Scale = 10 µm. (F) Graph representing the mean intensity of colocalization between LAMP1 and VAMP8. The mean intensity of colocalized dots was measured as in Figure 1H. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ns = nonsignificant. (G) HeLa cells cotransfected with FLAG-Stx17 and GFP-VAMP8 were either left untreated or treated with EACC. Scale = 10 µm. (H) Graph representing the mean intensity of colocalization between Stx17 and VAMP8. The mean intensity of colocalization was measured as in Figure 1H. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. *, $P < 0.05$. (I) HeLa cells transfected with FLAG-Stx17 and HA-VPS33A or only HA-VPS33A were either left untreated or treated with EACC. IP was performed using FLAG-tagged magnetic beads and the levels of HA-VPS33A and FLAG-Stx17 were checked by immunoblotting. (J) HeLa cells transfected with FLAG-Stx17 and GFP-VAMP8 or FLAG-Stx17 and empty GFP vector were either left untreated or treated with EACC. IP was performed using control agarose beads or GFP-Trap beads and the levels of GFP-VAMP8 and FLAG-Stx17 were checked by immunoblotting.

We show that EACC causes a massive accumulation of LC3-II over and above that of starvation-induced autophagy. Using various experimental approaches, we deduce that the increase in LC3-II is due to a block in autophagic flux rather than autophagy induction. Additionally, upon probing each step in the process of autophagy, we narrow down the action of EACC to the penultimate step of autophagic flux, that is, fusion of autophagosomes with lysosomes resulting in accumulation of autophagosomes. By further systematic analysis of stage-specific components of autophagy and lysosomal machinery, we conclude that EACC selectively renders autophagosome "fusion incompetent"' but does not affect the ability of lysosomes to fuse with other incoming traffic.

It is suggested that the fusion step proceeds temporally by first loading Stx17 on autophagosomes followed by SNAP29 recruitment. This Qabc SNARE complex is stabilized by ATG14. Subsequently, successful fusion ensues when SNARE pairing (Qa Stx17, Qbc SNAP29, and the lysosomal R-SNARE VAMP8) is promoted by small GTPase RAB7 and tethering complex HOPS (Itakura *et al.*, 2012; Guo *et al.*, 2014; Jiang *et al.*, 2014; Takats *et al.*, 2014; Diao *et al.*, 2015).

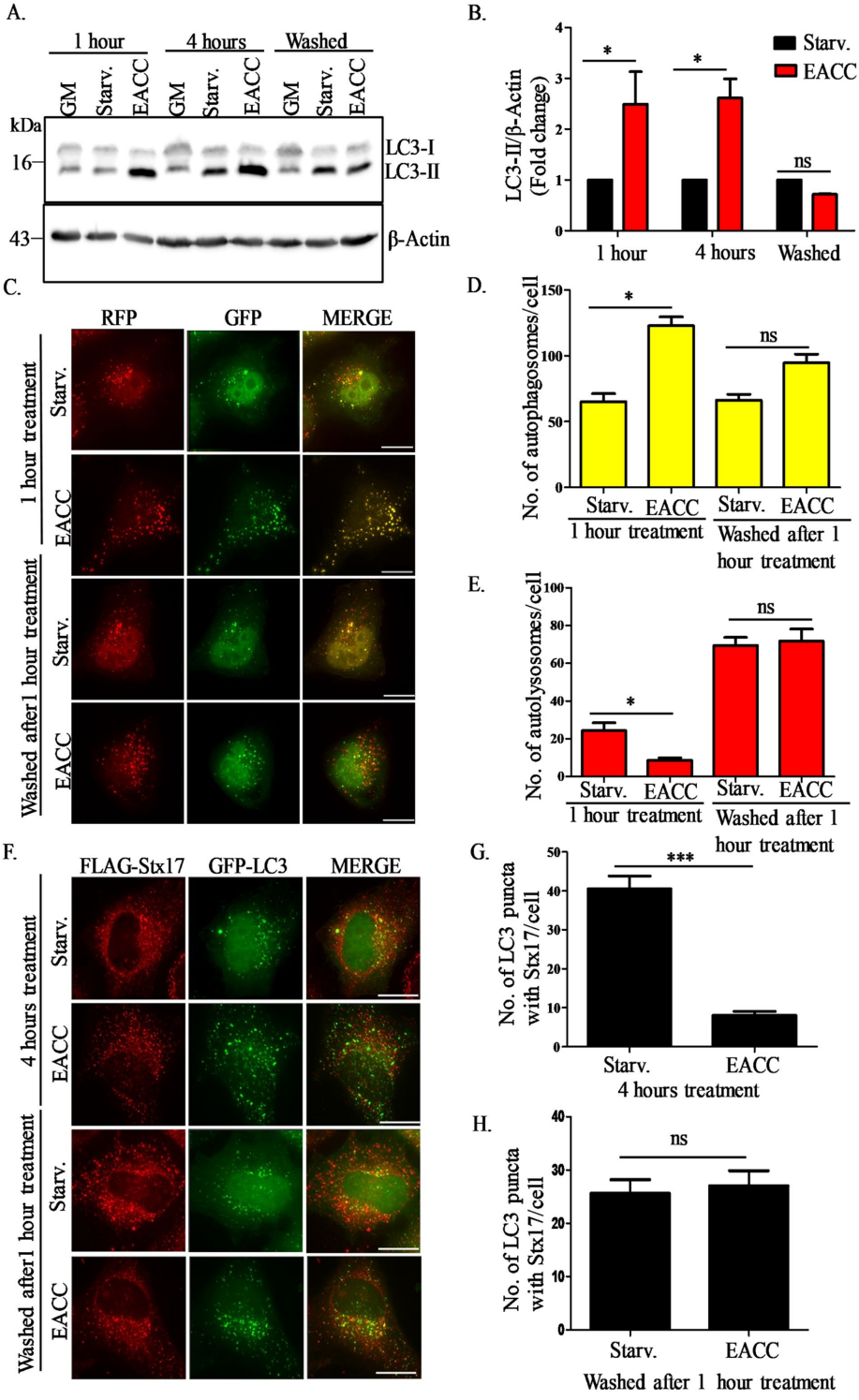


**FIGURE 6:** The action of EACC is reversible. (A) We divided EACC-treated cells into three subgroups. In the first group, cells in starvation media were treated with EACC for 1 h and lysates were collected. In the second group, after a similar treatment with EACC for 1 h, cells were washed with DPBS and kept in starvation medium without EACC for 3 h and lysates were collected. In the third group, the treatment with EACC was allowed to go on for 4 h and lysates were collected after that. All the lysates were probed for LC3B-II expression. (B) Relative levels of LC3-II:β-actin in untreated vs. treated samples were quantitated for three independent experiments. *, $P < 0.05$; ns = nonsignificant (two-way ANOVA, replicate means compared with Bonferroni posttest). (C) HeLa cells were transfected with tandem-tagged mRFP-GFP-LC3 construct for 48 h and treatment was carried out as explained above in A. Scale: 15 μm. (D, E) The autophagosomes ($RFP^+/GFP^+$ structures) and autolysosomes ($RFP^+/GFP^-$ structures) per cell in various treatment conditions were counted as mentioned in Figure 1F. Data shown represent the number of autophagosomes ($RFP^+/GFP^+$) and autolysosomes ($RFP^+/GFP^-$) for a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. *, $P < 0.05$; ns = nonsignificant. (F) HeLa cells transfected with FLAG-Stx17 and GFP-LC3 were treated with EACC (10 μM) as explained above and immunostained with anti-FLAG antibody. Scale: 10 μm. (G, H) Graph represents the number of LC3 puncta colocalizing with Stx17. The colocalized dots were counted as mentioned in Figure 2B. Data shown here represent a minimum of 45 cells from three independent experiments plotted as mean ± SEM. Statistical significance was analyzed by Student's unpaired *t* test. ***, $P < 0.001$; ns = nonsignificant.

The striking feature of EACC-mediated block of autophagic flux is impaired Stx17 loading onto autophagosomes. To the best of our knowledge, there is no other report suggesting any chemical modulator of autophagy that can selectively prevent Stx17 translocation thereby rendering autophagosomes "fusion incompetent." The exact mechanism by which Stx17 is translocated onto complete autophagosomes is not very clear. A recent report suggested that Stx17 recruitment to autophagosomes occurs via interaction with a small GTPase IRGM and mammalian ATG8 proteins (Kumar *et al.*, 2018). Although we have not checked whether EACC can affect interaction between Stx17 and IRGM, we propose that identification of Stx17-binding partners in the presence or absence of EACC could give a clue regarding the target of EACC as well as help in identification of any other accessory factors that might be involved in Stx17 recruitment on autophagosomes. Furthermore, we also showed that the action of EACC is reversible. The block in autophagic flux is eliminated after washing out EACC because Stx17 is now able to translocate to autophagosomes and participate in further fusion events. Hence, due to the reversible nature of its action, EACC can be used as a useful tool to study Stx17 trafficking.

To determine the rate of autophagic flux, lysosomal inhibitors like BafA1 and chloroquine are routinely used. Unfortunately, these treatments are not ideal as they not only can impair lysosomal function but impede all other lysosomal pathways including the endo-lysosomal trafficking. Our results also show that the action of EACC is specific to autophagosomes and it does not affect lysosomal pH, function, or endocytic trafficking. It also does not affect the localization of lysosomal SNAREs

or RABs. Additionally, even the well-known early inhibitors of autophagy such as wortmannin and 3-methyl adenine are promiscuous as they block all phosphatidylinositol 3-kinase–dependent signaling pathways thereby resulting in a plethora of side effects. In such scenarios, inhibiting Stx17 translocation by using EACC, which leads to a specific block in autophagy, might be a cleaner way to perform autophagic flux experiments. In fact, silencing Stx17 expression is recommended as a desired attribute for selectively inhibiting autophagic flux (Hegedus *et al.*, 2013). In conclusion, molecules like EACC can fill the lacuna that exists in the field due to lack of specific autophagy inhibitors.

## MATERIALS AND METHODS

### Cell culture

HeLa cells were maintained in growth medium composed of DMEM (Sigma-Aldrich; D5648) supplemented with 3.7 g/l sodium bicarbonate (Sigma-Aldrich; S5761) plus 10% fetal bovine serum (Life Technologies; 10270-106) and 100 U/ml penicillin and streptomycin (Life Technologies; 15140-122) at 5% $CO_2$ and 37°C. Autophagy was induced by washing cells with DPBS (Sigma-Aldrich; D5773) and incubating them in starvation media/Earle's balanced salt solution (Sigma-Aldrich; E7510) made to 1× and supplemented with 2.2 g/l sodium bicarbonate for 2 h until otherwise stated.

### Plasmids

Plasmids used in the study were as follows: ptfLC3 (mRFP-GFP-LC3; Addgene plasmid #21074) and pmRFP-LC3 (Addgene plasmid #21075) were gifts from Tamotsu Yoshimori (Osaka University). FLAG-Stx17 (Addgene plasmid #45911) and FLAG-SNAP29 (Addgene plasmid#45915) were gifts from Noburu Mizushima (The University of Tokyo). GFP-VAMP8 was a gift from Thierry Galli (Institute of Psychiatry and Neuroscience of Paris [IPNP]) (Addgene plasmid #42311; Paumet *et al.*, 2000), mCherry-DFCP1 was a gift from Do-Hyung Kim (University of Minnesota) (Addgene plasmid #86746; Kim *et al.*, 2015), and HA-hATG14 was a gift from Noburu Mizushima (Addgene plasmid #24294; Itakura *et al.*, 2008). Plasmid-containing HA-VPS33A was a kind gift from Mahak Sharma, IISER Mohali. Myc-Stx17 plasmid was a kind gift from Viktor Korolchuk, Newcastle University. GFP-LC3 plasmid was generated in the lab by excising out mRFP fragment from mRFP-GFP-LC3 plasmid.

### Antibodies and reagents

The following primary antibodies were used: LC3 (MBL; M152-3), LC3B (Sigma-Aldrich; L7543), β-actin (CST; 4970), LAMP1 (CST; 9091), p62/SQSTM1 (Abcam; ab56416), β-tubulin (DHSB; E7), cathepsin B (Cloud Clone; PAC964Hu01), EGFR (Santa Cruz Biotechnology; sc-03), phospho-P70S6K (T389; CST; 9234), total P70S6K (CST; 9202), phospho-4EBP1 (CST; 2855), total 4EBP1 (CST; 9452), phospho-ULK1 (S757; CST; 6888), ATG14 (CST; 5504), ATG5 (CST; 12994), ATG16L1 (CST; 8089), WIPI2 (Abcam; ab105459), Stx17 (Sigma-Aldrich; HPA001204), FLAG (Sigma-Aldrich; F3165), FLAG (Sigma-Aldrich; F1804), HA (CST; 3724), Myc (Abcam; ab9106), RAB7 (CST; 9367), GFP (Roche; 11814460001), mouse immunoglobulin G (IgG) (Genei, IGP3). Secondary antibodies used were goat anti-mouse IgG (H+L) HRP (horseradish peroxidase) conjugate (Bio-Rad; 1721011), goat anti-rabbit IgG (H+L) HRP conjugate (Biorad; 1706515). Fluorescent secondary antibodies used were Atto 633 (goat anti-rabbit IgG; Sigma-Aldrich; 41176), Atto 488 (goat anti-rabbit IgG; Sigma-Aldrich; 41057), Alexa 647 (goat anti-mouse IgG; Invitrogen; A21236), Alexa 405 (goat anti-mouse IgG; Invitrogen; A31556).

Chemicals used in this study were EACC (Life Chemicals; F1358-0554), bafilomycin A1 (Sigma-Aldrich; B1793), actinomycin D (Sigma-Aldrich; A1410), cycloheximide (Sigma-Aldrich; C7698), and EGF (Thermo Fisher Scientific; PHG0311L). LysoTracker Deep Red (L12492) was purchased from Thermo Fisher Scientific.

### Immunoprecipitation

For immunoprecipitation assays, cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$, 150 mM NaCl, 0.5% NP-40) supplemented with protease inhibitor/phosphatase inhibitor cocktails for 30 min at 4°C and centrifuged at 13,000 × *g* for 15 min. Protein (500 µg to 1 mg) was incubated with specific primary antibody at 4°C (2 h to overnight) on a tube rotator followed by incubation with protein G dynabeads (Invitrogen; #10004D) for 2 h at 4°C. The beads were washed three times with ice-cold PBS and the proteins were eluted from washed beads by boiling for 5 min in 2× Laemmli sample buffer and processed for immunoblot analysis.

For immunoprecipitation with GFP-Trap beads (Chromotek), cells were lysed in lysis buffer recommended by the manufacturer (10 mM Tris-HCl, pH 7.2, 0.5 mM EDTA, 150 mM NaCl, 0.5% NP-40) supplemented with protease inhibitor/phosphatase inhibitor cocktails for 30 min at 4°C and centrifuged at 13,000 × *g* for 15 min. Protein (1 mg) from the supernatant was used, and immunoprecipitation was performed by following the manufacturer's instructions.

### Immunoblotting

Following appropriate treatments, cells were washed with ice-cold PBS. Cells were then lysed in 100 µl of sample buffer (10% wt/vol SDS, 10 mM dithiothreitol, 20% vol/vol glycerol, 0.2 M Tris-HCl, pH 6.8, 0.05% wt/vol bromophenol blue) and then collected using a rubber cell scraper. The lysates were boiled at 99°C for 15 min and stored at −20°C. Immunoblotting was performed using standard methods.

Blots were incubated overnight with the above-mentioned primary antibodies. Secondary antibody used at 1:10,000 was goat anti-mouse (Bio-Rad; #172-1011) or goat anti-rabbit antibody (Bio-Rad; #172-1019) conjugated to HRP. Blots were developed by using enhanced chemiluminescence (ECL) substrate (Bio-Rad; #170-5061) and images captured using auto capture or series capture program in a Gel documentation system (Syngene G-Box; UK). ImageJ software (National Institutes of Health [NIH]) was used for quantitation of band intensities.

### Immunofluorescence

An appropriate number of cells were plated on top of coverslips placed in 60-mm cell culture dishes for transfection. The following day, transfection was done on a 60-mm dish with HeLa cells at 60–70% confluency. Cells were transfected using 5 µl of lipofectamine 2000 (Invitrogen; 11668-019) and 2.5 µg of DNA (2:1 ratio) diluted in 100 µl of OPTI-MEM (Invitrogen; 31985-070) separately. At 48 h posttransfection, cells were either left untreated or treatment with EACC was done for 2 h. Starvation was induced by treating cells with Earle's balanced salt solution (EBSS). After treatment, cells were fixed in 4% paraformaldehyde and permeabilized using 0.25% Triton X-100. Overnight incubation with primary antibody was done at 4°C. Excess antibody was washed with PBS and coverslips were incubated with appropriate fluorescent secondary antibody. The coverslips were mounted with Vectashield antifade reagent (H-1000/ H-1200; Vector Laboratories). Imaging for HeLa cells was carried out using a DeltaVision microscope, GE (Olympus 60X/1.42, Plan ApoN, excitation and emission filter Cy5, FITC, DAPI, and TRITC, polychroic Quad).

### CellTiter-Glo cell viability assay

Toxicity of the compound was monitored by CellTiter-Glo cell viability assay (Promega; G7570). HeLa cells were counted and equal numbers (1500 cells/well) were plated in a 384-well plate in growth medium. The following day, different concentrations of EACC ranging from 100 nM to 100 μM were mixed in starvation media, added onto the cells, and incubated for 5 h. After 5 h, CellTiter-Glo Reagent was added to each well and luminescence measured using Varioskan Flash (Thermo Fisher Scientific).

### EGFR trafficking

HeLa cells were plated on six-well plates and allowed to attach. The following day, cells were washed with PBS and then starved in DMEM (serum-free media) for 3 h. Pretreatment with EACC was carried out for 1 h, following which cells were pulsed with 100 ng/ml EGF and samples were collected at 0, 1-, 2-, and 3-h intervals.

### Colocalization analysis and mean intensity calculation

ImageJ software (NIH) was used to calculate the mean intensity of staining or mean intensity of colocalization. Images were opened using the split channel plug-in. In the case of colocalization, a colocalization plug-in in the analyze tool was used to obtain the colocalized area between two channels as a separate window. The intensity was measured using the analysis measure plug-in in analysis tools. A cell counter plug-in was used to count the colocalized structures.

### Statistical analysis and image preparation

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Statistical analyses were performed by comparing the means using the paired/unpaired Student *t* test or two-way analysis of variance (ANOVA) followed by the Bonferroni posttest to compare replicate means by row. Images were prepared using SoftWoRx software (GE Healthcare). Some fluorescent MIP images had their brightness and contrast modified equally in control and treatment conditions just for the purpose of visualization.

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REVIEW
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# Chemical Screening Approaches Enabling Drug Discovery of Autophagy Modulators for Biomedical Applications in Human Diseases

*Prashanta Kumar Panda[1†], Alexandra Fahrner[1†], Somya Vats[1,2], Elena Seranova[1], Vartika Sharma[3], Miruna Chipara[1], Priyal Desai[1], Jorge Torresi[1,4], Tatiana Rosenstock[1,4], Dhiraj Kumar[3] and Sovan Sarkar[1]**

[1] Institute of Cancer and Genomic Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom, [2] Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India, [3] Cellular Immunology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India, [4] Department of Physiological Science, Santa Casa de São Paulo School of Medical Sciences, São Paulo, Brazil

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**Edited by:**
Brian Storrie,
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Vytaute Starkuviene,
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Satoshi Kametaka,
Nagoya University, Japan

***Correspondence:**
Sovan Sarkar
s.sarkar@bham.ac.uk

[†] These authors have contributed equally to this work

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Autophagy is an intracellular degradation pathway for malfunctioning aggregation-prone proteins, damaged organelles, unwanted macromolecules and invading pathogens. This process is essential for maintaining cellular and tissue homeostasis that contribute to organismal survival. Autophagy dysfunction has been implicated in the pathogenesis of diverse human diseases, and therefore, therapeutic exploitation of autophagy is of potential biomedical relevance. A number of chemical screening approaches have been established for the drug discovery of autophagy modulators based on the perturbations of autophagy reporters or the clearance of autophagy substrates. These readouts can be detected by fluorescence and high-content microscopy, flow cytometry, microplate reader and immunoblotting, and the assays have evolved to enable high-throughput screening and measurement of autophagic flux. Several pharmacological modulators of autophagy have been identified that act either via the classical mechanistic target of rapamycin (mTOR) pathway or independently of mTOR. Many of these autophagy modulators have been demonstrated to exert beneficial effects in transgenic models of neurodegenerative disorders, cancer, infectious diseases, liver diseases, myopathies as well as in lifespan extension. This review describes the commonly used chemical screening approaches in mammalian cells and the key autophagy modulators identified through these methods, and highlights the therapeutic benefits of these compounds in specific disease contexts.

**Keywords: autophagy, autophagy reporter, autophagy substrate, autophagy modulator, screening method, neurodegenerative diseases, cancer, lifespan extension**

## INTRODUCTION

Macroautophagy, herein referred to as autophagy, is an intracellular degradation process essential for ensuring cellular homeostasis. This well-conserved catabolic process mediates the targeted degradation of unwanted or excess cytoplasmic materials, such as aggregation-prone proteins, pathogens and damaged organelles like mitochondria, amongst others (Ravikumar et al., 2010). This process is also involved in the bulk degradation of cytoplasmic macromolecules and recycling of the breakdown products especially during nutrient deprivation to provide energy homeostasis, thereby forming a crucial connection between anabolism and catabolism (Boya et al., 2013; Kaur and Debnath, 2015). Due to its vital function as a homeostatic regulator, impairment of the autophagy is implicated in several human pathologies including certain cancer, metabolic syndromes, infectious diseases, liver diseases, myopathies, aging and neurodegenerative disorders (Mizushima et al., 2008). Therefore, therapeutic modulation of autophagy holds great potential in the development of treatment strategies for these diseases (Rubinsztein et al., 2012).

Autophagy is evolutionarily-conserved from yeast to humans. The *de novo* formation of phagophores, the double-membrane structures that expand to form double-membrane vesicles called autophagosomes, require multiple autophagy-related (*Atg*) genes in the autophagic machinery, such as the Atg5-Atg12-Atg16 complex and the phosphatidylethanolamine-conjugated microtubule-associated protein 1 light chain 3 (LC3-II) (Kabeya et al., 2000; Mizushima et al., 2011; Ktistakis and Tooze, 2016). Maturation of autophagosomes into the degradative autolysosomes occurs either via the multi-step route involving the fusion of autophagosomes with late endosomes to form amphisomes which subsequently fuse with the lysosomes, or via the direct route involving the fusion between autophagosomes and the lysosomes (Nakamura and Yoshimori, 2017). The autophagic cargo engulfed by the autophagosomes are ultimately degraded in the acidic autolysosomes by the lysosomal hydrolases, which are only active at the low pH maintained by the vacuolar-type $H^+$-ATPase (V-ATPase) on the lysosomal membrane (Saftig and Klumperman, 2009). Finally, the breakdown products are recycled and utilized as inputs to cellular metabolism for energy generation (Rabinowitz and White, 2010). The rate at which this dynamic turnover of cellular contents occurs through the process of autophagy is referred to as autophagic flux. Autophagic flux encompasses all stages of autophagy which includes autophagosome formation, fusion with the lysosomes and cargo degradation in the autolysosomes (**Figure 1**).

Key upstream modulators of autophagy include the mechanistic target of rapamycin complex 1 (mTORC1) pathway, which promotes cellular biosynthesis and inhibits autophagy (Saxton and Sabatini, 2017). Regulation of autophagosome formation by mTORC1 is mediated via the ULK1–Atg13–FIP200 complex; mTORC1 suppresses autophagy under nutrient-rich conditions by phosphorylation-dependent inactivation of ULK1 and Atg13 (Mizushima, 2010; Zachari and Ganley, 2017). Various signals such as growth factors and nutrients impinge on mTORC1 to negatively influence autophagy (Kim and Guan, 2015). Conversely, during nutrient starvation, autophagy is promoted by inhibition of the mTORC1 activity (Carroll et al., 2014; Russell et al., 2014). Furthermore, ULK1 can be directly phosphorylated and activated by the energy sensor AMPK to stimulate autophagy (Egan et al., 2011; Kim et al., 2011). In addition, several mTORC1-independent pathways have been described where autophagy is negatively regulated by the elevation in intracellular inositol, $Ca^{2+}$ and nitric oxide levels, amongst others (Sarkar, 2013b). Molecular mediators of the late stage of autophagy involving autophagosome maturation include Rab7, SNAREs (N-ethylmaleimide-sensitive factor-attachment protein receptors), GABARAPs, BRUCE and Beclin1-interacting partners such as Atg14L, UVRAG and Ambra1 (He and Levine, 2010; Nguyen et al., 2016; Wang et al., 2016; Reggiori and Ungermann, 2017; Ebner et al., 2018). At a transcriptional level, autophagy is governed by the transcription factor EB (TFEB) (Settembre et al., 2011), which in itself is activated by lysosomal $Ca^{2+}$ (Medina et al., 2015).

