Insights into the regulatory mechanisms of xenophagy as revealed by chemical genetics

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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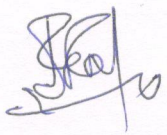
September 2020

CERTIFICATE

This is to certify that work described in this thesis entitled **"Insights into the regulatory mechanisms of xenophagy as revealed by chemical genetics"** is the result of investigations carried out by Ms. Veena Ammanathan in Autophagy laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my guidance and that the results presented here have previously not formed the basis for the award of any other diploma, degree or fellowship.



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DECLARATION

I hereby declare that the thesis entitled **"Insights into the regulatory mechanisms of xenophagy as revealed by chemical genetics"** is an authentic record of the research work carried out by me, under the guidance of Dr. Ravi Manjithaya at Autophagy laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore.

In keeping with the norm of reporting scientific observations, due acknowledgements have been given whenever work based on the findings of other investigators has been cited. Any omission owing to oversight or misjudgement is regretted.

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Acknowledgements

I would like to thank Dr. Ravi Manjithaya for guiding and supporting me in shaping my PhD project. He had been an encouraging mentor and always had confidence in my work. My project was like a puzzle that I enjoyed solving with his guidance. He was a huge inspiration for me to continue PhD after completing M.S in his laboratory. This has helped me in realizing my interest and potential in Research. Apart from project work, he has been very keen in motivating his students to learn various other facets like scientific writing, public speaking and many more that develops a student to fit well in the field. In whatever career I pursue in future, it will be a reflection of many things that I have learnt from him. I am also grateful to his family members- his mother, his wife Ms. Aparna Hebbar and Aditya for being a kind host during lab parties and for the memorable lab trips 😊.

I am extremely thankful to my lab members – Dr. Gaurav Barve, Dr. Sreedevi P, Dr. Piyush Mishra, Dr. Sunaina, Dr. Shashank, Dr. Aravind, Dr. Suresh, Dr. Malini, Dr. Somya Vats, Dr. Lakshmi Garimella, Dr. Mamta, Dr. Vidyadhara, Vidhya Jadhav, Lalitha and Viswa for having been wonderful seniors. We all have shared quality time in both discussing work as well as fun times during multiple lab trips and movie breaks. The good times continue with enthusiastic juniors- Akshaya, Mridhula, Cuckoo, Anushka, Ruchika, Aarti, Irine, Tejal, Dr. Keerti, Dr. Jayaprakash, Dr. Sajan, Tharun, Kaushik, Gokul, Neha, Harshadeep, Mallika, Gresshma, and Shahina. Their inputs have helped me to improve my work and presentation skills. Their presence also contributed greatly to the positive work environment.

I am grateful to Dr. Srikanth, RCB for sharing S. typhimurium strains that have been extremely critical for my work. I am also thankful to Dr. Deepak Saini and his student Mr. Vignesh N for helping me in live-cell microscopy experiments done during my M.S tenure. I would also like to thank Dr. Jayanta Haldar and his student Paramita Sarkar from JNCASR as well as Dr. Suresh Basagoudanava and his student Ranjitha from IVRI for their support in collaboration projects. Special thanks to Dr. Subba Rao, IISc for his suggestions and for helping us by providing reagents during the revision of manuscript.

I would like to thank all the MBGU and NSU faculty members of JNCASR for their coursework and valuable insights during annual review presentations. I am thankful to Dr. Ranga Uday kumar and Dr. James for their suggestions in improving my work.

It is always good to know there are a few people whom we can always depend on. In that way, I am lucky to have to Gaurav Barve, Sreedevi P, Akshaya, Mridhula and Cuckoo for being a constant source of encouragement and support. All the times we spent together in coffee and dinner times, shopping and movie breaks, jogging and health plans that we all ventured into, will always stay fresh in mind. I remember reading somewhere that "be happy that it happened rather than being sad its over!". I think it fits very well for us. As we all get busy settling in our careers, I will always look forward for an opportunity for a get together 😊😊.

I would like to thank my batchmates- Bhavna, Siddarth, Pallabi, Priya and Shubangini for all the fun times and serious group studies in the first year of our course. Next, I express my sincere gratitude towards my JNCASR friends – Aparna Dongre, Reshmi, Ruchika, Rishav, Pallabi, Bhavna, Siddarth and other JNCASR friends for sharing reagents, dinner and movie outings and many more. I would also

like to thank all my school and college friends for being there whenever I needed them.

Last but not the least, I would like to thank my parents and sister. They have borne the tough and difficult side of me during PhD. Without their support, I would not have reached till here. I am extremely grateful for their encouragement and dedicate my work to them. They are truly my anchor. I would also like to thank my grandparents, brother-in-law, in-laws and my husband Rakesh for their support. While others consciously supported, my little nephew Avish with his charming presence did the job. Born on the same year I joined Autophagy lab, every day was a little extra special with his smile 😊😊.

Finally, I am extremely happy for the experience and life lessons I gained these years with the support of the all the people mentioned and also thank the Almighty God for this destined opportunity.

Abbreviations

μm	micrometre
μM	micromole
A_{600}	Absorbance at 600 nm
OD	Optical density
ATG	AuTophaGy related
3-MA	3-methyladenine
ASC1	Apoptosis-associated speck-like protein containing a CARD
BafA1	Bafilomycin A_1
CFU	Colony-forming units
CLEAR	Coordinated Lysosomal Expression And Regulation
CD	Crohn's disease
DQ-BSA	Dye quenched-bovine serum albumin
EEA1	Early endosome antigen 1
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
FM4-64	Pyridinium,4-(6-[4-{diethylamino}cphenyl]-1,3,5-hexatrienyl)-1-(3[triethylammonio] propyl)-dibromide;
GFP	Green fluorescent protein

HTS	High throughput screening
IF	Immunofluorescence
IKKα	Inhibitor of nuclear factor-κB (IκB) kinase
IRGM	Immunity-related p47 guanosine triphosphatase
LAMP1	Lysosomal associated membrane protein 1
LC3	Microtubule associated protein 1 light chain 3
MAPK	Mitogen-activated protein kinase 1
MOI	Multiplicity of infection
mTOR	mechanistic Target of rapamycin kinase
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NOD1/2	Nucleotide-binding oligomerization domain ½
NDP52	Nuclear dot protein 52
OPTN	Optineurin
RFP	Red fluorescent protein
RLU	Relative luminescence units
SCVs	*Salmonella*-containing vacuoles
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean

TBK1	TANK binding kinase 1
TFEB	Transcription factor EB
TGF-β	Transforming Growth Factor- β
ULK1	Unc-51 like autophagy activating kinase
WB	Western blotting

Thesis synopsis

Insights into the Regulatory Mechanisms of Xenophagy as Revealed by Chemical Genetics

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Insights into the Regulatory Mechanisms of Xenophagy as Revealed by Chemical Genetics

Introduction

Macroautophagy (herewith autophagy) is an intracellular process in which a part of the cytoplasm is sequestered in double membrane vesicles called autophagosomes that eventually fuse with lysosomes resulting in degradation of its contents. This process occurs at basal level in normal cells whereas it gets induced under certain conditions like nutrient starvation, hypoxia, and infection. The cargo for degradation could be cytoplasmic long lived proteins, aggregated proteins, damaged or superfluous organelles, or intracellular pathogens (Morishita and Mizushima, 2019). The specialized type of autophagy involved in capturing and clearing of intracellular pathogens is known as xenophagy. The process of xenophagy provides defence mechanism against a broad spectrum of intracellular pathogens such as bacteria, virus and parasites (Jo et al., 2013).

Aim and scope of the study:

Xenophagy functions as a part of innate immune system eliminating a wide range of intracellular pathogens like bacteria, viruses and protozoans. However, pathogens have evolved ways to subvert xenophagy to facilitate their survival (Birmingham et al., 2006). Additionally, depending on the pathogen studied, the mechanism of xenophagy evasion differs mediated by the virulence genes of the pathogen. For example, bacterial infections predominantly evade capture by autophagosomes or prevent fusion with lysosomes whereas viral infections utilize autophagosomes as replicating niche. Hence, restoration of xenophagy block imposed by pathogens through genetic or pharmacological methods would enhance the clearance of intracellular pathogens and provide insights into host-pathogen mediated molecular events that control xenophagy (Kuo et al., 2015). Although investigations addressing the xenophagy mediated pathogen clearance are well-studied in the past few decades, molecular pathways that regulate the process are still underexplored.

With this brief introduction, the objectives for the current study are as follows,

1. Screening and validating small molecule chemical modulators that induce xenophagy.
2. Investigations on the mechanism of action of the compound to induce xenophagy.
3. Identifying the upstream regulatory pathways that impinge on xenophagy.

4. Development and standardization of high throughput xenophagy screening

The chapters are divided to accommodate the above mentioned objectives in the following manner.

Chapter 1: General introduction about autophagy and xenophagy.

Chapter 1 is the review of literature about the process of autophagy and xenophagy. In addition, the role of autophagy in health and disease with emphasis on intracellular infections will be discussed. Further, host-pathogen interactions that modulate xenophagy and the evasion strategies employed by the pathogens will be elaborated. Importantly, various genetic and pharmacological approaches to modulate xenophagy will be discussed in detail. Finally, the current status and the future perspective of the field will be summarized.

Chapter 2: Materials and methods

In this chapter the materials and methods used in this study are discussed. The materials used include lists of strains, plasmids and primers. The methods section contain details about the pilot screening of the compounds, HTS screening, wide-field and live cell fluorescence microscopy, immunofluorescence, electron microscopy, western blot analysis and gene expression analysis. In addition, the image processing, image quantitation and statistics details are described.

Chapter 3: Acacetin decreases *Salmonella* replication

Chapter 2 describes the pilot-scale screening of xenophagy inducers to decrease intracellular *Salmonella typhimurium*. The chapter also highlights the validation of the selected xenophagy inducer, acacetin. Further, the results pertaining to acacetin action are discussed. Acacetin decreases the intracellular *Salmonella* replication in cell lines such as epithelial and macrophage cell lines. Acacetin increases the recruitment of xenophagy proteins to intracellular bacteria enhancing cargo recognition. In addition to the xenophagy induction, acacetin induces general autophagy in mammalian cells. Results describing the autophagic flux and involvement of critical autophagy regulatory pathways like mTOR, ERK modulated by acacetin are discussed.

Chapter 4: Mechanism of action of acacetin.

Chapter 3 discusses the results pertaining to the mechanism of acacetin action. Investigations on cargo recognition mediated by acacetin, revealed enhanced phosphorylation of autophagy adaptor protein, p62 (SQSTM1). Additionally, fluorescent and electron microscopy images revealed increase in lysosomal biogenesis and autophagy. Following this observation, the involvement of crucial transcriptional regulator, TFEB in acacetin mediated autophagy induction is studied. Further, we show that induction of TFEB during *Salmonella* infection is beneficial, as it helps the host cells by increasing the proteolytic activity of lysosomes. This leads to decrease in the replication of *Salmonella* in *Salmonella* containing vacuoles (SCVs). The results obtained from *in cellulo* based assays were also verified *in vivo* mouse model of infection.

Chapter 5: Identification of upstream regulators of TFEB.

Chapter 4 describes the attempts made to identify the upstream regulator of TFEB. Preliminary results of gene expression analysis of acacetin treated cells will be discussed. Shortlisted candidate genes from gene expression analysis were tested for its ability to induce TFEB nuclear translocation in an image-based assay. Future work aimed at validating the putative regulators of TFEB obtained from the study will be discussed.

Chapter 6: Development of HTS to identify novel compounds.

Chapter 5 describes the development of HTS for xenophagy to identify novel compounds that regulate xenophagy. The pilot-scale screening described in chapter 1 was scaled up to test a large number of compounds/genes for their xenophagy effect. The development of HTS involves the generation of luminescent *Salmonella*, standardization of infection assay on 96-well microtitre plates. The proof-of-principle screening was carried out using a custom-made small molecule library comprising of 410 compounds was carried out. The results and future work pertaining to identification of novel xenophagy inducers will be elaborated.

Chapter 7: Discussion and future directions

Chapter 6 summarises the overall xenophagy-pathogen insights obtained by the study explained in the preceding chapters. Briefly, we have identified a novel small molecule modulator of xenophagy, acacetin. The compound functions by activating a transcriptional regulator, TFEB which is regulated by its phosphorylation status to induce lysosomal biogenesis and autophagy. Interestingly, the results of this study indicate that TFEB is kept transcriptionally inactive post *Salmonella* infection. Acacetin treatment during infection increased the proteolytically active lysosomes inhibiting intracellular *Salmonella* replication. We attempted to identify the upstream regulator of TFEB by gene expression analysis. The future work of the study includes validation of putative genes/pathways shortlisted from the gene expression analysis for its ability to modulate TFEB and xenophagy.

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Publications arising from this study:

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1. Restriction of intracellular Salmonella replication by restoring TFEB-mediated xenophagy.

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Sarika Chinchwadkar, Sreedevi Padmanabhan, Piyush Mishra, Sunaina Singh, S. N. Suresh, Somya Vats, Gaurav Barve, **Veena Ammanathan** and Ravi Manjithaya. J. Indian Inst. Sci. A Multidisciplinary Reviews Journal ISSN: 0970-4140 Coden-JIISAD 2017.

Patents:

1) Indian Patent Application No. 6596/CHE/2015 Title: Modulator and modulation of autophagy and applications thereof. Indian patent application filed.

Table of contents

Chapter 1

Introduction

Chapter 1

Introduction

1.1 Overview

Cells maintain homeostasis by a continuous process of synthesis and degradation of their constituent proteins and organelles. This facilitates the cell to remain as a dynamic entity responding rapidly to changing extracellular environment. The landmark discovery of lysosomes in 1955 by Prof. Christian de Duve led to the identification of pathways by which the cell degrades proteins and organelles (De Duve et al., 1955; De Duve and Wattiaux, 1966). The two major intracellular degradation systems include the Ubiquitin-Proteasome System (UPS) and macroautophagy.

UPS is a two-step process for degrading short-lived and soluble proteins in eukaryotes. It involves tagging of a substrate protein with multiple ubiquitin molecules through covalent attachment and subsequent degradation of the tagged protein by 26S proteasome. It is a tunnel-shaped protein complex made up of a 20S core particle and two 19S cap protein subunits. The process involves the action of three enzymes to conjugate ubiquitin to the lysine residues of the substrate- E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase). The ubiquitinated proteins are recognized by 19S cap protein and the proteolysis occurs in 20S core subunit (Myung et al., 2001).

Macroautophagy is an intracellular degradation process in which a part of the cytoplasm is sequestered in double-membrane vesicles called autophagosomes that eventually fuse with

lysosomes resulting in degradation of its contents (De Duve and Wattiaux, 1966). The process is constitutively active at basal levels whereas gets induced under conditions such as nutrient starvation, infection and hypoxia. The cargo for degradation could be cytoplasmic long-lived proteins, aggregated proteins, intracellular pathogens, damaged or superfluous organelles. It is an evolutionarily conserved process that is indispensable for maintaining cellular homeostasis (Meijer et al., 2007). The degraded products like amino acids and basic building blocks are recycled back to the cytoplasm and participate in cellular anabolic processes (Rabinowitz and White, 2010).

1.2 Autophagy: a historical perspective

The process of autophagy was first observed in electron micrographs (EM) of rat liver cells containing mitochondria encapsulated within 'dense bodies' containing lysosomal enzymes (Novikoff et al., 1956). Parallelly, other groups (Ashford and Porter, 1962; Clark, 1957; Novikoff and Essner, 1962) also observed cytoplasmic contents isolated in membranous compartments which were also positive for lysosomal enzymes. The process was termed as 'autophagy' by Christian de Duve in 1963 meaning "self-eating" (auto- self; phagy- eating) at the Ciba Foundation Symposium on lysosomes. Following this, Arstila and BF Trump spotted double-membranous structures containing cytoplasmic contents without hydrolytic enzymes indicating the existence of autophagosomes before lysosomal fusion (Arstila and Trump, 1968). Subsequent studies identified various signalling cues that induce the process. This includes induction by glucagon, nutrient starvation, and insulin levels emphasizing the strong relationship between autophagy and cellular metabolism (Deter et al., 1967; Mortimore et al., 1983). Among the first mechanistic insights into the process were the discoveries of autophagy inhibitors such as 3-methyl-adenine and wortmannin by their inhibitory activity on phosphatidylinositol 3-kinase (Blommaart et al., 1997; Seglen and Gordon, 1982). Despite

multiple studies, the molecular players involved in autophagy was not known. In this regard, the breakthrough discovery was made in the early 1990s by Prof. Y. Ohsumi's group where yeast was used as a model system to study autophagy (Takeshige et al., 1992). Using a yeast genetic screen, the group identified mutants that failed to accumulate autophagic bodies during starvation. Analyses of these mutants revealed 15 autophagy-related genes. Parallel studies by Klionsky's group identified Cytoplasm-to-vacuole targeting (Cvt) pathway which shares molecular players with macroautophagy. Cvt pathway is now considered as selective autophagy of vacuolar enzymes, aminopeptidase and mannosidase (Harding et al., 1995). A unified nomenclature was framed for naming the autophagy-related genes as ATGs. Furthermore, other ATG genes, as well as ATG orthologues in higher eukaryotes, were reported subsequently showing that the process is evolutionarily conserved (Kabeya et al., 2000; Meijer et al., 2007; Reggiori and Klionsky, 2002). Key discoveries in this regard that shifted the focus to mammalian autophagy include identification of Atg5-Atg12 conjugation system in mammals (Mizushima et al., 1998a; Mizushima et al., 1998b) and identification of homologue of yeast Atg8 referred to as microtubule-associated protein 1 light chain 3 (MAP1LC3, hereafter LC3) (Kabeya et al., 2000). LC3 serves as the sole marker for the process involved from autophagosome biogenesis to degradation. Hence, various assays follow LC3 for monitoring autophagic flux. Further studies showed that there are at least six Atg8 homologues present in mammalian cells: three members of LC3 subfamily and three members of gamma-aminobutyric receptor-associated protein (GABARAP) subfamily (Kabeya et al., 2004).

These landmark discoveries have contributed immensely to delineating the process in terms of both molecular details as well as its crosstalk with cellular metabolism. The 2016 Nobel Prize in Physiology or Medicine was awarded to Prof. Yoshinori Ohsumi for the discoveries in mechanisms of autophagy.

1.3 Types of autophagy

There are three types of mechanistically distinct autophagy seen in eukaryotic cells: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA) (**Figure 1.1**).

Microautophagy involves direct engulfment of cytoplasmic contents at the lysosomal surface by invagination, protrusion and septation. EM studies have revealed the extension of the lysosomal membrane to enwrap a "micro" portion of the cytoplasm (De Duve and Wattiaux, 1966; Oku and Sakai, 2018). A similar observation was made in yeast *Pichia pastoris* to degrade peroxisomes in glucose-rich media. Finger-like projections from vacuoles are seen to capture clusters of peroxisomes (Tuttle and Dunn, 1995). Genetic screens have identified molecular players involved in the process such as Atg18 (Guan et al., 2001) and Vac8 (Wang et al., 1998) both of which are needed for vacuolar membrane protrusion and proteins such as Atg4, Atg8 and Atg24 are required for regulating the process (Ano et al., 2005; Tamura et al., 2010).

On the other hand, CMA is a selective form of autophagy for a pool of cytosolic proteins. This selectivity is achieved by the presence of CMA-specific amino acid motif, KFERQ in the substrates. The process begins by unfolding of the substrate proteins by a cytosolic chaperone, Hsc70 (heat shock cognate protein 70KDa) and its co-chaperones such as Bag1, Hip and Hop (Cuervo and Dice, 1996). Following this, substrates are targeted to lysosomal surface initiating LAMP2A (Lysosome-associated membrane protein 2A) multimerization and CMA translocation-complex formation. Eventually, the substrates are translocated across the lysosomal membrane for degradation (Tekirdag and Cuervo, 2018). Although autophagy is conserved across all eukaryotes, CMA is observed only in mammals as LAMP2A is considered as CMA receptor which is absent in yeast, worms, and flies.

Among the other types, the macroautophagy (herein autophagy) is the well-studied process. It is a bulk degradation of cytoplasmic contents captured in double-membrane autophagosomes fusing with lysosomal compartments.

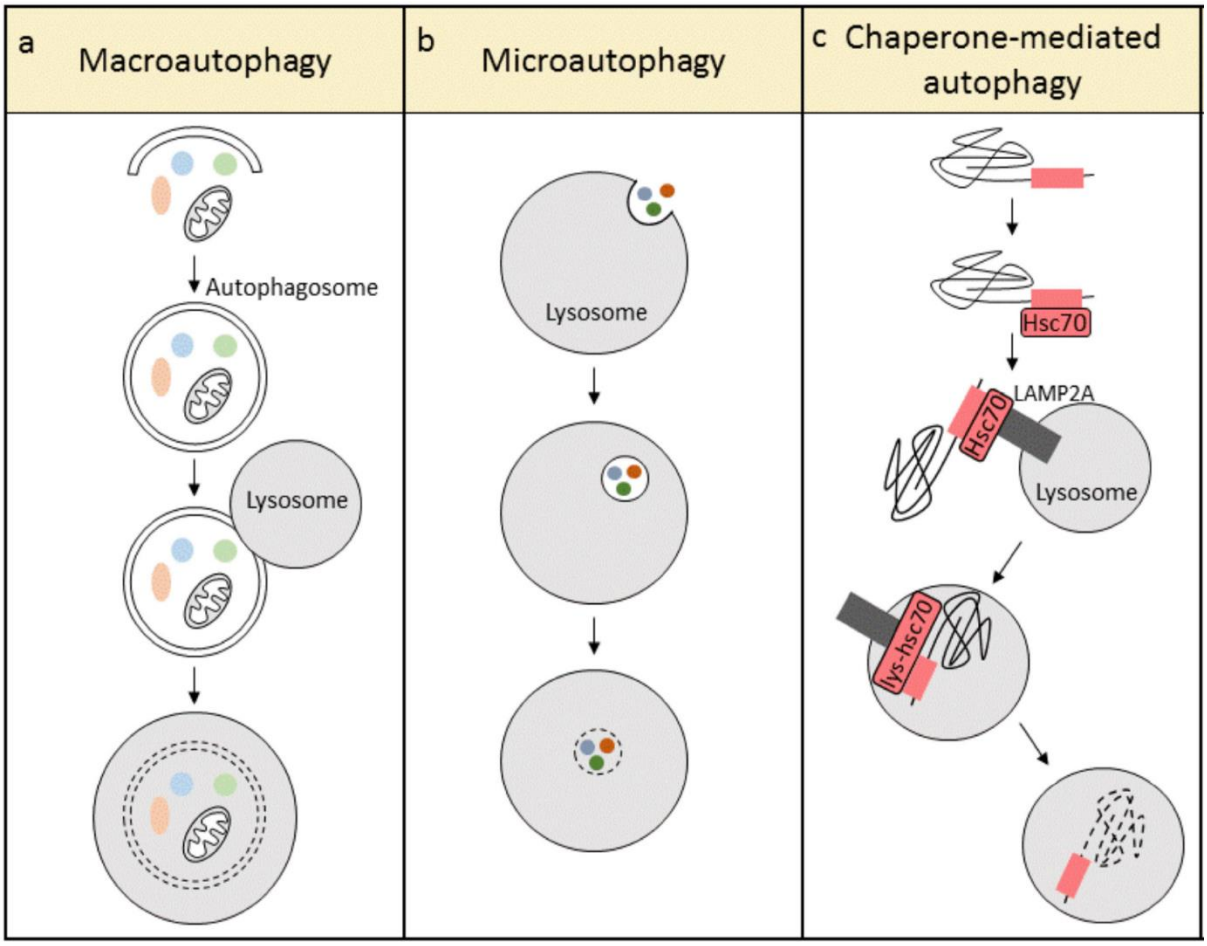

Figure 1.1. Types of autophagy pathways. (a) Macroautophagy captures cytoplasmic cargo in autophagosomes which eventually fuses with lysosomes to form autolysosomes and results in degradation of the substrates. (b) Microautophagy engulfs substrates at the vicinity of lysosomes. (c) Chaperone mediated autophagy selectively degrades protein substrates harbouring KFERQ motif. Major events include recognition of substrates by Hsc70 chaperone and translocating the substrates across the lysosomal membrane by LAMP2A. Reprinted with permission from Li et al., (Li et al., 2018).

1.4 Molecular mechanisms of autophagy

Autophagy is a multistep process involving several proteins at different steps in a sequential manner (Reggiori and Klionsky, 2013). Autophagy begins with an isolation membrane at pre-autophagosomal structure or phagophore assembly site (PAS) located closer to the vacuole in yeast (Tooze and Yoshimori, 2010). Following this, the phagophore expands to capture the cargo in a double-membrane vesicle called autophagosome. The autophagosomes then mature to fuse with lysosomes forming autolysosomes, where the autophagosomal contents are degraded. The degraded products are then exported back to the cytoplasm through lysosomal membrane permeases and transporters. These by-products are re-used by the cell as building blocks for anabolic processes.

At the molecular level, the process is orchestrated by a cascade of multiple proteins (Itakura and Mizushima, 2010). The process can be explained in different stages as follows (**Figure 1.2**).

a. Induction of autophagy

Nutrient starvation was the earliest identified stimuli for autophagy induction. The cellular pathway involved in nutrient sensing; Target of Rapamycin (TOR) complex, especially TOR complex C1 (TORC1) plays a major role in the regulation of the process. TORC1 intricately regulates Atg13 phosphorylation status to mediate its interaction with Atg1. In nutrient-rich conditions, the kinase activity of TOR causes Atg13 hyperphosphorylation preventing its complex formation with Atg1. When TOR kinase activity is inhibited by starvation or by pharmacological inhibitor such as rapamycin, Atg13 associates with Atg1 forming complex with Atg17, Atg31 and Atg29 (Noda and Ohsumi, 1998). The mammalian Atg1 homologues, Unc-51-like kinases 1 and 2 (ULK1/2) binds putative Atg17 homologue, focal adhesion kinase family interacting protein of 200 kDa (FIP200) during autophagy conditions. Additionally,

FIP200 deficient cells show impaired autophagosome formation (Hara et al., 2008). Further studies indicated that FIP200, apart from participating in autophagosome biogenesis, also interacts with Atg16L1 during the expansion of the autophagosomes (Nishimura et al., 2013). The mammalian ULK1/2 complex (ULK1/2-Atg13-FIP200-Atg101) phosphorylates and Beclin1 (yeast Atg6 homologue) and Ambra1 (activating molecule in Beclin 1-regulated autophagy) for downstream autophagy activation (Di Bartolomeo et al., 2010; Fimia et al., 2007; Russell et al., 2013). Subsequent studies identified energy-sensing AMPK pathway, ERK (extracellular signal-regulated kinase) and Akt signalling pathways to also play a critical role in regulating the process (Arico et al., 2001; Budovskaya et al., 2004; Kim et al., 2011; Liang et al., 2007; Russell et al., 2014).

b. Phagophore formation and nucleation

Phagophore formation occurs in yeast near the vacuole at the position referred to as pre-autophagosomal structure (PAS). However, there is no defined location for phagophore formation in mammalian cells. Studies suggest that it occurs at multiple cytosolic membranous structures such as ER, trans-Golgi network and late endosomes (Axe et al., 2008). Additionally, the membrane source for phagophore formation is not well understood. It is hypothesized that there is *de novo* synthesis of membranes needed for the process using cytosolic lipids. Atg17 is the first protein to get recruited to PAS followed by Atg31 and Atg29, forming a ternary complex (Ragusa et al., 2012). Additionally, Atg17 interacts with the kinase activity of Atg1 complex consisting of Atg13 (Reggiori and Ungermann, 2012). Atg1 complex, in turn, interacts with Atg9. Atg9 is the only transmembrane protein involved in autophagy, which is shown to shuttle between membrane sources and expanding phagophore to deliver membrane (Mari et al., 2010; Reggiori et al., 2005; Suzuki and Ohsumi, 2007). Atg23 and Atg27 are involved in the anterograde transport of Atg9 vesicles to PAS, whereas, Atg1, Atg2 and Atg18 are essential for the retrograde transport (Gomez-Sanchez et al., 2018; Mari et al., 2010; Nagy

et al., 2014; Reggiori et al., 2004). Elegant study by Yamamato *et al* showed that approximately three Atg9 vesicles (each containing 27 molecules of Atg9) are involved in each round of autophagosome formation (Yamamoto et al., 2012). Mammalian Atg9 homologue, Atg9A localizes to trans-Golgi network and late endosomes during nutrient-rich conditions and during autophagy conditions is trafficked from plasma membrane via clathrin-coated endosomes to recycling endosomes to autophagosome formation site (Puri et al., 2013).

Phagophore formation also requires the participation of class III phosphatidylinositol (PI)-3 kinase complexes especially complex-I which consists of Vps34 along with its binding partners, Atg14, Vps15 and Atg6 (Simonsen and Tooze, 2009). Atg14 directs the recruitment of complex-I to PAS. Vps34 utilizes PI as the substrate for generating phosphatidylinositol triphosphate (PI3P) which serves as a platform for binding FYVE domain-containing proteins like Atg18-Atg2 complex to PAS (Obara and Ohsumi, 2008, 2011; Obara et al., 2008; Suzuki et al., 2007). Other effector proteins that PI3P recruits in mammalian cells include WD-repeat-interacting phosphoinositide (WIPI) and double FYVE domain-containing protein 1 (DFCP1), both of which are needed for autophagosome biogenesis (Burman and Ktistakis, 2010). Phosphorylation of Beclin1 by ULK1/2 complex activates Vps34 kinase complex that binds either with regulatory proteins such as Atg14L, UVRAG (UV Radiation Resistance Associated), BIF1 (Bax-interacting factor 1), Ambra1, to promote autophagy or with Rubicon (RUN domain protein as Beclin-1 interacting and cysteine-rich containing) and Bcl-2, to inhibit autophagy in a context-dependent manner (Russell et al., 2013).

c. Expansion of the phagophore and cargo capture

There are two ubiquitin-like conjugation systems involved in the autophagy process: Atg7-Atg3-Atg10 and Atg5-Atg12-Atg16 (Kuma et al., 2002; Ohsumi and Mizushima, 2004). Atg7 acts like E1 ubiquitin-activating enzyme activating Atg12 in an ATP-dependent manner

(Tanida et al., 1999). Activated Atg12 is then acted upon by Atg10 (E2-like ubiquitin carrier protein) that potentiates covalent linkage formation with Atg5 (Mizushima et al., 2003). Further, the Atg5-Atg12 complex then conjugates with Atg16L1 to induce curvature into the growing phagophore (Mizushima et al., 1999; Mizushima et al., 1998a).

The second ubiquitin-like system is involved in the processing of Atg8. Full-length Atg8 upon activation is cleaved at its carboxyterminal exposing a glycine terminal by a cysteine protease, Atg4. This processed Atg8 is activated by Atg7 to form Atg8-I which is then transferred to E2-ubiquitin carrier protein, Atg3. Atg8 is then covalently attached to phosphatidylethanolamine (PE) by the action of ATG12-ATG5–ATG16L1 complex forming Atg8-II, making it compatible for membrane association (Ichimura et al., 2000; Nair et al., 2012). Atg8-II is found on both internal and external surfaces of the autophagosomes where it is known to mediate selection of cargo and hemifusion of the autophagosomal membranes. To support this phagophore expansion, several cellular compartments such as plasma membrane, ER, Golgi and mitochondria are proposed as membrane source (Axe et al., 2008; Hayashi-Nishino et al., 2009; Ravikumar et al., 2010; Wei et al., 2018). Recent reports also highlight the dynamic membrane interactions such as ER-mitochondria and ER-plasma membrane contact sites crucial for autophagosome biogenesis (Chan and Tang, 2013; Morel, 2020; Rowland and Voeltz, 2012).

d. Fusion with lysosomes

After completion of autophagosome formation, it is transported towards lysosomes for fusion. This vesicular movement takes place on microtubules in a plus-end directed manner potentiated by adaptor proteins such as Rab7 and FYCO1 (FYVE and coiled-coil (CC) domain-containing protein). Studies suggest that autophagosomes prior to fusion with lysosomes, fuse with early and late endocytic compartments which deliver membrane fusion machinery to

autophagosomes (Eskelinen, 2005). Autophagosomes are also shown to fuse with multi-vesicular structures forming amphisomes which finally fuse with lysosomes forming autolysosomes. Additionally, autophagosomes ideally have the same pH as that of cytosol and during the maturation process become acidic. Molecular genetic screens have identified proteins such as SNAREs (soluble N-ethylmaleimide-sensitive fusion (NSF) attachment protein receptors) (Vam3, Vam7, Vti1, Ykt6), Rab7 and members of HOPs (homotypic fusion and protein sorting) complex (Vps16, Vps18, Vps33, Vps39) to be involved in fusion machinery (Jani et al., 2016; Nair et al., 2011). After fusion with lysosomes, Atg8 present on the outer membrane of autophagosomes is recycled back into the cytoplasm. This recycling again requires the activity of Atg4 for removal of PE from Atg8 (Abreu et al., 2017; Nair et al., 2012). Continuous progression of autophagy also requires the activity of a PI3P-phosphatase, Ymr1, in the absence of which, the recycling of Atg proteins is perturbed (Cebollero et al., 2012b).

Recent studies have concentrated on understanding the biochemical properties of autophagy proteins by *in vitro* reconstitution of autophagosomes using yeast machinery proteins. Recombinant proteins along with membrane platforms are purified and studied to recapitulate the *de novo* formation of autophagosomes. Initial insights regarding membrane binding ability of Atg1 as well as Atg17 complex architecture was identified by *in vitro* reconstitution of protein complex comprising of Atg17, Atg31, Atg29, Atg1 and Atg13 (Ragusa et al., 2012). Further, a study by Rao *et al* identified that Atg17 physically interacts with Atg9 *in vitro* by employing artificial Atg9 vesicles and purified Atg1 complex proteins such as Atg1, Atg17 and Atg13 (Rao et al., 2016). Other key observations made using *in vitro* reconstitution studies include identification of Atg3 as membrane curvature sensor (Nath et al., 2014) as well as composition and activity of conjugation systems that are required for Atg8 lipidation (Hanada et al., 2007; Ichimura et al., 2004; Kaufmann et al., 2014). A recent study by Makarska *et al*

have carried out a near-complete *in vitro* reconstitution of autophagosome biogenesis. The study utilized Atg9 proteoliposomes and highlighted that Atg9 vesicles serve as a platform for recruiting autophagy machinery proteins (Sawa-Makarska et al., 2020).

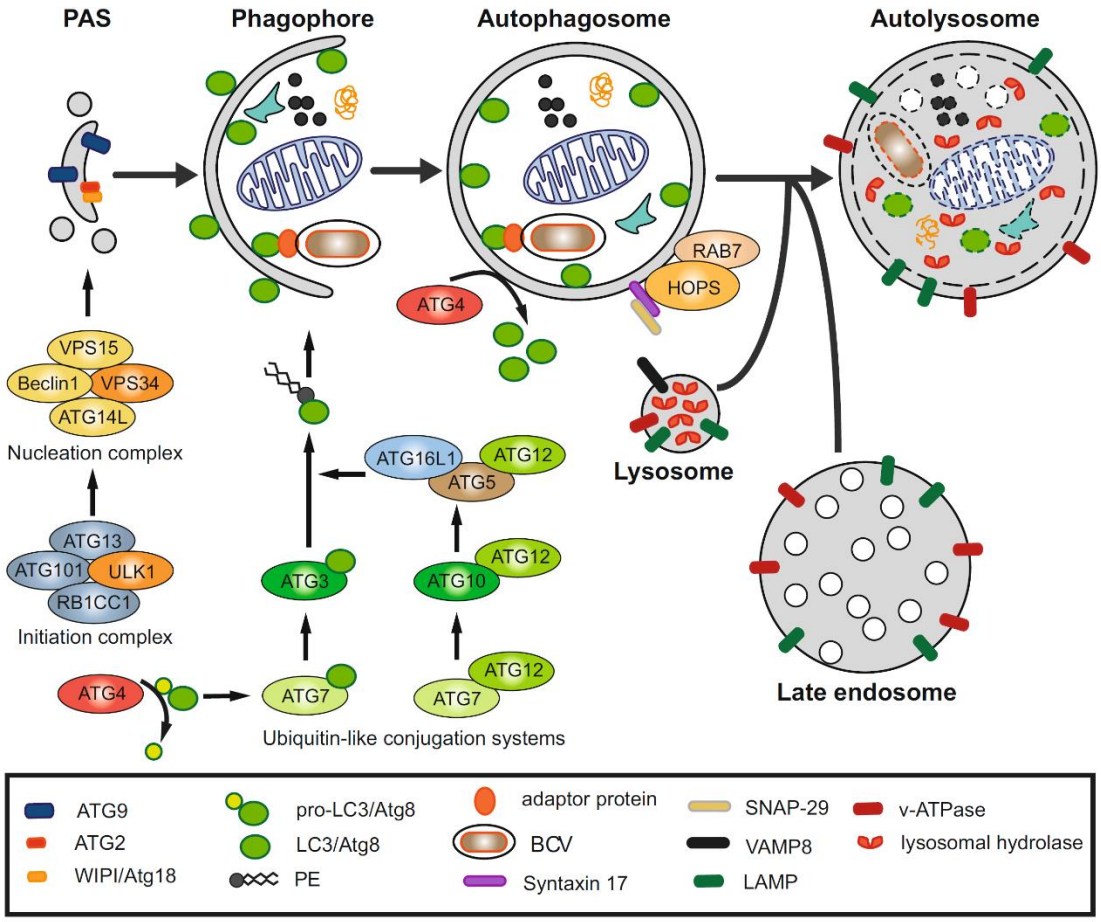

Figure 1.2. The process of autophagy and the molecular players participating at different stages. Image used with permission from Lippai and Szatmari (Lippai and Szatmari, 2017).

1.5 Transcriptional regulation of autophagy

Autophagy is constitutively active in basal levels in most cells of the body to maintain the homeostasis of the cell (Mizushima and Komatsu, 2011). However, the process can be upregulated transcriptionally by a variety of cellular stimuli including starvation, misfolded protein aggregation and intracellular pathogen invasion (Fullgrabe et al., 2014). Transcription

factors switch between “on” and “off” states in response to physiological signals to regulate the temporal expression of its downstream targets. Further, some transcription factors are employed to either exclusively upregulate or downregulate the autophagy process. Additionally, few known transcription factors can function as both activator and inhibitor of the process depending on the external cues or stimuli (**Figure 1.3 and Table 1.1**).

a. Master regulators: TFEB and ZKSCAN3

Transcription factor EB (TFEB) belongs to microphthalmia/transcription factor E (MiTF) family of transcription factors that are characterized by the presence of a basic Helix-Loop-Helix, a basic domain and a leucine zipper domain. The other members of the family include TFE3, TFEC and MiTF. Members of MiTF family bind to a 10 base-pair GTCACGTGAC consensus sequence. This consensus sequence was identified by the *in silico* study of Ballabio’s group in the promoter of lysosomal genes (Sardiello et al., 2009). Overexpression of TFEB in multiple cell lines induced co-ordinated induction of a large set of genes involved in lysosome biogenesis. The promoter sequence was hence referred to as Coordinated Lysosomal Expression And Regulation (CLEAR) motif (Settembre et al., 2011). Subsequent studies by the same group identified that TFEB in addition to lysosomal genes also induces the expression of autophagy genes (Palmieri et al., 2011; Martina et al., 2012; Puertollano et al., 2018).

A Zinc finger family DNA binding protein, ZKSCAN3 (zinc finger transcription factors harbouring Kruppel-associated box (KRAB) and SCAN domain) was identified by Chauhan *et al* as a repressor of lysosomal and autophagy genes (Chauhan et al., 2013). Interestingly, ZKSCAN3 functions in an opposing manner to TFEB and is kept inactivated during starvation. Transcription factors with the KRAB domain are typically known for repressing gene expression (Urrutia, 2003). ZKSCAN3 is predominantly localized in the nucleus and binds the KRDGGG consensus sequence on DNA. A large set of more than 60 genes related to lysosomal biogenesis and autophagy are reported to be repressed by ZKSCAN3. Additionally, silencing

of ZKSCAN3 activates TFEB suggesting that these two transcription factors function as master regulators of lysosomal biogenesis and autophagy genes.

b. FOXO

The forkhead family of transcription factors consist of four members in mammals, namely, FOXO1, FOXO3, FOXO4 and FOXO6. Activation of FOXO members is mediated in response to growth factors and insulin. Recent studies have identified FOXO3 to transcriptionally induce autophagy genes (Zhou et al., 2012). Similar to MiTF, FOXO3 also exhibits nucleo-cytoplasmic shuttling. In the presence of growth factors, Akt phosphorylates FOXO3 which leads to its cytoplasmic retention. Subsequent studies have shown that FOXO family proteins regulate autophagy in a transcription-independent manner. Acetylation of FOXO1 in the cytoplasm enhances its binding to Atg7 and favours autophagy initiation (Banreti et al., 2013).

c. E2F1 and NFkB crosstalk

E2 transcription factor (E2F) is well-studied for its role in regulating cell cycle mediated by its inhibitory interaction with retinoblastoma tumour suppressor protein. E2F family member, E2F1 regulates several genes involved in autophagy (Polager et al., 2008). Among the target genes, E2F1 regulates BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) to positively regulate autophagy. This regulation is mediated by disrupting the Bcl2-Beclin1 interaction thereby releasing Beclin1 making it available to induce autophagy. Additionally, Nuclear factor kappa-light-chain-enhancer of activated B-cells (NFkB) competes with E2F1 for repressing BNIP3 expression to mediate a tight regulation of the process (Trocoli and Djavaheri-Mergny, 2011).

A growing number of studies highlight the complex transcriptional gene network involved in regulating autophagy. Other key transcriptional factors involved in the autophagy process are summarized in the table below.

Table 1.1: Transcriptional regulation of autophagy

Name	Target genes	Activation	Autophagy effect	Reference
ATF4	*ATG5, ULK1*	ER stress, hypoxia	Induced	(Pike et al., 2013)
ATF5	*mTOR*	Oncogenic stress	Suppressed	(Sheng et al., 2011)
β-catenin	*SQSTM1*	Development, nutrient stress	Suppressed	(Petherick et al., 2013)
C/EBPβ	*BNIP3, LC3, ULK1*	Circadian and nutritional signalling	Induced	(Ma et al., 2011)
CHOP	*ATG5, LC3B*	ER stress, viral infection	Induced	(Rouschop et al., 2010)
CHF1	*ATG4, ATG9, ATG5, BNIP3, LC3*	Serum stimulation	Induced	(Yu et al., 2010)
E2F1	*ULK1, BNIP3, LC3A, ATG5*	Hypoxia	Induced	(Polager et al., 2008)
FOXO3	*ATG5, ATG12, ATG14, BECN1, BNIP3, LC3, VPS34, ULK1/2*	Starvation and oxidation stress	Induced	(Sanchez et al., 2012; Zhao et al., 2007)
GATA1	*LC3*	Cytokine signalling	Induced	(Kang et al., 2012)
HIF1	*BNIP3*	Hypoxia	Induced	(Zhang et al., 2008)
Jun	*BECN1, MAP1LC3B*	Pro-inflammatory cytokines, environmental stress	Induced	(Raingeaud et al., 1995)
NF-kB	*BCL2, BECN1, BNIP3, SQSTM1*	Cytokines, hypoxia, ER stress	Both	(Copetti et al., 2009)
p53	*ATG2, ATG4, ATG7, ATG10, ULK1*	DNA damage, activated oncogenes,	Induced	(Crighton et al., 2006; Kenzelmann Broz et al., 2013; Stambolic et al., 2001)
p63	*ATG3, ATG4, ATG5, ATG7, ATG9, ATG10, BECN1, LC3, ULK1*	DNA damage	Induced	(Huang et al., 2012)
p73	*ATG5, ATG7, UVRAG*	DNA damage	Induced	(Rosenbluth and Pietenpol, 2009)
SMAD	*ATG5, ATG7, BECN1*	Cytokines	Induced	(Pan et al., 2015)
PML-RAR	*BECN1, SQSTM1*	Constitutively active	Both	(Isakson et al., 2010)
SOX2	*ATG10*	Cancer	Induced	(Cho et al., 2013)
SREBP2	*LC3, ATG4B, ATG4D*	Lipid metabolism	Induced	(Seo et al., 2011)

Name	**Target genes**	**Activation**	**Autophagy effect**	**Reference**
STAT1	*ATG12, BECN1*	Myocardial infarction	Suppressed	(McCormick et al., 2012)
STAT3	*BCL2, BNIP3, ATG3, BECN1, CTSB, HIF1A*	Cytokines and growth factor	Suppressed	(Dauer et al., 2005)
TFE3	*ATG16L1, ATG9B, GABARAP-L1, WIPI, UVRAG*	Starvation	Induced	(Martina et al., 2014)
TFEB	*ATG4, ATG9, BCL2, SQSTM1, ATG5, UVRAG, WIPI, BECN1, LC3, GABARAP,*	Starvation, Protein aggregation, infection	Induced	(Palmieri et al., 2011; Settembre et al., 2011)
CREB	*ATG7, ULK1, TFEB*	Starvation	Induced	(Seok et al., 2014)
Ume6	*ATG8*	Starvation	Suppressed	(Bartholomew et al., 2012)
ZKSCAN3	*LC3, ULK1, WIPI*	Starvation	Suppressed	(Chauhan et al., 2013)

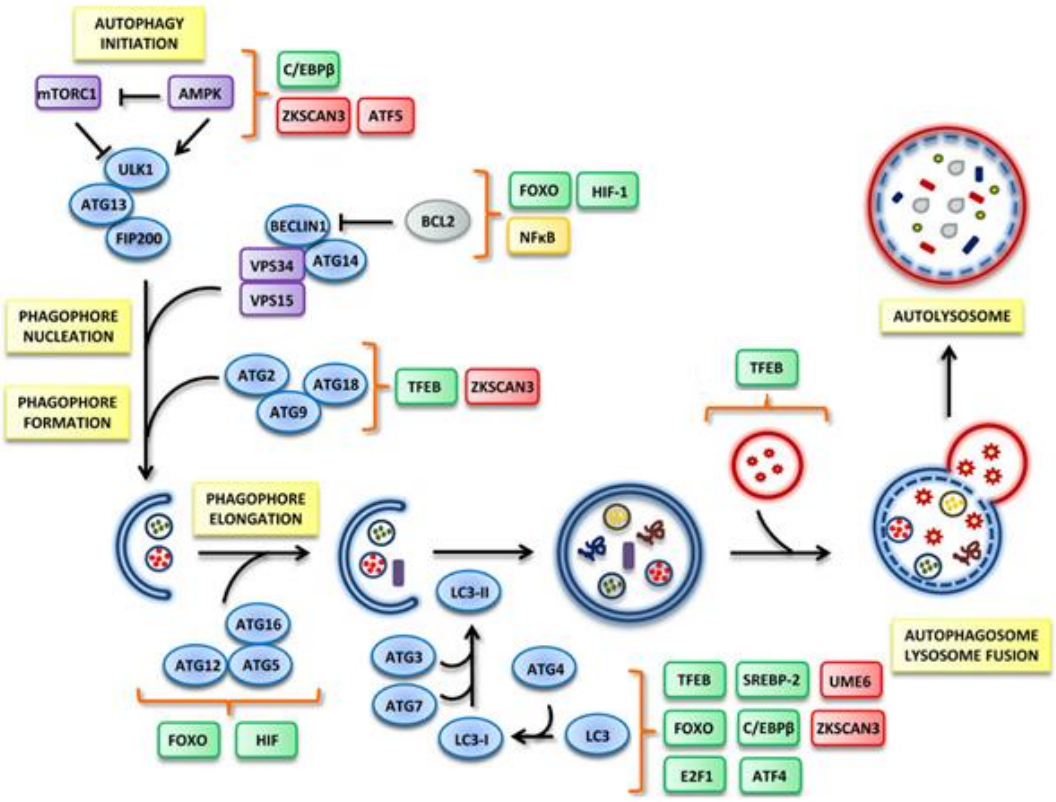

Figure 1.3. Transcriptional regulation of autophagy process. In response to induction stimuli, transcription factors are activated to regulate the expression of target genes. Colour coding indicates their effect on autophagy; labelled in green represents autophagy inducers, red represents autophagy inhibitors and yellow represents factors that function in both induction and inhibition of autophagy. Image used with permission from Chandra et al., (Chandra et al., 2016).