Chemical modulation of autophagy by targeting the mTOR-dependent and mTOR-independent pathways has proven to be of potential biomedical relevance due to therapeutic advantages, especially in neurodegenerative disorders as well as in diverse human pathological conditions such as in certain liver diseases, myopathies, infectious diseases, metabolic diseases, cancer and aging (Rubinsztein et al., 2012; Sarkar, 2013b; Levine et al., 2015). Hence, the discovery of potent small molecules regulating autophagy is of great interest. Here we review the chemical screening strategies for autophagy drug discovery, and highlight the potential benefits of autophagy modulators in human diseases.

## CHEMICAL SCREENING STRATEGIES FOR IDENTIFYING AUTOPHAGY MODULATORS

A number of *in vitro* screening methods have been designed for identifying compounds (Sarkar, 2013a; Joachim et al., 2015; Seranova et al., 2019). The assays are primarily based on the perturbations of autophagy reporters or autophagy cargoes as readouts (**Figure 1**), which can be measured via fluorescence or high-content imaging, immunoblotting, flow cytometry and microplate reader (Mizushima et al., 2010; Klionsky et al., 2016; **Figure 2** and **Table 1**). Some of these screening methods can be subjected to high-throughput applications. Below are descriptions of the common screening approaches in mammalian cells, and the identification and therapeutic benefits of key autophagy modulators.

## CHEMICAL SCREENING METHODS BASED ON AUTOPHAGY REPORTERS

Screening methods based on autophagy reporters are the most commonly used approaches to detect changes in the numbers of autophagosomes and autolysosomes (**Table 1**). The protein

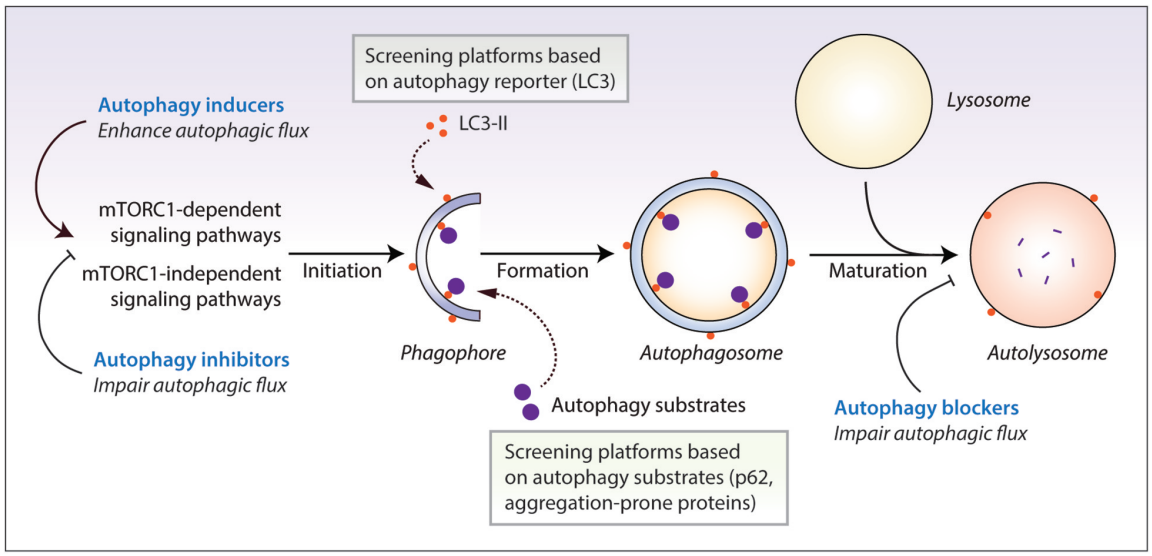


**FIGURE 1 |** Autophagy reporter and substrate based screening strategies and the impact of autophagy modulators at different stages of the autophagy process. Autophagy is regulated by the mechanistic target of rapamycin complex 1 (mTORC1) or mTORC1-independent pathways. This process initiates by the formation of phagophores that expand and engulf autophagy substrates to form autophagosomes, which then fuse with the lysosomes to form autolysosomes where the autophagic cargo is degraded. Autophagy inducers and inhibitors increase or decrease autophagosome formation, respectively, at the early stages of autophagy, whereas autophagy blockers prevent lysosomal degradation and/or autophagosome maturation at late stages of autophagy. Autophagic flux is thus enhanced by autophagy inducers but is retarded by autophagy inhibitors and blockers. Chemical screening methods for identifying autophagy modulators are commonly based on the readouts of perturbations in autophagy reporters such as LC3-II, or autophagy substrate clearance such as aggregation-prone proteins or p62/SQSTM1.

**TABLE 1 |** Chemical screening methods for identifying autophagy modulators in mammalian cells.

| Autophagy screening assays | Detection methods | Strengths | Limitations |
|---|---|---|---|
| GFP-LC3 | Fluorescence or high-content microscopy | (1) Simple readout easy to detect | (1) Can not distinguish between autophagy inducer and blocker |
| | | (2) High-throughput application | (2) Can not assess overall autophagic flux |
| mRFP-EGFP-LC3 | Fluorescence or high-content microscopy | (1) Can distinguish between autophagy inducer, inhibitor and blocker | (1) Assay depends on proper acidification of the lysosomes that can be affected by lysosomotrophic agents |
| | | (2) Measures autophagosome flux | (2) Can not precisely assess overall autophagic flux as it does not measure cargo clearance. |
| | | (3) High-throughput application | |
| GFP-LC3-RFP-LC3ΔG | Fluorescence or high-content microscopy, Flow cytometry, Microplate reader | (1) Measures overall autophagic flux | (1) Can not distinguish between autophagy inhibitor and blocker |
| | | (2) Versatile detection methods | (2) Homologous recombination of two LC3 sequences could result in non-degradable GFP-LC3ΔG |
| | | (3) High-throughput application | |
| Inducible p62-fLuc or GFP-p62 | Microplate reader, Flow cytometry | (1) Measures clearance of autophagic cargo indicating overall autophagic flux | (1) Can not distinguish between autophagy inhibitor and blocker |
| | | (2) Possible high-throughput application | (2) Transcriptional changes in leaky p62 transgene could affect readout |
| Inducible EGFP-HDQ74 or HA-α-syn$^{(A53T)}$ | Immunoblotting | (1) Measures clearance of autophagic cargo indicating overall autophagic flux | (1) Can not distinguish between autophagy inhibitor and blocker |
| | | | (2) High-throughput analysis not possible |

*The detection methods, strengths and limitations of the autophagy reporter and substrate based screening assays are highlighted.*

reporter that is widely used to study autophagy is microtubule-associated protein 1 (MAP1) light chain 3 (LC3). The nascent LC3 is cleaved at its C-terminal arginine residue by Atg4 to form the cytoplasmic LC3-I, which is then post-translationally conjugated with phosphatidylethanolamine at its C-terminal glycine residue by Atg7 to form the autophagosome-associated LC3-II (Kabeya et al., 2000). The lipidated LC3-II remains associated to the autophagosomes throughout their lifespan, and is present on

both the outer and inner membranes. Following the maturation of autophagosomes with lysosomes to form autolysosomes, the LC3-II on the inner surface is degraded whereas the LC3-II on the outer surface is delipidated and removed by Atg4B for recycling (Tanida et al., 2004). A number of fluorescent-tagged reporters of LC3, such as GFP-LC3 (Kabeya et al., 2000), mRFP-GFP-LC3 (Kimura et al., 2007) and GFP-LC3-RFP-LC3ΔG (Kaizuka et al., 2016), have been used to study autophagy and undertake chemical screening.

## Identification of Autophagy Modulators by GFP-LC3 Screening Method

The most common LC3-based reporter that has been used in several studies is GFP-LC3, which labels autophagosomes, autolysosomes as well as phagophores (Kabeya et al., 2000). For the GFP-LC3 screening method, image-based analysis is done by quantifying the $GFP^+$ puncta per cell to measure perturbations in autophagosome number. In general, an autophagy inducer as well as an autophagy blocker will increase GFP-LC3 puncta whereas an autophagy inhibitor will decrease GFP-LC3 puncta (**Figure 2**). A number of high-throughput and small-scale screens have been undertaken with this strategy that has been also utilized to assess the key hits arising from other screening methods; and some of the primary chemical screens utilizing GFP-LC3 readout are highlighted below.

Using GFP-LC3 as the primary screening method in a stable human glioblastoma H4 cell line, an image-based chemical screen with 480 bioactive compounds was performed wherein the number, size and intensity of GFP-LC3 spots were taken into consideration while selecting potent autophagy modulators (Zhang et al., 2007). Compounds were treated at 3–12 μM concentrations for 24 h. This screen identified 8 autophagy inducers, which included a number of FDA-approved drugs such as fluspirilene, trifluoperazine, pimozide (antipsychotic drugs), niguldipine, nicardipine, amiodarone (drugs used for cardiovascular conditions) and loperamide (used in diarrhea). While fluspirilene, trifluoperazine are dopamine antagonists, the other drugs are $Ca^{2+}$ channel antagonists that lower intracellular $Ca^{2+}$; all of which induced

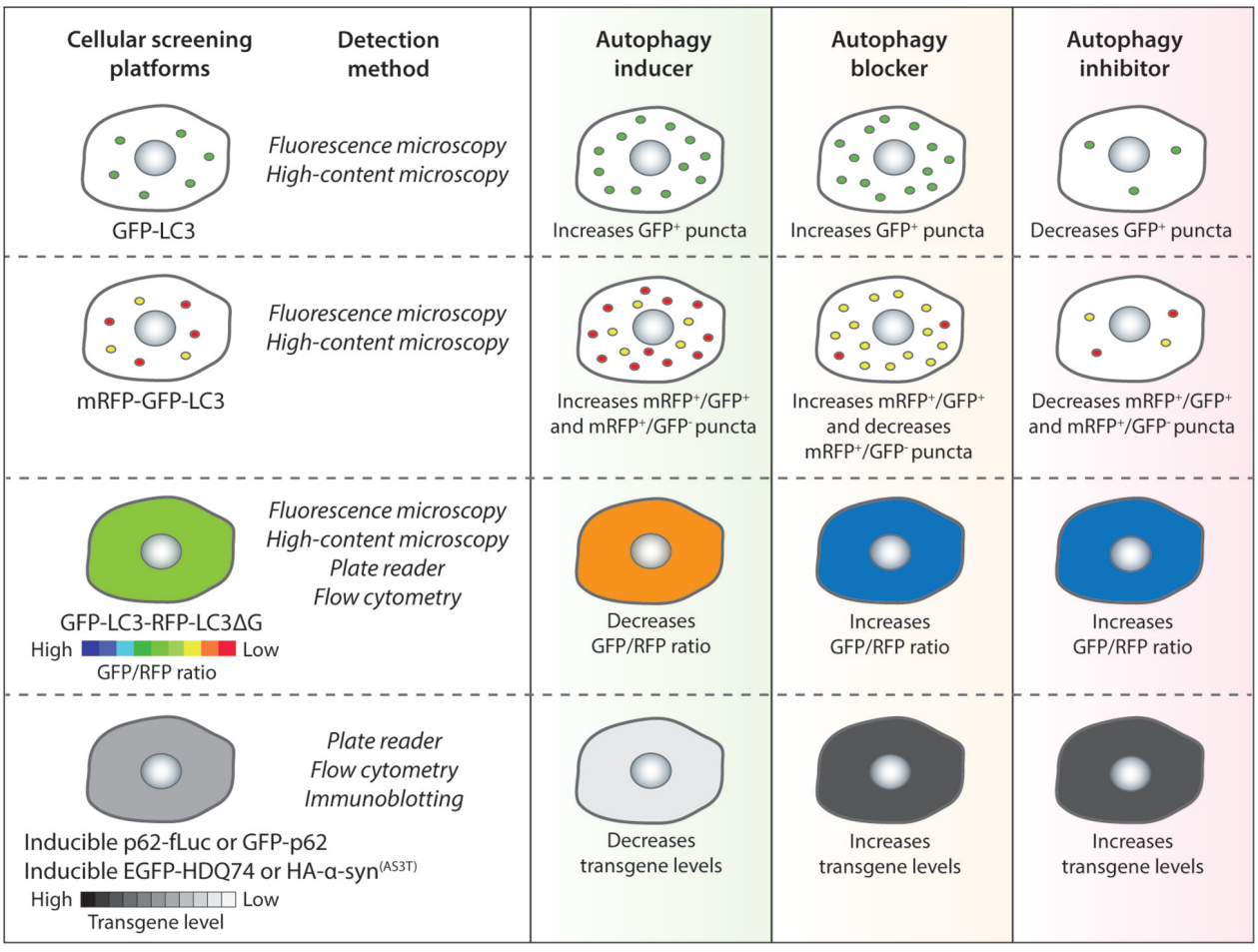


**FIGURE 2 |** Autophagy chemical screening strategies in mammalian cells. Chemical screening methods that are commonly used for identifying autophagy modulators are based on autophagy reporters (LC3) or autophagy substrates (p62 or aggregation-prone proteins). The detection methods for the respective assays and the expected readouts for autophagy inducers, blockers or inhibitors are indicated as a general guidance.

autophagy independently of mTOR (Zhang et al., 2007). Another image-based chemical screen was performed with a library of 3584 pharmacologically active compounds in human breast cancer MCF-7 cells stably expressing GFP-LC3 (Balgi et al., 2009). Treatment of compounds was done at ∼15 μM concentration for 4 h. This screen identified 3 FDA-approved drugs such as perhexilene, niclosamide and amiodarone, as well as rottlerin, as autophagy inducers; all of which were shown to inhibit mTORC1 (Balgi et al., 2009). However, other screens have reported amiodarone ($Ca^{2+}$ channel antagonist) to act independently of mTORC1 for inducing autophagy at a much lower dose than what is required to inhibit mTORC1 (Williams et al., 2008); and likewise, perhexilene is a $Ca^{2+}$ channel blocker that could be also mTOR-independent. Furthermore, one of the largest chemical screens for identifying autophagy modulators was undertaken in HeLa cells stably expressing GFP-LC3 with 59541 stereochemically and skeletally diverse compounds derived from diversity-oriented synthesis (Kuo et al., 2015). Compounds were treated for 4 h in 8-point dose with a maximal concentration of 10 μM. Several hits were subjected to a secondary screen at 10 μM concentration from which BRD5631 was identified as the potent autophagy inducer along with other hits like BRD2716 and BRD34009; all of which did not affect mTOR activity. Interestingly, the hit rate in the primary screen for compounds having an alkyl amine was higher than that for all of the compounds. This effect was augmented by the additional presence of a single lipophilic group, such as diphenyl alkyne, biphenyl, cyclohexane or naphthalene (Kuo et al., 2015). While the above screens were undertaken in immortalized human cell lines, another chemical screen was done with 1280 pharmacologically active compounds in mouse embryonic fibroblasts (MEFs) stably expressing GFP-LC3 (Li et al., 2016). Compounds were treated at 0.02–46 μM concentrations for 16 h in the presence or absence of chloroquine (autophagy blocker) to determine their effects on autophagic flux. Out of the 27 autophagy inducers identified, few were characterized further. These include anti-psychotic drugs such as indatraline hydrochloride (dopamine inhibitor), chlorpromazine hydrochloride and fluphenazine dihydrochloride (dopamine receptor antagonists). Fluphenazine was found to inhibit mTORC1 whereas indatraline and chlorpromazine were mTOR-independent (Li et al., 2016).

Although GFP-LC3 is a straightforward, widely-used screening assay, its inability to distinguish between autophagosomes and autolysosomes is a major inadequacy of this reporter. Accumulation of autophagosomes can occur either due to induction of autophagosome formation (by autophagy inducers) or due to block in autophagosome maturation (by autophagy blockers) in the early and late stages of autophagy, respectively (Rubinsztein et al., 2009). Since autophagy is a dynamic, multi-step process, it is imperative to measure autophagosome flux in order to assess the status of autophagy. Therefore, the hits from the primary GFP-LC3 screen are subjected to rigorous secondary assays (such as autophagosome formation and maturation, and autophagic substrate clearance, amongst others) (Mizushima et al., 2010; Klionsky et al., 2012) for characterizing autophagy modulators.

## Identification of Autophagy Modulators by mRFP-GFP-LC3 Screening Method

In order to overcome the problem of the GFP-LC3 reporter, a tandem fluorescent-tagged mRFP-GFP-LC3 reporter can be employed to determine autophagosome maturation for distinguishing between the autophagosomes and the autolysosomes. This mRFP-GFP-LC3 reporter is pH-sensitive. When overexpressed in cells, the autophagosomes exhibit both mRFP and GFP signals, whereas the autolysosomes emit only mRFP signal because the acid-labile GFP signal is quenched in the acidic environment (Kimura et al., 2007). For the mRFP-GFP-LC3 screening method, image-based analysis is done by quantifying the $mRFP^+$ and $GFP^+$ puncta per cell to measure perturbations in the number of autophagosomes ($mRFP^+/GFP^+$) and autolysosomes ($mRFP^+/GFP^-$). In general, an autophagy inducer (acting at early stage) will increase autophagosomes and autolysosomes, an autophagy inhibitor (acting at early stage) will decrease both these compartments, whereas an autophagy blocker (acting at late stage) will increase autophagosomes and decrease autolysosomes (**Figure 2**). Alternative versions of the mRFP-GFP-LC3 reporter have been described that may provide better readouts. These include replacing mRFP with mCherry that has superior photostability over mRFP (Pankiv et al., 2007), and substituting GFP with mWasabi that is more acid-sensitive than GFP (Zhou et al., 2012).

This pH-sensitive reporter has been primarily utilized as a secondary screening strategy following primary screens utilizing the more simpler GFP-LC3 method. In a high-throughput screen with 59541 compounds in GFP-LC3 platform, 400 screen hits were subjected to additional screening in stable HeLa cells expressing mCherry-GFP-LC3 (Kuo et al., 2015). These compounds were treated at 10 μM concentration for 24 h, after which 250 compounds increased (putative inducers) and 80 compounds decreased (putative inhibitors/blockers) the number of $mCherry^+/GFP^-$ autolysosomes. Following further characterization, potent mTOR-independent autophagy inducers identified were BRD5631, BRD2716, and BRD34009 (Kuo et al., 2015). In another study, HeLa cells stably expressing mRFP-GFP-LC3 was subjected to three drug libraries such as the Prestwick Chemical Library, Microsource Spectrum 2000 library and Johns Hopkins Library that encompass 3791 compounds including FDA-approved drugs and bioactive molecules (Chauhan et al., 2015). Compounds were treated at 10 μM concentration for 4 h. However, high-content image analysis was done based only on GFP-LC3 puncta and total integrated area per cell, but not together with mRFP-LC3 that was utilized later during secondary characterization. 80 compounds were identified, out of which 55 were novel and 25 were previously reported as autophagy modulators. Further characterization of the hits including the mRFP-GFP-LC3 analysis identified flubendazole as a novel autophagy inducer that is also an antihelminthic drug. Flubendazole was shown to impact on dynamic and

acetylated microtubules to inhibit mTOR and disrupt Bcl2-Beclin 1 complex for inducing autophagy (Chauhan et al., 2015). More recently, a primary screen with mRFP-GFP-LC3 has been performed in U343 glioma cell spheroids (3D tumor spheroids) by dynamic live-cell imaging (Pampaloni et al., 2017). A subset of the Enzo Life Sciences Screen-Well Natural Compounds library comprising of 94 compounds were used at 1, 12.5, and 50 μM concentrations, followed by long-term time-lapse fluorescence imaging over 24 h at an interval of 1 h. Instead of measuring puncta formation, this study quantified the readout based on the ratio of mRFP and GFP emission intensities over time. Apart from validating this approach with the Enzo Life Sciences Screen-Well Autophagy library consisting of known autophagy modulators, the screen with selected natural compounds identified six potent autophagy inducers and four inhibitors. The autophagy-inducing natural compounds include PI-103, nonactin, valinomycin, quercetin, ivermectin, and harmine (Pampaloni et al., 2017).

The mRFP-GFP-LC3 reporter or its alternative versions can be subjected to high-throughput image-based screens to analyse autophagosome flux. This assay requires proper acidification of the lysosomes that could be affected by lysosomotrophic agents. However, autophagic substrate clearance along with other secondary assays should be assessed following the primary screen in order to assess the overall autophagic flux.

### Identification of Autophagy Modulators by GFP-LC3-RFP-LC3ΔG Screening Method

A novel autophagy probe, GFP-LC3-RFP-LC3ΔG, has been recently developed for evaluating autophagic flux that can be used for high-throughput screening approaches (Kaizuka et al., 2016). When overexpressed in cells, the Atg4 family proteases can cleave this reporter into equimolar amounts of GFP-LC3 and RFP-LC3ΔG. While GFP-LC3 on the autophagosomes is degraded or recycled after fusion with the lysosomes, RFP-LC3ΔG cannot be lipidated due to a deletion in its C-terminal glycine and thus remains in the cytosol serving as an internal control. This GFP-LC3-RFP-LC3ΔG reporter can be subjected to both qualitative (by ratiometric imaging via fluorescence microscopy) and quantitative (via microplate reader or flow cytometry) analyses by measuring the fluorescence of GFP-LC3 and RFP-LC3ΔG, and then calculating the GFP/RFP ratio (Kaizuka et al., 2016). Autophagy inducers are expected to decrease GFP/RFP ratio by enhancing autophagic flux, whereas autophagy inhibitors or blockers will increase GFP/RFP ratio by reducing autophagic flux (**Figure 2**).

Two chemical screens employing the GFP-LC3-RFP-LC3ΔG screening method have been undertaken using a selected library of 34 known autophagy-regulating compounds and 1054 approved drugs under basal or starvation conditions in HeLa cells stably expressing this reporter (Kaizuka et al., 2016). The GFP/RFP ratio was calculated from fluorescence measurement via a microplate reader. For the first screen with known autophagy-regulating compounds, cells were treated for 6, 12 or 24 h with concentrations previously shown to modulate autophagy. A number of known autophagy modulators, but not all, acted as expected primarily after 12 or 24 h treatment. Specifically, autophagy inducers such as rapamycin (Blommaart et al., 1995) and Torin 1 (Thoreen et al., 2009) decreased GFP/RFP ratio whereas autophagy blockers like bafilomycin A1 (Yamamoto et al., 1998) and chloroquine (Seglen et al., 1979) increased GFP/RFP ratio (Kaizuka et al., 2016). For the second screen with approved drug library, cells were treated for 24 h at 10 μM concentration with few exceptions at 5 μM. The screen hits included 47 autophagy-inducing drugs (comprising of certain anti-cancer drugs, antibiotics and cardiotonic drugs) and 43 autophagy inhibitory drugs. Although many of these hits were previously reported, 13 inducers and 18 inhibitors/blockers were identified as novel autophagy modulators, of which some of the novel autophagy inducers were adefovir pivoxil, methyltestosterone, norethisterone, oxaprozin, and zidovudine (Kaizuka et al., 2016). This GFP-LC3-RFP-LC3ΔG probe has been demonstrated to be capable of measuring basal and induced autophagic flux in Zebrafish and in tissues of transgenic mice (Kaizuka et al., 2016), and is thus valuable for monitoring autophagic flux *in vivo*.

Although this reporter can be used for high-throughput applications and *in vivo* studies to measure the overall autophagic flux, it is not ideal for investigating the distinct stages of autophagy such as autophagosome formation and maturation. Importantly, the two LC3 sequences of GFP-LC3-RFP-LC3ΔG in retrovirally transfected cells can undergo homologous recombination, which will generate GFP-LC3ΔG that is incapable of being degraded by autophagy. In addition, the expression levels of this reporter define the accuracy of the readout, and hence analysis in different cell lines or tissues will require comparable expression (Kaizuka et al., 2016; Geng and Klionsky, 2017).

## CHEMICAL SCREENING METHODS BASED ON AUTOPHAGY SUBSTRATES

In addition to the screening approaches based on LC3 reporters, autophagy substrate clearance has also been utilized as a primary screening assay for identifying autophagy modulators (**Table 1**). This method measures the autophagic cargo flux, which together with LC3-based secondary assays for autophagosome flux can indicate the overall autophagic flux.

### Identification of Autophagy Modulators by Clearance of Aggregation-Prone Proteins

A number of neurodegeneration-associated aggregation-prone proteins are predominantly degraded by autophagy (Menzies et al., 2017), and hence screening methods can be based on their clearance as readouts (Sarkar, 2013a). The well-established substrates undergoing autophagic degradation include mutant huntingtin (with expanded polyglutamine repeats) and mutant α-synuclein (A53T or A30P mutants) associated with Huntington's and Parkinson's disease,

respectively (Webb et al., 2003; Ravikumar et al., 2004). Since the steady-state level of proteins is not ideal for accurately reflecting any impact on their degradation, stable inducible cell lines are required for analyzing autophagic substrate clearance where the transgene product is temporally synthesized by doxycycline followed by treatment with compounds after the expression is turned off (Wyttenbach et al., 2001; Webb et al., 2003; Sarkar et al., 2009). In general, autophagy inducers will enhance the clearance of aggregation-prone proteins, whereas autophagy inhibitors or blockers will retard their clearance (**Figure 2**).