1.6 Selective autophagy

Autophagy, initially perceived as a process for bulk degradation of cytoplasmic contents was subsequently understood to also selectively degrade cargoes such as superfluous or damaged organelles, aggregated proteins, and invading pathogens to maintain cellular homeostasis (Rogov et al., 2014). Mechanistically, both bulk (non-selective) and selective autophagy utilizes the same core proteins required for autophagosome formation, maturation and fusion with lysosomes. Additionally, in selective autophagy, there is involvement of a subset of proteins referred to as receptor/adaptor proteins which bridge the cargo to autophagosomes (Rogov et al., 2014). The mechanism of cargo recognition can be majorly of two kinds- ubiquitin-dependent and ubiquitin independent. In the ubiquitin-dependent mechanism, the cargoes are tagged with ubiquitin (Korolchuk et al., 2010). Further systemic studies identified that K63-linked ubiquitin chains give specificity to autophagy-mediated capture as opposed to K48-linked ubiquitin chains that are targeted to proteasomal mediated degradation. Ubiquitinated cargoes are then recognized by autophagy specific adaptor proteins. These adaptor proteins serve as a bridge to link the ubiquitinated cargoes to growing autophagosomes by interacting with Atg8 homologue proteins. As mentioned earlier, there are six Atg8 homologues present in mammalian cells, LC3A, LC3B, LC3C, GABARAP, GABARAPL1, GABARAPL2/GATE16. Adaptor proteins are characterized by the presence of conserved LC3 Interacting Region (LIR) and ubiquitin-binding domain. Analysis of multiple LIRs revealed the core consensus sequence [W/F/Y] XX [L/I/V], where X is any amino acid (Birgisdottir et al., 2013). There are also certain autophagy proteins (Atg1/ULK1, Atg3) that are not

conventional adaptors but contain LIR for interaction with Atg8 family proteins. Further, by analysing the multiple LIRs, their preference in selective binding to LC3s and GABARAPs was determined defining the GABARAP Interaction Motif (GIM) sequence- (W/F)-(V/I)-X_2-V (Wirth et al., 2019). For example, PLEKHM1-GIM binds GABARAP with multifold higher affinity than LC3 (Rogov et al., 2017). In addition to ubiquitin-mediated selective autophagy, there are also adaptor proteins present on the organelles that can recruit LC3 for autophagosome formation independently of ubiquitin (Khaminets et al., 2016). Similar to adaptor proteins that bind Atg8 family proteins, there are also Atg5-binding adaptor proteins such as TECPR1, ALFY and Atg16L1 for cargo selection (Chen et al., 2012; Filimonenko et al., 2010). However, the recruitment of these ATG5-binding proteins to cargo remains unclear. Studies indicate that cargo localized ATG5 and its binding partners, ATG12-ATG16L1 further recruit ULK1 complex initiating autophagosome formation. Depending on the cargoes that are recognized (**Table 1.2**), the selective autophagy can further be classified as aggrephagy (autophagy of aggregated proteins), mitophagy (autophagy of mitochondria), ribophagy (autophagy of ribosomes), xenophagy (autophagy of intracellular pathogens), lipophagy (autophagy of lipids).

Table 1.2: Types of selective autophagy and their adaptor proteins

Selective Autophagy	Cargo	Adaptor proteins	Reference
Cytoplasm to vacuole targeting (cvt)	Precursor aminopeptidase and α-mannosidase	Atg19p, Atg34p	(Scott et al., 2001) (Hutchins and Klionsky, 2001)
Mitophagy	Damaged/superfluous mitochondria	Atg32, Atg33, Nix, Bnip3	(Lazarou et al., 2015)
Xenophagy	Intracellular pathogens	p62, NDP52, OPTN, TAX1BP1	(Deosaran et al., 2013; Thurston et al., 2009; Zheng et al., 2009)
Nucleophagy	Nuclear envelope	NVJI, Atg39	(Mochida et al., 2015)
Aggrephagy	Aggregated proteins	p62, OPTN, NBR1, Cue5, TOLLIP	(Korac et al., 2013; Pankiv et al., 2007)
Ribophagy	Ribosomes	Ubp3, Bre5	(Cebollero et al., 2012a)
Lysophagy	Damaged lysosomes	Galectin3, NDP52	(Hasegawa et al., 2015)
Reticulophagy	Endoplasmic reticulum	FAM134B, Atg40	(Cebollero et al., 2012a; Khaminets et al., 2015)
Pexophagy	Superfluous Peroxisomes	Atg30, Atg36	(Deosaran et al., 2013; Farre et al., 2008; Manjithaya et al., 2010)
Lipophagy	Lipid droplets	P62	(Singh et al., 2009)
Ferritinophagy	Ferritin	nuclear receptor coactivator 4 NCOA4	(Mancias et al., 2014)
Zymophagy	Zymogen	p62	(Grasso et al., 2011)
Clockophagy	Circadian clock proteins	p62	(Liu et al., 2019; Yang et al., 2019)
Mid body disposal	Mid body during cytokinesis	p62, NBR1	(Pohl and Jentsch, 2009)

1.7 Xenophagy

Xenophagy refers to autophagic capture of non-host entities ("xeno" – foreign "phagy"- eating) such as intracellular pathogens. In xenophagy, diverse intracellular pathogens including bacteria, viruses and protozoans (Ling et al., 2006) are captured by double membranous autophagosomes for degradation in autolysosomes. Mounting evidence suggests that xenophagy functions as an innate immune mechanism of the host (Deretic and Levine, 2009).

Although autophagy was identified in 1963, the first evidence of xenophagy came from the study by Rikihisa in 1984, where polymorphonuclear leukocytes (PMNs) incubated with pleomorphic bacteria, *Rickettsiae* showed accumulation of double-membranous autophagosome like structures containing bacteria. These structures were also positive for the lysosomal enzyme, acid phosphatase showing that it is a degradative compartment for the entrapped bacteria (Rikihisa, 1984). Subsequent studies showed that xenophagy is active against diverse intracellular infections such as bacteria, virus, fungi, and protozoa (Ahmad et al., 2018; Mauthe et al., 2016; Mori et al., 2018; Sancho-Shimizu and Mostowy, 2018) (**Figure 1.4**). The earliest genetic evidence that xenophagy restricts microbial replication was observed during Sindbis virus infection. The study by Liang *et al* showed that overexpression of Beclin1 protected mice against Sindbis virus encephalitis and reduced neuronal death (Liang et al., 1998). Further, protozoans such as *Toxoplasma gondii* replicates in parasitophorous vacuoles preventing its fusion with lysosomes. However, autophagy induction is shown to overcome the fusion block and enhances its fusion with lysosomes (Andrade et al., 2006; Ling et al., 2006). Furthermore, the role of xenophagy during other protozoan infections such as *Plasmodium spp* and *Leishmania spp* are also reported (Agop-Nersesian et al., 2017; Besteiro et al., 2006). The interaction of various pathogens with xenophagy process is summarized in the table below (**Table 1.4**). Additionally, xenophagy is now studied in multiple host systems such as

Dictyostelium discoideum (Otto et al., 2004), *Caenorhabditis elegans*, *Drosophila* (Kuo et al., 2018), plants (Kershaw and Talbot, 2009) and mammalian cells.

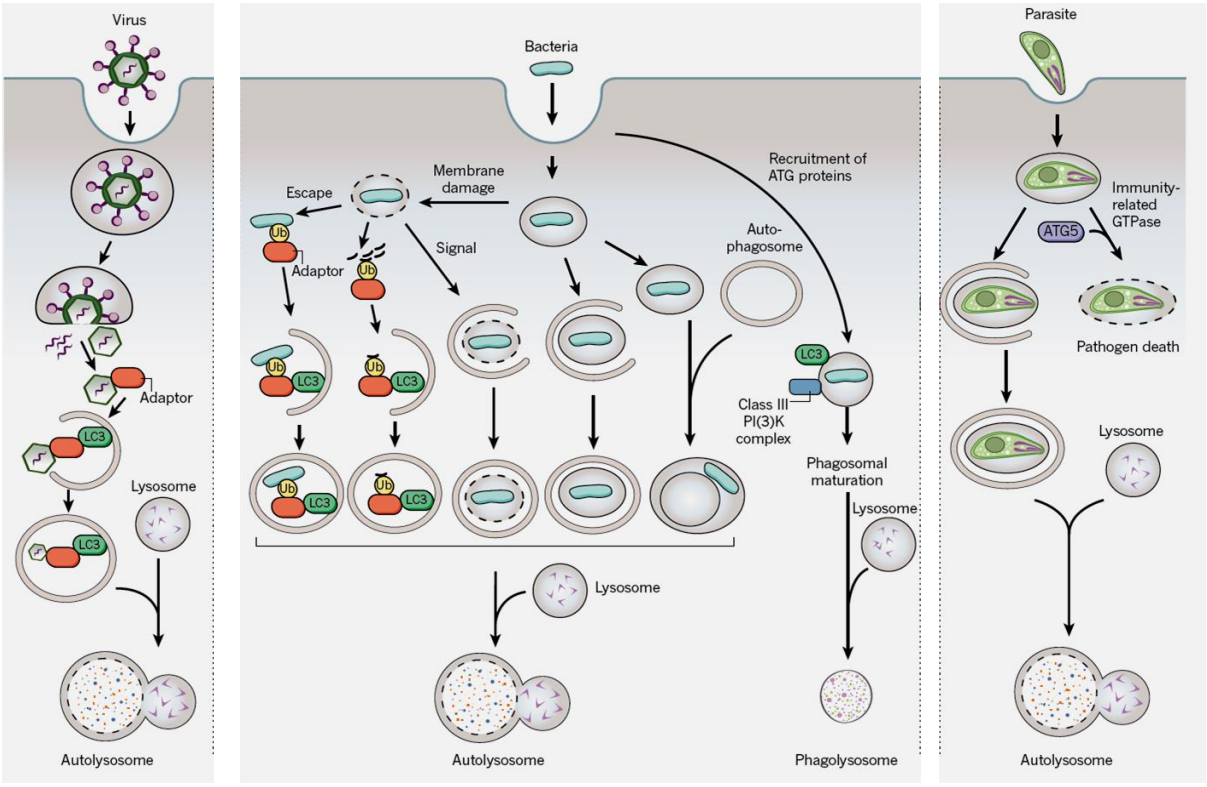

Figure 1.4. The process of xenophagy during virus, bacteria, and parasite infections. Image used with permission from Levine et al., (Levine et al., 2011)

Table 1.4: Role of xenophagy during intracellular infections. Table adapted from Ammanathan et al., (Ammanathan et al., 2020).

Pathogen	Disease	Host/pathogen effectors
Streptococcus pyogenes	Pharyngitis, Rheumatic fevers, Skin infections.	Streptolysin causes the escape of bacteria to cytosol, where it is captured by xenophagosomes [36].
Staphylococcus aureus	Skin infection, sinusitis, food poisoning	Xenophagy enhances the survival of *S. aureus* due to inflammasome degradation [37].
Salmonella typhimurium	Gastroenteritis	*Salmonella* escaping from endosomes into cytosol are captured by xenophagy [15].
Mycobacterium tuberculosis	Tuberculosis	Xenophagy induction leads to INF production which is shown to inhibit *M. tuberculosis* replication [38].
Legionella pneumophila	Legionellosis- fever, muscle pain and shortness of breath	Induces xenophagy in type IV system secretion (T4SS, also known as the secretion system Dot/Icm)-dependent manner [39].
Shigella flexneri	Shigellosis- diarrhoea and abdominal pain	Xenophagy is activated by the recognition of IcsA (surface protein) by ATG5 [17].
Helicobacter pylori	Peptic ulcers and gastritis	Xenophagy block caused by *H. pylori* facilitates in its intracellular survival [40].
Listeria monocytogenes	Listeriosis- gastroenteritis (immuno-compromised patients can get meningitis)	Listeriolysin causes the escape of bacteria to cytosol, where it is captured by xenophagosomes. At later time points of infection, *Listeria* escapes xenophagy by expressing ActA protein that forms actin tail and mediates bacterial movement within cells. [41].
Coxiella burnetii	Q fever	*C. burnetti* can survive and replicate in the harsh environment of large, acidified phagolysosome-like vacuoles [42].
Porphyromonas gingivalis	Periodontitis and infection of gastrointestinal tract and colon.	Bacteria get sequestered in xenophagosomes, but it evades the fusion with lysosomes [43].
Parvovirus B19	Erythema infectiosum	Increase in LC3 II levels during virus replication is observed and inhibition of autophagy led to more virus-induced cell death [44].
Poliovirus	Poliomyelitis	Inhibition of autophagy decreased poliovirus yield [45].
Hepatitis C	Jaundice	HCV induces autophagic vacuoles and recruitment of LC3 and Apg5 [46].
Influenza A	Influenza	The virus causes accumulation of autophagosomes through preventing fusion to lysosomes [47].
Human immunodeficiency virus (HIV)	Acquired Immune Deficiency Syndrome (AIDS)	HIV Gag-derived proteins co-localizes and interacts with the autophagy factor LC3 and is seen to be degraded [18].
Varicella zoster virus (VZV)	Varicella (Chicken pox)	VZV induces xenophagy at later time points of infection [48].
Epstein-Barr virus(EBV)	Infectious mononucleosis	EBV blocks autophagic flux during re-activation from latency [49].
Herpes Simplex Virus (HSV)	Herpes encephalitis	HSV protein γ34.5 inhibits xenophagy by binding to Beclin1 [50].
Sindbis virus	Sindbis fever	Binds to Beclin1 inhibiting xenophagy [51].

1.7.1 Non-canonical xenophagy: LC3 associated phagocytosis (LAP)

LAP is a form of non-canonical xenophagy that shares most of the canonical xenophagy machinery proteins along with few distinct molecular players. The key feature that differentiates both the processes is single-membraned LAPosome mediated capture of intracellular pathogens. Similar to canonical xenophagy, class-III PI3K mediated PI3P generated on LAPosome recruit LC3. Eventually, the LC3 bound LAPosomes fuse with lysosomes for degradation of the captured pathogens. Studies identified Rubicon, as a protein needed exclusively for LAP but not canonical xenophagy (Martinez et al., 2015). Rubicon acts as a negative regulator of autophagy by binding to Vps34 or Rab7 (Matsunaga et al., 2009; Nakamura et al., 2019; Zhong et al., 2009). Clearance of *L. monocytogenes, Saccharomyces cerevisiae* is impaired in LAP-deficient macrophages highlighting the importance of LAP (Sanjuan et al., 2009; Yang et al., 2012a). Additionally, during viral infection, Rubicon also binds IRF3 and IRF7 to negatively regulate interferon signalling and facilitate viral replication (Kim et al., 2017; Wan et al., 2017). *In vivo* studies suggest that Rubicon deficiency leads to increased levels of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-12 in response to infection (Yang et al., 2012a; Yang et al., 2012b). These studies suggest LAP as a critical process regulating immunotolerance.

1.7.2 Xenophagy during bacterial infectious diseases

Bacteria enter host cells predominantly by a process referred to as phagocytosis (in case of phagocytic cells) or endocytosis (in case of non-phagocytic cells). Conventionally, phagosomes fuse with lysosomes for degradation of the contents. However, pathogens, in order to establish intracellular infection, have evolved mechanisms to overcome phagocytosis - mediated degradation in lysosomes. This phagocytic evasion is achieved by using multiple

strategies by different pathogens (Desai and Kenney, 2019; Ottemann and Kenney, 2019). One of the common modes of evasion is by preventing the fusion of phagosomes with lysosomes. Pathogens impose "phagosome arrest" by modifying the phagosomes into its replicating niche. The modified phagosomes are not recognized by host cells as conventional vesicles for fusion with lysosomes. This is seen in the case of pathogens such as *Mycobacterium tuberculosis*, *Salmonella typhimurium* among others (Gutierrez et al., 2004). Alternatively, pathogens such as Group A *Streptococcus, Listeria monocytogenes, Shigella flexneri* secrete lysins to damage phagosomes and enter cytosol for their replication (Ogawa and Sasakawa, 2006; Tan et al., 2018). Similarly, *S. typhimurium* secretes various effectors for facilitating invasion and intracellular survival. The well-studied effectors that are involved in xenophagy evasion include SseL, SifA SsaV, SsrB (Mesquita et al., 2012, Ganesan et al., 2017, McGourty et al., 2012). Recent proteomic studies revealed profiling of *Salmonella* secretome highlighting several novel effectors affecting *S. typhimurium* pathogenesis (Cheng S et al., 2017).

The process of xenophagy is shown to target the pathogens replicating in the cytosol as well as in arrested or damaged phagosomes (Knodler and Celli, 2011; Yuk et al., 2012). The pathogenic cargo is ubiquitinated for subsequent recognition by xenophagy adaptor proteins such as p62, NBR1, OPTN, TAX1BP1. However, there are also ubiquitin-independent mechanisms involved as seen in case of damaged phagosomes. Membrane damage exposes glycans present on the inner surface of phagosomes to the cytosol. Glycans act as damage-associated molecular patterns (DAMPs) that are recognized by galectins. Galectins are beta-galactoside-binding proteins that are known to recruit xenophagy adaptor proteins (NDP52) and eventually lead to LC3-mediated xenophagosome capture (Thurston et al., 2012; Yuk et al., 2012). The process of xenophagy is now proven by many groups as a defence mechanism against a huge number of intracellular pathogens in both phagocyte and non-phagocyte cells. For example, in bacterial pathogens such as Group A *Streptococcus*, there is increased

intracellular replication in host cells that are devoid of functional xenophagy suggesting its importance in eliminating pathogens (Nakagawa et al., 2004). Another well-studied pathogen for its interaction with xenophagy is *M. tuberculosis*. Induction of autophagy by nutritional stress, rapamycin, or vitamin D3 increases the fusion of *Mycobacterium* replicating in arrested phagosomes with autophagosomes for degradation (Gutierrez et al., 2004; Yuk et al., 2009).

Given the importance of xenophagy, pathogens have evolved mechanisms to overcome autophagy-mediated capture. There is a continuous battle between the host and pathogen during infection. One of the well-studied examples is seen in the case of *S. flexneri*, which post invasion ruptures phagosomes to prevent its fusion with lysosomes. Additionally, to prevent xenophagy-mediated capture, *S. flexneri* secretes IcsB that directly binds to Atg5 preventing xenophagosome formation (Krokowski and Mostowy, 2016). Furthermore, the anti-viral role of xenophagy captures viral components for degradation to lysosomes. Studies on viruses such as Sindbis virus and HIV highlight the protective role of xenophagy. However, many viruses are known to manipulate autophagy for their advantage enabling viral replication. For example, Kaposi's sarcoma herpes virus and Herpes simplex virus-1 target Beclin-1 to inhibit autophagosome formation (Jordan and Randall, 2012).

1.7.3 Xenophagy in immunity and inflammation

The functional role of xenophagy is multifaceted, ranging from cell-autonomous defences to organismal immune system encompassing innate and adaptive immune responses (Deretic and Levine, 2009) (**Figure 1.5**).

1.7.3.1 Xenophagy in innate immunity

Extensive research in the past few decades have shown capturing of intracellular pathogens by xenophagy for degradation. Further studies on the mechanism of xenophagy identified

immune-related signalling molecules to regulate the process. For example, host cells express pattern recognition receptors (PRRs) such as Toll-like Receptors (TLRs) and nucleotide-binding oligomerization domains (NOD)-like receptors (NLRs). These receptors recognize pathogen patterns such as lipopolysaccharides or peptidoglycans to activate signalling cascades that induce xenophagy among other antimicrobial responses. For instance, the study by Xu *et al* showed that xenophagy is induced post TLR4 activation during Mycobacterial infection, specifically, TRIF-dependent TLR4 signalling (Xu et al., 2007). Similar to TLRs, the role of NLRs to induce xenophagy is also known. Nod1 and Nod2 detect peptidoglycan of bacteria and recruits Atg16L1 to the site of bacterial entry and enhance subsequent capture by xenophagosomes (Travassos et al., 2010). These signalling pathways induce initiation of xenophagosome formation and recruitment of machinery proteins to capture intracellular bacteria. Adaptor proteins such as p62, NDP52, TAX1BP1, OPTN recognize ubiquitinated bacteria for subsequent recruitment of LC3 (Mostowy et al., 2011; Zheng et al., 2009).

Xenophagy can also be activated by reactive oxygen species (ROS) commonly produced following TLR and Fcγ receptor stimulation (Shi and Kehrl, 2008). Another known immune mediator IL-β, a proinflammatory cytokine produced in response to PAMPs and DAMPs activate downstream xenophagy pathway. Apart from the intracellular role of degrading pathogens, xenophagy also dampens proinflammatory responses such as type I IFN, IL-1β, IL-18 (Deretic and Levine, 2018; Levine et al., 2011).

1.7.3.2 Xenophagy in adaptive immunity

In addition to innate immune signalling, the role of xenophagy in inducing adaptive immune signalling is also extensively studied. As xenophagy leads to degradation of pathogens in lysosomes, it enhances antigen presentation by major histocompatibility complex class-II (MHC-II) (Gannage and Munz, 2009). This autophagy mediated antigen presentation plays a

role in thymic selection of T cell repertoire (Nedjic et al., 2008). Other functions of xenophagy in adaptive immunity mediate the "fine-tuning" of immune responses. For example, the production of Th1 cytokines (TNFα and IFNγ) during infection induce xenophagy while Th2 cytokines (IL-4, IL-3) inhibit the process (Harris et al., 2007). On a broader level, autophagy plays a crucial role in maintaining homeostasis of immune-related cell types such as T-cells, B-cells and intestinal granulocytes known as Paneth cells. For instance, autophagy is reported essential for the removal of mitochondria during T-cell maturation (Pua et al., 2009) and autophagy inhibition shows a significant defect in B-cell development with increased cell death (Miller et al., 2008).