Independent studies using a stable inducible PC12 cell line expressing EGFP-tagged mutant huntingtin (EGFP-HDQ74) identified mTOR-independent autophagy inducers such as trehalose (Sarkar et al., 2007a) as well as inositol-lowering agents (lithium, carbamazepine, valproic acid, L-690330) (Sarkar et al., 2005) and nitric oxide synthase inhibitors (L-NAME) (Sarkar et al., 2011). These studies also identified autophagy inhibitory compounds such as agents increasing inositol or inositol 1,4,5-trisphosphate ($IP_3$) levels (myo-inositol, prolyl endopeptidase inhibitor 2) (Sarkar et al., 2005) and nitric oxide donors (DEA NONOate, DETA NONOate) (Sarkar et al., 2011). Utilizing stable inducible PC12 cell line expressing hemagglutinin (HA)-tagged A53T α-synuclein (HA-α-syn$^{(A53T)}$) as the primary screening method, a chemical screen was undertaken with 72 hits arising from an yeast screen involving 50729 compounds (Sarkar et al., 2007b). Cells were treated with compounds at 2 mg $mL^{-1}$ concentration for 24 h after the initial doxycycline-induced synthesis of the transgene product (A53T α-synuclein), followed by immunoblotting analysis to measure its clearance. A number of novel autophagy modulators were identified which enhanced the autophagy substrate clearance. These include 4 small molecule enhancers of rapamycin (SMERs) and 13 small molecule inhibitors of rapamycin (SMIRs), of which SMER10, SMER18, and SMER28 were characterized to be autophagy inducers acting independently of mTOR. Further screening of the chemical analogs of these SMERs identified 18 additional autophagy inducers, such as 1 SMER10, 7 SMER18 and 10 SMER28 analogs that are capable of enhancing substrate clearance; although not substantially better than the respective parent compounds (Sarkar et al., 2007b). Another screen also utilizing a stable inducible PC12 cell line expressing HA-tagged A30P α-synuclein (HA-α-syn$^{(A30P)}$) was undertaken with a library of 253 compounds including FDA-approved drugs and pharmacological probes (Williams et al., 2008). Drug treatment was done at 1 μM for 24 h after the synthesis of the transgene product, followed by immunoblotting analysis. This study elucidated a cyclic mTOR-independent autophagy pathway with multiple drug targets, in which cAMP regulates $IP_3$ levels that impact on calpain activity, which in turn activates $G_{s\alpha}$ that regulates cAMP levels. Some of the autophagy-inducing compounds identified include L-type $Ca^{2+}$ channel blockers (verapamil, loperamide, amiodarone), calpain inhibitors (calpastatin), ATP-sensitive $K^+$ channel agonist (minoxidil), cAMP reducing agents (rilmenidine, clonidine) and inositol lowering agents (valproic acid), whereas $Ca^{2+}$ channel openers [(±)-Bay K8644] and agents elevating cAMP (dibutyryl cAMP, forskolin) and cytosolic $Ca^{2+}$ (thapsigargin) levels were autophagy inhibitory (Williams et al., 2008). In addition to these immunoblotting based methods, the effects of autophagy modulators on autophagy-dependent clearance of EGFP-tagged mutant huntingtin aggregates can be validated by fluorescence microscopy in wild-type (*Atg5*$^{+/+}$) and autophagy-deficient (*Atg5*$^{-/-}$) mouse embryonic fibroblasts (MEFs) (Kuma et al., 2004; Sarkar et al., 2009).

Although autophagic clearance of aggregation-prone proteins is informative for autophagic flux, only low-throughput approaches are possible that creates a major hurdle for high-throughput applications. Nonetheless, this method could be used as a secondary assay for characterization of selected hits arising from screens with LC3-based reporters.

## Identification of Autophagy Modulators by p62/SQSTM1 Clearance

An alternative approach to the clearance of aggregation-prone proteins is to monitor the autophagic degradation of a known autophagy substrate, p62/SQSTM1, which also functions as an adaptor protein during selective autophagy for recruiting specific autophagic cargo to the autophagosomes (Bjorkoy et al., 2005; Pankiv et al., 2007). Similarly, to the method involving aggregation-prone proteins, screening approaches based on p62 clearance would ideally require a stable inducible cell line where the transgene product is temporally expressed before the treatment with compounds. The p62 reporters, such as GFP-p62 (Larsen et al., 2010) or luciferase-tagged p62 (Brown et al., 2016; Min et al., 2018), could be utilized for medium- to high-throughput screens by flow cytometry or microplate reader (for analyzing p62 levels) or by fluorescence imaging (for analyzing p62 aggregates). Genetic screens have been undertaken with p62-based reporters (Pietrocola et al., 2015; Strohecker et al., 2015; DeJesus et al., 2016; Hale et al., 2016), and therefore, similar chemical screening approaches are also possible. In addition, analyzing the steady-state levels of endogenous p62 by immunoblotting is often used as a secondary assay for characterization of autophagy modulators (Klionsky et al., 2012). It is expected that an autophagy inducer will decrease p62 levels or aggregates, whereas an autophagy inhibitor or blocker will cause its accumulation (**Figure 2**). Recently, an assay based on LC3B-II and p62 time-resolved fluorescence resonance energy transfer (TR-FRET) has been described to monitor autophagy independent of any exogenous labels. This method is based on the proximity of the donor and the acceptor antibodies of LC3-II and p62, in which autophagy inducers increase LC3-II signal and decrease p62 signal, autophagy inhibitors do not display any turnover of either signals, whereas autophagy blockers will increase LC3-II signal without any turnover of p62 signal (Bresciani et al., 2018).

Although p62 is a specific autophagy substrate in most mammalian cell lines (Klionsky et al., 2012), its autophagic degradation should be confirmed in the cell-type and the time-points to be used in the screens. Moreover, transcriptional upregulation of p62 has been reported during some instances of autophagy activation, such as under prolonged starvation or with certain pharmacological inducers (Klionsky et al., 2012;

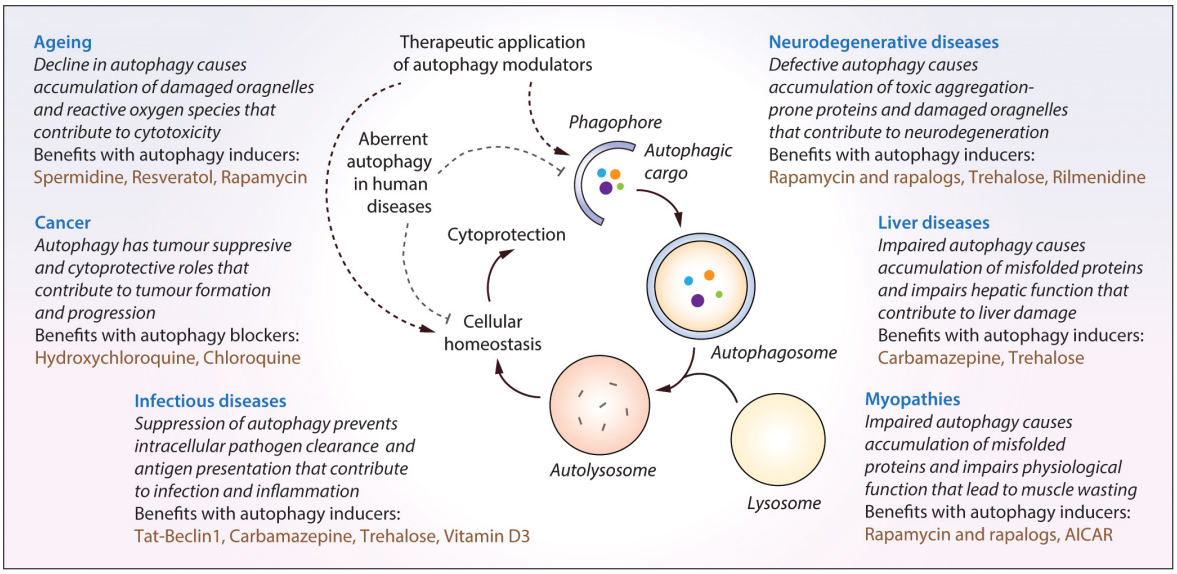


**FIGURE 3 |** The impact of malfunctioning autophagy and the therapeutic benefits of autophagy modulators in diverse human diseases. Autophagy is implicated in diverse human diseases due to its vital role in maintaining cellular homeostasis. Defective or aberrant autophagy contributes to the cytotoxicity underlying many pathological conditions whereas pharmacological upregulation of autophagy is beneficial in various transgenic models. Key autophagy modulators exerting therapeutic benefits in neurodegenerative disorders, cancer, infectious diseases, liver diseases, myopathies and lifespan extension, as well as the impact of malfunctioning autophagy in these contexts, are highlighted.

Sahani et al., 2014; Kuo et al., 2015), and therefore, any perturbation in p62 protein levels needs to be accompanied by qPCR assessment of its mRNA levels.

## BIOMEDICAL APPLICATIONS OF AUTOPHAGY MODULATORS IN HUMAN DISEASES

Autophagy plays an essential role for tissue homeostasis and cellular survival by removing unwanted materials like malfunctioning aggregated proteins and damaged organelles from the cells; however, deregulation of this process could contribute to cytotoxicity (Mizushima et al., 2008). Autophagy dysfunction has been implicated in the pathogenesis of diverse human diseases (Levine and Kroemer, 2008; Jiang and Mizushima, 2014), and therefore, therapeutic exploitation of autophagy is of potential biomedical relevance (**Figure 3**). A number of independent studies and chemical screens have identified several autophagy modulators, which have been shown to impart beneficial effects in various transgenic disease models (**Table 2**; Rubinsztein et al., 2012; Sarkar, 2013b; Levine et al., 2015). Some of the key studies in specific disease contexts are highlighted below.

## AUTOPHAGY MODULATORS IN NEURODEGENERATIVE DISEASES

Basal autophagy in the brain is critical for maintaining cellular homeostasis in post-mitotic cells like neurons, which is evident from the genetic studies in mice where brain-specific deletion of essential autophagy genes resulted in neurodegenerative phenotypes (Hara et al., 2006; Komatsu et al., 2006). Particularly, autophagy is the primary degradation pathway for several aggregation-prone proteins associated with neurodegeneration (Rubinsztein, 2006; Nixon, 2013). However, defective autophagy has been reported in several neurodegenerative diseases, including neurodegenerative lysosomal storage disorders, and is considered a major causative factor for neurodegeneration (Nixon, 2013; Sarkar, 2013b; Menzies et al., 2017; Seranova et al., 2017). Therefore, induction of autophagy for enhancing the clearance of mutant aggregation-prone proteins is considered a potential treatment strategy. The therapeutic benefits of autophagy inducers have been robustly demonstrated in the context of neurodegeneration where upregulation of autophagy was protective in several *in vitro* and *in vivo* transgenic models of neurodegenerative diseases (Rubinsztein et al., 2012; Sarkar, 2013b; Levine et al., 2015; Seranova et al., 2017). Stimulating autophagy with mTOR inhibitors like rapamycin or its analogs had beneficial effects in fly and mouse models of Huntington's disease, Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia (FTD), spinocerebellar ataxia type 3 (SCA3) and prion disease (Ravikumar et al., 2004; Berger et al., 2006; Sarkar et al., 2008; Menzies et al., 2010; Spilman et al., 2010; Cortes et al., 2012; Wang et al., 2012; Ozcelik et al., 2013; Jiang et al., 2014). Likewise, several mTOR-independent autophagy inducers such as, but not limited to, lithium, carbamazepine (inositol lowering agents), rilmenidine (cAMP reducing agent), trehalose (AMPK activator), SMERs and BRD5631 have been shown to be protective in fly, Zebrafish, mouse or induced pluripotent stem cell (iPSC) models of AD,

FTD, HD, amyotrophic lateral sclerosis (ALS) and Niemann-Pick type C1 (NPC1) disease (Sarkar et al., 2005, 2007a,b; Fornai et al., 2008; Williams et al., 2008; Rose et al., 2010; Zhang et al., 2011, 2018; Shimada et al., 2012; Wang et al., 2012; Li et al., 2013; Maetzel et al., 2014; Kuo et al., 2015). The most widely used mTOR-independent autophagy inducer *in vivo* is trehalose (Sarkar et al., 2007a), a disaccharide that stimulates autophagy by inhibiting SLC2A family of glucose transporters and activating AMPK (DeBosch et al., 2016), which in turn can directly influence the phosphorylation of the autophagy-initiating kinase ULK1 (Egan et al., 2011; Kim et al., 2011). Remarkably, trehalose had beneficial effects in mouse models of AD, PD, HD, FTD, SCA17, ALS, as well as cellular and iPSC-derived neuronal models of prion and NPC1 disease, respectively (Tanaka et al., 2004; Aguib et al., 2009; Rodriguez-Navarro et al., 2010; Schaeffer et al., 2012; Castillo et al., 2013; Du et al., 2013; Zhang et al., 2014; Chen et al., 2015; Tanji et al., 2015). Additional autophagy-inducing agents reported to be cytoprotective in neurodegenerative models such as HD, PD, ALS, FTD and Lafora disease include Tat-Beclin 1 peptide, calpastatin, verapamil, metformin, AUTEN-67, AUTEN-99, 6-Bio and fluphenazine (Ma et al., 2007; Williams et al., 2008; Shoji-Kawata et al., 2013; Barmada et al., 2014; Berthier et al., 2016; Billes et al., 2016; Papp et al., 2016; Kovacs et al., 2017; Suresh et al., 2017). A combinatorial approach in enhancing autophagy has been shown with rapamycin and mTOR-independent autophagy inducers such as lithium, trehalose or SMERs. Higher efficacy was achieved via the additive effects of dual treatment on autophagy induction and cytoprotection in cell and fly models of HD than the effects of single compounds (Sarkar et al., 2007a,b, 2008).

## AUTOPHAGY MODULATORS IN CANCER

The ability of autophagy in the maintenance of metabolic homeostasis has drawn considerable attention as a potential target for cancer therapy via its pro-survival and pro-death mechanisms (Rabinowitz and White, 2010; Levy et al., 2017). Autophagy plays tumor suppressive role by mitigating oxidative stress, removing superfluous mitochondria and preventing DNA damage and genome instability; and on the other hand, shows pro-tumor activity by preventing the induction of tumor suppressors, increasing resistance to apoptosis and maintaining tumor metabolism through recycling of nutrients (Mathew et al., 2007; Galluzzi et al., 2015; Kimmelman and White, 2017). Depending on the cancer context and the opposing effects of autophagy, either inhibitors or inducers of autophagy could be exploited for cancer therapy (Galluzzi et al., 2017; Levy et al., 2017). Since autophagy promotes tumorigenesis in most contexts, inhibition of autophagy has gathered considerable interest for cancer therapy. Accumulating evidence demonstrate that autophagy inhibitors/blockers exerted therapeutic benefits in cancer models. The clinically- approved autophagy inhibitors chloroquine or hydroxychloroquine (HCQ), which impair lysosomal acidification and block autophagic flux (Murakami et al., 1998; Boya et al., 2005), caused tumor shrinkage in preclinical studies; and thus HCQ being more potent with lesser side-effects is used in ongoing clinical trials either alone or in combination with other treatments (Briceno et al., 2003; Amaravadi et al., 2007; Cook et al., 2014; Chude and Amaravadi, 2017; Levy et al., 2017; Onorati et al., 2018). Autophagy inhibitory compounds, such as Lys05 and ROC-325, which exhibited anti-tumor activity in mice have been suggested to be more potent than HCQ (McAfee et al., 2012; Carew et al., 2017). In addition, autophagy inhibitors preventing autophagosome formation such as ATG4B antagonists (compounds NSC185058 and UAMC-2526), Vps34 (vacuolar protein sorting protein 34) inhibitor (compound SAR405), ULK1 (Unc-51-like kinase 1) inhibitor (compound SBI-0206965), USP10/USP13 (ubiquitin-specific peptidases) inhibitor (Spautin-1) and agents causing transcriptional inhibition of autophagy genes (pyrvinium pamoate), also exerted anti-proliferative and anti-tumor effects in cellular and *in vivo* models of cancer (Liu et al., 2011; Deng et al., 2013; Akin et al., 2014; Ronan et al., 2014; Shao et al., 2014; Egan et al., 2015; Kurdi et al., 2017). On the contrary, various chemical agents or natural products exerting antiproliferative or anti-tumor activity either alone or in combination with chemotherapeutic agents could induce autophagy or autophagic cell death, which include Torin 1, AC-73, MC-4, metformin, silibinin, Abrus agglutinin, curcumin, liensinine, spermidine, vitamin D3, and imatinib (Buzzai et al., 2007; Ertmer et al., 2007; Wang et al., 2008; Thoreen et al., 2009; Qian et al., 2011; Francipane and Lagasse, 2013; Law et al., 2014; Jiang et al., 2016; Pietrocola et al., 2016; Panda et al., 2017; Son et al., 2018; Spinello et al., 2018).

## AUTOPHAGY MODULATORS IN INFECTIOUS DISEASES

Autophagy plays an important role in innate defense mechanism by removing intracellular pathogens; a process termed xenophagy (Levine et al., 2011; Deretic et al., 2013). The role of autophagy in regulating intracellular infections initially emerged through studies on *Mycobacterium tuberculosis* (*Mtb*) (Gutierrez et al., 2004; Singh et al., 2006). Subsequently, several other bacterial pathogens like *Salmonella* and *Listeria*, and viral pathogens like HIV and Dengue were shown to utilize host autophagy pathways for their own advantage (Jia et al., 2009; Kyei et al., 2009; Yoshikawa et al., 2009; Heaton and Randall, 2010). A genome-wide siRNA screen to identify host factors required for intracellular *Mtb* survival within macrophages revealed that a large number of host factors acted via regulation of autophagy to help the bacteria (Kumar et al., 2010). Induction of autophagy with rapamycin, carbamazepine, SMER28, and vitamin D3 were shown to prevent bacterial survival or HIV replication in macrophages (Gutierrez et al., 2004; Floto et al., 2007; Yuk et al., 2009; Kumar et al., 2010; Campbell and Spector, 2011, 2012; Schiebler et al., 2015). Notably, carbamazepine reduced bacterial burden, improved lung pathology and stimulated adaptive immunity in mice infected with multidrug-resistant *Mtb* (Schiebler et al., 2015). Rapamycin also controlled viral and

**TABLE 2 |** Therapeutic benefits of autophagy modulators in diverse human diseases.

| Diseases | Selected autophagy modulators | Mechanisms of autophagy modulation | Therapeutic benefits in animal and iPSC models |
|---|---|---|---|
| Neurodegenerative diseases | Rapamycin, CCI-779 (Inducers) | Inhibition of mTORC1 (Blommaart et al., 1995; Ravikumar et al., 2004) | HD flies (Ravikumar et al., 2004; Sarkar et al., 2008), FTD flies (Berger et al., 2006), HD mice (Ravikumar et al., 2004), AD mice (Spilman et al., 2010), FTD mice (Wang et al., 2012; Ozcelik et al., 2013; Jiang et al., 2014), SCA3 mice (Menzies et al., 2010), Prion disease mice (Cortes et al., 2012) |
| | Lithium (Inducer) | Reduction of inositol and $IP_3$; mTORC1-independent (Sarkar et al., 2005) | HD flies (Sarkar et al., 2008), AD mice (Zhang et al., 2011), FTD mice (Shimada et al., 2012), ALS mice (Fornai et al., 2008) |
| | Carbamazepine (Inducer) | Reduction of inositol and $IP_3$; mTORC1-independent (Sarkar et al., 2005) | AD mice (Li et al., 2013), FTD mice (Wang et al., 2012), ALS mice (Zhang et al., 2018), NPC1 patient iPSC-derived neurons (Maetzel et al., 2014) |
| | Trehalose (Inducer) | mTORC1-independent (Sarkar et al., 2007a); Inhibition of SLC2A and activation of AMPK (DeBosch et al., 2016) | HD mice (Tanaka et al., 2004), AD mice (Du et al., 2013), PD mice (Tanji et al., 2015), FTD mice (Rodriguez-Navarro et al., 2010; Schaeffer et al., 2012), SCA17 mice (Chen et al., 2015), ALS mice (Castillo et al., 2013; Zhang et al., 2014), NPC1 patient iPSC-derived neurons (Maetzel et al., 2014) |
| | Rilmenidine, Clonidine (Inducers) | Reduction of cAMP; mTORC1 independent (Williams et al., 2008) | HD mice (Rose et al., 2010), HD zebrafish (Williams et al., 2008), HD flies (Williams et al., 2008) |
| | Verapamil (Inducer) | Reduction of $Ca^{2+}$; mTORC1 independent (Williams et al., 2008) | HD zebrafish (Williams et al., 2008), HD flies (Williams et al., 2008), NPC1 patient iPSC-derived neurons (Maetzel et al., 2014) |
| | SMER28 (Inducer) | Mechanism unknown; mTORC1 independent (Sarkar et al., 2007b) | HD flies (Sarkar et al., 2007b) |
| | BRD5631 (Inducer) | Mechanism unknown; mTORC1 independent (Kuo et al., 2015) | NPC1 patient iPSC-derived neurons (Kuo et al., 2015) |
| | Metformin (Inducer) | Activation of AMPK (Buzzai et al., 2007) | HD mice (Ma et al., 2007), LD mice (Berthier et al., 2016) |
| | 6-Bio (Inducer) | Inhibition of mTORC1 signaling (Suresh et al., 2017) | PD mice (Suresh et al., 2017) |
| | AUTEN-67, AUTEN-99 (Inducers) | Inhibition of MTMR14 (Papp et al., 2016; Kovacs et al., 2017) | HD flies (Billes et al., 2016; Papp et al., 2016; Kovacs et al., 2017), PD flies (Kovacs et al., 2017) |
| Cancer | Chloroquine, Hydroxychloroquine (Blockers) | Mechanism unknown; Impairment of lysosomal acidification and autophagosome-lysosome fusion (Murakami et al., 1998; Boya et al., 2005) | *Myc/p53ER*$^{TAM}$ induced lymphoma mice (Amaravadi et al., 2007), mice bearing MCF7-RR and LCC9 ER+ breast cancer xenografts (Cook et al., 2014) |
| | Lys05, ROC-325 (Blockers) | Mechanism unknown; Impairment of lysosomal acidification and autophagosome-lysosome fusion (McAfee et al., 2012; Carew et al., 2017) | Mice bearing c8161 melanoma, 1205Lu melanoma and HT-29 colon cancer xenografts (McAfee et al., 2012), mice bearing 786-0 RCC xenografts (Carew et al., 2017) |
| | NSC185058, UAMC-2526 (Inhibitors) | Inhibition of ATG4B (Akin et al., 2014; Kurdi et al., 2017) | Mice bearing Saos-2 osteosarcoma xenograft (Akin et al., 2014), Mice bearing HT29 colorectal tumor xenograft (Kurdi et al., 2017) |
| | Pyrvinium pamoate (Inhibitor) | Mechanism unknown; Reduction in *Atg* gene expression; mTORC1 independent (Deng et al., 2013) | Mice bearing 4TI mammary carcinoma xenograft (Deng et al., 2013) |
| | Torin 1 (Inducer) | ATP-competitive inhibition of mTORC1 (Thoreen et al., 2009) | Mice bearing Tu12 and Tu22 colon cancer xenografts (Francipane and Lagasse, 2013) |
| Infectious diseases | Tat-Beclin 1 (Inducer) | Interaction with the negative autophagy regulator GAPR-1 (Shoji-Kawata et al., 2013) | Mice infected with chikungunya or West Nile virus (Shoji-Kawata et al., 2013), murine or human macrophages infected with *L. monocytogenes* bacteria and HIV (Shoji-Kawata et al., 2013) |
| | Vitamin D3 (Inducer) | Increase in Beclin 1 (Wang et al., 2008); Increase in *Atg* gene expression (Yuk et al., 2009) | Human macrophages infected with *M. tuberculosis bacteria* or HIV or coinfection (Yuk et al., 2009; Campbell and Spector, 2011, 2012) |