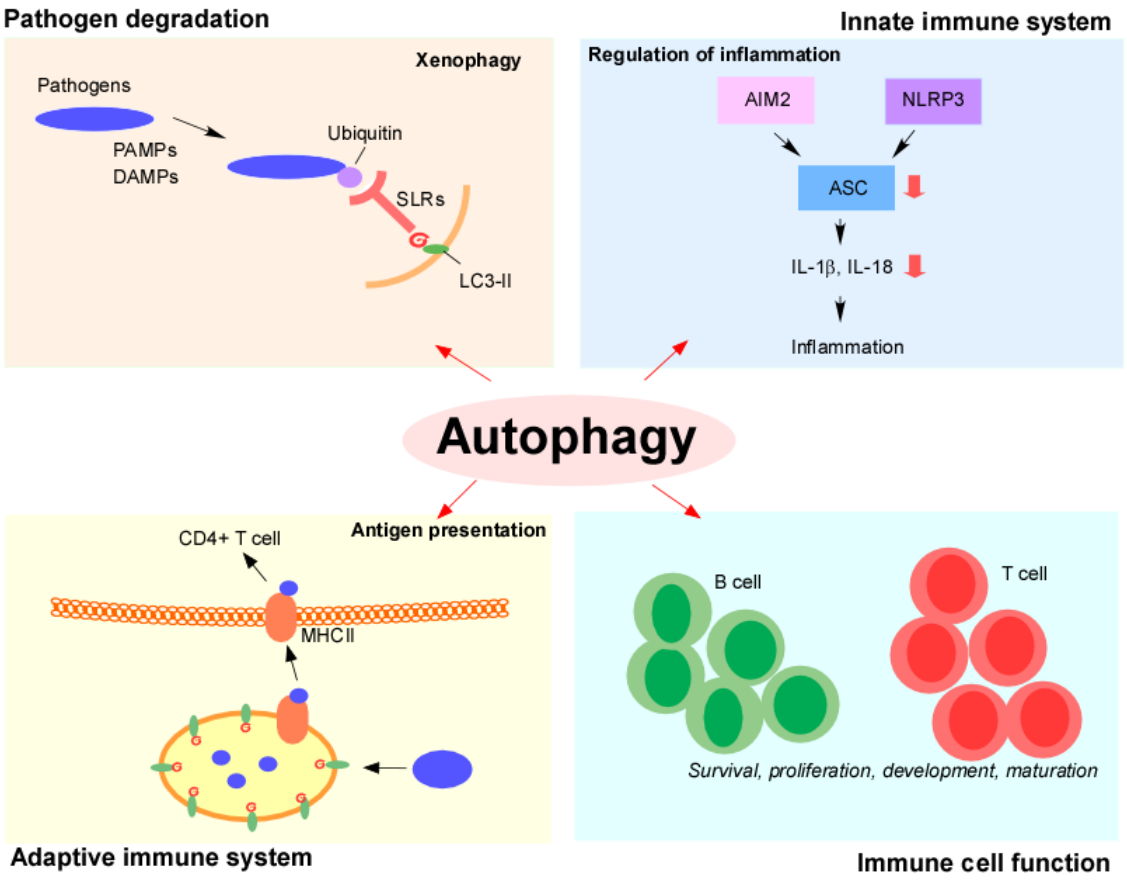

Figure 1.5. The known functions of autophagy in immunity and inflammation during infection. Clockwise from right i) Xenophagy – Intracellular pathogens are captured by double-membraned xenophagosomes for fusion with lysosomes. ii) Autophagy degrades ASC and IL-1β to regulate inflammation in response to infection. iii) Xenophagy mediated degradation of pathogens in lysosomes increases the antigen presentation mediated by MHC-II receptor. iv) Autophagy plays a crucial role in T-cell and B-cell development and maturation. Image used with permission from Jang et al., (Jang et al., 2019).

1.7.3.3 Xenophagy in inflammation

Inflammation is an immune response that acts as a double-edged sword, by controlling infection but when over exuberantly activated, leads to tissue damage. Recent studies probe the role of xenophagy in inflammatory diseases including infectious diseases and Crohn's disease among others. Xenophagy benefits in controlling inflammation by degrading inflammation-promoting microbes such as *Helicobacter pylori, Listeria spp. and Shigella spp* (Watson et al., 2019). Additionally, the role of autophagy in regulating inflammation is especially evident in relation to Atg16. Mice deficient in Atg16L1 produced significantly high levels of IL-1β and IL-18 both being potent inflammatory cytokines in response to TLR activation. Subsequent studies highlighted a key role of autophagy in degrading inflammasome, a multiprotein complex involved in the production of pro-inflammatory cytokines such as IL-1β and IL-18. This protein complex is assembled in the cytosol in response to sensing DAMPs and PAMPs to induce proinflammatory immune responses. The canonical inflammasome consists of inactive pro-caspase-1. Oligomerization leads to autoproteolysis forming active caspase-1 in turn processing pro-IL-1β and IL-18 generating biologically active cytokines. Other components of inflammasome include NLR family members (NLPR3 or NLPR4), apoptosis-associated speck-like protein containing a CARD (ASC) and AIM-like receptors (ALRs) (Saitoh and Akira, 2016). Studies have shown that ASC gets selectively degraded by autophagy post ubiquitination and p62 binding leading to suppression of inflammasome activation (Shi et al., 2012). Additionally, autophagy is also

shown to reduce IL-1β secretion by degrading pro-IL-1β during nutrient starvation (Harris et al., 2011). Autophagy also mediates the turnover of damaged mitochondria to prevent the accumulation of ROS. This prevents against damaged-mitochondria mediated activation of NLRP3 inflammasome.

1.7.3.4 Autophagy during inflammatory diseases

Recent studies have highlighted the role of autophagy during disease progression of certain inflammatory diseases (Lee et al., 2017; Qian et al., 2017). One of the well-studied examples is the involvement of autophagy in Crohn's disease (CD). It is a chronic inflammatory bowel disease characterized by ulceration and neutrophil influx in intestinal epithelia leading to persistent inflammation (Rubin et al., 2017). The underlying cause of the disease is both environmental and genetic. Genome-wide association studies (GWAS) shortlisted ATG16L1 T300A as the causative mutation for the disease (Boada-Romero et al., 2016). Interestingly, the mutation does not perturb the general autophagy process but displays deficits in intracellular bacterial clearance. Studies have also identified single nucleotide polymorphisms in other autophagy-related genes such as NOD2 and immunity-related p47 guanosine triphosphatase (IRGM) in CD (Quaglietta et al., 2007; Rufini et al., 2015). Subsequent studies on IRGM showed a protective role during various intracellular infections. For instance, treatment of IFN induces IRGM during Mycobacterial infection to activate autophagy (Gutierrez et al., 2004). Further studies on the mechanism revealed that IRGM induces core autophagy machinery by interacting with ULK1 and Beclin1 to initiate autophagosome formation (Chauhan et al., 2015b, 2016).

Furthermore, the involvement of autophagy in diseases caused by lung inflammation such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) are reported. Mechanistically, mutation of CF transmembrane conductance regulator (CFTR), CFTR-F508

deletion showed inflammation of airway epithelial cells. This leads to Beclin1 downregulation and subsequent autophagy inhibition leading to protein aggregation and lung inflammation (Leung et al., 2017; Luciani et al., 2010). In case of COPD, there is inhibition of autophagy observed in lung biopsies of patients, although the mechanism of inhibition is not known (Vij et al., 2018).

Other autophagy loci implicated in causing genetic predisposition towards chronic inflammatory diseases include IRGM, DRAM1 (systemic lupus erythematosus), ATG5 (asthma, rheumatoid arthritis), CLEC16A (multiple sclerosis) (Martin et al., 2012; Orozco et al., 2011; Ramos et al., 2011; Schuster et al., 2015; Yang et al., 2013).

1.8 Xenophagy in therapeutics

Pharmacological or nutritional supplements to modulate xenophagy have shown beneficial effects in multiple clinical conditions (Galluzzi et al., 2017). For example, compounds such as rapamycin and trehalose which is known for inducing general autophagy flux was also seen to induce xenophagy (Donia et al., 2010). Alternatively, many studies have employed high content screening platforms to identify novel xenophagy compounds. For instance, during *Mycobacterium* infection, compounds such as nortriptyline, nitazoxanide, carbamazepine, valproic acid, naturally occurring trehalose and vitamin D3 have been identified by several groups (Lam et al., 2012; Schiebler et al., 2015; Sundaramurthy et al., 2013; Yuk et al., 2009). Similarly, many pharmacological screens for xenophagy of HIV is also reported. Compounds such as rapamycin, flubendazole, trehalose, SMER28 and Vitamin D3 are shown to induce xenophagy for restricting the viral replication (Campbell and Spector, 2011; Chauhan et al., 2015a; Donia et al., 2010; Floto et al., 2007). Pioneering work by Beth Levine's group identified autophagy-inducing peptide, Tat-Beclin1. It was constructed by fusing region of Beclin1 with Tat1 protein of HIV. This peptide prevented the replication of viral and bacterial

pathogens (Shoji-Kawata et al., 2013). Furthermore, many phenolic compounds such as resveratrol, quercetin, biochanin A present in certain plants have been shown to induce xenophagy of *S. typhimurium* (Al Azzaz et al., 2018; Domiciano et al., 2017; Zhao et al., 2018). Another interesting study by Kim *et al* indicated a novel strategy of studying the xenophagy potential of known antibiotics such as isoniazid and pyrazinamide. This study highlighted that the antibiotics although exhibit direct antibacterial effect, also significantly induces xenophagy for its overall efficacy against *M. tuberculosis* (Kim et al., 2012). Despite extensive screening strategies developed, most compounds exhibit limited specificity towards the autophagy process. For example, rapamycin which robustly induces autophagy also affects cellular growth and proliferation. Alternatively, certain autophagy modulators such as BafA1 also lead to general blockage of vesicular trafficking (Galluzzi et al., 2017). It is therefore, essential to identify specific and clinical viable autophagy modulators. Additionally, xenophagy mediated pharmacological intervention requires a detailed understanding of the aetiology of the disease. This is because certain pathogens are susceptible to xenophagic degradation by chemical inducers of the process while others utilize xenophagy for their intracellular survival and hence inhibition of the process is beneficial. Therefore, in most cases, pharmacological intervention during xenophagy is species-specific.

1.9 Aims of the study

Although the regulatory mechanisms of general autophagy are well studied, the upstream pathways that modulate xenophagy and how the xenophagic flux is regulated is not completely understood. Modulating the xenophagic flux either by genetic or chemical biology means is a commonly used approach to study the process. In this study, we have employed a combination of both approaches to understand the process and its regulatory mechanisms.

To study the process, we utilised *Salmonella typhimurium* as the model pathogen, as its involvement in xenophagy is clearly established. Pilot-scale screening for xenophagy specific chemical modulators designed to reduce intracellular *S. typhimurium* replication was carried out and the results were summarized in *Veena, MS thesis, 2016*. The shortlisted small molecule candidate is used as the tool in this study to further probe their mechanism of action to induce xenophagy. By understanding the pathways/genes targeted by the compound, it would provide key insights into the regulatory mechanisms that modulate the process. By employing this chemical-genetic approach, we enlisted the following objectives for the study.

Objectives:

- Identification and validation of acacetin as a novel xenophagy inducer.
- Exploring the molecular mechanism of acacetin to induce autophagy/xenophagy.
- Understanding the upstream regulatory pathways of xenophagy modulated by acacetin.
- Development of high throughput screening assay to identify novel xenophagy inducers

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Chapter 2

Materials and methods

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2.1 Cell Culture

HeLa, U1752 and RAW 264.7 cell lines were maintained in growth medium comprising of Dulbecco's modified Eagle's medium (DMEM)(D5648, Sigma-Aldrich) supplemented with 3.7 g/L sodium bicarbonate, 10% fetal bovine serum (FBS) (PAN, 3302-P121508) and 100 units/ml of penicillin and streptomycin (Sigma-Aldrich, P4333) at 5% CO2 and 37 °C. ATG5$^{-/-}$ HeLa cells was a kind gift from Prof. Richard Youle, NIH, USA (Lazarou et al., 2015).

2.2 Bacterial Strains and construction

The bacterial strains used in the study are WT *Salmonella typhimurium* SL1344, mCherry expressing *Salmonella typhimurium* SL1344 (kind gift from Prof. CV Srikanth, RCB, India) (Mohapatra et al., 2019). and bioluminescent pAKlux1 expressing *Salmonella typhimurium* SL1344. They were grown in Luria Bertani media at 37 °C.

2.2.1 Generation of luminescent *S. typhimurium*

S. typhimurium was electroporated with pAKlux1 (ampicillin resistant) construct to obtain bioluminescent bacteria. For the process, electrocompetent *S. typhimurium* was prepared by resuspending 200 ml of 0.5 A_{600} of bacterial cells in 50 ml of ice cold 10% glycerol and centrifuged at 2500 *g* for 15 minutes at 4 °C. This was followed by resuspending the pellet in 1ml of ice cold 10% glycerol and centrifuged at 2500 *g* for 15 minutes at 4 °C. The pellet was finally resuspended in 200 µl of ice cold 10% glycerol. The electrocompetent cells were added to 100 ng of pAKlux1 and was pulsed at 2500 V and 5 ms time-constant using BioRad electroporator. The pulsed cells were allowed to recover by diluting in 1ml of SOC media

(tryptone 2 g, yeast extract- 0.5 g, NaCl- 0.05 g, $MgSO_4$- 0.24 g, and KCl 18.6 mg) for 1 hour at 37 °C. Finally, 100µl of bacterial cells was plated on ampicillin containing LB plates. The colonies were confirmed by measuring luminescence at 460 nm using micro-titre plate reader, Varioskan Flash.

2.3 Chemicals and reagents

The chemicals used in the study include 3-Methyl Adenine (M9281, Sigma-Aldrich), wortmannin (W1628, Sigma-Aldrich), acacetin (00017, Sigma-Aldrich), Bay11-7082 (B5556, Sigma-Aldrich), U0126 (U120, Sigma-Aldrich), Bafilomycin A1 (11038, Cayman chemical). Reagents used in the study include, trypsin EDTA (59418C, Sigma-Aldrich), EBSS (E7510, Sigma-Aldrich) OPTI-MEM (31985-070, Invitrogen) and FITC-Dextran (46945, Sigma-Aldrich), DQ-BSA Red (D12051, Thermo Fisher Scientific), FM 4–64 (F34653, Thermo Fisher Scientific), LysoTracker Deep Red (L12492, Thermo Fisher Scientific), CIP (M0290S, New England Biolabs), triton X-100 (MB031, HiMedia Laboratories), Vectashield antifade reagent with or without DAPI (Vector laboratories, H-1000/H-1200), paraformaldehyde (P6148, Sigma-Aldrich), lipofectamine 2000 (11668019, Invitrogen), chemiluminescence substrate (170–5061, Clarity Bio-Rad), PVDF membrane (1,620,177, BioRad,), TRIzol (15596–026, Ambion), gentamycin (EAI03089, Abbott), Phosphate buffer saline solution (PBS) (D6773, Sigma-Aldrich), bovine serum albumin (A7906, Sigma-Aldrich), dimethyl sulfoxide (D8418, Sigma-Aldrich).

2.4 Antibodies

The primary antibodies used in the study are as follows: anti-TFEB antibody (4240), anti-RPS6KB1/p70S6K antibody (9208), anti-p-RPS6KB1/p70S6K (9202) antibody, anti-p-EIF4EBP1 antibody (2855), anti- EIF4EBP1 antibody (9452), anti-LAMP1 antibody (9091), anti- TBK1/NAK antibody (3504), anti-p-TBK1/NAK Ser172 antibody (5483), anti-EEA1

antibody (3288), anti-ACTB/β-actin antibody (4970), anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP; 7074) were purchased from Cell Signalling Technology. Anti-SQSTM1/p62 (ab56416) was purchased from Abcam. Anti-p-SQSTM1/p62 Ser403 (D343-3) was purchased from MBL. Anti-TUBB/β-tubulin antibody (DSHB-C1-377) was purchased from Developmental Studies Hybridoma Bank. Anti-H3 was a kind gift from Prof. Tapas Kundu, JNCASR. Anti-LC3B (L7543, Sigma-Aldrich), anti-p62 (PM045, MBL), anti-β-tubulin (E7-c, Biogenuix), Anti-rabbit IgG, HRP linked antibody (7074P2, CST), Atto 663 (18620, Sigma-Aldrich), Atto 488 (62197, Sigma-Aldrich).

2.5 Plasmid constructs and siRNA

Table 2.1: List of plasmids used in this study

Mammalian expression plasmids (Addgene)				
S. No.	**Plasmid name**	**Catalogue No.**	**Depositing lab**	**Reference**
1.	ptfLC3	21074	Tamotsu Yoshimori	(Kimura et al., 2007)
2.	LAMP1-RFP	1817	Walther Mothes	(Sherer et al., 2003)
3.	pCMV5-FLAG-Smad1	14044	Joan Massague	(Liu et al., 1996)
4.	HA-SnoN	10908	Bob Weinberg	(Sun et al., 1999)
5.	TLR4-YFP	13018	Doug Golenbock	Unpublished
6.	pcDNA3-RelB	20017	Stephen Smale	Unpublished
7.	Myc-Smurf2	13678	Ying Zhang	(Zhang et al., 2001)
8.	BAMBI-Bio-His	51866	Gavin Wright	(Sun et al., 2015)
9.	NOD1-EGFP	131206	Thomas Kufer	Unpublished
10.	HA-hTRAF3	44032	Shao-Cong Sun	(Liao et al., 2004)
11.	GADD45	24929	Xin Wang	(Yang et al., 2000)
12.	pcDNA3-FLAG-Fos WT	8966	John Blenis	(Murphy et al., 2002)

Mammalian expression plasmids (Addgene)				
S. No.	**Plasmid name**	**Catalogue No.**	**Depositing lab**	**Reference**
13.	BDNF-SEP	83955	Ryohei Yasuda	(Harward et al., 2016)
14.	pEGFP-N1-TFEB	38119	Shawn Ferguson	(Roczniak-Ferguson et al., 2012)
15.	pcDNA3-FLAG-Rheb-N153T	19997	Fuyuhiko Tamanoi	(Urano et al., 2007)
16.	pCMV6-XL4 FLAG-BMP2-Fc	115771	Davide Comoletti	Unpublished
17.	pMSCV-FlagBcl10	18718	Jon Ashwell	(Wu and Ashwell, 2008)
18.	pcDNA3 Flag-humanTRAF6	66929	Michael Karin	Unpublished
19.	Flag-SIRT1	1791	Michael Greenberg	(Brunet et al., 2004)
20.	pXJ40 GADD34 Wt (1-674aa)	75478	Shirish Shenolikar	(Choy et al., 2015)
21.	THBS1-bio-His	53417	Gavin Wright	(Sun et al., 2015)
22.	IKK1-EYFP	111206	Johannes A. Schmid	Unpublished
23.	GFP-p38alpha	86832	Rony Seger	(Zehorai and Seger, 2014)
24.	pCMV6-XL4 FLAG-BMP2-Fc	115771	Davide Comoletti	Unpublished
25.	pcDNA3-HA-RAPGEF2	110162	Daniele Guardavaccaro	(Magliozzi et al., 2013)
Silencing constructs (Sigma-Aldrich)				
	scramble esiRNA	SIC001	TFEB esiRNA	EHU059261
Bacterial expression plasmids (Addgene)				
	pAKlux1.1	14073	Attila Karsi	(Karsi et al., 2006)

2.6 Protocols used in the study

2.6.1 Immunofluorescence assay

HeLa cells (~60% confluency) were seeded on coverslips and was allowed to attach overnight. Cells were transfected with plasmid construct(s) using lipofectamine 2000 as per the manufacturer's instructions. Briefly, for a 60 mm dish with cells, reaction mixture containing 2.5 µg of maxi prep grade DNA and 5 µl of lipofectamine (1:2 ratio) diluted in 100 ul of OPTI-MEM was added to cells. Forty-eight hours post transfection, compound treatment was carried out for the indicated time points. Following this, cells were processed for immunofluorescence analysis

Fixation: Cells were fixed using 4% paraformaldehyde (PFA) for 20 minutes at room temperature (RT) kept in dark.

Washes: The cells were washed using 1X PBS for 10 minutes at RT kept in dark.

Permeabilization: The cells were permeabilized 0.25% triton X-100 for 20 minutes at RT kept in dark.

Washes: The cells were washed using 1X PBS for 10 minutes at RT kept in dark.

Primary antibody staining: Primary antibody/antibodies (1:500 dilution) were diluted in 0.2% Bovine Serum Albumin (BSA) in PBS. The cells were incubated with antibody for overnight at 4 °C in dark. In case of probing for more than one protein simultaneously, antibodies can be coupled if the species used to raise the antibody is different.

Washes: After 12-16 hours, cells were washed three times using 1X PBS for 5 minutes each at RT kept in dark.

Secondary antibody staining: Appropriate secondary antibody/antibodies (1:200 dilution) were diluted in 0.2% Bovine Serum Albumin (BSA) in PBS. The cells were incubated with antibody for 1-2 hours at RT in dark. In case of probing for more than one protein simultaneously, antibodies can be coupled if the species used to raise the antibody is different.

The antibodies coupled together were previously studied for cross reactivity and background fluorescence by using 'no primary antibody' control.

Washes: After 12-16 hours, cells were washed three times using 1X PBS for 5 minutes each at RT kept in dark.

Mounting: The cover slips were mounted on glass slides using Vectashield antifade reagent with or without DAPI. Following this, the slides were imaged for fluorescence microscopy analysis or stored at 4°C in dark for future use.

2.6.2 Lysosome staining

a. LysoTracker Deep Red staining

LysoTracker Deep Red fluorescent dye at a final concentration of 100 nM was added to HeLa cells. Post 20 minutes of incubation at 37 °C, the cells were fixed using 4% PFA for 20 minutes. Following this, cells were washed with PBS and permeabilized using 0.25% triton X-100. The cover slips were mounted using Vectashield antifade reagent with or without DAPI.

b. DQ-BSA processing assay

HeLa cells were seeded on cover slips and allowed to attach overnight. DQ-BSA (10 µg/ml) was added to HeLa cells or HeLa cells post *S. typhimurium* infection for 2 hours. In the presence of acacetin (50 µM), the DQ-BSA processing was allowed for 4 hours and fixed using 4% PFA for 20 minutes. For antibody staining, permeabilization with 0.25% triton X-100 for 20 minutes was carried out. This was followed by anti-LAMP1 primary antibody (1:500 dilution) incubation overnight at 4 °C and appropriate secondary antibody (1:200 dilution) incubation for 1 hour at room temperature. The cover slips were mounted using Vectashield antifade reagent with or without DAPI.

2.6.3 Immunoblotting analysis

Cells were seeded in 6-well plates and allowed to attach overnight. Following appropriate treatments, cells were collected in 1X Laemmli buffer using cell scrapper until the sample

buffer becomes viscous. Following this, the samples were boiled for 10 minutes at 95 °C. Samples were stored at -80 °C until electrophoresed on SDS-PAGE (gel percentage varying from 8% to 15% depending on the protein of interest) and transferred onto PVDF membrane. The blots were incubated with 5% skimmed milk for 1 hour at room temperature for blocking non-specific antibody binding. Primary antibody (1:1000 dilution) was prepared in 0.2% BSA in 1X PBS. After incubation with primary antibody overnight at 4 °C the blots were washed three times for 5 minutes each in 1X PBST. Following this, appropriate HRP conjugated secondary antibody (1:10,000 dilution) was prepared in 5% skimmed milk and incubated with secondary antibody for 1 hour at room temperature, signals were obtained using chemiluminescence substrate and the image was acquired using gel documentation system (G-box, Chemi XT 4, Syngene, USA). The band intensities were quantified using ImageJ software (NIH).

2.6.4 TFEB silencing

HeLa cells were seeded on 6-well plates and allowed to attach overnight. The following day, cells were transfected with either scramble control or esiRNA-TFEB using lipofectamine 2000. Briefly, reaction mixture containing 1000 ng of esiRNA-TFEB or scramble control diluted in 100 µl OPTI-MEM was added to HeLa cells. After 48 hours of incubation, cells were treated with acacetin. After 2 hours of compound treatment, cells were processed either for immunoblotting analysis or immunofluorescene assay as explained in the previous sections.

2.6.5 Cytoplasm-nucleus fractionation of TFEB

HeLa cells were seeded on 100 mm dish (~60% confluency) and allowed to attach overnight. This was followed by transfection of GFP-TFEB using lipofectamine 2000 as previously explained. Post 48 hours of transfection, the cells were treated with acacetin. After 2 hours of compound treatment, cells were trypsinized and collected in 1X PBS. Following this, cells were lysed using a buffer containing 0.8 M sucrose, 150 mM HCl, 5 mM $MgCl_2$, 6 mM β

mercaptoethanol and 0.5% NP-40. The samples were centrifuged at 10,000 *g* for 5 minutes at 4 °C. The supernatant containing cytoplasmic fraction was separated from the nuclear pellet. The nuclear pellet was washed twice using the same lysis buffer to remove any cytoplasmic contamination. The separated fractions were prepared for immunoblotting analysis as explained previously. Samples were electrophoresed on SDS-PAGE (8% or 10% gel percentage in order to visualize both phosphorylated and total TFEB protein bands) and transferred onto PVDF membrane. After incubation with anti-TFEB antibody overnight at 4°C and HRP conjugated secondary antibody for 1 hour at room temperature, signals were obtained using chemiluminescence substrate and image was acquired using gel documentation system (G-box, Chemi XT 4, Syngene, USA).

2.6.6 Gene expression analysis

HeLa cells were seeded on 60 mm dishes and allowed to attach overnight. The following day, cells were treated with acacetin for 2 hours. Cells were lysed to isolate total RNA using 1ml of TRIzol reagent. After homogenization, samples were centrifuged at high speed (12000 *g* at 4 °C for 5 minutes). Clear supernatant collected after centrifugation was added to 200 µl chloroform. This was followed by 2-3 minutes of incubation and proceeded to centrifugation at 12,000 *g* at 4 °C for 15 minutes. The aqueous layer containing RNA was collected for precipitation of RNA. The samples were then treated with 0.5 ml of isopropanol and incubated for 10 minutes. Centrifugation at 12,000 *g* at 4 °C for 10 minutes precipitated white gel-like RNA pellet. The pellet was washed using 1 ml of 75% ethanol for 5 minutes at 7500 *g* at 4 °C and resuspended the RNA in 30 µl of RNase free water. The samples were then subjected to DNase treatment for 2 hours at 37 °C. The reaction mixture contained 4 µg of isolated RNA, 1X DNase buffer, DNase enzyme and RNase free water in a total reaction volume of 20 µl. The DNase reaction was terminated by adding 2 µl of 50 mM EDTA and heated at 65 °C for 15 minutes.

a. RT-PCR:

Reverse transcription (RT) was carried out to convert isolated RNA into cDNA using Taqman reverse transcription kit (Applied Biosystems, N8080234). Briefly, the 20 µl reaction mixture contained 1 µg of DNase treated RNA diluted with RT enzyme, 10X RT buffer, 100 mM dNTPs, and 10X random primers. Autophagy and lysosomal specific gene primers which were previously reported (Sardiello et al., 2009) were purchased from Sigma-Aldrich. The housekeeping gene ACTB was used as normalizing control to calculate the fold change.

Table 2: List of primers used in this study

Gene	Forward (5' to 3')	Reverse (5' to 3')
ACTB	CATCATGAAGTGTGACGTGGAC	CTTGATCTTCATTGTGCTGGGTG
LC3B	ACCGTGTGATCAGTAAGATTCC	GTGACCACTCACATGGGATATAG
BECN1	CCCGTGGAATGGAATGAGATTA	CCGTAAGGAACAAGTCGGTATC
BCL2	AGATGGAGCATGAATGGTACTG	TCTGTGCTCAGCTTGGTATG
TFEB	CCAGAAGCGAGAGCTCACAGAT	TGTGATTGTCTTTCTTCTGCCG
LAMP1	ACGTTACAGCGTCCAGCTCAT	TCTTTGGAGCTCGCATTGG
SQSTM1	GCACCCCAATGTGATCTGC	CGCTACACAAGTCGTAGTCTGG

The following program for RT-PCR was used

Initial denaturation 95 °C	5 minutes
Denaturation 95 °C	30 seconds
Annealing 60 °C	30 seconds
Extension 72 °C	30 seconds
Final extension 72 °C	5 minutes
Step 2 to 4 repeated for 40 cycles	

b. Microarray analysis:

Clariom D Affymetrix microarray technology from ThermoScientific was used for microarray analysis. The experiment was carried out for two biological replicates with two technical replicates each. The data analysis post-run was carried out using Applied Biosystems Transcriptome Analysis Console (TAC) Software 4.0. The foldchange of above 2 with p-value of less than 0.05 was set as threshold for shortlisting genes.

2.6.7 Intracellular infection assay

Single colony of *S. typhimurium* was grown for 6 hours at 37 °C in a shaking incubator. Secondary culture (0.2% inoculum) was grown overnight in micro-aerophilic conditions. U1752 or HeLa (WT and ATG5$^{-/-}$) or RAW264.7 cell lines were infected at a multiplicity of infection (MOI) of 200 for 1 hour. The cells were treated with media containing 40 μg/ml gentamycin for 1 hour to kill the extracellular bacteria. The cells were then treated with the compound and incubated for 4 hours. Finally, the mammalian cells were lysed using lysis buffer (0.1% SDS, 1% triton X-100, 1X PBS). The intracellular *S. typhimurium* were plated on Luria Broth plates and incubated at 37 °C. After 12-16 hours of incubation, the Colony Forming Unit (CFU) were counted.

2.6.8 Cell viability assay

HeLa cells were seeded on a 384-well plate and treated with indicated concentrations of acacetin. After 15 hours of compound treatment, cell viability was measured using

luminescence-based Cell Titre Glo cell viability assay kit (G7572, Promega) using a micro-titre plate reader (Varioskan Flash, Thermo Fisher Scientific, USA).

2.6.9 Animal studies

All procedures carried out in the study were approved by JNCASR and Vipragen Biosciences animal ethics committee. BALB/c mice (6–8 weeks of age, female) were distributed into three groups namely- uninfected, infected, and infected along with acacetin treatment. Acacetin (20 mg/kg) was administered intraperitoneally to the infected with acacetin treatment group. The compound treatment continued for next 7 days with one injection a day. Other two groups were injected with vehicle solvent, dimethyl sulfoxide (D8418, Sigma-Aldrich). Infection (10^{10}*Salmonella* diluted in PBS) was done through oral gavage for the second and third group on the first day. Food and water were available *ad libitum.* All animals were sacrificed on the seventh day post infection after 2 hours of compound injection and the organs (liver, spleen and intestine) were processed for plating and immunohistochemistry.

2.6.10 Immunohistochemistry

Harvested tissues were washed with 40 µg/ml gentamycin to remove extracellular bacteria.

Fixation: Tissues were fixed using 4% PFA overnight at room temperature (RT).

Washes: The cells were washed using 1X PBS for 10 minutes at RT.

Processing: The tissues were exposed to increasing gradient of cryoprotectant, sucrose from 10% to 15% to finally 30% sucrose. The samples begin to sink in sucrose. After they are sunk, the samples are ready for cryo-sectioning.

Washes: The cells were washed using 1X PBS for 10 minutes at RT.

Sectioning: The cryostat was set at a temperature of -20 °C. All equipment (cutting blade, foreceps) used for sectioning was also prechilled to -20 °C before use. The tissues were embedded using cryoembed onto the block-holder. Serial sections of 40 µm thicknes were

collected on gelatin coated slides. Briefly, solution containing 3% gelatin and 0.5% Chromium Potassium Sulphate maintained at 65 °C was used to coat the glass slides.

Antigen retrieval: The sections were immersed in Sodium Citrate buffer (pH 6) at 65 to70 °C for 20 minutes intermittently using a coplin jar. The slides are allowed to remain in the buffer until temperature falls below 60 °C.

Equilibration: The Slides were washed using PBSTx (0.1 M PBS containing 0.1% triton x-100) for 30 minutes.

Blocking: Sections were incubated in blocking buffer (2% BSA and 1% goat serum made in PBSTx) for 4 hours at RT.

Washes: Sections were washed three times using 1X PBSTx for 10 minutes each at RT.

Primary antibody staining: Primary antibody/antibodies (1:500 dilution) were diluted in 0.2% Bovine Serum Albumin (BSA) in PBST. The cells were incubated with antibody for overnight at 4°C in dark. In case of probing for more than one protein simultaneously, antibodies can be coupled if the species used to raise the antibody is different.

Washes: After 12-16 hours, cells were washed three times using 1X PBS for 5 minutes each at RT kept in dark.

Secondary antibody staining: Appropriate secondary antibody/antibodies (1:200 dilution) were diluted in 0.2% Bovine Serum Albumin (BSA) in PBST. The cells were incubated with antibody for 1-2 hours at RT in dark. In case of probing for more than one protein simultaneously, antibodies can be coupled if the species used to raise the antibody is different. The antibodies coupled together were previously studied for cross reactivity and background fluorescence by using ‘no primary antibody’ control.

Washes: After 4 hours, cells were washed three times using 1X PBS for 5 minutes each at RT kept in dark.

Mounting: The cover slips were mounted on glass slides using Vectashield antifade reagent with or without DAPI. Following this, the slides were imaged for fluorescence microscopy analysis or stored at 4 °C in dark for future use. Images were acquired using Olympus FV 3000 (1.25X objective was used for imaging entire liver section, 40X objective was used for observing LC3-II puncta).

2.6.11 Luciferase-based infection assay for small molecule screening

HeLa cells were seeded in 96-well plate and were allowed to attach overnight. The following day, HeLa cells were infected with *S. typhimurium* expressing bioluminescent construct at a MOI of 100 for 1 hour. This was followed by treatment with gentamycin and small molecule compounds of a custom-made library (410 compounds). After 11 hours of incubation, HeLa cells were lysed and intracellular luminescent reading was measured (Varioskan Flash, Thermo Scientific, USA). The experiment was performed in triplicate for each compound and in two different concentrations (12.5 μM and 25 μM) with rapamycin (8 μM) as a positive control in each plate was used.

2.6.11.1 Data normalization using HTS corrector

The excel file containing RAW luminescent values was uploaded in HTS corrector. A background trend based on each well value was evaluated using the option "*Background evaluation*" in "*Analysis"* tab. Background subtraction was done to get the normalized values.

2.6.11.2 Data visualization using R studio application

A directory was opened in R studio to save the excel sheet containing normalized luminescent values in .csv format. The packages used to get the HTS plot include plyr, tidyr, ggplot2, dyplyr (for box plot), forcats, tidyverse, hrbrthemes and viridis (for colours and patterns).

2.7 Microscopy

a. Fluorescence microscopy

Images were acquired on the widefield Delta Vision microscope (29,065,728, API, GE, USA) using DAPI, FITC, TRITC, Cy5 filters with Olympus 60X/1.42 NA objective. For every experiment, quad filters, 'Z' sections and acquisition parameters were maintained constant.

b. Electron microscopy

HeLa cells treated with acacetin for 2 hours were further processed for electron microscopy. In case of infection assay, HeLa cells post 6 hours of *S. typhimurium* infection were trypsinized and washed with 1X PBS. Cells were fixed using 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 hour at 4 °C. Sample processing for Transmission Electron Microscopy was carried out in Christian Medical College, Vellore, India. Briefly, fixed cells were dehydrated in ethanol series and embedded in epoxy resin (TAAB laboratory and microscopy, CY212 KIT, E009). Ultra-thin sections were stained and observed using Tecnai, G2 F-30 with a point resolution of 2.2 Å.

2.7.1 Image analysis

Fluorescence images acquired using widefield microscope were deconvolved (nearest neighbour algorithm) using Delta Vision SoftWorx software. For TFEB translocation assays, intensity of single Z-stack with largest DAPI structure was measured. For colocalization analysis, individual Z stacks were analysed using "Colocalization" plugin with "colocalization highlighter" option in ImageJ (NIH). The number of colocalized events were counted using "cell counter" plugin of ImageJ. Whereas, for experiments that required counting total puncta inside cells, projected images (collapsed Z-stacks) were used for quantification. However, all representative images are projected images for better clarity.

2.8 Statistical analysis

Graphs were plotted and the significance levels were tested using appropriate statistical test in GraphPad Prism (GraphPad software). Statistical tests were performed by comparing the means using Unpaired Student t-test or One/Two-way ANOVA followed by the Bonferroni test. 3 biological replicates were considered for every analysis unless mentioned otherwise.

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Chapter 3

Identification and validation of acacetin as a novel xenophagy inducer

The content of this chapter is published in *Autophagy* journal.

Restriction of intracellular *Salmonella* replication by restoring TFEB-mediated xenophagy.

Ammanathan V, Mishra P, Chavalmane AK, Muthusamy S, Jadhav V, Siddamadappa C, Manjithaya R. Autophagy. 2019 Nov 19:1-14. doi: 10.1080/15548627.2019.1689770

Chapter 3

Identification and validation of acacetin as a novel xenophagy inducer

3.1 Overview

In the introduction, we have described in detail the role of autophagy in maintaining cellular homeostasis. The process gained importance because of its intricate connection with health and disease. Autophagy degrades therapeutically relevant cargoes such as aggregated proteins, intracellular pathogens, and damaged organelles. As described earlier, dysfunctional autophagy can lead to the progression of diseases such as neurodegeneration, cancer, microbial infections, cardiovascular diseases among others.

In this study, we have explored the selective autophagy of intracellular pathogens referred to as xenophagy. It is considered as a part of the innate immune mechanism of the host cell in response to intracellular infection. The process of xenophagy is implicated during diverse bacterial, viral, and parasitic infections (Deretic and Levine, 2009). However, for the current study, we have explored antibacterial xenophagy using *Salmonella typhimurium* as the pathogen model system.

3.2 *Salmonella typhimurium* as a model system for xenophagy

S. typhimurium is a rod-shaped, flagellated, Gram-negative bacterium that is a major causative agent for gastroenteritis referred to as salmonellosis in humans and typhoid-like disease in mice. *S. typhimurium* is a member of genus *Salmonella enterica*, with more than 2500 serotypes reported. The pathogen harbours two type-III secretion systems (T3SS), which are encoded by Salmonella pathogenicity islands, SPI-I and SPI-II, responsible to impart virulence to the bacteria (Castanheira and Garcia-Del Portillo, 2017; Hansen-Wester and Hensel, 2001). The T3SS forms a protein complex referred to as "molecular syringe" for translocation of bacterial proteins called effectors inside host cytoplasm. Genes encoded by SPI-I is required for invasion of host cells, while intracellular survival and replication are dependent on genes encoded by SPI-II (Hensel, 2000; Steele-Mortimer et al., 2002). Briefly, effector proteins translocated inside host cells lead to drastic changes such as reorganization of cytoskeletal proteins to bring about membrane protrusion and finally mediating its own engulfment (Ly and Casanova, 2007; Srikanth et al., 2011; Srikanth et al., 2010). After invasion, the effector proteins hijack host endocytic pathway as explained below for their intracellular survival.

3.2.1 The intracellular lifestyle of *S. typhimurium*

S. typhimurium enter inside host cells either by phagocytosis (in phagocytic cells) or *Salmonella* mediated invasion (in non-phagocytic cells), During the course of infection, phagosomes are rich in early endosomal markers in the initial time points which are replaced by late endosomal markers after few hours of post-infection (p.i). *Salmonella* replicates inside phagosomes by converting it into its replicating niche referred to as *Salmonella* containing vesicles (SCVs) (Castanheira and Garcia-Del Portillo, 2017; Krieger et al., 2014; Drecktrah et al., 2007; Vorwerk, 2015, Liss et al., Cell Host Microbe, 2017). SCVs also develop membranous structures called as *Salmonella* induced filaments (Sifs) (Knuff and Finlay, 2017).

The SCVs provide an ideal niche for persistent *Salmonella* survival as compared to the lethal cytosolic environment of macrophages (Sindhwani et al., 2017; Tuli and Sharma, 2019). However, the cytoplasm of epithelial cells provides abundant nutrients for the bacteria and is conducive for robust replication (Brumell et al., 2002; LaRock et al., 2015; Malik-Kale et al., 2012) (**Figure 3.1**).

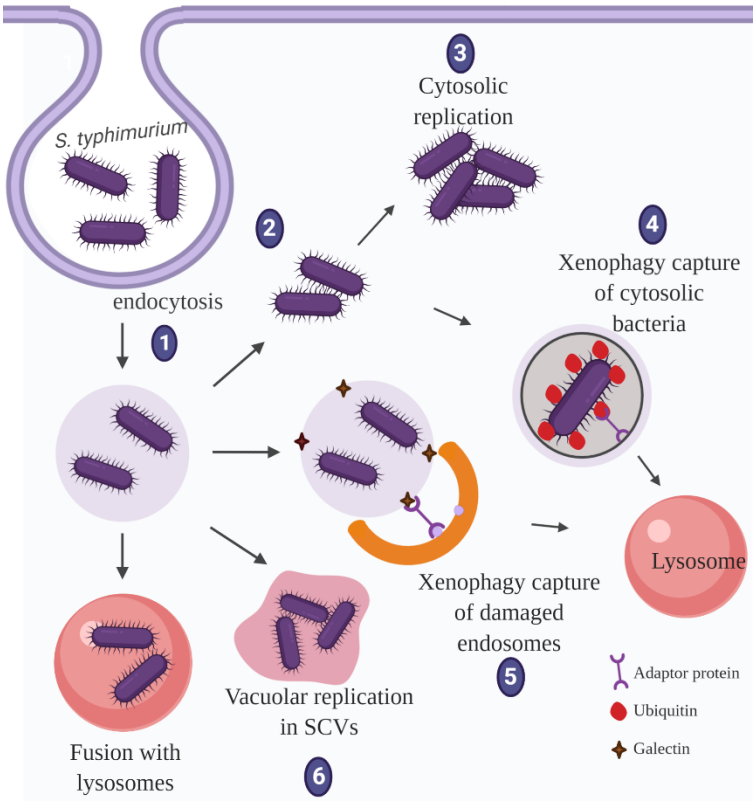

Figure 3.1. The intracellular lifestyle of *S. typhimurium*. Upon entry into host cells (1) by *Salmonella* mediated invasion, *S. typhimurium* can exist in one of the following populations. (2) Enter cytosol by damaging endosomes where (3) rapid replication occurs in the cytosol. (4) Cytosolic ubiquitinated bacteria are recognized by xenophagy proteins for autophagy-mediated degradation. (5) In a ubiquitin independent manner, damaged bacteria-containing endosomes exposing galectins on the surface are captured by xenophagy. (6) Persistent survival occurs in *Salmonella* containing vacuoles. Image created using BioRender.com.

3.2.2 Interaction of *S. typhimurium* with xenophagy machinery

The interactions of *S. typhimurium* with the process of xenophagy is well studied and reported (Siqueira et al., 2018; Wu et al., 2020). Firstly, entry of bacteria inside host cells by disruption of membrane integrity leads to loss of amino acids from the cell and causes starvation. This inhibits the nutrient responsive kinase, mTOR which in turn leads to activation of autophagy (Tattoli et al., 2012). Following invasion, about 30% of intracellular *S. typhimurium* is reported to be recognized by xenophagy machinery in the initial stages of infection (1-1.5hours p.i.) (Birmingham et al., 2006). Subsequent studies showed that *S. typhimurium* is recognized by xenophagy machinery by two mechanisms. The first involves a mechanism similar to other selective autophagy cargoes where intracellular bacteria targeted to xenophagy are ubiquitinated. Following this, adaptor proteins recognize the ubiquitinated bacteria to bridge with xenophagosomes for degradation. The second mechanism for *S. typhimurium* capture is by recognizing the damaged SCVs resulted due to pore formation by SPI-II T3SS (Birmingham and Brumell, 2006; Kreibich et al., 2015). If this damage caused to SCVs are sufficient to allow access to ubiquitin ligases, the vacuolar bacteria are ubiquitinated (Perrin et al., 2004). Alternatively, damaged SCVs expose glycans which is usually present on the inner surface of SCVs. This glycans are recognized by galectin 8, a cytoplasmic lectin that in turn recruit autophagy adaptors to SCVs (Thurston et al., 2012).

The recognition of intracellular *S. typhimurium* by adaptor proteins play a crucial role in restricting bacterial replication. Major adaptor proteins implicated in xenophagy of *S. typhimurium* include p62, NDP52, TAX1BP1 and OPTN (Mostowy et al., 2011; Tumbarello et al., 2015; von Muhlinen et al., 2010; Zheng et al., 2009). Silencing of adaptors such as NDP52 and OPTN is shown to weaken xenophagy of *S. typhimurium* leading to enhanced bacterial replication (von Muhlinen et al., 2012; Wild et al., 2011). Elaborate studies on

mechanistic aspects of autophagy-mediated cargo recognition identified that that post-translational modification in UBA domain of adaptor proteins can enhance its affinity to bind ubiquitin. For example, phosphorylation of p62 at position Serine 403 that is present in its UBA domain increases the binding affinity by to bind ubiquitin by multiple folds. Similarly, phosphorylation event in LIR sequence that binds LC3 also enhances recognition. This is seen in OPTN (Serine 177 position) to increase its ability to bind the cargoes. Studies indicate that although adaptor proteins such as p62 and NDP52 act in the same pathway to capture *S. typhimurium*, they are not redundant as knockdown of either adaptor significantly reduces LC3 recruitment to *Salmonella* (Cemma et al., 2011). Despite multiple xenophagic proteins involved, there is a drastic reduction in the recognition of *S. typhimurium* post 2 hours (Cemma et al., 2011). These studies suggest that xenophagy mediated recognition of *S. typhimurium* is an early event and pathogen employ strategies to overcome xenophagy at later stages of infection.

3.2.3 Mechanisms employed by *S. typhimurium* to escape xenophagy

Although intracellular xenophagy is induced by multiple strategies, *S. typhimurium* has evolved mechanisms to evade the capture and lysosomal degradation. As mentioned before, recognition of *S. typhimurium* by xenophagy adaptor proteins is short-lived occurring immediately after infection. Certain *S. typhimurium* factors have been identified to prevent this xenophagic recognition. For example, *S. typhimurium* effector protein, SseL is a deubiquitinase that prevents its ubiquitination and subsequently prevents recognition by xenophagic adaptors (Mesquita et al., 2012). Other effectors such as SsaV and SsrB disrupts Sirt1/LKB1/AMPK pathway leading to activation of mTOR and thereby inhibiting autophagy (Ganesan et al., 2017). Besides, studies have also shown this mTOR activation is amplified by recruitment of focal adhesion kinase to SCVs (Owen et al., 2014). Additionally, *S. typhimurium* replicating in

SCVs express SifA effector, which perturbs the trafficking of proteases to lysosomes. Therefore, the lysosomal activity of the infected cells is compromised providing a niche for bacteria to replicate in SCVs (McGourty et al., 2012). Further, effectors such as SseG and SseF are involved in maintaining the membrane integrity of SCVs thereby supporting bacterial replication (Deiwick et al., 2006). Furthermore, study by Feng *et al* showed that SseG and SseF also inhibits ULK1 activation by blocking Rab 1A activity to generate PI3P. This leads to decreased autophagy potential in the host cells (Feng et al., 2018).