*(Continued)*

**TABLE 2 |** Continued

| Diseases | Selected autophagy modulators | Mechanisms of autophagy modulation | Therapeutic benefits in animal and iPSC models |
|---|---|---|---|
| | Carbamazepine (Inducer) | Reduction of inositol and $IP_3$; mTORC1-independent (Sarkar et al., 2005) | Human macrophages infected with *M. tuberculosis bacteria* or coinfection with HIV (Schiebler et al., 2015), mice infected with multidrug-resistant *M. tuberculosis bacteria* (Schiebler et al., 2015) |
| | Trehalose (Inducer) | mTORC1-independent (Sarkar et al., 2007a); $PI(3,5)P_2$ agonist, activation of TRPML1 $Ca^{2+}$ channel (Sharma et al., 2017) | Human macrophages infected with *M. tuberculosis bacteria* or coinfection with HIV (Sharma et al., 2017), PBMCs from HIV patients (Sharma et al., 2017) |
| | Flubendazole (Inducer) | mTORC1 inactivation; nuclear translocation of TFEB (Chauhan et al., 2015) | Human dendritic cells infected with *HIV,* and HeLa cells infected with *E. coli bacteria* (Chauhan et al., 2015) |
| | Nitazoxanide (Inducer) | Inhibition of mTORC1 signaling (Lam et al., 2012) | Human acute monocytic leukemia cells or PBMCs infected with *M. tuberculosis bacteria* (Lam et al., 2012) |
| | Nortriptyline (Inducer) | Mechanism unknown | Human macrophages infected with *M. tuberculosis bacteria* (Sundaramurthy et al., 2013) |
| Liver Disease | Carbamazepine (Inducer) | Reduction of inositol and $IP_3$; mTORC1-independent (Sarkar et al., 2005) | AATD mice (Hidvegi et al., 2010), NAFLD and AFLD mice (Lin et al., 2013), FSD patients (Puls et al., 2013), AATD patient iPSC-derived hepatic cells (Choi et al., 2013), NPC1 patient iPSC-derived hepatic cells (Maetzel et al., 2014) |
| | Lithium, Valproic acid (Inducers) | Reduction of inositol and $IP_3$; mTORC1-independent (Sarkar et al., 2005) | AATD patient iPSC-derived hepatic cells (Choi et al., 2013) |
| | Trehalose (Inducer) | mTORC1-independent (Sarkar et al., 2007a); Inhibition of SLC2A and activation of AMPK (DeBosch et al., 2016) | NAFLD mice (DeBosch et al., 2016) |
| | Rapamycin (Inducer) | Inhibition of mTORC1 (Blommaart et al., 1995) | NAFLD mice (Lin et al., 2013), NPC1 patient iPSC-derived hepatic cells (Maetzel et al., 2014) |
| Myopathies | Rapamycin, CCI-779 (Inducers) | Inhibition of mTORC1 (Blommaart et al., 1995; Ravikumar et al., 2004) | Collagen type VI muscular dystrophy mice (Grumati et al., 2010), *LMNA* cardiomyopathy mice (Choi et al., 2012; Ramos et al., 2012) |
| | AICAR (Inducer) | Activation of AMPK (Buzzai et al., 2007) | DMD mice (Pauly et al., 2012) |
| | Simvastatin (Inducer) | Inhibition of Rac1-mTOR pathway (Wei et al., 2013) | DMD mice (Whitehead et al., 2015) |
| Lifespan extension | Spermidine (Inducer) | Inhibition of histone acetyltransferase and increase in *Atg* gene expression (Eisenberg et al., 2009) | Flies (Eisenberg et al., 2009), worms (Eisenberg et al., 2009), mice (Eisenberg et al., 2016) |
| | Resveratrol (Inducer) | Activation of SIRT1 (Morselli et al., 2010) | Flies (Wood et al., 2004), worms (Wood et al., 2004; Morselli et al., 2010), mice (Baur et al., 2006) |
| | Rapamycin (Inducer) | Inhibition of mTORC1 (Blommaart et al., 1995) | Flies (Bjedov et al., 2010), mice (Harrison et al., 2009) |

*Autophagy modulators have shown beneficial effects in a number of transgenic disease models, such as but not limited to, neurodegenerative disorders, cancer, infectious diseases, liver diseases and myopathies as well as in lifespan extension. Selected examples of autophagy modulators are highlighted in specific pathological contexts. AATD, α1 antitrypsin deficiency; AD, Alzheimer's disease; AFLD, Alcoholic fatty liver disease; ALS, Amyotrophic lateral sclerosis; AMPK, 5′ adenosine monophosphate-activated protein kinase; Atg, Autophagy-related genes; cAMP, 3′,5′-cyclic adenosine monophosphate; DMD, Duchenne muscular dystrophy; FSD, Fibrinogen storage disease, FTD, Frontotemporal dementia; GAPR-1, Golgi-associated plant pathogenesis-related protein 1; HD, Huntington's disease; HIV, Human immunodeficiency virus; $IP_3$, Inositol 1,4,5-trisphosphate; iPSC, Induced pluripotent stem cells; LD, Lafora disease; LMNA, Lamin A/C gene; MTMR14, Myotubularin related protein 14; mTORC1, Mechanistic target of rapamycin complex 1; NAFLD, Non-alcoholic fatty liver disease; NPC1, Niemann-Pick type C1 disease; PBMC, Peripheral blood mononuclear cells; PD, Parkinson's disease; $PI(3,5)P_2$, Phosphatidylinositol 3,5-bisphosphate; RCC, Renal cell carcinoma; SCA, Spinocerebellar ataxia; SIRT1 Sirtuin 1; SLC2A, Solute carrier 2A; TRPML1, Transient receptor potential cation channel mucolipin subfamily member 1.*

bacterial pathogens both *in vitro* and *in vivo* (Donia et al., 2010). In an integrated chemical and RNAi screening for modulators of intracellular mycobacteria, one of the top three compounds was nortriptyline which significantly suppressed *Mtb* survival within macrophages and induced autophagy (Sundaramurthy et al., 2013). Other compounds limiting bacterial or HIV infections through activation of autophagic flux were nitazoxanide (anti-protozoan drug) and flubendazole (antihelminthic drug) (Lam et al., 2012; Chauhan et al., 2015). Similarly, the naturally occurring disaccharide trehalose, a potent mTOR-independent enhancer of autophagy in diverse cell-types (Sarkar et al., 2007a), can also induce autophagy and xenophagy in *Mtb*-infected

macrophages that resulted in the killing of bacteria (Sharma et al., 2017). In this study, trehalose was found to act as a PI(3,5)$P_2$ (phosphatidylinositol *3,5*-bisphosphate) agonist for activating the lysosomal $Ca^{2+}$ channel TRPML1 (Sharma et al., 2017), which in turn released lysosomal $Ca^{2+}$ that caused nuclear translocation of TFEB to induce autophagy (Medina et al., 2015). Excitingly, trehalose also seemed to be effective during HIV-*Mtb* co-infection and limits *Mtb* survival by reversing the HIV-mediated block in autophagy flux (Sharma et al., 2017). Similarly, vitamin D3 could also kill *Mtb* during HIV co-infection by inducing autophagy (Campbell and Spector, 2012). Several host factors currently being tested for anti-*Mtb* therapeutics function by regulating host autophagy and xenophagy. For example, inhibition of host Src kinase by the compound AZD0530 induced autophagy and lysosomal maturation to clear *Mtb* (Chandra et al., 2016). A pioneering anti-infective, autophagy-inducing agent is Tat-Beclin 1, which is a peptide representing a region of the autophagy regulator Beclin 1 that interacts with the HIV-1 accessory protein NEF, and this domain is linked with the HIV-1 Tat transduction domain to make it cell permeable (Shoji-Kawata et al., 2013). Tat-Beclin 1 prevented the replication of a number of viral and bacterial pathogens *in vitro* in autophagy-dependent manner, as well as induced autophagy and anti-viral activity in mice infected with chikungunya or West Nile virus (Shoji-Kawata et al., 2013). Thus, it is evident that regulators of autophagy and xenophagy have tremendous potential for novel therapeutics against various infectious diseases. It is now clear that within an infected host cell, there is a possibility of uncoupling between homeostatic autophagy and anti-bacterial xenophagy (Chandra et al., 2015; Sharma et al., 2018). Therefore, it is desirable to perform chemical screening pertaining to infection-specific xenophagy flux for identifying novel regulators of bacterial/viral survival within the host cells through the autophagy pathway.

## AUTOPHAGY MODULATORS IN LIVER DISEASES

Liver autophagy is essential for various hepatic functions and is implicated in various liver conditions including α1-antitrypsin (AAT) deficiency, non-alcoholic fatty liver disease (NAFLD), hepatocellular carcinoma and viral hepatitis (Rautou et al., 2010; Ueno and Komatsu, 2017). Chemical modulation of autophagy has been shown to have beneficial effects in some of these diseases. Carbamazepine, an mTOR independent autophagy inducer acting by reducing inositol levels (Sarkar et al., 2005), reduced hepatic load of mutant α1-antitrypsin Z and hepatic fibrosis in a mouse model of AAT deficiency (Hidvegi et al., 2010), as well as decreased hepatocellular aggregate-related toxicity in patients suffering from fibrinogen storage disease (Puls et al., 2013). A high-throughput drug screen in hepatocyte-like cells derived from iPSC lines of patients with AAT deficiency also revealed inositol-lowering autophagy-inducing agents, such as carbamazepine, lithium, and valproic acid, in facilitating the clearance mutant AAT (Choi et al., 2013). Carbamazepine as well as the mTOR inhibitor rapamycin also rescued dysfunctional autophagic flux and improved cell viability in hepatic-like cells differentiated from patient-derived iPSC lines of Niemann-Pick type C1 (NPC1) disease (Maetzel et al., 2014). In addition, autophagy induction with trehalose, carbamazepine, rapamycin or hydrogen sulfide reduced steatosis, lipid accumulation and liver injury in high-fat diet-induced NAFLD in mice (Lin et al., 2013; Sun et al., 2015; DeBosch et al., 2016). Furthermore, the anti-diabetic drug metformin, which indirectly inhibits mTOR, induced SIRT1-mediated autophagy in primary hepatocytes and ameliorated hepatic steatosis *in vivo* (Song et al., 2015). Overall, these studies indicate that activation of autophagy via inhibition of mTOR, lowering inositol levels or with trehalose are effective modes of inducing autophagy in the liver.

## AUTOPHAGY MODULATORS IN MYOPATHIES

Basal autophagy is required for maintaining muscle mass and myofiber integrity (Masiero et al., 2009), and thus deregulation of muscle autophagy is implicated in myopathies and muscular dystrophies (Sandri et al., 2013). Sustained activation of mTORC1 in skeletal muscle of TSC1-deficient mice could cause late-onset myopathy related to suppression of autophagy (Castets et al., 2013). Upregulation of autophagy, primarily by inhibiting the mTORC1 pathway, has been reported to have beneficial effects in certain transgenic disease models. Autophagy induction by rapamycin or low-protein diet increased myofiber survival and attenuated dystrophic phenotype in a mouse model of collagen type VI muscular dystrophy (Grumati et al., 2010). Likewise, activation of autophagy by dietary changes or with the AMP-activated protein kinase (AMPK) agonist, AICAR (5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside), improved dystrophic phenotypes in mouse models of Duchenne muscular dystrophy (DMD) (De Palma et al., 2012; Pauly et al., 2012). A potential role of simvastatin, which has been reported to induce autophagy by inhibiting the Rac1-mTOR pathway (Wei et al., 2013), has been suggested in improving the physiological function of skeletal muscle in DMD transgenic mice (Whitehead et al., 2015). In addition, rapamycin or its analog, temsirolimus, ameliorated cardiomyopathy and improved skeletal and cardiac muscle function in mouse models of *LMNA* (lamin A/C gene) cardiomyopathy that recapitulate Emery-Dreifuss muscular dystrophy (EDMD) (Choi et al., 2012; Ramos et al., 2012).

## AUTOPHAGY MODULATORS IN LIFESPAN EXTENSION

The functionality of autophagy declines with aging (Rubinsztein et al., 2011), and thus restoring adequate autophagy is considered a possible anti-aging strategy for lifespan extension. There are a number of lifespan expanding strategies, and in many of such approaches, autophagy acts as a common denominator

for promoting longevity (Madeo et al., 2010; Hansen et al., 2018). Pharmacological treatment with autophagy inducers has been linked to increasing longevity in transgenic *in vivo* models (Madeo et al., 2015). Lifespan extension via induction of autophagy with naturally- occurring polyamines such as spermidine, which is an acetyltransferase inhibitor, was shown in yeast, flies, worms and mice (Eisenberg et al., 2009, 2016); and likewise also reported with the natural phenol resveratrol, which is a deacetylase activator, in yeast, flies, worms as well as in mice on high-fat diet (Howitz et al., 2003; Wood et al., 2004; Baur et al., 2006; Morselli et al., 2010). Although both spermidine and resveratrol impacts on the acetylproteome, stimulation of autophagy by resveratrol requires the nicotinamide adenine dinucleotide-dependent deacetylase sirtuin 1 (SIRT1) whereas the effect of spermidine was SIRT1 independent (Morselli et al., 2010, 2011). Inhibition of mTOR by rapamycin also extended lifespan in yeast, flies and mice (Alvers et al., 2009; Harrison et al., 2009; Bjedov et al., 2010; Lamming et al., 2013). In addition, lifespan extension in multiple organisms including mice and apes could be achieved by caloric restriction, which is a physiological inducer of autophagy via AMPK activation, mTORC1 inhibition and SIRT1 activation (Mair and Dillin, 2008; Colman et al., 2009; Mercken et al., 2014; Mattison et al., 2017). In some of these studies reporting lifespan extension by autophagy activation, the role of autophagy has been specifically determined by abolishing the anti-aging effects via knockdown of essential autophagy genes (Madeo et al., 2015; Nakamura and Yoshimori, 2018).

## CONCLUSION

The methodologies for measuring autophagy have evolved over the past decade and it is now feasible to undertake high-throughput chemical screens for identifying modulators of autophagic flux. A number of pharmacological modulators of autophagy have been identified via screening approaches or individual studies; some of which have been demonstrated to exert therapeutic benefits in diverse human diseases. Most of the key autophagy modulators have been identified either by the GFP-LC3 screening method in HeLa cells or via assessing the clearance of aggregation-prone proteins in inducible PC12 cell lines. While analysis of changes in autophagosome number with GFP-LC3 reporter requires shorter treatment period (such as 8–24 h), analysis of clearance of aggregation-prone proteins requires longer treatment duration (such as 24–72 h) depending on the nature of the transgene product. Following the primary screen, it is pertinent to characterize the high-confidence screen hits with secondary autophagy assays because there are no single assays to determine autophagic flux. These normally include analysis of autophagosome formation with bafilomycin $A_1$ via immunoblotting with anti-LC3 antibody, analysis of autophagosome maturation with mRFP-GFP-LC3 reporter, and analysis of autophagy substrate (p62) clearance via immunoblotting with anti-p62 antibody (Mizushima et al., 2010; Klionsky et al., 2016).

Although the methods described in this review are those that have been generally used in the field, alternative autophagy assays could also be employed for chemical screening. One potential approach is the use of Keima, a fluorescent acid-stable protein that exhibits bimodal excitation spectra in neutral and acidic pH, such as in autophagosomes and autolysosomes, respectively (Katayama et al., 2011). The cumulative fluorescence readout can be used to measure bulk autophagic flux. This protein can also be utilized for selective autophagic flux, such as with mitochondria-targeted Keima to measure mitophagy (Katayama et al., 2011; Sun et al., 2017). However, Keima-based assays solely depend upon the lysosomal acidity and thus cannot be performed in fixed cells where the pH gradient across lysosomal membranes is lost. In addition, other screening approaches could be based on fluorescent-tagged early markers of autophagy initiation, such as with WIPI-1 (Proikas-Cezanne and Pfisterer, 2009) and DFCP1 (Axe et al., 2008); however, these methods will not capture the late events of autophagy pathway involving autophagosome maturation and cargo degradation.

For the therapeutic exploitation of autophagy modulators, mTOR-independent autophagy inducers are generally favorable and considered to have lesser side-effects than the mTOR inhibitors like rapamycin. This is because mTOR controls vital cellular functions like cell growth and translation and thus its inhibition can lead to undesirable side-effects unrelated to autophagy induction. For clinical translation to patients, it is important to determine the efficacy and penetrance of the autophagy modulators in the target organs. Future directions could include identifying specific inducers of autophagy acting at the level of autophagic machinery rather than the upstream signaling pathways.

## AUTHOR CONTRIBUTIONS

PP, AF, SV, DK, and SS wrote the manuscript. ES and SS made the figures. PP and SS made the tables. PP, AF, SV, ES, VS, MC, PD, JT, TR, DK, and SS reviewed the manuscript.

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REVIEW ARTICLE

# Dual role of autophagy in hallmarks of cancer

Shikha Satendra Singh[1,2] · Somya Vats[3] · Amelia Yi-Qian Chia[2] · Tuan Zea Tan[1] · Shuo Deng[4] · Mei Shan Ong[4] · Frank Arfuso[5] · Celestial T. Yap[4] · Boon Cher Goh[1,2,6,7] · Gautam Sethi[2,8] · Ruby Yun-Ju Huang[1,9,10] · Han Ming Shen[4,11] · Ravi Manjithaya[3] · Alan Prem Kumar[1,2,7,12,13]

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## Abstract

Evolutionarily conserved across eukaryotic cells, macroautophagy (herein autophagy) is an intracellular catabolic degradative process targeting damaged and superfluous cellular proteins, organelles, and other cytoplasmic components. Mechanistically, it involves formation of double-membrane vesicles called autophagosomes that capture cytosolic cargo and deliver it to lysosomes, wherein the breakdown products are eventually recycled back to the cytoplasm. Dysregulation of autophagy often results in various disease manifestations, including neurodegeneration, microbial infections, and cancer. In the case of cancer, extensive attention has been devoted to understanding the paradoxical roles of autophagy in tumor suppression and tumor promotion. In this review, while we summarize how this self-eating process is implicated at various stages of tumorigenesis, most importantly, we address the link between autophagy and hallmarks of cancer. This would eventually provide a better understanding of tumor dependence on autophagy. We also discuss how therapeutics targeting autophagy can counter various transformations involved in tumorigenesis. Finally, this review will provide a novel insight into the mutational landscapes of autophagy-related genes in several human cancers, using genetic information collected from an array of cancers.

## Introduction

In all eukaryotic cells, autophagy occurs at a basal rate during normal growth conditions and is involved in degradation and removal of damaged, dead or superfluous organelles, and misfolded proteins [1]. In this evolutionarily

Shikha Satendra Singh and Somya Vats contributed equally to this work.

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✉ Ravi Manjithaya
ravim@jncasr.ac.in

✉ Alan Prem Kumar
csiapk@nus.edu.sg
phcapk@nus.edu.sg

1 Cancer Science Institute of Singapore, National University of Singapore, Singapore, Singapore

2 Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

3 Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, Karnataka, India

4 Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

5 Stem Cell and Cancer Biology Laboratory, School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, WA, Australia

6 Department of Haematology-Oncology, National University Hospital, National University Health System, Singapore, Singapore

7 National University Cancer Institute, National University Health System, Singapore, Singapore

8 School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, WA, Australia

9 Department of Obstetrics and Gynaecology, National University Health System, Singapore, Singapore

10 Department of Anatomy, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

11 NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore, Singapore

12 Curtin Medical School, Faculty of Health Sciences, Curtin University, Perth, WA, Australia

13 Medical Science Cluster, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

conserved intracellular degradation pathway, cytoplasmic components are captured in double-membrane vesicles called "autophagosomes" and delivered to lysosomes for degradation. The degradative products such as amino acids and sugars are recycled back into the cytoplasm. All the steps of autophagy starting from the formation of the autophagosomes, its maturation and fusion with the lysosomes, degradation of cargo, and the subsequent release of macromolecules into the cytosol are collectively defined as the autophagic flux [2]. Thus, autophagic flux determines the dynamic turnover of cellular components via autophagy. There are two kinds of autophagy: general (non-selective) and selective autophagy. General autophagy engulfs a portion of the cytoplasm as cargo, packs it into autophagosomes, and delivers it to lysosomes for degradation. On the other hand, selective autophagy, as the name suggests, recognizes a specific cargo, such as damaged organelles, protein aggregates, intracellular pathogens, etc., but utilizes the same core machinery as used for general autophagy [3].

Defects in autophagy have been associated with susceptibility to genomic damage, metabolic stress, and, importantly, tumorigenesis [4]. Autophagy has been reported to either inhibit or promote tumorigenesis, suggestive of a context-dependent role of autophagy in cancer. In this review, in the first section we summarize the normal physiological roles of autophagy, mechanism of autophagy, as well as the paradoxical role of autophagy in cancer. In the second section of this review, we elaborate on how autophagy plays a role in several hallmarks of cancer, and emergence of autophagy as a therapeutic target for both cancer prevention and therapy.

## Physiological roles of cellular autophagy

Autophagy acts as an intracellular recycling system, and some of the physiological roles associated with autophagy include the following.

### Maintenance of cellular homeostasis

In the absence of stress, basal autophagy serves a housekeeping function, maintaining cellular homeostasis and quality control of cellular components by facilitating the clearance or turnover of long-lived or misfolded proteins, protein aggregates, and damaged organelles [5]. Autophagy as a quality control process is immensely important. For example, in neurons accumulation of protein aggregates and damaged organelles occurs due to dysregulation of autophagic flux, giving rise to several neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amylotrophic lateral sclerosis. This highlights the hazards of improper cellular waste turnover [6, 7].

### Orchestrating stress responses

In times of cellular stress, such as nutrient starvation, oxidative stress, hypoxia, or infection, autophagy plays a cytoprotective or an adaptive role.

### Nutrient stress

During nutrient starvation, autophagy breaks down macromolecules such as DNA/RNA, carbohydrates, proteins, and triglycerides. Hence, nucleosides, amino acids, sugars, and free fatty acids are then available for de novo synthesis of biomolecules or for generation of ATP to power cellular functions via the tricarboxylic acid (TCA) cycle and other metabolic processes [8].

### Hypoxia

Autophagy is induced to mitigate the stress caused by low levels of oxygen [9]. Hypoxia inducible factor-1α (HIF-1α), which is the primary transcription factor activated by oxygen deficiency, mediates this response by increasing the expression of BCL-2 interacting protein 3 (BNIP3), which induces autophagy by disrupting the Bcl-2/Beclin1 interaction [10].

### Infection

In addition to the role of autophagy in cellular homeostasis, it also has a role in containing intracellular microbial infections. Autophagy captures invading pathogens such as *Mycobacterium tuberculosis, Salmonella typhimurium*, and Group A *Streptococcus*, and delivers them to the lysosomes for degradation. This sequestration of intracellular pathogens is an example of selective autophagy called xenophagy [11–13].

### Oxidative stress and mitochondrial damage

Excessive free radical production in the body can damage biomolecules and organelles. Oxidative stress can activate the PERK-eIF2α-ATF4-CHOP pathway and induce autophagy [14]. Increased reactive oxygen species (ROS) production can also activate mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase1 (JNK1) [15], which induce autophagy by phosphorylating Bcl-2. Phosphorylated Bcl-2 cannot form a complex with Beclin-1. This allows Beclin-1 to participate in the VPS34 complex formation. Activity of the VPS34 complex is essential for assembly of pre-autophagosomal structure and autophagy

induction [16, 17]. Also, in order to limit ROS damage, autophagy clears damaged mitochondria by their selective sequestration, i.e. mitophagy [18, 19].

## Mechanism of autophagy

As mentioned above, both general and selective autophagy utilize the same core machinery. This section explains the mechanism of this core machinery and highlights the role of adaptor proteins, which provide cargo specificity in selective autophagy.

Mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that can sense signals from different stimuli such as amino acids, energy levels, oxygen, growth factors and stress [20]. It detects presence or absence of nutrients in the cell and hence regulates cell growth and division. In mammals, two complexes of mTOR, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), have distinct localization and functions. In the presence of amino acids and growth factors, mTORC1 inhibits autophagy by preventing activation of Unc-51-like autophagy-activating kinase-1 (ULK1) and Unc-51-like autophagy-activating kinase-2 (ULK2), by phosphorylating both ULK1 and ULK2 [21]. mTORC1 is targeted to the lysosome, by the Ragulator-Rag complex, which is essential for mTORC1 activation. These membrane-bound compartments (lysosomes) contain mTOR activators like Rheb. Activation of Rheb and in turn mTOR is kept in check by the TSC1/2 complex, which is a GTPase-activating protein (GAP) for Rheb. This function of TSC1/2 complex is regulated by protein kinase B (Akt), which phosphorylates and inactivates it to maintain an active mTORC1 [22, 23].

On the other hand, autophagy initiation, in the case of nutrient and energy stress, is mediated by the key energy sensor 5′ AMP activated protein kinase (AMPK). AMPK directly activates ULK1 by phosphorylation at Ser555 and Ser777 among other sites [21]. It also activates the TSC1/2 complex, which negatively regulates mTOR [21, 24]. Thus, with evidence suggesting autophagy induction upon nutrient deprivation [25, 26] and mTOR inhibition [27, 28], it is clear that there is an inverse relationship between autophagy induction and mTOR activation.

### Induction of autophagy-isolation membrane nucleation

One of the earliest events during the process of autophagy induction is the assembly of the ULK1 complex, comprising ULK1, Atg13, Atg101, and FIP200 [29]. At the same time, an isolation membrane known as the phagophore is formed by membrane contributions from various organelles, including the endoplasmic reticulum, golgi apparatus, and mitochondria [30]. The ULK1 complex translocates to the phagophore, promoting the assembly of the class III phosphoinositide 3-kinase (PI3K) complex, which consists of Vps34, p150, Beclin-1, Atg14L, and Autophagy and Beclin1 Regulator 1 (AMBRA1) [31]. TheVps34/PI3K produces an autophagosome-specific pool of phosphatidylinositol3-phosphates (PI3Ps) that recruit downstream effectors, thereby driving the nucleation of the isolation membrane [32].

### Isolation membrane elongation and autophagosome completion

Following nucleation, the elongation of the isolation membrane involves two steps of Atg12–Atg5 conjugation and LC3 processing [29]. The Atg12–Atg5 conjugation is mediated by E1 and E2 ligases, Atg7 and Atg10 [33]. Next, the Atg12–Atg5 conjugate binds to Atg16L and this complex helps in phagophore elongation by recruitment of LC3-II to the membrane [34]. The WD-repeat PtdIns(3)P effector protein I2 (WIPI2) family of protein binds Atg16L, enabling the localization of the Atg12–Atg5–Atg16L complex to the autophagosomal membrane [35]. LC3 present in the cytoplasm is cleaved by cysteine protease Atg4 to generate a C-terminal-exposed glycine residue [36]. Phosphatidylethanolamine (PE) is attached to the exposed glycine with the help of E1 and E2 ligases, Atg7 and Atg3, to generate lipidated LC3 on the autophagosomal membranes. Mammalian Atg8s like LC3 are present on the autophagosome during and after its formation. LC3 has been well studied as an autophagosomal marker [5, 37, 38]. Isolation membrane elongation, sequestration of cargo, and closure of the membrane result in the completion of double-membrane autophagosome.

### Lysosomal fusion and degradation of cargo

Upon autophagosome completion, autophagosomes either directly fuse with lysosomes to form single-membrane autolysosomes or initially fuse with late endosomes to form amphisomes and later fuse with lysosomes [39]. Similar to any other membrane fusion event, the autophagosome–lysosome fusion is orchestrated by Rabs, soluble *N*-ethylmaleimide-sensitive factor attachment proteins (SNAREs), and tethers. Small G-protein Rab7, autophagosomal SNARE syntaxin17 (Stx17), and the membrane tethering complex HOPS are essential for autophagosome–lysosome fusion [40–43]. Lysosomal function and acidification are also necessary for autophagosome–lysosome fusion. Late-stage autophagy inhibitors BafilomycinA1 and Chloroquine (CQ) inhibit fusion by affecting acidification [44, 45]. Bafilomycin A1 inhibits autophagic flux by independently inhibiting

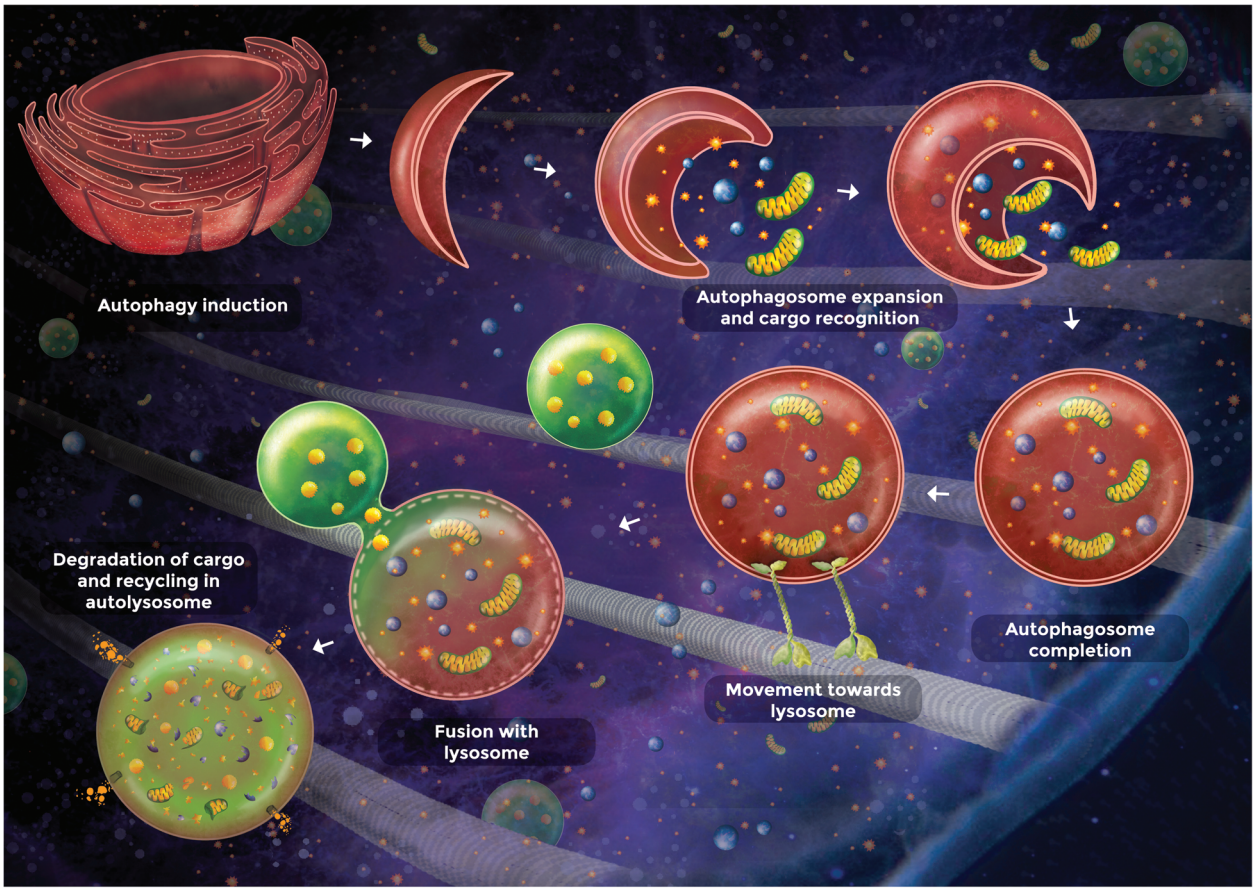


**Fig. 1** The process of autophagy. The process of autophagy begins with the initiation of isolation membrane at the phagophore assembly site (PAS). The isolation membrane expands, captures cargo like misfolded proteins, and dead or damaged organelles, and then both the ends of the isolation membrane join to form an autophagosome. The autophagosomes then move on with the microtubules towards lysosomes for fusion, i.e. autolysosome formation. In the autolysosomes, autophagosomal cargo is degraded by lysosomal enzymes and biomolecules are recycled back to the cytoplasm

V-ATPase-dependent acidification, while Chloroquine is a lysosomotropic agent that prevents endosomal acidification [46, 47]. Lysosome-associated membrane proteins 1 and 2 (LAMP1 and LAMP2), which protect lysosomal membrane from self-digestion, are also important for maintaining the structural integrity of lysosomes [48]. Lastly, the completion of the autophagic process requires degradation of cargo inside lysosomes by enzymes such as cathepsins, and release of biomolecules in the cytosol for reuse (Fig. 1) [6, 40].

The additional cargo specificity in selective autophagy occurs due to the role of adaptor proteins like p62, neighbor of BRCA1 (NBR1), nuclear dot protein 52 kDa (NDP52), Optineurin (OPTN), Nix, and BNIP3. They provide the required selectivity by binding to cargo on one end and LC3 via their LC3-interacting region (LIR) on the other end. Cargos destined for selective autophagy are recognized by adaptor proteins in an ubiquitin-dependent (p62, NBR1 and NDP52) or independent manner. Example of ubiquitin-independent cargo binding includes mitophagy mediated by adaptor proteins like Nix, BNIP3 and FUN14 domain containing 1 (FUNDC1), which reside on the outer mitochondrial membrane and can directly link damaged mitochondria to the autophagosome via LIR [49, 50].

## Transcriptional and epigenetic regulation of autophagy

The transcriptional and epigenetic regulation of autophagy has piqued interest in the past few years. The most well-reported transcriptional activator of autophagy is the transcription factor EB (TFEB), which is also termed as the master regulator of lysosomal genes. TFEB translocates to the nucleus upon starvation, where it enhances the expression of several autophagy and lysosomal genes [51]. Interestingly, ZKSCAN3, a zinc finger family DNA-binding protein, is the functional antithesis of TFEB. It represses the expression of a large number of autophagy and lysosomal genes, including LC3 and WIPI2 [52].

In addition, there are reports suggesting roles for Forkhead box containing protein O1 (FOXO1), HIF1 and Activating transcription factor 4 (ATF4) as transcriptional activators, whereas β-catenin and Signal transducer and activator of transcription 1 (STAT1) act as transcriptional repressors of autophagy genes [53].

Epigenetic mechanisms regulate gene expression often by post-translational modification (phosphorylation, methylation, acetylation, etc.) of histones in order to activate or repress transcription. In this context, several histone modifications that can either increase or decrease autophagic flux have been reported. One of the histone markers that regulate expression of autophagy genes is H3R17 dimethylation. During glucose starvation, AMPK gets activated, which leads to downregulation of SKP2-SCF E3 ubiquitin ligase complex. This event allows stabilization of coactivator-associated arginine methyltransferase 1 (CARM1), which would be otherwise targeted for proteasome-mediated degradation by the SKP2-SCF complex. This stabilized CARM1 can now bring about global H3R17 dimethylation, which is essential for proper autophagy induction [54]. Ellagic acid, a polyphenol that blocks CARM1-mediated H3R17 dimethylation, can also prevent autophagy induction [55].

Deacetylation of histone H4K16 by the deacetylase Sirtuin1 (SIRT1) is associated with autophagy induction [56]. SIRT1 also deacetylates nuclear LC3, thereby allowing it to move to the cytoplasm, where it can participate in autophagy [57].

Negative epigenetic regulation of autophagy during nutrient-rich conditions is brought about by two methyl transferases, G9a and enhancer of zeste homolog 2 (EZH2). While G9a dimethylates H3K9 and directly suppresses autophagy genes [58], EZH2 trimethylates H3K27 to inhibit expression of negative regulators of mTOR such as TSC2. Active mTOR therefore keeps autophagic flux at basal levels [59]. Subsequent roles of epigenetic control of autophagy in disease states, particularly in cancer, will be discussed later.

## Bipolar nature of autophagy in cancer

The complex and paradoxical role of autophagy in modulating cancer progression has been widely studied. The determination of tumor cell fate by autophagy depends on the cancer type, stage, and genetic context [60]. As a physiological quality control process, autophagy exerts a cytoprotective effect by removing misfolded proteins, damaged organelles and ROS, hence limiting the genomic damage that leads to aberrant mutations and ultimately cancer. However, as cancer progresses, the stress-mitigating properties of autophagy are hijacked by tumor cells to meet the heightened metabolic requirements necessary for tumor survival and rapid proliferation [61, 62].

### Tumor-suppressive role of autophagy

Autophagy has been widely established as a tumor-suppressive mechanism, as defective autophagy has been associated with genomic instability, tumorigenesis, and malignant transformation [61, 63]. For instance, mice having monoallelic deletion of the autophagy-related gene *beclin1* develop spontaneous tumors. Allelic loss of *beclin1* was also observed in 40 to 75% of breast, ovarian, and prostate cancers [3, 64, 65]. Therefore, evidence suggests the role of Beclin-1 as a tumor suppressor. It is known that Beclin-1 induces autophagy by binding and activating Vps34 through an evolutionarily conserved domain. This domain with which Beclin-1 binds to Vps34 is also required for its tumor-suppressive activity [66]. Another mechanism by which Beclin-1, an autophagy-promoting protein, regulates tumor-suppressive functions is through its regulators like UVRAG and Bax-interacting factor-1 (Bif-1). Both UVRAG and Bif-1 act as positive regulators of Beclin-1, enhancing binding between Beclin-1 and Vps34, resulting in increased autophagy [67]. Monoallelic deletion or mutations of UVRAG and decreased expression of Bif-1 have been observed in several cancers, including colon, gastric, breast, prostate and bladder cancers [63].

Also, mice with deficiency of *Atg5* and *Atg7* develop liver tumors due to mitochondrial damage and oxidative stress [68]. Another candidate autophagy protein acting as a tumor suppressor is ATG4C, which mediates autophagosome formation through processing of LC3/ATG8. Mice lacking *Atg4* have higher cases of chemically induced fibrosarcoma [69]. However, the effect of ATG4 as a tumor suppressor is relatively weaker than that of ATG5 and ATG7, most probably due to the presence of other ATG4 isoforms that might compensate for the loss of ATG4C. Therefore, further studies are needed to understand the role of ATG4 isoforms in tumor suppression.

Dysregulation of several members of the PI3K/Akt pathway also plays a significant role in the impairment of autophagy and tumorigenesis [70, 71]. Phosphatase and tensin homolog (PTEN) has been recently shown to promote autophagy in HT-29 colon cancer cells [72]. This phosphoinositide's phosphatase activity inhibits the Akt survival pathway and thus induces autophagy. Thus, loss-of-function mutations of *PTEN* gene or constitutive activation of Akt leads to inhibition of autophagic activity [72]. Additionally, accumulation of p62 aggregates due to autophagy inhibition can cause cytotoxicity, oxidative stress, and DNA damage. This phenomenon is seen in several cancers and is associated with poor prognosis [69, 73]. Thus, aberrant PI3K/Akt signaling or loss of tumor

suppressor *PTEN* can be a cause of decreased autophagy in malignant cells [69, 72, 73]. These evidences suggest that autophagy is an important mechanism that suppresses tumor initiation and, when impaired, may lead to tumorigenesis.

### Tumor-promoting role of autophagy

In established tumors, autophagy tries to fulfill the high metabolic demands of the constantly proliferating tumor cells. It has been shown to protect tumor cells from metabolic stress-induced necrosis. Autophagy-mediated macromolecular degradation results in recycling of basic building blocks, which fuel the elevated metabolism of cancer cells [74]. In addition, poor oxygen supply triggers HIF-1α-dependent and -independent autophagy induction (hypoxia), which also contributes to tumor survival [10]. Therefore, unsurprisingly, pharmacological autophagy inhibition or genetic knockdown of essential autophagy genes can result in apoptotic tumor cell death. For example, genetic studies in mice have shown that autophagic gene *FIP200* deletion can reduce the growth of mammary tumors [75]. Cancers harboring activating *HRAS* or *KRAS* mutations are heavily dependent on autophagy (autophagy addiction) and have a high basal rate of autophagy even in growth conditions [69, 76]. Inhibiting autophagy by genetic and pharmacological means in these cells, has shown tumor regression in pancreatic cancer xenografts and genetic mouse models [62, 69, 77].

Thus, by enhancing stress tolerance and providing an alternative avenue through which cancer cells can power their massive nutrient and energy demands, autophagy is well-regarded as a mechanism for tumor cell survival.

## Autophagy and the hallmarks of cancer

Cancer initiation and progression is associated with several molecular and biochemical changes in cells that eventually contribute towards oncogenesis. These cellular traits, which convert a normal cell to a malignant one, are considered the hallmarks of cancer [78, 79].

The aforementioned studies suggest that autophagy has context-dependent roles in cancer, and hence it has been associated with several hallmarks of cancer.

### Sustained proliferation: independence from growth signals and cell cycle control

Sustained proliferation is one of the key features of tumor cells [79]. All normal cells have mechanisms in place to check unwarranted proliferation, which goes haywire in cancer. Tumor cells achieve limitless replicative potential by escaping cell cycle arrest due to loss of regulators *p53* and/or *RB* (retinoblastoma-associated), and aberrant growth signaling [78]. Autophagy maintains genome integrity in cells and prevents tumor initiation. Deletion of tumor suppressor *PTEN* and upregulation of the PI3K/Akt/mTOR pathway, which is rampant in many cancers, might be the cause for decreased cytoprotective autophagy and unchecked proliferation [80]. mTOR signaling inhibits the pro-autophagic protein AMBRA1, which regulates cell proliferation by dephosphorylating c-myc [81]. These examples insinuate that ablation of autophagy can increase the risk of tumors [64, 68].

However, the case of established tumors is quite complex and here the role of autophagy in modulating cell proliferation is highly context-dependent. High levels of autophagy are often observed in cancers with *KRAS* and *BRAF* driver mutations. Pancreatic ductal adenocarcinomas (PDAC) have activating *KRAS* mutations and high basal levels of autophagy. This elevated autophagy is essential for PDAC tumor growth and sustenance, and inhibiting it results in tumor regression [77]. Similarly, inhibiting autophagy by *Atg7* deletion in a *BRAF*-driven lung cancer model showed tumor regression and reversal of malignancy [82]. There are also examples that contradict the proliferative roles of high autophagy in tumor cells. For instance, studies show that autophagy inducers, rapamycin and its derivatives, which are very well-known inhibitors of mTOR, can also inhibit mTOR-dependent cell proliferation by inducing cell cycle arrest in mantle cell lymphoma and MDA-MB-231 breast cancer cells [83, 84]. Taken together, the evidence shows that autophagy regulates proliferation in a context-dependent manner. These studies, linking coordination of autophagy with proliferation, support a dual function of autophagy in one of the crucial hallmarks of cancer.

### Sustained angiogenesis

The ability to form new blood vessels from the existing vasculature (i.e. angiogenesis) is crucial for tumors because they cannot grow beyond a few millimeters in size without blood supply. To maintain growth, tumors switch from avascular to a vascularized stage; this transition is called the "angiogenic switch" [85]. Tumor cells, either by themselves or by stimulating nearby cells, produce vascular endothelial growth factor (VEGF) and other growth factors that bind to receptors on endothelial cells and initiate signaling cascades to stimulate the growth of new blood vessels. Angiogenesis provides a supply of nutrients to cancer cells, eventually aiding in tumor invasion, growth, and metastasis [78, 86]. Taking into consideration the critical role of neovascularization in tumor growth, the current therapeutic focus is on developing various angiogenesis inhibitors. One such inhibitor is bevacizumab, which inhibits VEGF signaling and

has been approved by the FDA for use in glioblastoma [87]. This drug limits endothelial cell proliferation and induces apoptotic tumor cell death. When cells lose their vascular supply, they become hypoxic and induce autophagy via HIF-1α-mediated signaling [88]. The elevated levels of autophagy help tumor cells to survive oxygen stress and become resistant to lack of blood supply. Hence, due to its pro-survival effects, autophagy is one of the mechanisms responsible for poor efficacy and acquired resistance in anti-angiogenesis therapy. The use of autophagy inhibitors along with an anti-angiogenesis drug often potentiates apoptotic cell death [88–90].

A distinct role of autophagy in angiogenesis came from a study in neuroblastoma cells that showed that autophagy can inhibit angiogenesis by degrading gastrin-releasing peptide (GRP), which has a pro-angiogenesis role [91]. Autophagy induction by cells upon anti-angiogenesis treatment has been widely reported; however, the signaling events regulating this relationship are not clear and further probing is needed in this regard.

## Tissue invasion and metastasis

Tumor cells have the capability of metastasis, i.e. invading and colonizing new tissues by entering the vasculature and/or the lymphatics. In primary tumor cells, autophagy mediates hypoxia and nutrient stress and prevents tumor cell necrosis and inflammation. It thereby reduces the recruitment of macrophages at the primary tumor site, which is necessary for induction of metastasis [92]. Another mechanism by which autophagy inhibits epithelial–mesenchymal transition (EMT) is by degrading p62/SQSTM1 and its cargo TWIST1, which is a transcription factor that promotes EMT [93].

When the process of invasion and metastasis begins, the cells lose contact with neighboring cells, detach from the extracellular matrix, undergo EMT, and become motile. In order to do so, they have to outclass physiological mechanisms that are in place to check such dysplastic migration and growth. A type of apoptotic cell death known as anoikis [94] is initiated when cells lose attachment to the extracellular matrix. Tumor cells, in order to overcome anoikis and to cope up with the stress involved with detachment, induce autophagy that provides "anoikis resistance" [95]. Studies have identified that inducing autophagy by starvation results in increased metastasis and invasion of hepatocellular carcinoma cells, regulated by TGF-β/Smad3 signaling [96]. A connection between autophagy, TGF-β signaling, and metastasis was also seen in colorectal cancer, where microRNA mediated suppression of Smad2 by interrupting autophagy, decreasing tumor cell survival, and decreasing the invasion potential [97].

When the detached tumor cells reach distant sites, they may remain dormant until they establish new contacts with the extracellular matrix. At this stage, autophagy helps in their survival, the mechanism of which is largely unknown. The first example in this regard was shown in ovarian cancer cells, where the tumor suppressor gene *ARHI* was able to induce autophagy and increase tumor cell dormancy. Dormancy of tumor cells is an impediment in cancer therapy because it helps them survive rigorous anti-cancer treatments that focus mainly on proliferating cells. Hence, in order to understand if dormancy can be modulated, we need to study its connection with autophagy in greater detail [92].

Collectively, autophagy initially prevents occurrence of invasion and metastasis by inhibiting tissue necrosis and inflammation. Once the tumor cells detach from the ECM, high rates of autophagy help them avoid apoptotic cell death and maintain dormancy in a distant, hostile microenvironment until they settle and form new colonies. Hence, any therapeutic interventions using autophagy modulators will depend on the stage of metastasis and require further in vivo studies [92, 95].

## Evading cell death

Apoptosis is a highly regulated, programmed cell death cascade that occurs in multicellular organisms [4]. The continuous proliferation of tumor cells is not only due to their independence of growth signals but also because they can evade apoptotic cell death even after DNA damage, genome instability, and oncogene activation. There are several mechanisms by which cancer cells escape death. p53, the "guardian of the genome" [98], initiates apoptosis in cells if the DNA damage is beyond repair. Loss of p53, which is found in a multitude of cancers, makes cells resistant to apoptosis. All cells have a set of pro- and anti-apoptotic proteins that regulate apoptosis. Overexpression of Bcl-2, an anti-apoptotic protein, is a pro-survival strategy employed by several cancers such as leukemia, prostate, breast, small-cell, and non-small-cell lung cancers [99, 100]. Bcl-2 also has a role in autophagy, wherein it binds and sequesters Beclin-1 to prevent autophagy induction [101].

In cancers with apoptosis defects, chemotherapy that aims at killing cells might not prove very beneficial. Moreover, autophagy is induced in most cells treated with chemotherapy drugs. For such cancers that do not succumb to apoptotic cell death, an alternative form of cell death, namely autophagic cell death, has been suggested. This type of cell death due to excessive autophagy is caspase-independent and is distinct from apoptosis [102]. Glioblastomas are very well known for their aggressiveness and resistance to apoptosis. There are reports showing

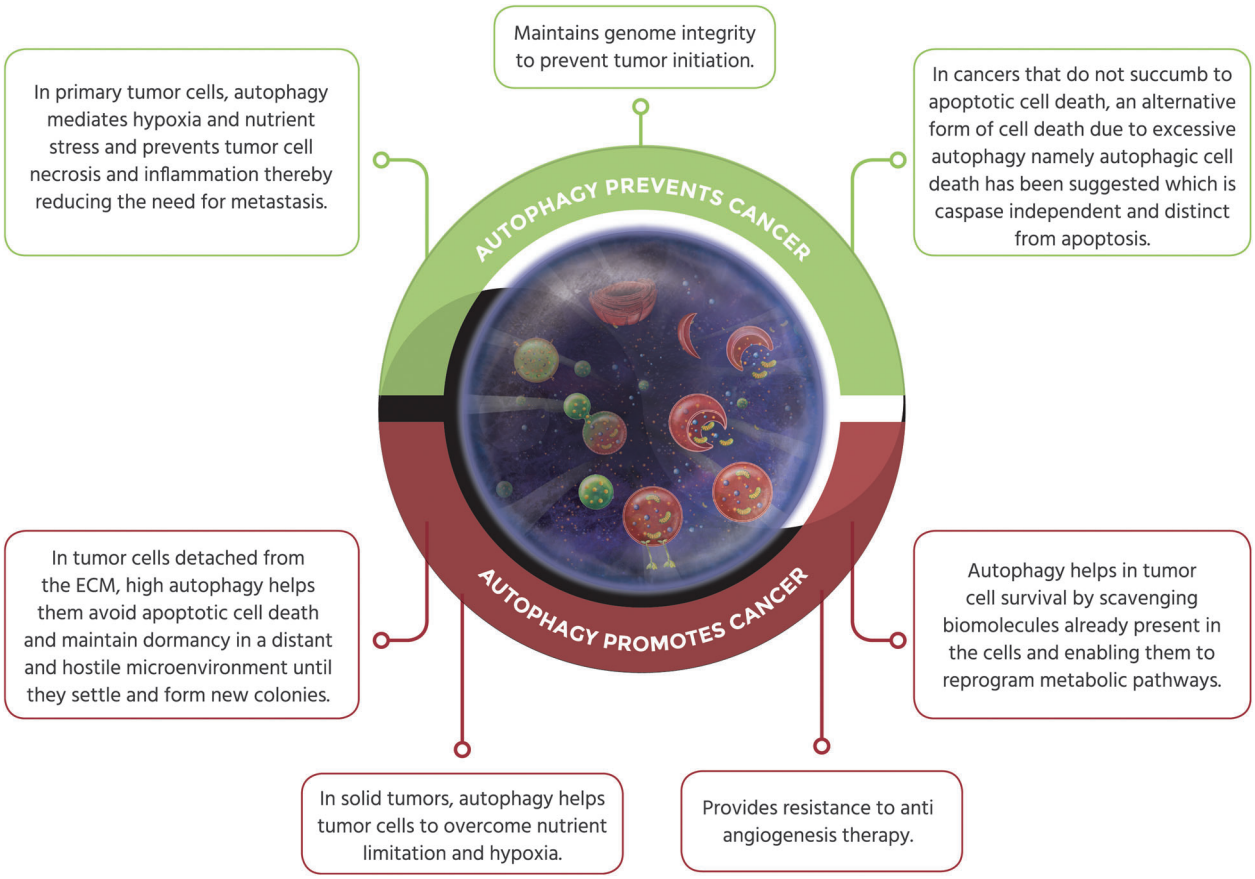


**Fig. 2** The bipolar nature of autophagy in cancer. Autophagy has a complex and dual role in cancer. It maintains genomic integrity to prevent mutations that lead to tumorigenesis. In primary tumor cells, it prevents necrosis and metastasis. Excess autophagy can also be an alternate death mechanism in apoptosis-resistant cancers. In tumor cells detached from the extracellular matrix, autophagy prevents anoikis and enables their survival. It helps the tumor cells overcome lack of oxygen and nutrients, reprograms their metabolism, and provides resistance to anti-angiogenesis

that several chemotherapy regimens involving temozolomide, arsenic oxide, cannabinoids, or 4-hydroxy tamoxifen can induce autophagy but not apoptosis and can cause autophagic cell death and tumor regression, which might be potentiated even more by blocking autophagosome–lysosome fusion [103–106]. However, whether this autophagic induction is a last attempt by cells to survive or whether it is the cause of cell death still remains a conundrum.