3.3 Rationale of the study

As explained in the earlier section, the interaction of *S. typhimurium* with xenophagy is well-known and the pathogen is shown to overcome host xenophagy to establish intracellular infection. It, therefore, provides an ideal pathogen model system to modulate xenophagy. We hypothesized that chemical modulation of xenophagy to restore the block imposed by *S. typhimurium* can provide mechanistic insights into the process.

In pursuit of identifying novel chemical modulators of autophagy, our laboratory had previously performed a yeast-based high throughput screening (HTS). Briefly, firefly luciferase containing peroxisomal targeting signal, SKL driven under POT1 (Peroxisomal Thiolase-I) promoter was used to label peroxisomes for monitoring autophagic flux (Sakai et al., 2006). The principle of the HTS assay involves induction of peroxisome biogenesis by growing yeast in fatty acid-containing media. The rates of degradation of luciferase targeted to peroxisomes were monitored after autophagy induction and in the presence of compounds. Those compounds that induced more than 50% peroxisome degradation were shortlisted as putative enhancers of autophagy (Mishra et al., 2017).

Owing to the conserved nature of autophagy, the hits obtained in yeast-based primary screen were further tested for their potential in mammalian cells to induce general and selective

autophagy. Here we present a pilot-scale secondary assay for screening the autophagy inducers for their ability to induce xenophagy. The assay is based on quantitating the reduction of intracellular *S. typhimurium* in the presence of the compounds tested.

3.4 Results:

3.4.1 Pilot-scale screening for xenophagy inducers

The set of autophagy inducers shortlisted from the HTS were further tested for their ability to clear intracellular pathogens. The screening for xenophagy involves monitoring *Salmonella typhimurium* after intracellular infection of host epithelial cell line, U1752, in the presence of compounds until 6 hours p.i (**Figure 3.2A**). The compounds that induced pexophagy in yeast and autophagy in mammalian cells were further tested for their ability to induce xenophagy. However, most of the compounds tested were not able clear intracellular *S. typhimurium* (**Figure 3.2B** & Veena, MS thesis, 2016). Acacetin, a plant-derived flavonoid from ENZO library of natural compounds, showed a two-fold decrease in the intracellular pathogen numbers as observed by colony-forming-unit (CFU) assay. The acacetin-mediated reduction in bacteria was tested in other cell lines such as HeLa epithelial and RAW 264.7 macrophages. Consistent results were obtained in all the tested mammalian cell lines (**Figure 3.2C** & Veena, MS thesis, 2016).

Further, the toxicity of the compound was tested using Cell Titre-Glo cell viability assay. It is a luminescent based method to determine the number of viable cells in culture by measuring the amount of ATP generated by metabolically active cells. The CellTitre Glo reagent utilizes the ATP from the viable cells to fuel the reaction of luciferin in the reagent to form oxyluciferin. We used various concentrations of acacetin and even at 100 µM concentration, the compound did not show any significant loss in cell viability for the 15 hours tested (**Figure 3.3A** & Veena,

MS thesis, 2016). Further, the compound showed a dose-dependent effect in decreasing the intracellular pathogen burden and was most effective at 50 µM concentration of the compound for 6 hours (Figure 3.3B & Veena, MS thesis, 2016). Therefore, for further experiments, acacetin at 50 µM was used for a maximum period of 6 hours.

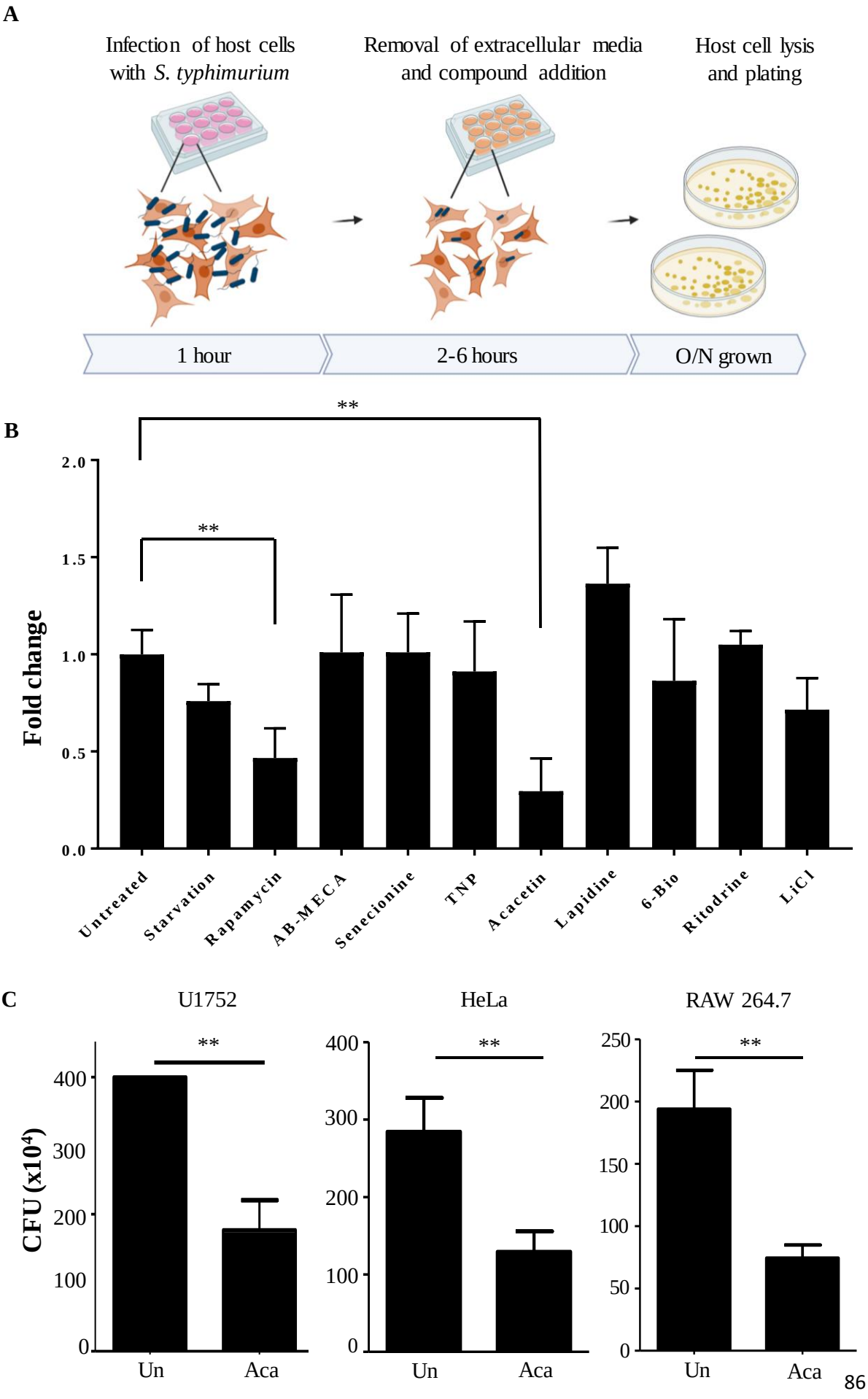
A
Infection of host cells with S. typhimurium
Removal of extracellular media and compound addition
Host cell lysis and plating
1 hour
2-6 hours
O/N grown
B
Fold change
2.0
1.5
1.0
0.5
0.0
**
**
Untreated
Starvation
Rapamycin
AB-MECA
Senecionine
TNP
Acacetin
Lapidine
6-Bio
Ritodrine
LiCl
C
U1752
HeLa
RAW 264.7
CFU (x10⁴)
400
300
200
100
0
250
150
50
**
Un
Aca

Figure 3.2. Pilot-scale secondary screening assay to identify xenophagy inducers. (A) Schematic of intracellular infection assay. Epithelial or macrophage cell lines were infected with *S. typhimurium* for one hour. This was followed by the removal of extracellular bacteria in the presence of gentamycin. The infected host cells were treated with the compounds and incubated until 6 hours p.i. Finally, the host cells were lysed, and the intracellular bacteria were plated on Luria Bertani agar plates. (B) Graph showing the fold change values for compounds tested in the pilot-scale screening. Starvation (HBBS media) and rapamycin (8 μM) were used as positive controls. (C) Graph showing CFU values indicating intracellular *S. typhimurium* in U1752 epithelial cells, HeLa epithelial cells and RAW 264.7 macrophages. Statistical analyses on three independent experiments were performed using unpaired student's two-tailed t-test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

3.4.2 Validation of acacetin as a xenophagy inducer

Before proceeding to xenophagy assays, we performed certain validation experiments to understand the effect of acacetin on host cells as well as on *S. typhimurium*. In this regard, it is possible that acacetin may impart an antibacterial effect like conventional antibiotics, by directly targeting bacterial components besides its role in inducing autophagy flux. To investigate this aspect, *S. typhimurium* growth in extracellular Luria Bertani broth in the presence of acacetin was monitored. It was noticed that there was no lag in the growth kinetics of acacetin treated samples compared to that of untreated cells. However, gentamycin used as antibiotic control showed a decrease in the growth of *S. typhimurium* (**Figure 3.3C** & Veena, MS thesis, 2016).

Next, we questioned the essentiality of autophagy for the acacetin-mediated decrease in intracellular *S. typhimurium* growth. This was done by performing intracellular infection in autophagy-deficient $ATG5^{-/-}$ HeLa cell line. We observed that acacetin was non-functional in this autophagy-deficient cell line (**Figure 3.3D** & Veena, MS thesis, 2016). To further confirm

this observation, chemical inhibitors of autophagy such as 3-Methyladenine (3-MA) and Wortmannin (Wort) were used in the presence and absence of acacetin. Acacetin failed to decrease the bacterial burden as observed by both CFU quantitation and microscopy analysis when co-treated with chemical inhibitors (**Figure 3.3E-G** & Veena, MS thesis, 2016). These results highlight that functional autophagy is essential for acacetin-mediated xenophagy effect.

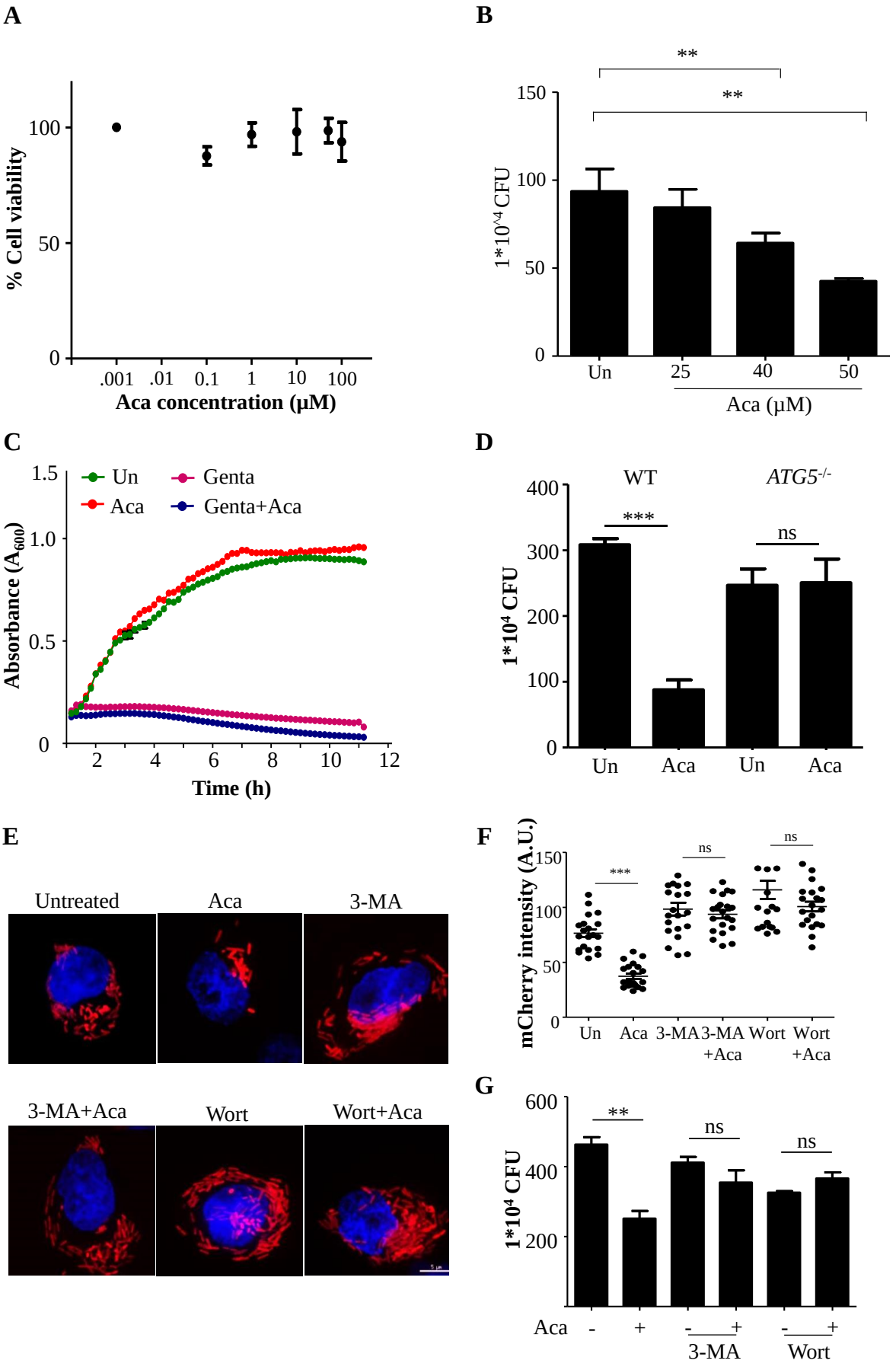
A
% Cell viability
100
50
0
.001 .01 0.1 1 10 100
Aca concentration (µM)
B
**
**
150
100
50
0
1*10^4 CFU
Un 25 40 50
Aca (µM)
C
Un
Genta
Aca
Genta+Aca
Absorbance (A600)
1.5
1.0
0.5
0
2 4 6 8 10 12
Time (h)
D
WT
ATG5-/-
***
ns
400
300
200
100
0
1*10^4 CFU
Un Aca Un Aca
E
Untreated
Aca
3-MA
3-MA+Aca
Wort
Wort+Aca
F
mCherry intensity (A.U.)
***
ns
ns
150
100
50
0
Un Aca 3-MA 3-MA +Aca Wort Wort +Aca
G
**
ns
ns
600
400
200
1*10^4 CFU
Aca - + - + - +
3-MA
Wort

Figure 3.3. Acacetin functions in an autophagy-dependent manner to decrease intracellular *S. typhimurium*. (A) Cell viability assay. Graph indicating the percentage of HeLa cell viability post acacetin treatment in increasing concentrations (0.001 to 100 µM), for 15 h. (B) Graph showing CFU indicating intracellular *S. typhimurium* infection treated with acacetin in a dose-dependent manner. (C) Growth curve of *S. typhimurium* in Luria Bertani broth containing acacetin. (D) Graph showing CFU indicating intracellular *S. typhimurium* in HeLa cells and ATG5$^{-/-}$ HeLa cells after acacetin treatment (N=3). (E-F) Representative microscopy images and quantitation of intracellular mCherry expressing *S. typhimurium* in HeLa cells after various treatments like acacetin, Wort and 3-MA. Scale bar: 5 µm. A.U. represents arbitrary units. (G) Graph showing intracellular CFU of *S. typhimurium* in HeLa cells treated with compounds such as acacetin, wortmannin and 3-MA (N=3). Statistical analyses on three independent experiments were performed using One-way ANOVA with Bonferroni test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

Furthermore, there is a close interconnection between the endocytic and autophagy pathways, both pathways sharing certain effector proteins and lysosomes as the ultimate destination. Endocytosis is an intracellular trafficking pathway which begins with the uptake of extracellular cargo in vesicles known as endosomes that eventually fuse with lysosomes for degradation of the cargo. To study the effect of acacetin on endocytosis, we temporally followed endocytic cargoes such as FM4-64 (pyridinium,4-(6-[4-{diethylamino}phenyl]-1,3,5-hexatrienyl)-1-(3[triethylammonio] propyl)-,dibromide) dye and FITC (fluorescein isothiocyanate) labelled Dextran beads. The rate of cargo uptake after acacetin treatment was temporally quantitated which did not change in the presence of acacetin (**Figure 3.4**). Furthermore, trafficking of FITC-Dextran to lysosomes was studied by analysis colocalization between early endosomal marker, EEA1 and lysosomal stain, LysoTracker Deep Red (LysoT). Acacetin did not affect the uptake and the temporal recruitment of endocytic markers suggesting that the compound does not have any significant effect on endocytosis (**Figure 3.5**).

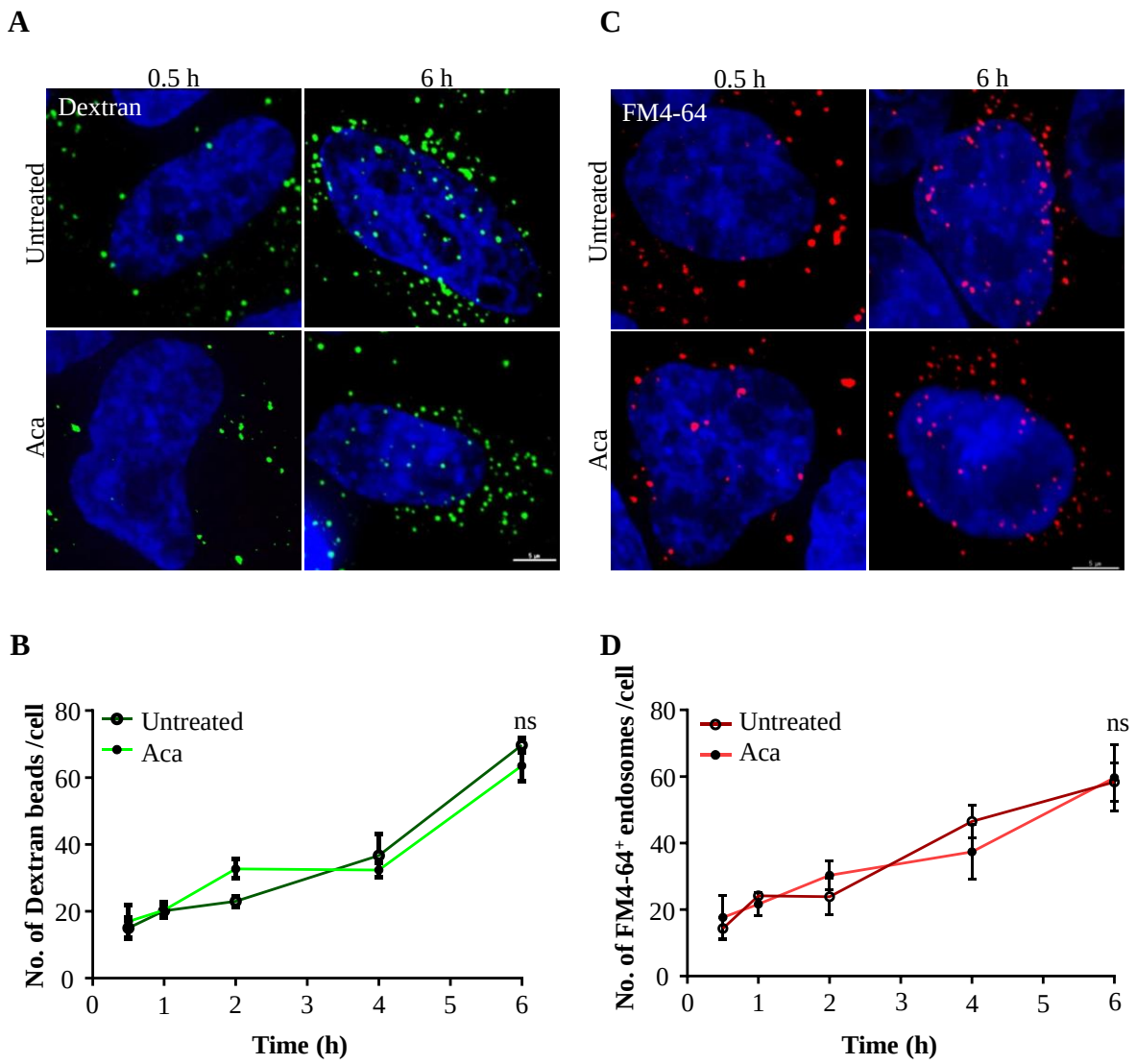

Figure 3.4. Acacetin does not affect the uptake of endocytic cargoes. Representative microscopy images of HeLa cells treated with (A) FITC-Dextran or (C) FM 4-64 at indicated time points. Scale bar: 5 µm. (B and D) The graphs represent the rate of uptake of FITC-Dextran and FM 4-64 post acacetin treatment (n=25, N=3). Quantification of microscopy images were performed on projected images. The concentrations of FITC-Dextran and FM 4-64 dye used are 0.1 mg/ml and 5 µM respectively. Statistical analyses on three independent experiments were performed using Two-way ANOVA with Bonferroni test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

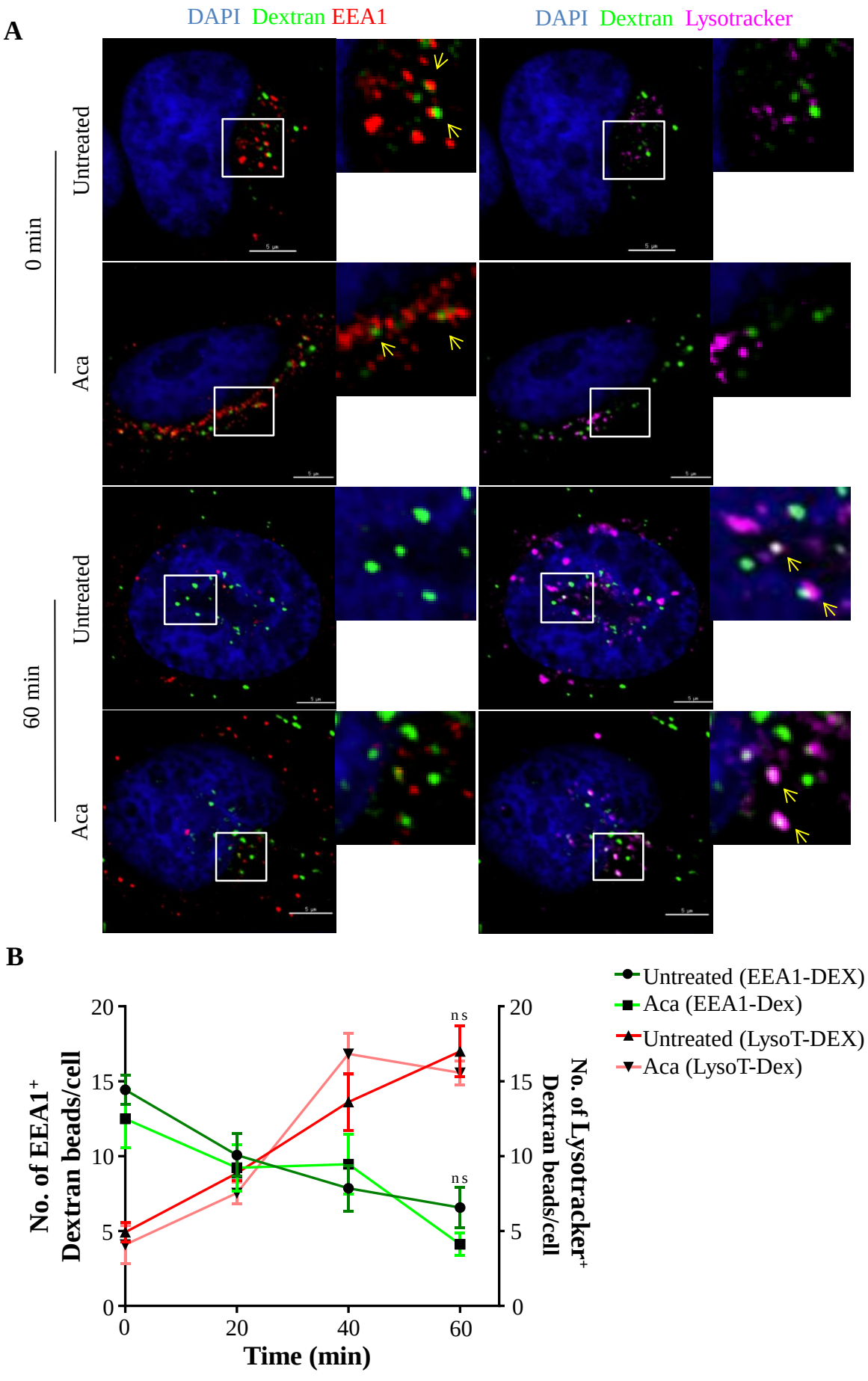
A
DAPI Dextran EEA1
DAPI Dextran Lysotracker
0 min
60 min
Untreated
Aca
Untreated
Aca
B
Untreated (EEA1-DEX)
Aca (EEA1-Dex)
Untreated (LysoT-DEX)
Aca (LysoT-Dex)
No. of EEA1+ Dextran beads/cell
No. of Lysotracker+ Dextran beads/cell
Time (min)
ns
ns
0
5
10
15
20
0
20
40
60