Hence, autophagy may be a mode of cell death in apoptosis-deficient tumors, but, considering the complexity involved, this might not hold true for all cancers.

## Re-programming metabolism

Tumor cells adapt and change their metabolic pathways according to the microenvironment, which enables them to survive [100]. In contrast to normal cells, tumor cells obtain energy by aerobic glycolysis rather than oxidative phosphorylation, a phenomenon termed as the “Warburg effect”. This adaptation eliminates the need of oxygen for ATP production [107]. The pyruvate produced during glycolysis is converted to lactate, not acetyl CoA, hence causing a deficiency of TCA cycle substrates. This is an acquired characteristic of tumors as a result of mitochondrial impairment and it also helps them survive hypoxic conditions due to the lack of a proper blood supply. In the absence of pyruvates, cells will need other substrates to run the TCA cycle for ATP production, and autophagy can provide this by scavenging biomolecules already present in the cells. In *RAS*-driven cancers, cells utilize excessive glucose for glycolysis by increasing the expression of glucose transporters. Cancers with *RAS* mutations are also addicted to glutamine as a substrate for the TCA cycle. Other amino acids generated by breakdown of proteins are also utilized in the liver for gluconeogenesis or for ATP synthesis via the TCA cycle. Fatty acids can produce energy by getting converted to acetyl CoA, and entering the TCA cycle. Autophagy also abrogates ROS toxicity due to damaged mitochondria. Thus, the role of autophagy is

important in reprogramming metabolic pathways and enabling tumor cell survival [108].

Overall, autophagy is oncogenic at certain stages of tumor development and tumor-suppressive at other stages, and analysis of the role of autophagy in contribution towards several hallmarks of cancer will certainly provide insights into the realistic prospect of cancer therapy by modulating autophagy in a context-dependent manner (Fig. 2).

## Targeting autophagy for cancer therapeutics

Autophagy as a physiological process keeps cells healthy and suppresses tumorigenesis, but in established tumors it helps in survival by mitigating stress. Hence, the decision to target autophagy for pharmacological intervention has to be well supported by preclinical data regarding the role and status of autophagy in a particular cancer.

### Autophagy inhibitors

Enhanced autophagy has been implicated as a mechanism adapted by tumor cells to survive and acquire resistance against chemotherapy. In this scenario, inhibition of autophagy either by genetic or by pharmacological means has been extensively shown to sensitize tumor cells to anti-cancer therapy [88, 109]. Small-molecule inhibitors of autophagy can either be used alone or in combination therapies along with anti-cancer drugs.

Genetic ablation of autophagy function by deletion of *Atg5*, *Atg7* or *beclin1* has been shown to revert the acquired resistance against tamoxifen in ER-positive breast cancer cells [110]. The combinatorial treatment consisting of autophagy inhibitor 3-MA and trastuzumab in HER2-positive breast cancer cells can increase the potency of chemotherapy [111]. A high level of autophagy is also associated with cisplatin resistance in ovarian cancer cells, and Atg5 deletion in these cells induces apoptotic cell death [112].

In the current scenario, CQ/HCQ is the only autophagy inhibitor that has been approved by the FDA for clinical trials [113]. CQ is an anti-malarial drug that inhibits autophagosome–lysosome fusion by affecting lysosomal acidification [114]. Autophagy induction by HIF-1α makes glioblastoma cells resistant to the anti-angiogenesis drug bevacizumab, which can be reverted by *Atg7* deletion or CQ treatment [88]. Inhibition of autophagy by 3-MA or *beclin1* deletion sensitizes hepatocellular carcinoma cells to chemotherapy [115]. CQ treatment in HT29 colorectal cancer cells sensitizes them to anti-angiogenesis and DNA-damaging chemotherapy drugs [116].

There are ample examples that conclude that autophagy inhibitors, when used in concert with anti-cancer drugs, can sensitize chemoresistant cells and potentiate apoptotic cell death, eventually inhibiting tumor survival [80]. These findings hold immense clinical importance because acquired resistance by tumors is the biggest bottleneck in cancer chemotherapy.

### Autophagy inducers

As autophagy is a cell survival process, most cells treated with chemotherapy drugs exhibit autophagy induction. This autophagy induction is a last attempt at survival by tumor cells, which can be tackled by a combination therapy of autophagy inhibitors with chemotherapeutic drugs. However, excessive autophagy induction upon cytotoxic drug treatment or by using autophagy inducers could also lead to autophagic cell death. Analogs of the mTOR inhibitor rapamycin, such as temsirolimus and everolimus, alone or in conjunction with chemotherapy drugs showed an anti-proliferative effect in mantle cell lymphoma and acute lymphoblastic leukemia by inducing cell cycle arrest and excessive autophagy, which might be the cause of tumor cell death [84, 117]. Glioblastoma cells that are resistant to apoptotic cell death succumbed to a combination chemotherapy of anti-cancer drug temozolomide with dasatinib, both of which induce autophagy [118]. Similarly, HDAC inhibitors have also shown autophagy-inducing potential as one of its anti-cancer effects [119, 120]. One of the most successful therapeutic interventions with the autophagy inducer rapamycin was in terms of its anti-angiogenesis effects correlating with hampered VEGF production and downstream signaling. Everolimus has been approved for use by the FDA as an angiogenesis inhibitor in renal cell carcinoma, advanced breast cancer, and pancreatic neuroendocrine tumors [121, 122]. However, whether the induction of autophagy as a result of mTOR inhibition by everolimus contributes to its anti-angiogenesis ability is not clear.

Therefore, although the current clinical focus is on using autophagy inhibitors in combination with chemotherapeutic/cytotoxic drugs, the contradictory role of autophagy in suppressing tumor growth at the initiation stage, and whether excessive autophagy can lead to cell death, should still be considered from a therapeutic viewpoint. With several small molecules exhibiting autophagy activating ability, more attention is needed to understand the importance of deploying autophagy inducers based on the progression of cancer.

Thus, with a focus on both autophagy inducers and inhibitors for cancer therapy, there is a need for further understanding how autophagy can be modulated to achieve better clinical outcomes. Also, recent investigations have highlighted the role of some autophagy modulators in cancer therapy, most of which are in

**Table 1** List of autophagy modulators currently in clinical trials

| Clinical trials | Mechanism/target of chemotherapy drugs | Current state | Type of cancer | ClinicalTrials.gov identifier |
|---|---|---|---|---|
| Sirolimus or vorinostat with HCQ (Phase I) | mTOR and HDAC inhibitor | Ongoing | Advanced solid tumors | NCT01266057 |
| Vorinostat with HCQ (Phase I) | HDAC inhibitor | Ongoing | Advanced solid tumors | NCT01023737 |
| Gemcitabine/abraxane with HCQ (Phase I/II) | Nucleoside analog /antimicrotubule agent | Ongoing | Pancreatic adeno carcinoma | NCT01506973 |
| Paclitaxel, carboplatin and bevacizumab with HCQ (Phase II) | Microtubule disrupting agents/ inhibitor of DNA synthesis/VEGF-A inhibitor | Ongoing | Non-small lung cancer | NCT01649947 |
| FOLFOX and bevacizumab with HCQ (Phase I/II) | Folinic acid/thymidylate synthase inhibitor/inhibition of DNA synthesis/VEGFA inhibitor | Ongoing | Colorectal cancer | NCT01206530 |
| Vorinostat with HCQ versus regorafenib (Phase II) | HDAC inhibitor/ Multi kinase inhibitor | Ongoing | Refractory metastatic colorectal cancer | NCT02316340 |
| MLN9708 and vorinostat (Phase I) | Proteasome inhibitor/ HDAC inhibitor | Ongoing | Advanced p53 mutant malignancies | NCT02042989 |
| Temsirolimus with HCQ (Phase I) | Cell cycle arrest | Ongoing | Refractory solid tumors | NCT00909831 |
| CQ (Phase I/II) | | Completed | Breast ductal carcinoma | NCT01023477 |
| RAD001 with HCQ (Phase I/II) | mTOR inhibitor | Ongoing | Renal cell carcinoma | NCT01510119 |
| Navitoclaxandabiraterone acetate with or without HCQ (Phase II) | Bcl2 inhibitor/Androgen synthesis inhibitor | Completed | Refractory prostate cancer | NCT01828476 |
| Sunitinib malate with HCQ (Phase I) | Receptor tyrosine kinase inhibitor | Ongoing | Advanced solid tumors | NCT00813423 |
| CQ (Phase II) | | Ongoing | Breast cancer | NCT02333890 |
| Sorafenib with HCQ (Phase I) | Multi kinase inhibitor | Completed | Refractory or relapsed solid tumors | NCT01634893 |
| Dabrafenib and trametinib with HCQ (Phase I/II) | BRAF inhibitor/MEK inhibitor | Ongoing | Advanced BRAF mutant melanoma | NCT02257424 |
| Velcade and cyclophosphamide with CQ (Phase II) | Proteasome inhibitor/ DNA replication inhibitor | Completed | Refractory or relapsed multiple myeloma | NCT01438177 |
| Cisplatin and etoposide with CQ (Phase I) | DNA replication inhibitor/ topoisomerase inhibitor | Completed | Stage 4 Small cell lung cancer | NCT00969306 |
| MK2206 with HCQ (Phase I) | Akt inhibitor | Ongoing | Advanced solid tumors (Prostate, melanoma or kidney cancer) | NCT01480154 |
| Enzalutamide with metformin hydrochloride | Anti-androgen | Ongoing | Prostate cancer | NCT02339168 |
| CQ (Phase II) | | Ongoing | Glioblastoma and astrocytoma | NCT02432417 |
| Gemcitabine and abraxane with or without HCQ (Phase II) | Nucleoside analog /Antimicrotubule agent | Ongoing | Pancreatic cancer | NCT01978184 |

**Table 2** Data on TCGA array performed on liver, lung, breast, ovarian, colorectal, pancreatic, and prostate cancer samples. The list of autophagy genes was taken from HADb (Human Autophagy Database (http://www.autophagy.lu/index.html). Several autophagy-related gene mutations were identified in this array as listed. The genes are listed and formatted differently (bold, italics, bold italic, underline, [a]) according to the stage of autophagy

| Genes involved in autophagy | Type of cancer (frequency of mutation) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Colon | Breast | Pancreatic | Ovarian | Prostate | Lung | Liver |
| **ULK1** | 0.45% | 0.72% | 2.67% | – | – | 2.08% | 1.01% |
| **ULK2** | 1.79% | 0.72% | – | 0.32% | – | 2.36% | 3.03% |
| **ULK3** | 1.35% | 0.51% | 1.33% | – | – | 0.28% | – |
| **FIP200** | 3.59% | 1.43% | 3.33% | 2.53% | 0.30% | 4.31% | 1.52% |
| **ATG13** | 0.45% | – | – | – | – | – | – |
| **ATG101** | – | 0.31% | 0.67% | 0.32% | – | – | – |
| **WIPI1** | 1.79% | 0.41% | 2.00% | 0.90% | 0.90% | 0.97% | – |
| **WIPI2** | 0.90% | 0.31% | 1.33% | 0.63% | – | 1.11% | – |
| **PIK3R4** | 7.17% | 1.13% | 3.33% | 1.58% | 0.30% | 4.72% | 2.02% |
| **PIK3C3** | 4.48% | 0.82% | 1.33% | 0.32% | 0.30% | 2.92% | – |
| **WDFY3** | 14.80% | 2.76% | 6.00% | 0.95% | 1.51% | 10.42% | 3.54% |
| **WDR45** | 0.90% | 0.41% | – | 0.32% | – | 1.25% | 0.51% |
| **ZFYVE1** | 2.69% | 0.51% | | 0.32% | 0.30% | 2.36% | 1.01% |
| **BECN1** | 1.79% | – | 1.33% | 0.32% | – | 0.83% | 0.51% |
| **AMBRA1** | 2.24% | 1.74% | 2.00% | 0.32% | 0.30% | 1.81% | 2.02% |
| **BCL2** | 0.45% | – | – | – | – | 1.39% | 1.01% |
| **RUBICON** | 1.79% | 1.02% | 4.00% | 0.32% | – | 2.78% | 0.51% |
| **RAB5A** | – | – | 0.67% | – | – | 0.42% | – |
| *ATG2A* | 3.59% | 1.33% | 2.67% | 0.63% | 0.90% | 3.33% | 1.52% |
| *ATG2B* | 4.93% | 1.02% | 4.00% | 0.32% | 0.60% | 6.11% | 3.03% |
| *ATG3* | 0.45% | 0.31% | 0.67% | 0.95% | – | 0.97% | 0.97% |
| *ATG4A* | 1.79% | 0.72% | – | – | 0.30% | 0.69% | – |
| *ATG4B* | 0.45% | 0.10% | – | – | – | 0.56% | 1.01% |
| *ATG4C* | 2.69% | – | – | 0.32% | 0.30% | 0.56% | 0.51% |
| *ATG4D* | 2.24% | 0.51% | 0.67% | – | 0.30% | 1.25% | 0.51% |
| *ATG5* | 1.79% | 0.51% | – | – | – | 0.56% | 1.01% |
| *ATG7* | 1.79% | 0.72% | 2.00% | – | – | 2.08% | – |
| *ATG9A* | 2.24% | 0.61% | 1.33% | – | – | 1.39% | – |
| *ATG9B* | 1.35% | 1.23% | 0.67% | 1.58% | – | 2.08% | – |
| *ATG10* | 0.45% | – | 0.67% | – | – | 0.42% | 1.01% |
| *ATG12* | – | 0.10% | – | – | 0.30% | – | – |
| *ATG16L1* | 2.24% | 0.72% | – | – | – | 1.53% | 0.51% |
| *ATG16L2* | 0.45% | 0.51% | – | – | – | 0.97% | 0.51% |
| *MAP1LC3A* | 0.45% | – | 1.33% | – | – | 0.28% | – |
| *MAP1LC3B* | – | – | – | – | – | 0.28% | – |
| *MAP1LC3C* | 0.45% | 0.10% | 0.67% | 0.32% | – | 2.22% | – |
| *GABARAPL1* | – | 0.20% | 0.67% | 0.32% | – | 0.14% | – |
| *GABARAPL2* | – | 0.20% | 0.67% | 0.32% | – | 0.14% | – |
| *RAB1A* | – | 0.20% | – | – | – | 0.14% | 0.51% |
| *RAB11A* | 0.90% | 0.31% | 0.67% | – | – | 0.14% | – |
| *RAB33B* | 0.90% | – | – | – | 0.60% | 0.28% | – |
| *NBR1* | 1.35% | 0.41% | 0.67% | – | – | 1.11% | 2.53% |
| *SQSTM1* | 0.90% | 0.51% | 2.67% | 0.32% | – | 0.97% | – |

**Table 2** (continued)

| Genes involved in autophagy | Type of cancer (frequency of mutation) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Colon | Breast | Pancreatic | Ovarian | Prostate | Lung | Liver |
| ***KIF5B*** | 5.38% | 0.61% | 1.33% | 0.32% | 0.60% | 1.39% | – |
| UVRAG | 0.90% | 0.41% | 2.67% | – | – | 1.39% | 0.51% |
| RAB24 | 0.90% | 0.10% | 0.67% | – | 0.60% | 0.14% | 0.51% |
| RAB7A | 0.90% | – | 0.67% | 0.32% | – | 0.42% | 0.51% |
| LAMP1 | 1.79% | 0.10% | 1.33% | – | – | 1.11% | 1.01% |
| LAMP2 | 0.90% | 0.92% | 1.33% | 0.32% | – | 0.83% | – |
| CTSB[a] | 2.69% | 0.31% | 1.33% | – | – | 0.42% | 1.01% |
| CTSD[a] | 1.79% | 0.10% | – | – | 0.30% | 1.11% | 0.51% |
| CTSL1[a] | 1.79% | 0.41% | – | 0.95% | – | – | 0.51% |

Bold indicates autophagy induction, italic indicates autophagosome expansion, cargocapture and completion, bold italic indicates movement towards lysosomes, underline indicates fusion with lysosomes and values denoted by [a] indicated degradation of cargo in autolysosomes

ongoing clinical trials (Table 1). These autophagy modulators under clinical investigation are either used alone or in combination with chemotherapeutic drugs for several cancers in order to achieve a better clinical outcome.

## Small-molecule modulators of epigenetics can modulate autophagy in cancer

As discussed in the preceding sections, autophagy can be transcriptionally and epigenetically regulated. Protein methyltransferase CARM1 is a positive regulator of autophagy, whereas EZH2 is a negative regulator of autophagy. CARM1 overexpression in breast and prostate cancers leads to transcription activation in several genes [123]. Ellagic acid, an inhibitor of CARM1-mediated H3R17 methylation, decreases the proliferation and malignant potential of ovarian carcinoma cells by potentially inhibiting autophagy and enhancing apoptosis [124]. EZH2 is overexpressed in several cancer types, such as breast, prostate, colon, gastric, bladder, liver, melanoma and lymphomas [123]. As mentioned earlier, H3K27 trimethylation by EZH2 inhibits autophagy by transcriptional repression of negative regulators of mTOR like TSC2. TSC2 is also a very well-studied tumor suppressor. Hence, EZH2 activity is associated with decreased activity of tumor suppressors and cancer-preventing autophagy. S-Adenosyl-L-methionine (SAM) competitive inhibitors such as UNC1999 and GSK343 can inhibit EZH2 activity and induce autophagic cell death in human colorectal cancer cells [125]. Several small molecules with the potential to regulate epigenetic enzymes related to autophagy are available in the market, but a lot remains to be studied in order to form a direct connection between their action and autophagy, especially in case of a complex disease like cancer.

## Identification of autophagy gene mutations in cancers

As mentioned previously, autophagy-related gene mutations play a role in either sensitizing the cells towards chemotherapeutic drugs or contributing towards promoting tumorigenesis. For example, *beclin1* deletion sensitizes hepatocellular carcinoma cells to chemotherapy [115], and recent studies showed that *UVRAG* frameshift mutation, an autophagic tumor suppressor gene, promotes the progression of colorectal cancer [126]. Thus, unveiling autophagy-related gene mutations are gaining importance, and, to further gain insights into the mutational status of autophagy-related genes in tumor samples, we performed the cancer genome atlas (TCGA) array analysis on different tumor samples (Table 2). Briefly, the lists of autophagy genes were taken from the Human Autophagy Database (HADb) (http://www.autophagy.lu/index.html). Based on the previous reports on dependence of several cancers on autophagy gene mutations, the cancers under investigation for this study were hepatocellular carcinoma (Supplementary Table 1), colorectal carcinoma (Supplementary Table 2), breast carcinoma (Supplementary Table 3), ovarian serous cystadenocarcinoma (Supplementary Table 4), prostate adenocarcinoma (Supplementary Table 5), lung squamous cell carcinoma (Supplementary Table 6) and pancreatic adenocarcinoma (Supplementary Table 7). The mutation profiles of these genes of the above-stated cancers in TCGA cohort were obtained from Broad GDAC firehose mutation_packager_calls [127–132]. Several autophagy-related gene mutations were identified in this array. For example, *UVRAG* mutations were found not only in colorectal cancers but also in other cancers like hepatocellular, breast, prostate, pancreatic carcinoma, as well as lung squamous cell carcinoma and adenocarcinoma.

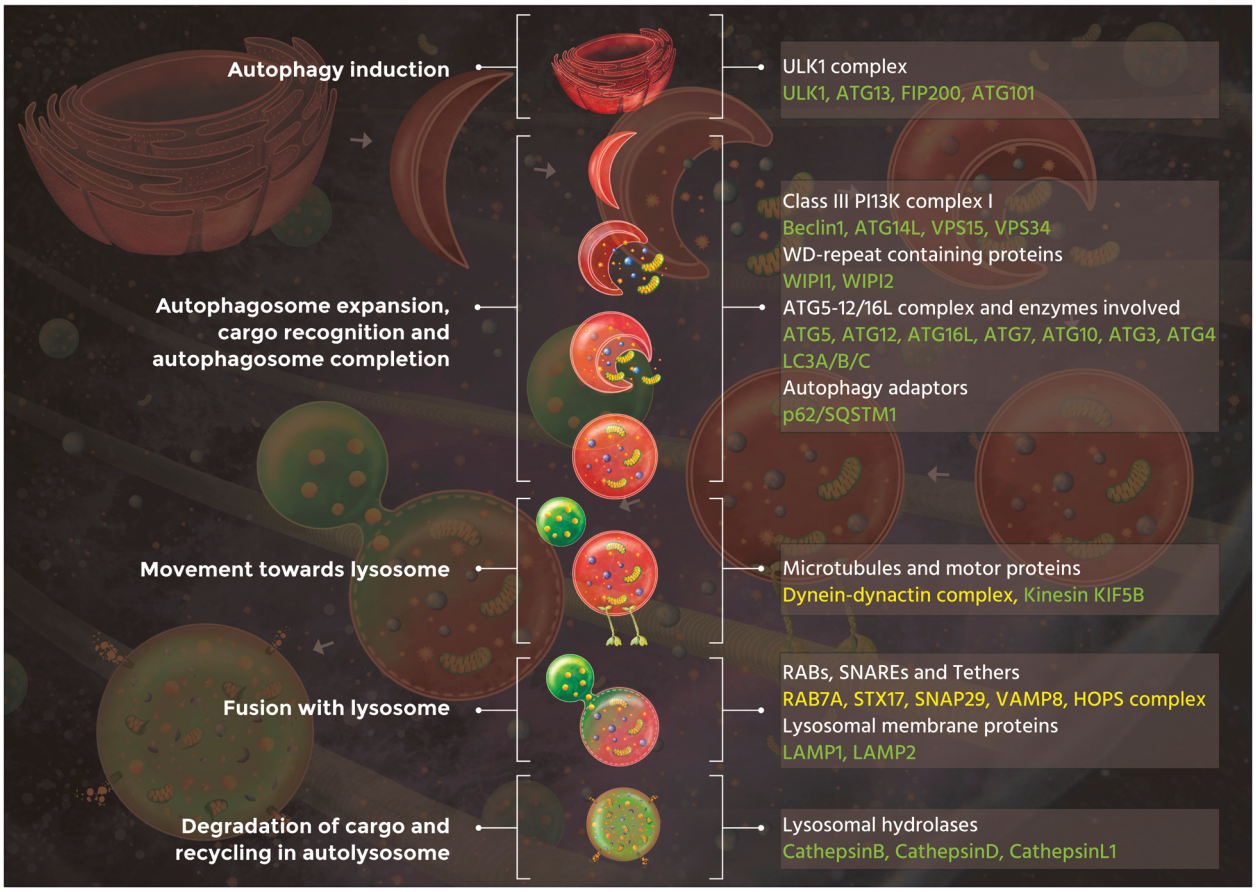


**Fig. 3** Autophagy-related gene mutations in cancer. This figure depicts autophagy genes participating at different steps of autophagy. The genes depicted in green color are the ones that were identified to be mutated in the TGCA array of lung, liver, colorectal, breast, ovarian, pancreatic, and prostate cancers. The non-mutated genes are depicted in yellow color. (For detailed information please refer to Table 2)

From these preliminary data, we found that several autophagy-related genes are altered in many of the above-mentioned cancers. Taken as a whole, our findings are in accordance with the previous reports on dependence of several cancers on autophagy-related gene mutations, with a highlight on new plausible gene mutations. Future studies are needed to address the roles of these mutations in tumorigenesis (Fig. 3).