Figure 3.5. Early and late events of endocytic trafficking are unperturbed by acacetin. (**A**) Representative microscopy images of single Z stack of HeLa cells treated with FITC-Dextran for 15 min and chased for indicated time points. The cells were also stained with LysoT for 15 min before fixation and then immunostained with EEA1. Yellow arrows indicate colocalized points of dextran beads with either EEA1 or LysoT. Scale bar: 5 µm. (**B**) The graph represents the number of colocalized events of $EEA1^{+}$Dextran beads and $LysoT^{+}$Dextran beads (n=25, N=3). Quantification of microscopy images were performed on individual Z slices. The concentrations of FITC-Dextran and LysoT used are 0.5 mg/ml and 100 nM respectively. Scale bar: 5 µm. Statistical analyses on three independent experiments were performed using Two-way ANOVA with Bonferroni test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

3.4.3 Acacetin enhances recruitment of receptor proteins to *S. typhimurium*

Xenophagy mediated capture of intracellular *S. typhimurium* was quantified by studying the temporal recruitment of p62 adaptor protein and xenophagosome membrane protein, LC3. HeLa cells post *S. typhimurium* infection was monitored at different time points through immuno-staining. It was seen that acacetin treated samples showed enhanced recruitment of p62 (**Figure 3.6A and 3.6B**) and LC3 (**Figure 3.6C and 3.6D**) with increasing time points as compared to the untreated infected samples where the recruitment was drastically decreased post two hours of infection. Further studies by colocalizing to vesicular marker such as LAMP1 will help in understanding the location of *Salmonella* (cytosolic or vacuolar) To understand the mechanism of p62 recruitment after acacetin treatment, we studied the phosphorylation in the UBA domain (S403) of p62 that has been associated with enhanced binding to ubiquitinated cargoes (**Figure 3.7A**). Furthermore, TBK1 is known to phosphorylate p62 at position S403. Additionally, the active form of TBK1 is phosphorylated at S172 within its activation loop. Thus, monitoring the levels and recruitment of phospho-p62 and phospho-TBK1 can be used to assess the effectiveness of cargo capture. As seen in **figure 3.7B and 3.7C**, there was increased recruitment of phospho-TBK1 and phospho-p62 to *S. typhimurium*. The protein levels of phospho-TBK1 and TBK1 was accessed after acacetin treatment. It was seen that

acacetin treated samples showed increase in phosphorylated TBK1 whereas the total TBK1 proteins levels did not change (**Figure 3.7D**).

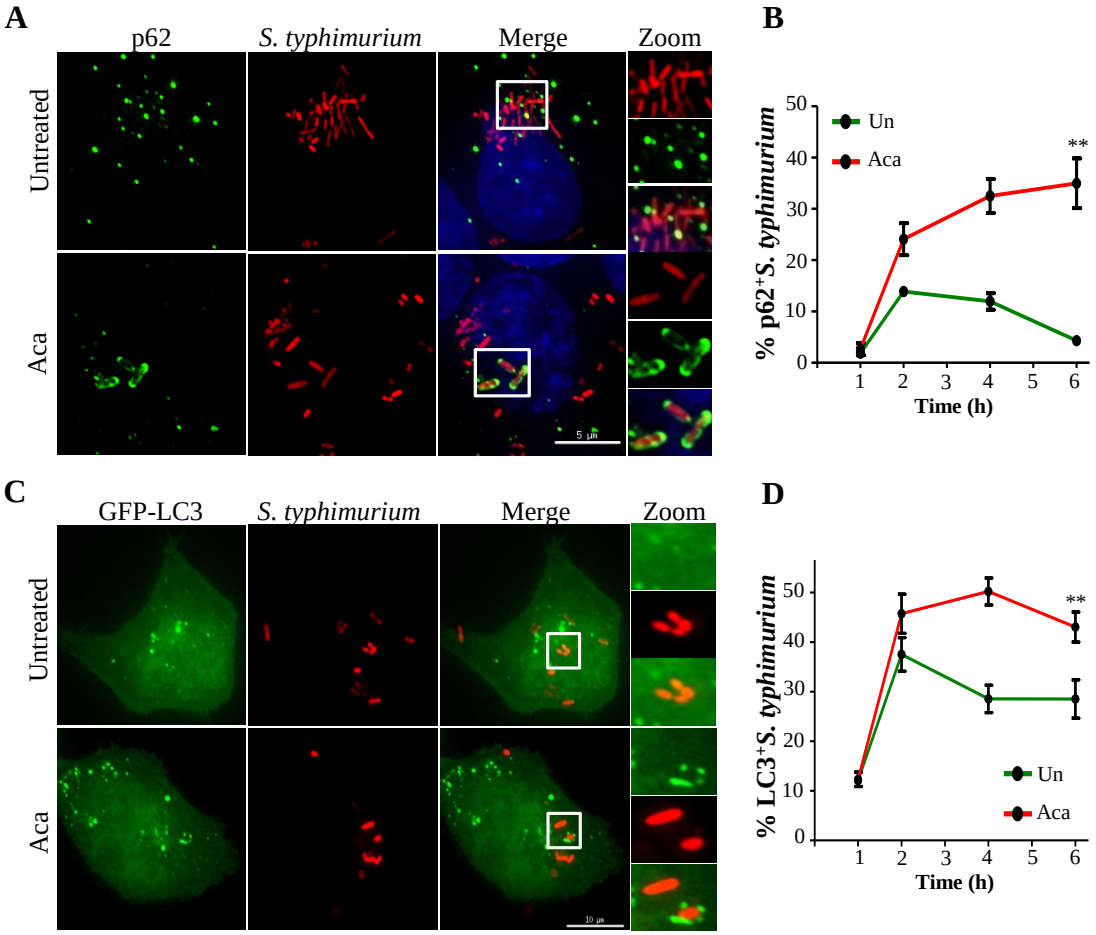

Figure 3.6. Acacetin enhances recruitment of receptor proteins to *S. typhimurium*. (**A**) Representative microscopy images of HeLa cells infected with mCherry expressing *S. typhimurium* and immunostained for p62 at 6 h p.i in the presence and absence of acacetin (n=25, N=3). Scale bar: 5 µm. (**B**) Graph representing the percentage recruitment of p62 to intracellular *S. typhimurium* induced by acacetin. (**C**) Representative microscopy images of GFP-LC3 transfected HeLa cells and infected with mCherry expressing *S. typhimurium* at 6 h p.i in the presence and absence of acacetin. Scale bar: 10 µm. (**D**) Graph representing the percentage recruitment of LC3 to intracellular *S. typhimurium* induced by acacetin. Statistical analyses on three independent experiments were performed using Two-way ANOVA with Bonferroni test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

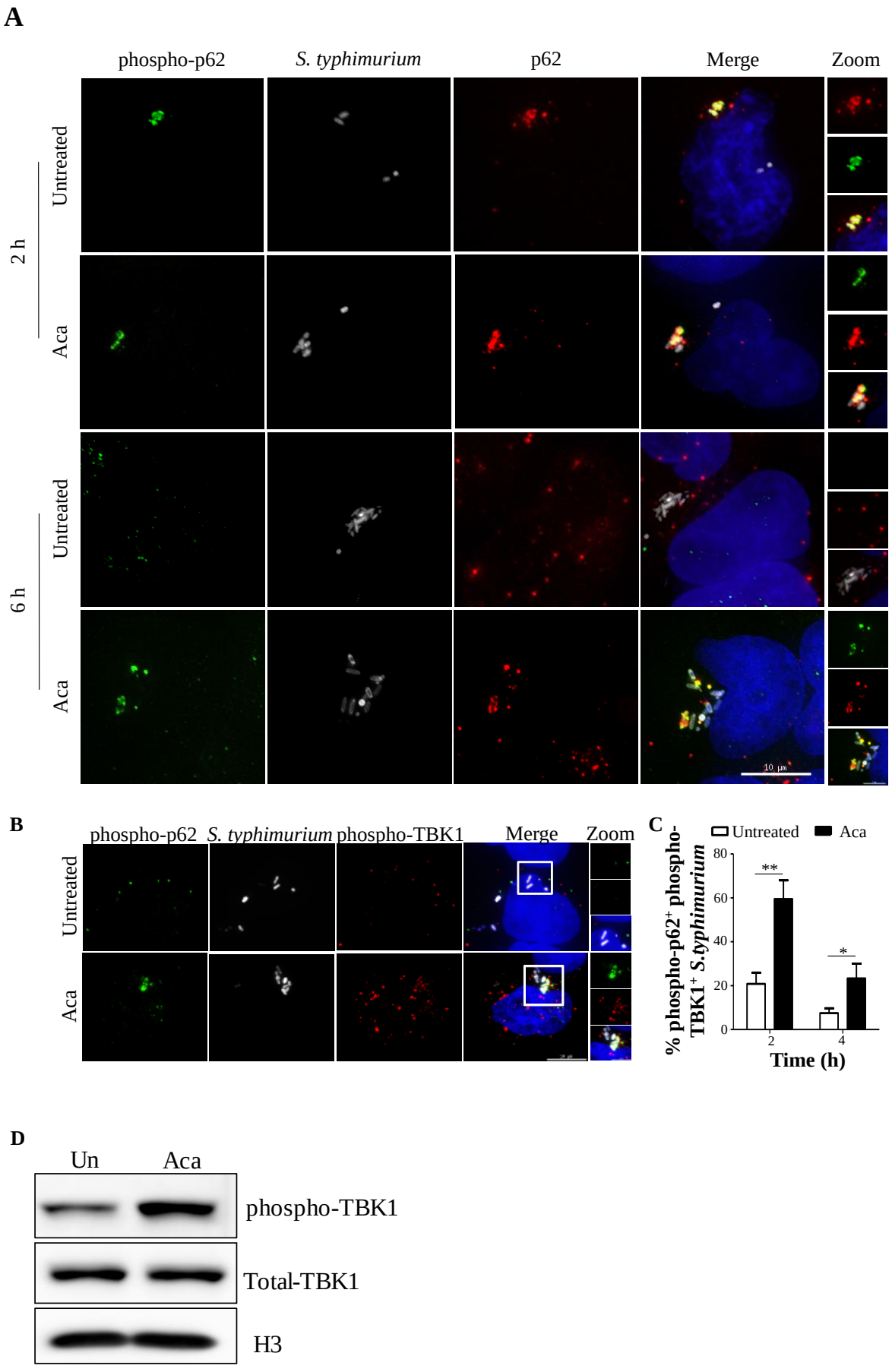
A
phospho-p62
S. typhimurium
p62
Merge
Zoom
2 h
6 h
Untreated
Aca
10 μm
B
phospho-p62
S. typhimurium
phospho-TBK1
Merge
Zoom
C
Untreated
Aca
% phospho-p62+ phospho-TBK1+ S.typhimurium
Time (h)
D
Un
Aca
phospho-TBK1
Total-TBK1
H3

Figure 3.7. Acacetin enhances recruitment of receptor proteins to *S. typhimurium*. (A) Representative microscopy images of HeLa cells stained for p62 and phospho-p62 at different time points p.i and treatment with and without acacetin. (B) Representative microscopy images of HeLa cells infected with mCherry expressing *S. typhimurium* and immunostained for phospho-TBK1 and phospho-p62 at 6 h p.i in the presence and absence of acacetin (n=25, N=3). Scale bar: 10 µm. (C) Graph representing the percentage recruitment of phospho-TBK1 and phospho-p62 to *S. typhimurium* induced by acacetin. Quantification of microscopy images was performed on individual Z slices. (D) Representative immunoblot of HeLa cells treated with acacetin and probed with anti-TBK1 and anti-p-TBK1 antibodies. Statistical analyses on three independent experiments were performed using Two-way ANOVA with Bonferroni test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

3.4.4 Acacetin induces autophagy in mammalian cell lines.

As mentioned earlier, acacetin was shortlisted for inducing autophagy in yeast. To quantitate the autophagic flux in a mammalian cell model, immunoblotting of HeLa cell line lysates treated with acacetin for 2 hours was carried out. An increase in LC3-II levels indicating activation of autophagic flux was observed (**Figure 3.8A and 3.8B**). Although LC3-II accumulation is a common indicator for autophagy modulation, it could be either due to autophagy induction or block in autophagic degradation. To verify if acacetin is a bonafide autophagy inducer, a co-treatment of bafilomycin A1 (BafA1) and acacetin was carried out. BafA1 is a well-known autophagy inhibitor which blocks degradation and leads to accumulation of LC3-II. Co-treatment of BafA1 (100 nM) and acacetin accumulate LC3-II levels over and above the accumulation resulted from BafA1 alone. This indicates that acacetin leads to induction of autophagy in addition to the inhibition imposed by BafA1 (**Figure 3.8C**). Besides LC3-II accumulation, p62 degradation was also enhanced post acacetin treatment, also indicating autophagy induction (**Figure 3.8D**). Additionally, to identify the step at which the autophagy flux is induced by acacetin, we used tandemly tagged RFP-GFP-LC3 plasmid transiently transfected in HeLa cells in the presence of acacetin for 2 hours. Compared to untreated cells, acacetin treated samples showed a significant increase in the number of

autolysosomes (**Figure 3.8E and 3.8F**). This increase in autolysosomes indicates enhanced fusion flux of autophagosomes with lysosomes.

In addition to inducing the formation of autolysosomes, acacetin treatment also showed an increase in the number of lysosomes marked by LAMP1. To differentiate between lysosomes and autolysosomes of the cell, we employed immunostaining of LC3 and LAMP1. It was noticed that in acacetin treated cells, there was an increase in both LAMP1 positive vesicles (indicating lysosomes) as well as LC3 and LAMP1 double positive vesicles (indicating autolysosomes) (**Figure 3.8G and 3.8H**). We further confirmed this observation by using electron microscopy (EM). EM analysis of acacetin treated HeLa cells exhibited an increase in electron-dense structures indicative of lysosomes (**Figure 3.9**).

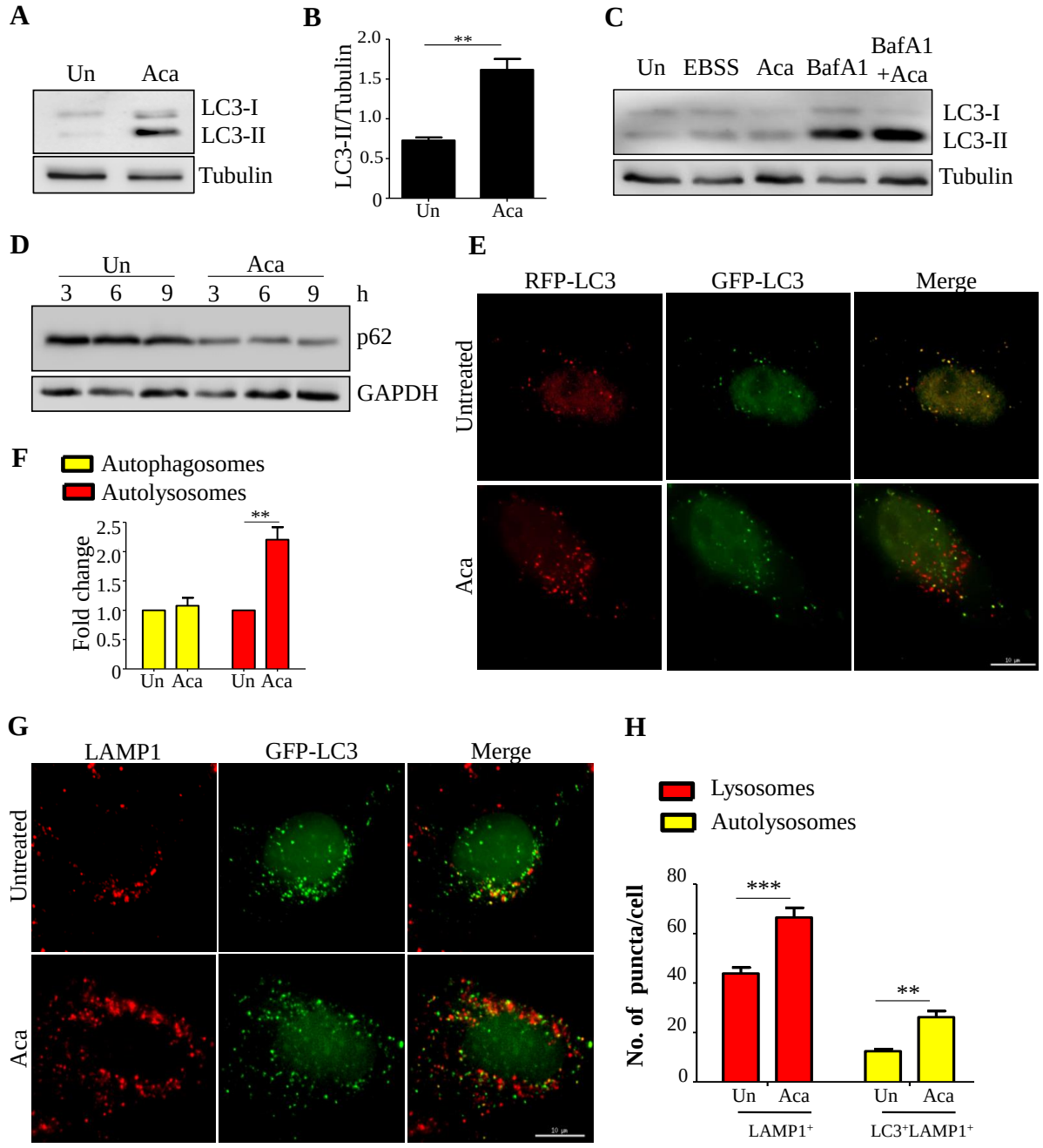

Figure 3.8. Acacetin induces autophagy and increases lysosomal population. (**A**) Representative immunoblot for LC3-I to LC3-II conversion in HeLa cells in the presence of the compound for 2 h. (**B**) Fold change in normalized LC3-II levels between growth condition and acacetin treatment were quantified (N=3). Statistical analyses on three independent experiments were performed using unpaired student's two-tailed t-test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM. (**C**) Representative immunoblot for LC3-II accumulation in the presence of acacetin only and acacetin with BafA1 (100 nM). (**D**) Representative immunoblot for p62 degradation post acacetin treatment. (**E**) Representative microscopy images for tandem RFP-GFP-LC3 transfected HeLa

cells treated with acacetin (50 µM) for 2 h. Yellow puncta correspond to autophagosomes whereas red puncta correspond to autolysosomes. Scale bar: 10 µm. (**F**) Fold change in autophagosomes and autolysosomes induced by acacetin were quantified (n=25, three independent experiments N=3). (**G**) Representative immunofluorescence microscopy images of HeLa cells stained for LAMP1 and LC3 after 2 h of acacetin treatment (n=25, N=3). Scale bar: 10 µm. (**H**) Fold change in lysosomes and autolysosomes induced by acacetin were quantified (n=25, N=3). Statistical analyses on three independent experiments were performed using One-way ANOVA with Bonferroni test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

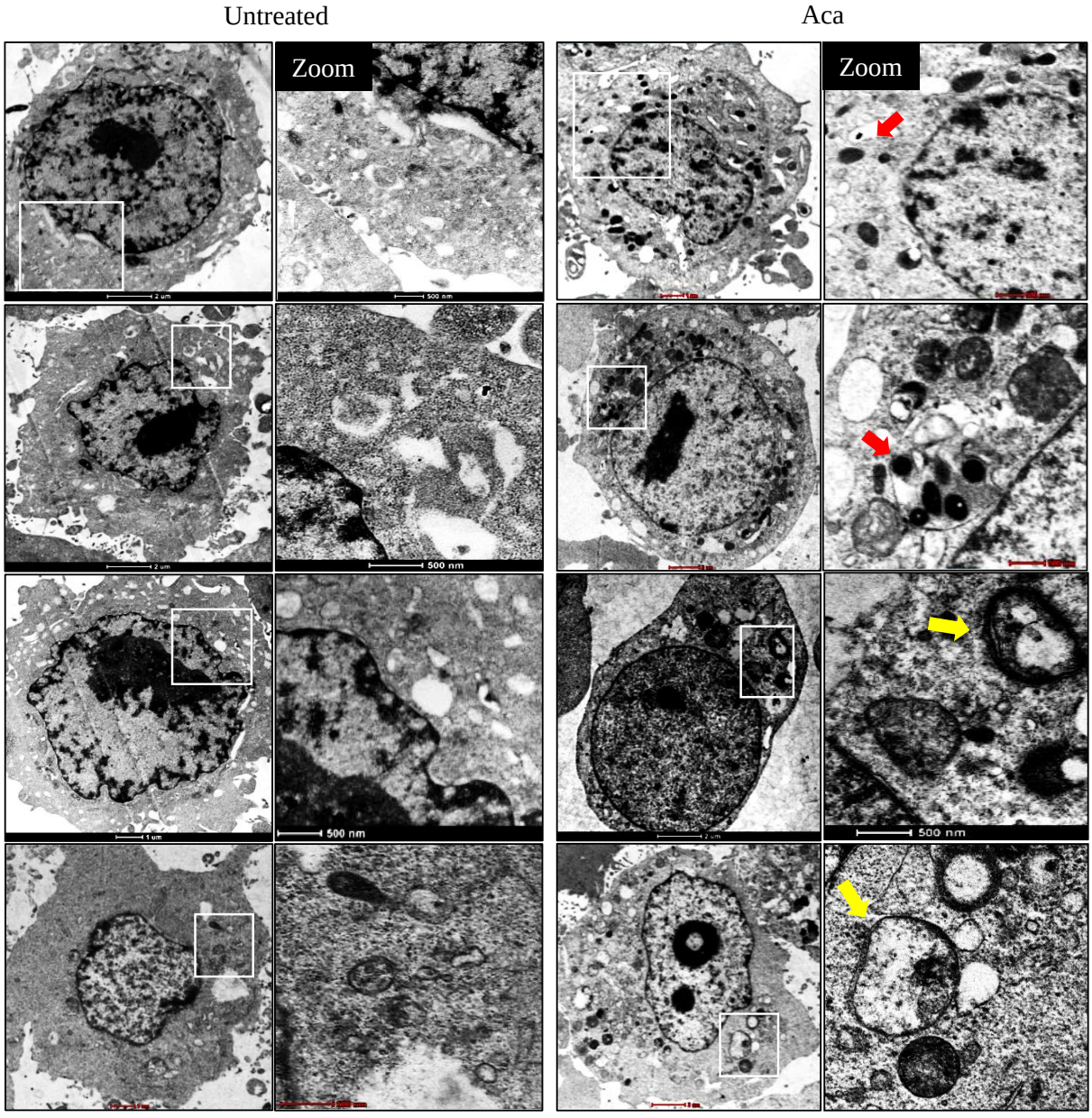

Figure 3.9. Acacetin induces autophagy and increases lysosomal population. Representative electron micrographs of acacetin treated HeLa cells. Electron dense structures in the zoomed-in panel

represent lysosomes (red arrow). Accumulation of multi-membrane vesicles and double-membrane vesicles in acacetin treated cells are indicated by yellow arrows.