## Conclusion

Autophagy has a significant impact on tumor initiation and promotion, with both tumor-suppressive and tumor-promoting roles. Interestingly, conventional cancer therapy has been reported to have chemoresistance as one of the primary limitations. The cytoprotective role of autophagy has been postulated as one of the mechanisms of resistance. Hence, combination therapy for cancer using autophagy inhibitors has brought into the limelight another important application of autophagy modulation. Currently, there are several clinical trials in progress involving HCQ and anti-cancer drugs. Although the role of CQ in autophagy inhibition is quite clear, it also has several off-target effects. As it affects lysosomal pH, it is not autophagy specific and also blocks the endocytic pathway. Moreover, a very high dose of CQ/HCQ (~600 mg/kg) is required to inhibit autophagy in the clinical setting, and even after that it is only moderately potent [133]. Hence, the need of the hour is to develop more potent and specific autophagy inhibitors. We also need to understand the potential long-term side effects of autophagy modulation on normal cells and on the whole organism.

Most importantly, further understanding the cellular and functional relevance of autophagy in the tumor microenvironment would aid in better translation of laboratory investigations into the clinical settings.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.



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# *Multifaceted Housekeeping Functions of Autophagy*

**Sarika Chinchwadkar, Sreedevi Padmanabhan, Piyush Mishra, Sunaina Singh, S. N. Suresh, Somya Vats, Gaurav Barve, Veena Ammanathan, et al**

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REVIEW ARTICLE

# Multifaceted Housekeeping Functions of Autophagy

*Sarika Chinchwadkar, Sreedevi Padmanabhan, Piyush Mishra, Sunaina Singh, S. N. Suresh, Somya Vats, Gaurav Barve, Veena Ammanathan and Ravi Manjithaya**

**Abstract** | Autophagy is an evolutionarily conserved intracellular degradation process in which cytoplasmic components are captured in double membrane vesicles called autophagosomes and delivered to lysosomes for degradation. This process has an indispensable role in maintaining cellular homeostasis. The rate at which the dynamic turnover of cellular components takes place via the process of autophagy is called autophagic flux. In this review, we discuss about the orchestrated events in the autophagy process, transcriptional regulation, role of autophagy in some major human diseases like cancer, neurodegeneration (aggrephagy), and pathogenesis (xenophagy). In addition, autophagy has non-canonical roles in protein secretion, thus demonstrating the multifaceted role of autophagy in intracellular processes.

## 1 Introduction

Autophagy, an intracellular evolutionarily conserved process, involves engulfment of unwanted proteins and organelles by double-membrane vesicles, called autophagosomes, which then fuse with the lysosomes/vacuole, and the engulfed cargo is subsequently degraded. It is a cell survival mechanism under stress conditions and it also play important roles in many other intra-cellular processes like protein and organelle turnover and transport of some of the vacuolar enzymes. This process can be divided into various steps, including autophagy induction, nucleation, autophagosome formation, maturation, fusion with the lysosomes/vacuole, degradation of the cargo, and recycling of the precursor molecules, such as amino acids, lipids, and nucleotides, back to the cytoplasm. Autophagy is a tightly regulated cellular mechanism and its flux varies depending on the cell type(s) of an organism. Autophagy is involved in various physiological roles, such as cellular homeostasis, embryonic development, antigen presentation, protein quality control, and maintenance of the amino-acid pool during starvation conditions. It is also implicated in various pathophysiological diseases, such as infection, cancer, diabetes, and neurodegeneration.

Although autophagy is predominantly a cytosolic event, the nucleus exerts a considerable control in the extent of autophagy response, especially during adverse conditions, such as starvation. Depending on the cargo it captures, autophagy is broadly classified as general and selective autophagy. For example, as a response to nutrient deprivation, general autophagy is triggered where it captures random portion of cytosol. In contrast, selective autophagy ensures specific capture of cytosolic cargo, such as damaged or superfluous organelles. When selective autophagy captures and degrades mitochondria, the process is termed as mitophagy. Similarly, autophagic degradation of peroxisomes (pexophagy), Golgi (golgiphagy), ER (ER-phagy), ribosomes (ribophagy), etc., have been documented.[1] The genes comprising the autophagy machinery are named as ATG (AuTophaGy related gene).[1]

## 2 Process of Autophagy

### *2.1 Autophagy Induction*

The initial characterization of autophagy flux with respect to involvement of molecular players was carried out in yeast extensively. Although recycling of the cytoplasmic contents happens at

**Autophagosomes**: The "Pac-Man" like double membrane vesicles involved in macroautophagy.

*Autophagy Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru 560 064, India*
**ravim@jncasr.ac.in*

steady state levels by basal autophagy, autophagy flux increases drastically when it is induced. Autophagy induction happens when the cells are under stress conditions, such as amino acid starvation[1] (Fig. 1). Alternatively, autophagy can also be induced using drugs, such as rapamycin,[2] which targets the TOR (Target of Rapamycin), a major serine-threonine kinase involved in nutrient sensing and cell growth regulation.[3] Both amino-acid starvation and rapamycin inhibit TOR activity and induce autophagy. Under the nutrient rich conditions, TOR is active and it negatively regulates kinase activity of Atg1 by hyper-phosphorylating Atg13 and thus disturbing the Atg1–Atg13 association, required for downstream processes of autophagy.[4] When autophagy is induced either by nutrient limitation or by rapamycin, TOR becomes inactive and does not phosphorylate Atg13 and thus increases affinity of Atg13 towards Atg1, further passing the signal for nucleation of different autophagy proteins (Fig. 1).

**Phagophore Assembly Site (PAS):** The site inside cells that gives birth to autophagosomes.

### 2.2 Nucleation of Autophagy Proteins

When autophagy is induced, nucleation of autophagy proteins takes place at a site called the pre-autophagosomal structure or phagophore assembly site (PAS) which is present near the vacuole. The very first autophagy-related protein (ATG) that is recruited at PAS is Atg17. Atg17 and Atg11 act as scaffold in general autophagy and selective autophagy, respectively.[5] In general autophagy, Atg17 interacts with Atg31 which then interacts with Atg29 and thus forms a ternary complex. Atg17 also interacts with Atg13 and thus links the trimer to Atg1.[6–8] Recent study showed that Atg1 tethers Atg9 vesicles at PAS.[9] Atg9 is a transmembrane protein required for autophagy, and its transport from peripheral sources, such as mitochondria, ER, to PAS is believed to be important for providing a membrane source for the formation of autophagosomes.[10, 11] Atg23 and Atg27 are involved in anterograde transport of Atg9, wherein Atg9 vesicles are brought to PAS.[12] Retrograde transport of Atg9 from PAS to peripheral

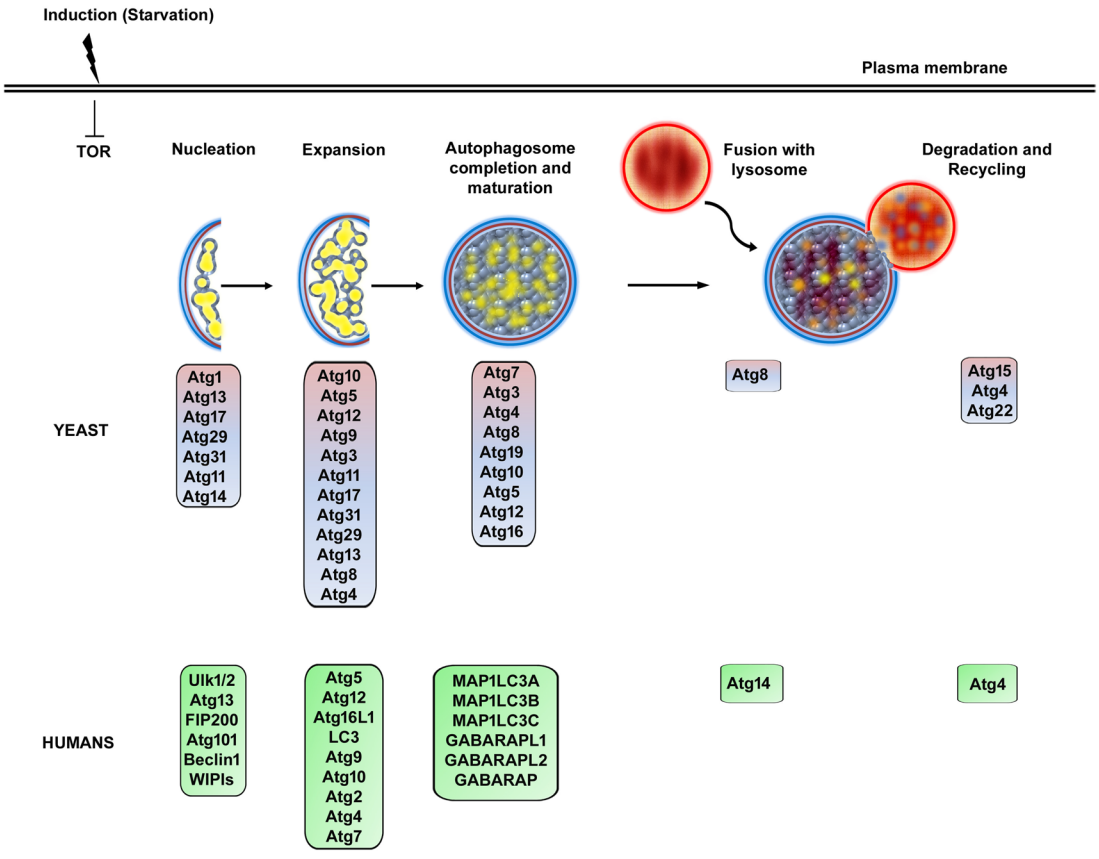


***Figure 1:*** Schematic demonstrating the various steps in the autophagy process. The yeast and human autophagy proteins involved in nucleation, expansion, autophagosome maturation and completion, fusion, and degradation processes are mentioned.

membrane sources require Atg1, Atg2, and Atg18.[13] Another complex important for PAS formation and initiation of autophagosomes is Class III PI3-K complex (VPS34, Atg6/VPS30, VPS15, and Atg14) which forms PI3P (Phosphatidylinositol-3-phosphate) that is present in the autophagosomal membranes.[14] Graef et al. in 2013 also have shown that the PAS containing multiple Atg proteins are tethered to ER exit sites. Localization of all these ATG proteins and the hierarchy of the complexes they form at the PAS have been determined. These orchestrated signaling events lead to a double membrane vesicle formation called an autophagosome [15] (Fig. 1).

### 2.3 Biogenesis, Maturation, and Completion of Autophagosomes

The initiation of the autophagosome biogenesis starts with formation of an isolation membrane at PAS. Atg8 is one of the important proteins that is present on the inner and outer membrane of the autophagosomes and it remains associated with the autophagosomes throughout the process of autophagy right from the formation of isolation membrane to the autophagosome degradation in the vacuole.[16] Atg8 is inserted in the autophagosomal membranes in the form of Atg8-PE (Phosphatidylethanolamine). Two ubiquitin-like conjugation systems help in the formation of Atg8-PE, the first being the Atg7–Atg3–Atg10 conjugation system and the second Atg5–Atg12–Atg16.[17] Atg4 is a cysteine protease that helps in conjugation of Atg8 with PE by cleaving the C-terminal Arg residue and exposes the Gly for conjugation. The recycling Atg8 from the Atg8-PE present at the outer membrane of the autophagosomes also requires Atg4 for the cleavage of PE from Atg8. Thus, Atg4 plays dual role of conjugation and recycling of Atg8.[18] As explained earlier, the membrane source for autophagosome formation is further contributed by transport of Atg9 vesicles along with Atg41.[19] Thus, Atg8, along with Atg4, Atg7–Atg3–Atg10 complex, and Atg5–Atg12–Atg16 help in autophagosome formation and maturation (Fig. 1). An important protein required for autophagosome completion is a PI3P phosphatase, Ymr1 in the absence of which recycling of the Atg proteins from the autophagosomal membrane is blocked and the Atg proteins remain associated with autophagosomes inside the cytoplasm.[20] Once the autophagosomes are completely formed, they are transported to the vacuole and are fused with the vacuole.

### 2.4 Fusion of Autophagosomes

As in the case of any vesicle destined to fuse with a membrane, autophagosomes also involve three major conditions for fusion with the vacuole—(1) interaction of Rab like GTPase, (2) tethering to the vacuole, and (3) SNARE-pair interactions leading to membrane fusion.

Ypt7, an yeast Rab GTPase, was shown to be involved in the homotypic vacuolar fusion along with Sec17 and Sec18.[21–23] Tethering of the vesicles is mediated by a complex called as the class C VPS complex or the Homotypic fusion and Vacuolar Protein Sorting complex also known as HOPS. HOPS consists of six subunits Vps18, Vps11, Vps16, Vps33, Vps39, and Vps41.[24–26] HOPS complex functions as an effector for Ypt7.[25]

A number of SNARE proteins also mediate the process of membrane fusion. Vam3 is a v-SNARE (also a syntaxin homologue) that localizes to the vacuolar membrane and has been shown to be important for both cytoplasm to vacuole delivery of Ape1 and for the fusion of autophagosomes to the vacuole.[27] Vam7 was later shown to be functioning together with Vam3 in vacuolar fusion.[28] Another v-SNARE Vti1 was reported to interact with Vam3 in both alkaline phosphatase pathway (Golgi-vacuole) and CVT pathway (one of the selective autophagy pathways). Along with these two other proteins which form a complex and function in the fusion step are Ccz1 and Mon1 which were identified in a screen of mutants defective in autophagy and CVT pathways.[29]

The fusion of outer membrane of the autophagosomes leads to the delivery of single membrane autophagic bodies into the vacuolar lumen which is then degraded.

### 2.5 Degradation of Autophagosomes and Its Contents

Takeshige et al. reported that yeast strain which was defective in vacuolar proteinases showed accumulation of autophagic bodies inside the vacuole.[2] Pep4 and Prb1 were the two mutants that accumulated autophagic bodies post starvation. Aut5/Cvt17 was identified to be an important component of the degradation machinery owing to its lipase activity.[30] Cvt17 was shown to be the lipase which degrades the membrane of the autophagic body in the vacuole.[31] Moreover, acidification of the yeast vacuoles was shown to be important for the degradation per se.[32]

**SNAREs:** Proteins involved in fusion of cytoplasmic vesicles.

**Tethering complexes-HOPS:** Tethering complexes-HOPS-Multi subunit protein complex that help anchoring autophagosomes and lysosomes.

**Phagophore/isolation membrane:** The beginning structure that grows into an autophagosome.

**CVT pathway:** Cytoplasm-to-Vacuole pathway that delivers proteins from cytoplasm to the vacuole.

**Autophagic bodies:** Single membrane vesicles inside yeast vacuoles as a result of autophagosome vacuole fusion.

### 2.6 Recycling of Degradation Products

One of the major roles of autophagy is to provide nutrients to the cell during nutrient limiting conditions. This requires not only degradation of part of cytoplasm but also effective recycling of the breakdown products to the cytoplasm. Aut4 which was later named as Atg22 was first identified to be involved in the degradation step as the mutants of Aut4 accumulated autophagic bodies in the vacuole.[33]

## 3 Autophagy in Higher Eukaryotes

The highly conserved nature of autophagy assisted in the identification of orthologs of yeast autophagy genes in mammals. As in yeast, autophagy in mammals is responsible for cellular homeostasis and quality control. Basal levels of autophagy in the cell remove misfolded proteins and damaged organelles. Induced autophagy, on the other hand, combats nutrient starvation, intracellular bacterial infection, oxidative stress, genomic damage, or accumulation of toxic protein aggregates (Fig. 2). The process of autophagy begins with the assimilation of tetrameric ULK1 complex comprising of ULK1, FIP200, Atg101, and Atg13 at the membrane nucleation site or 'Phagophore assembly site' (PAS). The ULK1 kinase activity is necessary for recruiting the Class III PI3-K complex I kinase, Vps34 along with regulatory subunits Beclin1, p150, Atg14L, and AMBRA1 at the PAS. The PI3P produced by Vps34 activity brings FVYE domain containing proteins, such as WIPI2 and DFCP1, to the nucleation site.[34, 35] Expansion of the phagophore is facilitated by Atg9 which brings membrane from various cellular organelles as well as the two conjugation systems; Atg5–Atg12–Atg16L and LC3.[36, 37] Ubiquitin like protein Atg12 is activated by E1 ligase Atg7, transferred to E2 ligase Atg10 and eventually conjugates with Atg5. The Atg5–Atg12 non-covalently binds to Atg16L and forms an Atg5–Atg12/Atg16L complex which is targeted to the PAS. The second conjugation system involves LC3, an ubiquitin like protein, which is generally present in the cytoplasm. It is cleaved by protease Atg4 to expose a C-terminal glycine which gets conjugated to phosphatidylethanolamine (PE) with the help of Atg7 and Atg3 which are E1 and E2 ligases, respectively. The PE conjugated LC3 binds to the inner and outer membranes of the expanding autophagosome.[38–40] The autophagosome cargo recognition and capture are facilitated by ubiquitin-binding adaptor proteins like p62/SQSTM1 which bind to polyubiquitinated cargo on one end and LC3 through the LC3 interacting region (LIR) on the other end.[41] Isolation membrane nucleation and elongation, cargo recognition and capture, and eventual closure result in the completion of double-membrane autophagosomes. Once completed, autophagosomes move along microtubules assisted by cytoskeletal motor proteins dynein and dynactin to fuse with lysosomes. The fusion of autophagosomes with lysosomes is mediated by small GTPases Rab7, autophagosomal SNARE Syntaxin17 (Stx17), lysosomal SNARE VAMP8, and tethering proteins of HOPS complex. Proper lysosomal function is important for autophagosome-lysosome fusion as autophagy inhibitors BafilomycinA1 and Chloroquine (CQ) inhibit fusion by affecting lysosomal pH. The end function of autophagic process is the degradation of cargo inside lysosomes by hydrolases like CathepsinB/D and recycling of biomolecules.[37, 42, 43]

**mTOR:** A protein that negatively controls autophagy.

## 4 Signaling Regulation of Autophagy

The highly conserved serine/threonine kinase mTOR (mammalian Target Of Rapamycin) senses nutrient signals in a cell and regulates its growth and division. Two complexes of mTOR, mTORC1, and mTORC2 are localized to different subcellular compartments. In the presence of amino acids and growth factors like Insulin-like growth factor (IGF), protein kinase B (PKB/Akt) is activated by phosphoinositide-dependent kinase-1 (PDK1). Akt phosphorylates TSC1 which blocks its interaction with TSC2, and hence, TSC1/2 complex is not formed which allows small GTPase Rheb to remain active. The mTORC1 complex is targeted to the lysosome by Ragulator-Rag complex where it is activated by Rheb and the active mTORC1, in turn, negatively regulates autophagy by inhibitory phosphorylation of ULK1 hence preventing ULK1 complex formation. During nutrient and metabolic stresses, the low levels of ATP in cells are sensed by AMPK which phosphorylates and activates TSC1/2 complex thereby inactivating Rheb and further mTORC1, hence allowing autophagy upregulation. AMPK also directly regulates autophagy independent of mTOR by phosphorylating and activating ULK1 independent of mTOR.[44, 45]

## 5 Transcriptional Regulation of Autophagy

Understanding the process of autophagy in an unabridged manner requires study of nuclear events that control autophagy along

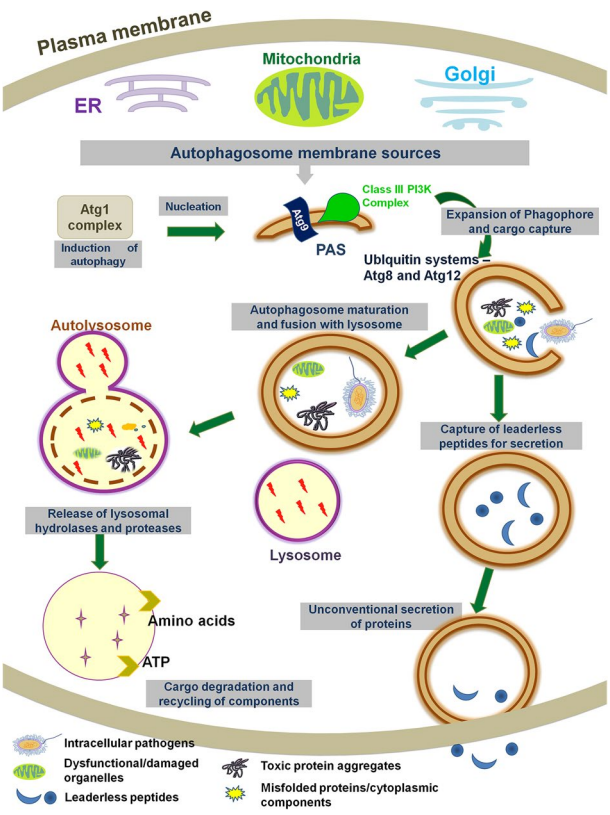


***Figure 2:*** Canonical and non-canonical autophagy flux: under basal levels, autophagy helps in maintaining the cellular homeostasis by getting rid of cellular waste and superfluous components. Stimulation through several factors, such as starvation, stress, or chemicals, leads to induction of autophagy. The initiation complex comprising of Atg1 complex and Class III PI3K complex along with several accessory proteins helps in nucleation at the site of autophagosome biogenesis also referred to as Pre-autophagosomal structure (PAS). Addition of membrane from several different sources leads to the expansion of autophagosomal membrane (phagophore). Atg9 along with accessory proteins is known to provide membrane to the developing phagophore from different sources, such as plasma membrane, endoplasmic reticulum, mitochondria, and Golgi. A ubiquitin ligase like system delivers Atg8 to the developing membrane and leads to the autophagosome expansion around the cargo and finally captures of the cargo. The cargo could be: (1) destined for degradation inside the lysosome through the canonical form of autophagy or; (2) could be secreted out of the cell through non-canonical function of autophagy referred to as unconventional protein secretion. (1) The cargo destined for degradation could comprise of cytoplasmic components like misfolded proteins, dysfunctional or damaged organelles or superfluous components under the basal levels of autophagy. However, autophagy also serves a cytoprotective role by getting rid of any intracellular pathogen or protein aggregates. The mature autophagosome along with its constituents fuses with the lysosome. Lysosomal enzymes act upon the cargo and degrade it into simpler building blocks like amino acids and ATP that are eventually pumped back into the cytosol to be reused by the cell. (2) Many newly synthesized or processed peptides could also be taken up by the autophagy machinery and delivered to the plasma membrane for secretion out of the cell. Such phenomenon of unconventional protein secretion through autophagy has been observed for several peptides that lack any conventional leader sequences for secretion.

with cytoplasmic process that unfold during autophagy. Nuclear regulation of autophagy is mediated by transcription factors, miRNAs, epigenetic marks, and histone modifications. These factors regulate both rapid and long-term responses to autophagy. More than about 20 transcription factors are now known to regulate autophagy.[46] Transcriptional regulation of autophagy can be via both mTOR-dependent and independent mechanisms. The first clue to the transcriptional regulation of autophagy came when in the yeast cells; Atg8 was found to be transcriptionally up-regulated via inactivation of the TOR signaling cascade.[16]

Studies by Settembre et al. gave new impetus to transcriptional regulation of autophagy. They

identified TFEB as the master positive regulator of autophagy. The two extensively studied major regulators of autophagy are TFEB and ZKSCAN3.[47, 48] TFEB is a basic-helix-loop-helix-leucine zipper transcription factor which is a master positive regulator of autophagy. It controls expression from nexus of genes involved in lysosome biogenesis (and function) and autophagy. It regulates the expression of genes that contain Coordinated Lysosomal Expression and Regulation (CLEAR) DNA sequences.[47] ZKSCAN3 is a zinc finger family protein that contains KRAB (KRuppel-Associated Box) and SCAN domains. Silencing of ZKSCAN3 shows induction in autophagy and lysosome biogenesis, while their presence down-regulates the expression of large array of genes involved in autophagy and lysosome biogenesis.[47, 48] TFEB and ZKSCAN3 play antagonistic role to each other in regulating expression of autophagy genes. Under nutrient rich conditions, mTORC1 in its active state phosphorylates TFEB on the lysosome membrane preventing it from entering the nucleus. This, in turn, prevents the activation of the genes harboring CLEAR DNA sequences. On the contrary, ZKSCAN3 has an antagonistic role. It is present in the nucleus where it down-regulates the expression of multitude of genes involved in autophagy and lysosome biogenesis. During starvation conditions, calcineurin dephosphorylates TFEB allowing it to enter the nucleus and positively regulate the expression of genes involved in autophagy and lysosome biogenesis. Concomitant to TFEB translocation to the nucleus, ZKSCAN3 is relocated to the cytoplasm releasing the negative control on the expression genes of autophagy and lysosome biogenesis.[49] Core autophagy genes transcriptionally regulated by TFEB are ATG4, ATG9, BCL2, LC3, SQSTM1, UVRAG, WIPI, and by ZKSCAN3 are ULK1 and WIPI, respectively.

Similarly there are other TFs, such as hypoxia inducing factor (HIF-1),[50] FOXO,[51] p53,[52] NF-κB,[53] and many others, that play a direct or indirect role in autophagy under different environmental stress conditions.

Transcriptional regulation of autophagy has also been addressed in the yeast model. Here, Ume6, Pho23, and Rph1/KDM4 are the three master transcriptional repressors of autophagy related genes in yeast.[54–56] Ume6 is associated with histone deacetylase complex which includes Sin3 and Rpd3, and negatively regulates the transcription of Atg8. Under nutrient replete conditions, the absence of any of these three components leads to an increase in Atg8, and consequently, autophagic activity is augmented. During autophagy, a protein kinase named Rim15 is responsible for phosphorylating Ume6, thereby dissociating it from Sin3 and Rpd3. The absence of Rim15 from cells leads to reduction in the synthesis of Atg8 at basal level. The authors have demonstrated Rim15 as a positive regulator of autophagy that acts upstream of Ume6 to regulate Atg8 synthesis.[54] Pho23 is another transcriptional repressor of autophagy that negatively regulates ATG9 and thus controls the frequency of autophagosome formation. It also down-regulates the expression of other autophagy-related genes, such as ATG7, ATG14, and ATG29. Studies show that deletion of PHO23 in yeast cells leads to an increase in the autophagosome formation and the number of autophagic bodies. This increase is possibly due to an increase in the levels of Atg9.[55] Rph1/KDM4 is a histone demethylase that negatively regulates the expression of ATG7, ATG8, ATG9, ATG14, and ATG29. It regulates autophagy in histone demethylase independent manner. In nutrient rich conditions, Rph1 keeps autophagy induction under check. However, under starvation, Rph1 phosphorylation by Rim15 causes partial degradation of this protein, thereby leading to induction of autophagy.[56] Thus, as in mammalian cells, yeast too has transcriptional machinery devoted to control expression of autophagy genes.

In many genetic and neurodegenerative diseases, autophagy becomes dysfunctional. Mechanisms that promote autophagy and mediate cellular clearance of toxic protein aggregates are being identified that serve as the novel therapeutic targets. For example, over expression of TFEB rescues cytoxicity of α-synuclein in rat model of Parkinson's disease[57] and also clears the polyQ Huntingtin protein.[58] Recently, HEP14 and HEP15 (small molecules) have been shown to increase biogenesis of lysosomes by activating TFEB. This increases the clearance of the cytotoxic aggregates from the cell and also increases the degradation of lipid droplets.[49] Thus, modulating the expression of TFs can help enhance autophagy which may be beneficial in alleviating disease conditions.

## 6 Autophagy in Disease

Dysfunctional autophagy is implicated in various diseases and disorders, such as cancer, intracellular infections, and neurodegeneration.

## 7 Cancer

The role of autophagy in maintaining cellular homeostasis is undeniably important and any perturbations in this can accumulate damaged organelles, oxidative stress, and misfolded proteins in a cell leading to genomic damage and even tumorigenesis. This concept was very elegantly proven in experiments with mice having deletion of essential autophagy genes like BECLIN, *ATG5*, and *ATG7* which made them prone to spontaneous tumors.[59] Beclin1 deletions were also identified in human breast, prostate, and ovarian cancer samples.[60] However, understanding the role of autophagy in cancer is not as simple as that. Autophagy can also provide survival advantage to tumor cells in a solid tumor which are facing nutrient limitation and hypoxia. Cancers, such as pancreatic and lung cancer, have been shown to have high basal levels of autophagy. On gene deletion of essential autophagy genes, tumor regression occurred in these cells. Hence, the role of autophagy in cancer is complex and requires an understanding of the stage and type of cancer. It definitely prevents the onset of tumorigenesis by limiting genomic damage but may be pro-cancer in established tumors.[61–63]

## 8 Xenophagy

Autophagy, apart from serving as a metabolic pathway providing building blocks like amino acids during conditions of nutritional stress, is also involved in degrading intracellular pathogens. The process of capturing and eliminating intracellular pathogens by autophagy is called as xenophagy. The process of xenophagy provides a broad spectrum of defense mechanism to capture bacterial, viral, and protozoan pathogens. Plethora of studies in recent times has shown that xenophagy acts as a part of innate immune system against huge number of intracellular pathogens in both phagocytic and non-phagocytic cells.

Although the conventional autophagy was discovered in 1963 by de Duve,[64] xenophagy remained unknown until electron micrographs of guinea pig polymorphonuclear leukocytes (PMNs) infected with *Rickettsiae* (Gram-negative pleomorphic bacteria) showed autophagosome like structures containing bacteria.[65] Following this, notable discoveries on xenophagy in Group A *Streptococcus*,[66] *Mycobacterium*,[67] *Salmonella*,[68] *Shigella*,[69] HIV,[70] Sindibis virus,[71] *Toxoplasma*[72] showed that xenophagy is a conventional defense mechanism of host against various pathogen types.

### 8.1 Pathogen Capture by Xenophagy

Post entry, some pathogens escape into cytosol to prevent fusion with lysosomes. This also provides them with sufficient nutrition from the cytosol to replicate efficiently.[73] These cytosolic pathogens are targeted by xenophagy machinery that captures them in double membrane vesicles (xenophagosomes) and delivers them to the lysosomes.[74]

Recognition of cargo for xenophagic capture occurs via ubiquitination of the pathogens which, in turn, is recognized by autophagy adaptor proteins like p62, NDP52, Optineurin, and NBR1. These adaptors bridge interactions with the ubiquitin and the autophagy machinery by interacting with LC3. This enables autophagosome formation around the pathogen.[75] Pathogen-specific adaptor proteins like septins (in case of *Shigella* and *Listeria*) and Tecpr1 (in case of *Shigella*) are also shown to recruit autophagy machinery to the pathogens.[76, 77]

*Salmonella enterica* serovar Typhimurium is a well-studied pathogen that gets restricted by xenophagy. Inside the host cells, *Salmonella* can reside either inside membrane bound endosomes or enter into cytosol by rupturing the endosomes. There are temporal changes in the intracellular *Salmonella* replicating niche in terms of morphology and recruitment of host factors. At later time points (6–8 h p.i), membrane bound endosomes develop into replicative vesicles for salmonella called as Salmonella Containing Vacuole (SCVs) which is characterized by its tubular structure. Adaptors like p62, NDP52, and optineurin recognize ubiquitin positive *Salmonella,* and NDP52 also recognizes galectin that are bound to damaged *Salmonella* containing endosomes. In a ubiquitin independent pathway, *Salmonella* gets captured to autophagosomes through diacylglycerol present on SCVs. Almost 25–30% of intracellular bacteria are shown to be captured by autophagosomes at early time points like 1 h post infection and the recruitment drastically falls at later points.[68] One of the speculated reasons for surpassing xenophagy is translocation of *Salmonella* virulence effectors, especially sseL which has deubiquitinase activity that could essentially prevent the ubiquitination of the pathogen. Another reason being repression of autophagy by *Salmonella* at later time points through mTOR activation.[78, 79]

The mechanism of subversion differs between pathogens. Another example is in the case of *Shigella flexneri* which causes shigellosis can escape from the phagosome/endosome and move within the host cells by directing actin polymerization

using its *vir*G gene. VirG is an outer membrane protein that accumulates on one end of the bacterium and mediates bacteria's polar movement. It is also known to be the target of autophagy machinery via interaction with Atg5. Recent studies have shown that an effector protein of *Shigella*, IcsB, acts as anti-Atg5-binding protein, by having a strong affinity for the same binding region on VirG as that of Atg5. Hence, mutants of icsB are captured by autophagosomes more rapidly.[69] Thus, although xenophagy exists, it is suppressed/subverted by most pathogens to evade detection and capture.

Impairment of xenophagy is also known to play role in the chronic infection of Crohn's disease. Genome Wide Association Studies (GWAS) have provided evidence for the contribution of two autophagy genes, ATG16L1,[80] and immunity-related GTPase M (IRGM) in the disease pathogenesis.[81] Subsequent studies show that single-nucleotide polymorphism occurring at ATG16L1 (T300A) does not impair the general autophagy process but show deficits in intracellular bacterial clearance.[82]

### 8.2 Signaling Pathways of Xenophagy

Recent studies have shed light on signaling pathways that lead to xenophagy activation even prior to ubiquitination of pathogens. Pattern recognition receptors are host proteins of immune system that recognize pathogen products initiating anti-microbial signals. These receptors could be either membrane bound (e.g., Toll-like receptors) or cytoplasmic (e.g., NOD-like receptors). Both are shown to play role in inducing xenophagy.[83, 84] IRGM is human gene shown to interact with NOD2 during infection, and together, they recruit Ulk1 and Beclin1 to initiate autophagy.[85] Similarly, membrane bound TLR4 has been shown to be involved in LPS-induced xenophagy. This activation also facilitates incorporation of VPS34 to autophagy vesicle formation.

Among other genetic factors that regulate xenophagy, TFEB, a mammalian transcription factor whose role is well studied in lysosomal biogenesis gets activated during *Staphylococcus aureus* infection in a pathogen-specific manner, while a similar effect is not seen in *E.coli* infection. In addition to lysosomes biogenesis, HLH30 (*Caenorhabditis elegans* homolog of TFEB) is also shown to induce number of autophagy genes, such as Atg2, Atg16, ULK1, among others. TFEB activation also seems to increase the tolerance to bacterial infection by prolonging the life span of infected *C.elegans* in comparison to autophagy mutants.[86]

In addition to the immediate innate response that xenophagy elicits, considerable research has been done to find its contribution to adaptive immunity in macrophages and antigen presenting cells. Atg5-deficient dentritic cells show reduced MHC class II representation of antimicrobial peptides and this, in turn, also affects the T-cell priming.[87] These cells also show reduced IL2 and interferon gamma production in response to viral infections.

These studies suggest that xenophagy is a conserved innate immunity pathway that pathogens evade to establish infection. Thus, enhancing xenophagy that rescind the block imposed by the pathogens would enhance the host immunity to fight against infectious agents. In this direction, screening for compounds that could enhance clearance of intracellular pathogens by xenophagy has been done for pathogens like *Toxoplasma* and *Mycobacterium*.[88, 89]

## 9 Aggrephagy

One of the hallmarks of life threatening neurodegenerative diseases is neuronal death caused by accumulation of misfolded toxic protein aggregates, such as α-synuclein, β-amyloid, huntingtin polyQ repeats, FUS, and TDP43. Cellular proteostasis involving the clearance of superfluous cellular organelles and other cargos, including toxic proteins, is maintained through the chaperones, the Ubiquitin–Proteasome System (UPS), and the autophagy pathways.[90] Chaperone and UPS functions are choked by the misfolded protein aggregates. Misfolded proteins are substrates for autophagy.[91] A selective autophagy pathway, aggrephagy, is a cellular degradation mechanism to clear the toxic, misfolded proteins. Recent studies highlight the importance of autophagy in maintaining organismal homeostasis. Brain-specific autophagy knockout mice (Atg5) accumulate p62 protein aggregates in neurons, and subsequently manifest neurodegenerative phenotypes, illustrating the vital role of basal autophagy for aggregate clearance.[92]

Autophagy is dysfunctional in neurodegenerative disease pathologies.[91] Thus, restoring autophagy through pharmacological approaches using small molecules has been reported to have beneficial neuroprotective effects.[93–95]

## 10 Non-canonical Roles of Autophagy

Besides the canonical role of cellular homeostasis and degradation, autophagy process also has some moonlighting functions which are underexplored. Involvement of autophagy machinery is seen in several contexts which do not involve capture and delivery of the cargo to the lysosome for degradation via a double membraned autophagosome. Such non-canonical autophagy processes include LC3-Associated Phagocytosis (LAP) and autophagy mediated unconventional protein secretion are two such examples. These non-canonical functions were explicitly put forth in a recent review by the pioneers in the field.[96] Some of the pleiotropic functions of autophagy include their role in cell survival and apoptosis, cellular transport, secretion, signaling, transcriptional and translational responses, membrane organization, and microbial pathogenesis.

The non-canonical roles can be looked upon from two diverse perspectives:

1. As macroautophagy involves formation of vesicles and membranous structures, these could be harnessed by other cellular and non-cellular processes.
2. Moonlighting functions of Atg proteins.

### 10.1 Harnessing Autophagy Machinery for Other Cellular Processes

The prime role of autophagy is turnover and is accompanied by the process of dynamic membrane biogenesis.[97, 98] The double layered autophagosome membrane formation to entrap cargoes is an orchestrated, dynamic process with the involvement of several Atg proteins and requires PI3-K activity. This property has been elegantly exploited by the pathogens that infect mammalian cells. Virus and bacteria have evolved mechanisms not only to evade the degradative action of autophagy but also to hijack the host autophagy machinery for their multiplication. In this section, we will focus only on the non-canonical role of autophagy proteins in microbial pathogenesis. LC3 in mammals mediates the recruitment of the substrates onto the autophagosomes via their LC3-interacting regions (LIR). Some of the examples that utilize the Atg proteins besides their degradative functions are discussed below:

1. Influenza A virus redirects LC3-conjugated membranes meant for autophagy to the cell surface for budding of stable viruses.[99] The ion-channel matrix protein of the virus (M2) recruits the central player of autophagosomal membrane or the landing pad of cargo receptor, LC3, inhibiting the fusion to lysosomes, thereby aiding in the transport of virions to the plasma membrane.[100]
2. In *Mycobacterium tuberculosis* infection, Atg5 is found to play a unique role of protection by preventing PMN-mediated immunopathology. Knockout studies support an additional, ATG16L1 independent role of ATG5 in protecting the mice from *M. tuberculosis* infection.[101]
3. Another study from an unbiased siRNA screen has indicated the involvement of ATG13 and FIP200 in the picornavirus replication that is independent of their canonical autophagy functions.[102] The host and the viruses exploit the autophagy machinery along with the autophagy-related membranous structures to either restrict or enhance viral replication that is non-canonical of the autophagy functions. Autophagy proteins, including Beclin1, LC3, Atg4B, Atg5, Atg7, and Atg12, positively regulate the Hepatitis C viral replication,[103] whereas in murine norovirus, some of the autophagy proteins are required by the IFN-γ activated macrophages to inhibit viral replication complex.[104] Non-involvement of ULK complex distinguishes the non-canonical from canonical autophagy.[105] There is a general notion that a single ATG gene deletion leads to specific block in the autophagy process, but the above-mentioned examples provide evidence that the Atg proteins also exhibit many of the non-canonical roles during viral infection.[106]
4. In Mouse Hepatitis Virus (MHV) infection, as unlipidated LC3 (LC3-I) promotes viral replication in Double-Membrane Vesicles (DMVs) without utilizing ATG5[107] and LC3-II,[108] it suggests that the canonical autophagy is not involved. Detailed analysis of the vesicles indicates that the DMVs are another LC3-presenting membrane that is distinct from the canonical double membrane autophagosomes.
5. Zikavirus, a member of the Flaviviridae family, causes microcephaly affecting the central nervous system.[109] This virus produces a variety of intracytoplasmic inclusions termed as "virus factories" in the infected cells. The zika virus infected skin fibroblasts demonstrate that the virus not only blocks the autophagic flux but also hijacks the

**Virions:** Virus particles.

**Non-canonical autophagy:** Moonlighting functions of autophagy such as those involved in protein secretion.

**Macroautophagy:** An intracellular mechanism to capture, degrade and recycle unwanted, damaged or surplus cytoplasmic materials. Commonly referred as autophagy.

autophagic machinery for its own replication.[110, 111]

In all the above examples, we see that the ability to form membrane structures of the autophagy proteins is being exploited by the virions to promote their viral budding and replication, thereby aiding in their survival and infection.

### 10.2 Moonlighting Functions of Atg proteins

(i) Role in Unconventional Protein Secretion

Beyond its role of cellular self-eating and homeostasis, autophagy proteins also play an important role in unconventional protein secretion whose mechanism is not well elucidated.

The conventional secretory proteins enter endoplasmic reticulum via signal peptides, whereas the unconventional secretory proteins destined for secretion follow an alternate trafficking route. The process by which proteins that are devoid of canonical leader sequence still get secreted is termed as unconventional protein secretion.

Extensive studies of two main cargoes studied till this date have provided us clues on autophagy-mediated unconventional protein secretion.

1. First, the secretion of mature cytokine, IL1-β, is found to be controlled by the process of autophagy.[112] Its secretion is presumed to involve Rab proteins and MVBs.[113] The matured form of the IL1-β is released outside the cell after cleavage from its precursor form. Although Caspase-1 mediated IL1-β release is reported, elegant studies by Zhang et al, 2015 have demonstrated that the translocation of the unconventional secretory protein, IL1-β into a secretory vesicle, is mediated by autophagy, multivesicular bodies (MVBs), and Golgi-associated proteins (Golgi Reassembly Stacking Protein-GRASPs).
2. The second cargo is the Acyl-CoA-binding protein (Acb1) that gets secreted outside the cell by unconventional protein secretion upon starvation in yeast. Genetic studies in yeast[114] have demonstrated that Acb1 is unconventionally secreted via vesicles and are captured in a new compartment called CUPS (Compartment for Unconventional Protein Secretion).[115] These studies in yeast have revealed that the core autophagy machinery is a necessary requisite for autophagosome construction, suggesting that secretory autophagosomes must be formed. This secretion is found to be GRASP-dependent and autophagy-mediated, and plays an important role in peroxisome biogenesis providing some clues on membrane source for autophagosome biogenesis.[116]

Multiple lines of evidence demonstrate the interplay of autophagy and unconventional protein secretion in the clinical and pathophysiological context.

1. The GRASP-dependent unconventional secretion of CFTR, the Cystic Fibrosis Transmembrane conductance Regulator, demonstrates a physiological relevance of unconventional protein secretion in the cystic fibrosis disease. Autophagy-mediated trafficking of CFTR leads to proper insertion of the protein to the plasma membrane, whereas the transgenic overexpression of GRASP rescued the phenotype of the ΔF508-CFTR mice.[117]
2. Autophagy plays a significant role in polarized secretion of lysosomal contents in osteoclastic bone resorption.[118]
3. Impairment of autophagosome–lysosome fusion promotes tubulin polymerization-promoting protein (TPPP/p25α) to secrete α-synuclein, the hallmark protein of Parkinson's disease, in an unconventional manner.[119]
4. Another unconventionally secreted protein, Insulin Degrading Enzyme (IDE), was found to be mediated through autophagy-based unconventional secretion upon statin induction[120] and also has disease relevance in Alzheimer's disease.[121]
5. Secretion of β-amyloid aggregates formed in the Alzheimer's disease is also mediated by autophagy. Knockout studies in mice neuronal Atg7 was found to influence the β-amyloid secretion thereby affecting the plaque formation, a pathological hallmark of AD.[122]
6. Atg16L1 not only regulates cellular autophagy but also acts as Rab33A effector by secreting the hormone from the dense core vesicles of the neuroendocrine PC12 cells.[123] Another example of the combined role of Atg5, Atg7, Atg4B, and LC3 is observed in the polarized secretion of lysosomal contents (cathepsin) in the osteo-

clasts.[118] Defects in Atg4B and Atg5 in mice are found to manifest balance related disorders due to deficient secretion of otoconins by vestibular sensory cells in the inner ear.[124, 125]

(ii) Role in cell division:

The non-canonical role of autophagy proteins has gained significance, especially in microbial pathogenesis. The functional importance of localization of *Pf*Atg8 to apicoplast, a four membrane-bound non-photosynthetic plastid, provides clue for non-canonical function of autophagy in *Plasmodium falciparum.*[126] In the apicomplexan parasite *Toxoplasma gondii*, *Tg*ATG8 is vital for normal replication of the parasite inside the host cell. Recent studies have demonstrated that another key role of apicoplasts bound *Tg*ATG8 is involved in centrosome-driven inheritance of the organelle during cell division.[127]

In the Zika virus infected patients, microcephaly is brought about by the abnormal function of centrosomes affecting neural brain development.[128, 129] As this process is coupled with hijacked autophagy machinery, it is presumed that autophagy proteins are probably involved in cell division too.

(iii) Role in inflammatory disease control:

The LC3-Associated Phagocytosis (LAP) is one of the prime non-canonical functions of autophagy that is required for effective clearance of apoptotic cells.[130] In canonical autophagy, LC3 conjugates to the autophagosomal membranes facilitating maturation upon fusion with lysosomes. Rubicon, a Beclin-1-binding protein, is found to be required for LAP but not for canonical autophagy.[131] In Systemic Lupus Erythematosus (SLE), the pathogenesis is brought about by the defects in clearance of dying cells. LAP is found to inhibit autoinflammatory responses caused by dying cells implicating its link in inflammatory disease control of SLE.[105] Even in viral RNA-mediated infection, the immunostimulatory RNA (isRNA)-mediated type I interferon production is negatively regulated by the Atg12–Atg5 conjugate[132, 133] demonstrating its suppressor activity in the innate antiviral immune signaling aiding cell survival.

Studies reveal interplay between inflammasomes (multiprotein complex that activates caspase-1) and autophagy. While autophagy negatively regulates inflammasome activation, autophagy induction is dependent on the presence of specific inflammasome sensors. Autophagosomes degrade inflammasomes via the selective autophagic receptor p62 and autophagy plays a role in the biogenesis and secretion of the proinflammatory cytokine IL-1β.[134–138]

The involvement of the adaptor protein, ATG16L1, in the inflammatory bowel disease (Crohn's disease) is characterized by dramatic increase in commensal bacteria.[139] Deletion studies in ATG16L1-WD repeat domain and T300A mutant of mouse embryonic fibroblasts did not affect xenophagy or the normal autophagic function indicating its differential role in Crohn's disease.[140]

(iv) Role in lipidogenesis and development:

Lipid droplet formation in mammalian white adipocytes involves massive cytoplasmic remodeling within the cells. Besides the conventional roles in autophagy, several autophagy genes have been implicated to have "non-autophagy roles". For example, Atg2 and LC3 are also involved in lipid droplet biogenesis in mouse hepatocytes and cardiac myocytes,[141, 142] while knockout studies in mice for Atg5 and Atg7 have revealed their additional roles in adipogenesis.[143, 144] The mice fed with high fat diet in the Atg12 lacking pro-opiomelanocortin expressing neurons exhibited aggravated obesity which demonstrates an auxiliary function of Atg12 in diet-induced obesity.[145] In addition, Atg5-independent non-canonical autophagy generates autophagosomes in a Rab9-dependent manner. Such Atg5-independent autophagy is found to be required for iPSC reprogramming that mediates mitochondrial clearance.[146]

The versatility of the autophagy proteins in all the cellular processes opens new avenues to explore its moonlighting functions. It is imperative to understand the discrete functions of the autophagy proteins besides their central role in degradation and cellular homeostasis.

### *10.3 Open Questions in Autophagy*

Although the field has garnered much interest now with the award of the Nobel Prize to Prof. Yoshinori Ohsumi for his contributions to understanding the mechanism of autophagy, several autophagy-related frontiers remain unchallenged. Questions pertaining to understanding basal autophagy and the mechanisms that regulate it are still open. How various intracellular membrane sources contribute to autophagosome biogenesis and the factors that

govern autophagosome size and number is still an active area of research. In spite of identification of a conserved set of core autophagy proteins, their actual roles in autophagosome construction and mechanisms regulating autophagosome-lysosome fusion are not clear. The contribution of autophagy in cell death is controversial and the case of "cell death by over eating oneself" is highly debatable.[147, 148] Finally, restoration of impaired autophagy in several disease states via small molecule autophagy modulators has been shown to be promising in many cases, but bonafide and exclusive modulators are still elusive. Discovery of such small molecules will not only further our understanding of autophagy flux but will also fuel the tremendous therapeutic potential autophagy holds.

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**Sarika Chinchwadkar** is an IntPhD student doing her Masters thesis on novel genes involved in autophagy.

**Sreedevi Padmanabhan** is currently working as a SERB-National Post-Doctoral Fellow at the Autophagy Laboratory working on unconventional secretion.

**Piyush Mishra** did his M.Sc. in biomedical sciences from ACBR, Delhi University. Piyush is developing assays to study autophagy-related pathways and wants to apply them to discover new drugs (small molecules).

**Sunaina Singh** is an IntPhD student. Currently, she is working on the moonlighting functions of vesicular trafficking pathways in autophagy in yeast.

**S. N. Suresh** is an IntPhD student. His interests include studying neurodegenerative diseases using yeast as model organism.

**Somya Vats** is an IntPhD student interested in using small molecules as probes to understand autophagy flux.

**Gaurav Barve** completed his M.Sc. in microbiology from the Department of Microbiology, University of Pune. His interest lies in studying involvement of cytoskeletal elements in autophagy.

**Veena Ammanathan** is an IntPhD student studying xenophagy.

**Dr. Ravi Manjithaya** received his Ph.D. degree in posttranscriptional gene regulation from the Indian Institute of Science (Advisor: Prof. Rajan Dighe). He did his postdoctoral training in the autophagy-related pathways at the University of California, San Diego (Mentor: Prof. Suresh Subramani) before joining JNCASR as a Faculty Fellow in 2011. He was a Wellcome Trust-DBT Intermediate fellow (2011–16).