3.5 Discussion

S. typhimurium has evolved multiple mechanisms to evade restriction and enable its persistence inside host cells (Brumell and Grinstein, 2004). A multitude of *S. typhimurium* effectors especially factors of T3SS2 is shown to be involved to overcome xenophagy. In this study, we used chemical biology approach to induce host xenophagy during intracellular *S. typhimurium* infection. Since autophagy is an evolutionarily conserved process, the small-molecule compounds shortlisted in yeast-based screening for autophagy modulators were further tested for their ability to induce xenophagy. From the secondary screening for xenophagy, we identified acacetin for its potency to decrease intracellular *S. typhimurium* replication in multiple cell lines. Acacetin is an O-methylated flavone naturally found in certain plants such as members of Asteraceae family. Like other flavones, acacetin is known to exhibit anti-tumour and anti-inflammatory activities. Additionally, acacetin is known to activate ERK/PI3K/Akt pathway and cyclin signalling pathways promoting proliferation MCF-7 breast cancer cell line (Ren et al., 2018). Incidentally, parallel studies by Zhang *et al* also reported acacetin as an autophagy inducer along with other flavones tested. However, the study did not explore the mechanism of acacetin-mediated autophagy induction (Zhang et al., 2018).

In our study, validation experiments highlighted that acacetin does not directly target *S. typhimurium* like an antibiotic as seen by the unperturbed growth kinetics when grown in Luria Broth. Besides, various assays to study the effect of acacetin on HeLa cells were performed. Acacetin did not exhibit toxicity on HeLa cells at the concentration used in the study. More importantly, acacetin mediated decrease in *S. typhimurium* was abrogated when tested in an autophagy-deficient cell line or chemical inhibition of autophagy. Furthermore, by performing uptake assays and recruitment of markers onto endocytic cargo, we showed that acacetin does

not affect the kinetics of the endocytic pathway. All results highlighted that acacetin functions in a host-mediated mechanism especially requiring functional autophagy.

Following this, we measured the rate of xenophagic capture in the presence of acacetin. Adaptor proteins such as p62/SQSTM1, OPTN, NDP52 serve as bridging proteins to recruit ubiquitinated *S. typhimurium* to LC3 leading to the formation of double-membrane xenophagosomes. However, this recognition by adaptor proteins and subsequent recruitment of LC3 drastically decreases after 2 hours p.i. The molecular mechanisms that are involved in pathogen recognition are still not clear. Our study on acacetin mediated S. typhimurium capture showed an increase in the temporal recruitment of p62 and LC3. Further mechanistic investigations revealed post-translational modification of p62 in its UBA domain. This modification, phospho-S403 of p62 is predicted to form polar contacts with Lys6 and His68 of ubiquitin increasing their binding affinity (Matsumoto et al., 2015). Further, this phosphorylation event is catalysed by two kinases namely, CK2 (Matsumoto et al., 2011) and TBK1 (Pilli et al., 2012). Among the two kinases, acacetin treatment showed increased localization of TBK1 to intracellular *S. typhimurium*. TBK1 is a serine/threonine kinase well-studied for its role in anti-viral responses such as activation of IRF3 and NF-kB signalling. The study by Ritcher *et al* showed that activation of OPTN by TBK1 mediated phosphorylation led to signal amplification (Richter et al., 2016). Apart from p62, other autophagic adaptors recruited to *S. typhimurium* include NDP52, TAX1BP1, NBR1 and OPTN (Wild et al., 2011). Additionally, studies revealed that NDP52 specifically recruits LC3C to the intracellular *S. typhimurium* and is also involved in recognizing bacteria replicating in damaged endosomes (von Muhlinen et al., 2012). However, the involvement of other adaptor proteins in acacetin-mediated xenophagy is yet to be explored.

After establishing acacetin as a potent xenophagy inducer, we next measured the autophagic flux induced by acacetin. By performing LC3 processing assay, we saw an increased

accumulation of LC3-II which was further increased upon co-treatment with BafA1 going to confirm that acacetin indeed is an autophagy inducer. Further, to study the step at which acacetin induces autophagy flux, we used mRFP-GFP-LC3 reporter, which showed an increase in autolysosomes indicating an increase in autophagosome-lysosome fusion flux. Apart from inducing autophagy, immunostaining for the lysosomal marker, LAMP1, also showed an increase in general lysosome population of the cell. A similar observation was also seen in transmission electron micrographs of HeLa cells treated with acacetin. These results taken together establish acacetin as an inducer of xenophagy and suggest that there is an increase in both autophagosome-lysosome fusion and number of lysosomes in the cell. In the next chapter, we have looked at the upstream pathways that regulate the acacetin mediated xenophagy induction.

3.6 References

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Chapter 4

Molecular mechanism of acacetin to induce autophagy/xenophagy

The content of this chapter is published in *Autophagy* journal.

Restriction of intracellular Salmonella replication by restoring TFEB-mediated xenophagy.

Ammanathan V, Mishra P, Chavalmane AK, Muthusamy S, Jadhav V, Siddamadappa C, Manjithaya R. Autophagy. 2019 Nov 19:1-14. doi: 10.1080/15548627.2019.1689770

Chapter 4

Molecular mechanism of acacetin to induce autophagy/xenophagy

4.1 Overview

In the previous chapter, we elaborated our efforts on identifying novel xenophagy inducers and various validation assays carried out to access the xenophagy potential of the shortlisted compound, acacetin. Following this, we wanted to study the mechanism by which acacetin induces xenophagy flux. Microscopy analysis revealed enhanced autophagosome-lysosome fusion as well as an increase in the number of lysosomes in the cell (**Chapter 3, Figure 3.8 and 3.9**). This hinted the possibility of a global increase in gene network involved in lysosomal biogenesis and autophagy genes. Based on the available literature, we decided to investigate the role of Transcription factor EB (TFEB), a transcription factor well known for regulating the expression of genes involved in lysosomal biogenesis and autophagy pathways.

4.2 TFEB - master regulator of lysosomal biogenesis and autophagy genes

Lysosomes are organelles that contain digestive proteases to degrade macromolecules of the cell. It is involved in key cellular processes such as endocytosis, phagocytosis, apoptosis and autophagy. *In silico* study of Ballabio's group in the promoter of lysosomal genes identified GTCACGTGAC consensus sequence based on pattern discovery analysis (68 out of 96 genes analysed with high confidence $p<0.0001$). This palindromic 10 base pair sequence referred to as CLEAR element is located within 200 bp from the transcription start site. MiTF family members are known to bind sequences similar to CLEAR element. Overexpression of TFEB

among other MiTF family members induced maximal expression of lysosomal genes. Concomitantly, levels of lysosomal enzymes were also induced. Furthermore, microarray analysis after TFEB overexpression identified 291 genes upregulated highly enriched in lysosomal biogenesis and function (Sardiello et al., 2009). Another study from the same group also identified increased expression of autophagy genes inducing the formation of autophagosomes after TFEB overexpression. Additionally, there was an increased fusion of autophagosomes with lysosomes (Settembre et al., 2011).

Characterization of TFEB targetome revealed several insights into the integrated regulation of lysosomal biogenesis and function (Palmieri et al., 2011). *De novo* motif analysis of chromatin IP peaks revealed the existence of CLEAR element in -300 to +100 bp from lysosomal gene transcription start site. The analysis revealed 3468 genes containing CLEAR element and, in some cases, multiple CLEAR sites were present.

Regulation of TFEB activity is majorly achieved by its cellular localization (Sardiello et al., 2009). This spatial distribution is mediated by phosphorylation/dephosphorylation of TFEB on multiple residues. Phosphorylation of TFEB on conserved residues such as Ser211, Ser122, Ser142, and Ser138 leads to its sequestration in the cytoplasm. Cytoplasmic TFEB is recruited to the lysosomal surface through its direct interaction with mTOR (Martina et al., 2012). Conversely, upon TFEB dephosphorylation of the same residues, rapidly translocate TFEB to the nucleus to induce expression of its target genes (Puertollano et al., 2018) (**Figure 4.1**). Studies by multiple groups have identified kinases such as mTOR, ERK, GSK3β, Akt to be involved in phosphorylating TFEB (Palmieri et al., 2017; Parr et al., 2012; Roczniak-Ferguson et al., 2012). Similarly, a study by Medina *et al* identified a phosphatase, calcineurin to mediate dephosphorylation and subsequent nuclear translocation (Medina et al., 2015). Several cellular stimuli such as nutrient deprivation, pathogen invasion and misfolded protein response are

known to cause nuclear translocation of TFEB. Novel regulators of TFEB activation depending on the stimuli are recently identified. For example, cyclin-dependent kinases, CDK4/6 is reported to modulate TFEB activation during cell cycle (Yin et al., 2020). Similarly, autophagy-related GTPase, IRGM regulates TFEB localization in an mTOR-dependent manner in response to pathogens (Kumar et al., 2020). Apart from the regulation of TFEB mediated by its phosphorylation status, is also fine-tuned by micro RNA in the nucleus. A recent report identified miR-30b-5p to bind CLEAR element to suppress the expression of TFEB-dependent genes (Guo et al., 2020).

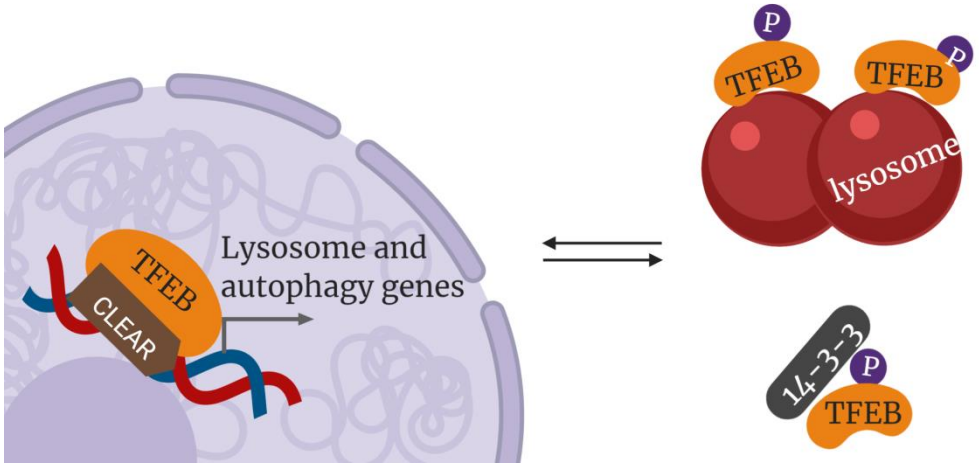


Figure 4.1. Schematic of TFEB activation. Multiple sites of TFEB (S122, S134, S138, S142, and S211) when phosphorylated is retained in the cytoplasm either on the lysosomal surface or bound to 14-3-3 protein. Conversely, dephosphorylation of TFEB on the same sites leads to its nuclear translocation to induce lysosomal and autophagy genes. Image created using BioRender.com.

4.2.1 TFEB during infection

TFEB is a stress-responsive transcription factor that is also shown to provide cytoprotective effects during infection (El-Houjeiri et al., 2019; Irazoqui, 2020). In response to lipopolysaccharide stimulus, macrophages exhibited TFEB accumulation in the nucleus and concomitant induction in host immune genes in addition to the known lysosomal and autophagy genes. Conditional knockout of TFEB in macrophages showed decreased expression of

proinflammatory cytokines and chemokines such as Colony Stimulating Factor (CSF2), interleukin 1β, IL2 and IL27 (Pastore et al., 2016). Additionally, the involvement of TFEB during pathogen-specific infections are reported. For instance, during *Mycobacterium* infection, host cells respond by inducing TFEB nuclear translocation. Mechanistically, a study by Singh *et al*, showed that exposure of interferon-gamma to *M. tuberculosis* infected macrophages leads to an increase in intracellular calcium levels activating calcineurin phosphatase. Calcineurin dephosphorylates TFEB in specific residues leading to nuclear translocation (Singh et al., 2018). Alternatively, pharmacological means of activating TFEB such as simvastatin and rifampin is shown to restrict *M. tuberculosis* replication by inhibiting mTOR (Bruiners et al., 2020; Bryk et al., 2020). Studies focussed on identifying pathogenic factors in modulating TFEB, revealed the involvement of cell wall lipid component, sulfolipid of *M. tuberculosis* to activate TFEB (Sachdeva et al., 2020). Other bacterial infections that replicate in lysosome-derived vacuoles such as *Coxiella burnetti* modulates MITF members, TFEB and TFE3. Studies analysing the genetic remodelling p.i also indicate global changes in TFEB gene network. The study by Giansanthi *et al* indicated modulation of mTOR and TFEB signalling pathways in response to enterovirus infection (Alirezaei et al., 2020; Giansanti et al., 2020).

The role of TFEB during *S. typhimurium* infection is limited. The study by Visvikis *et al* showed that TFEB mediated antimicrobial genes provides cytoprotection during *S. typhimurium* infection (Visvikis et al., 2014). Additionally, a study in *C. elegans* identified the role of protein kinase D (PKD) to activate TFEB in response to intracellular *S. typhimurium* and *Staphylococcus aureus* (Najibi et al., 2016).

4.3 Results

4.3.1 Acacetin treatment results in activation of TFEB

Acacetin treated samples led to an increase in both lysosomal and autolysosomal population of the cell. As TFEB is implicated as a master regulator of lysosomal and autophagy biogenesis, we suspected its involvement in acacetin mediated autophagy/xenophagy induction. We tested this hypothesis by treating HeLa cells with acacetin for 2 hours and studying the nuclear and cytoplasmic localization of TFEB. As seen in **figure 4.2A and 4.2B** there was an increase in the nuclear TFEB accumulation of the acacetin treated samples. Additionally, immunoblotting using total TFEB antibody also revealed a lower molecular band indicative of dephosphorylated TFEB in acacetin treated samples (**Figure 4.2C and 4.2D**). Similarly, cytoplasm and nuclear fractionation of acacetin treated HeLa cells showed increased accumulation of TFEB in nuclear fraction compared to the untreated sample (**Figure 4.2E**). Following this, we studied the functional readout of translocation which is the transcript levels of selected TFEB target genes. As seen in **figure 4.2F** acacetin treatment for 2 hours in HeLa cells showed significant increase in certain TFEB target genes. ASC1 (apoptosis-associated speck-like protein containing a CARD) gene does not harbour CLEAR motif and was used as the negative control.

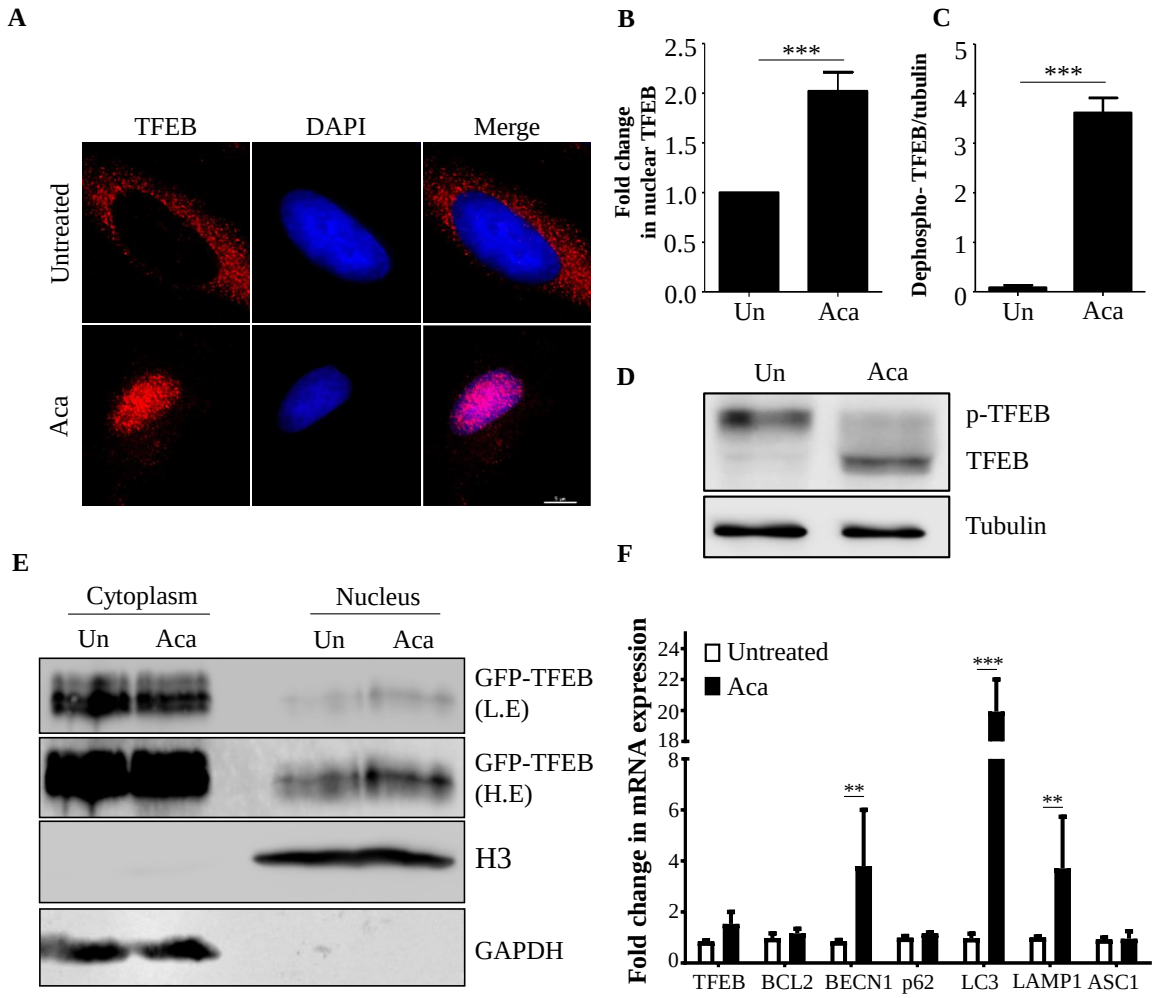


Figure 4.2: Acacetin enhances nuclear translocation of TFEB. (**A**) Representative microscopy images of HeLa cells treated with and without acacetin for 2 h and immunostained for TFEB. Scale bar: 5 µm. (**B**) Fold change in nuclear TFEB intensity induced by acacetin were quantified (n=50, N=3). Quantification of microscopy images was performed on individual Z slices. (**C**) The graph represents the fold change in the dephosphorylated form of TFEB caused by acacetin (N=3). (**D**) Representative immunoblot for HeLa cells treated with acacetin and probed for TFEB. Molecular weight shift in TFEB band corresponds to dephosphorylated TFEB. β-tubulin was used as a loading control. (**E**) Representative immunoblot of cytoplasmic-nuclear fractionation indicating GFP-TFEB levels in nucleus and cytoplasm. H3 and GAPDH were used as nuclear and cytoplasm loading controls respectively. 2X concentration of nuclear fraction was loaded compared to cytoplasmic fraction. S.E and L.E represent short and long exposure respectively. (**F**) Fold change in mRNA levels of indicated TFEB target genes related to autophagy and lysosomal pathways post 2 h of acacetin treatment (N=3). Statistical analyses on three independent experiments were performed using unpaired student's two-tailed t-test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

Further, to visualize the intracellular *S. typhimurium* populations, we resorted to electron microscopy. Electron micrographs of infected HeLa cells showed both vacuolar and cytosolic population (**Figure 4.3A**). Whereas, after 6 hours of acacetin treatment, host-induced capture of *S. typhimurium* was seen with reduced bacterial numbers. Additionally, electron-dense lysosomes were seen at close proximity to intracellular bacteria after acacetin treatment (**Figure 4.3B**).

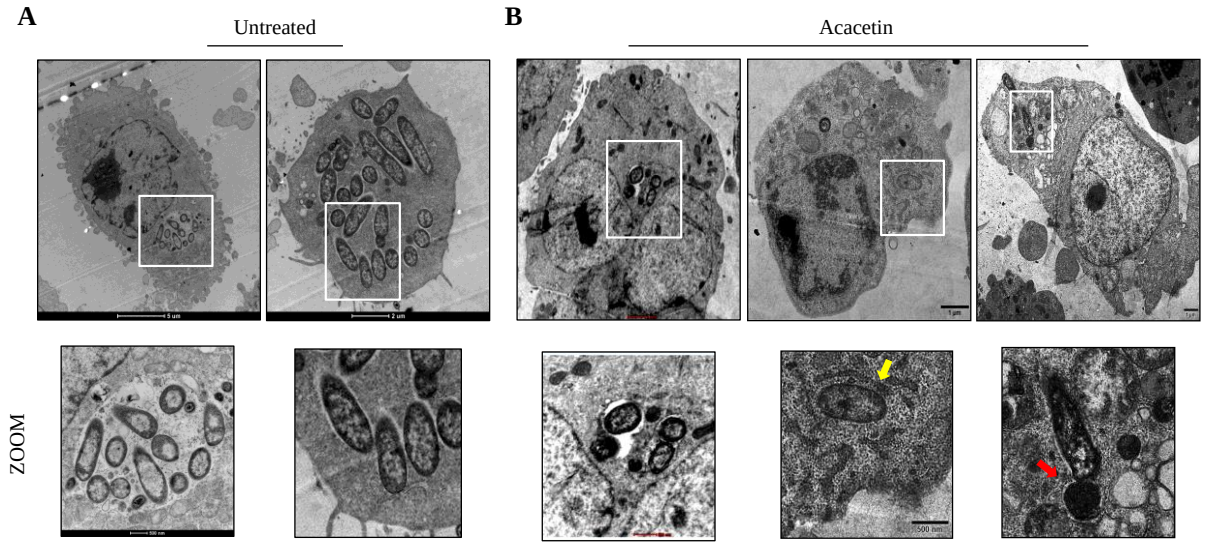


Figure 4.3. Representative electron micrographs of *S. typhimurium* infected HeLa cells with and without acacetin. (**A**) Untreated infected samples show vacuolar and cytosolic replication. (**B**) Acacetin treated samples show electron-dense lysosomes (red arrow) and host-mediated capture (yellow arrow) of *S. typhimurium.*

4.3.2 Acacetin treatment results in enhanced capture of *S. typhimurium* in a TFEB-dependent manner

We further investigated the phosphorylation status of TFEB during *S. typhimurium* infection. We noticed that the overall levels of TFEB increased during infection, especially the higher molecular weight band which is indicative of phosphorylated TFEB (**Figure 4.4A**). To test if this band was indeed p-TFEB, we treated the infected lysates with calf intestine phosphatase (CIP). As seen in **figure 4.4B**, CIP treated samples showed accelerated migrating bands of

TFEB suggesting that infection results in maintaining TFEB in the phosphorylated state. Contrastingly, acacetin treatment on HeLa cells during infection showed an increase in dephosphorylated TFEB (**Figure 4.4C and 4.4D**).

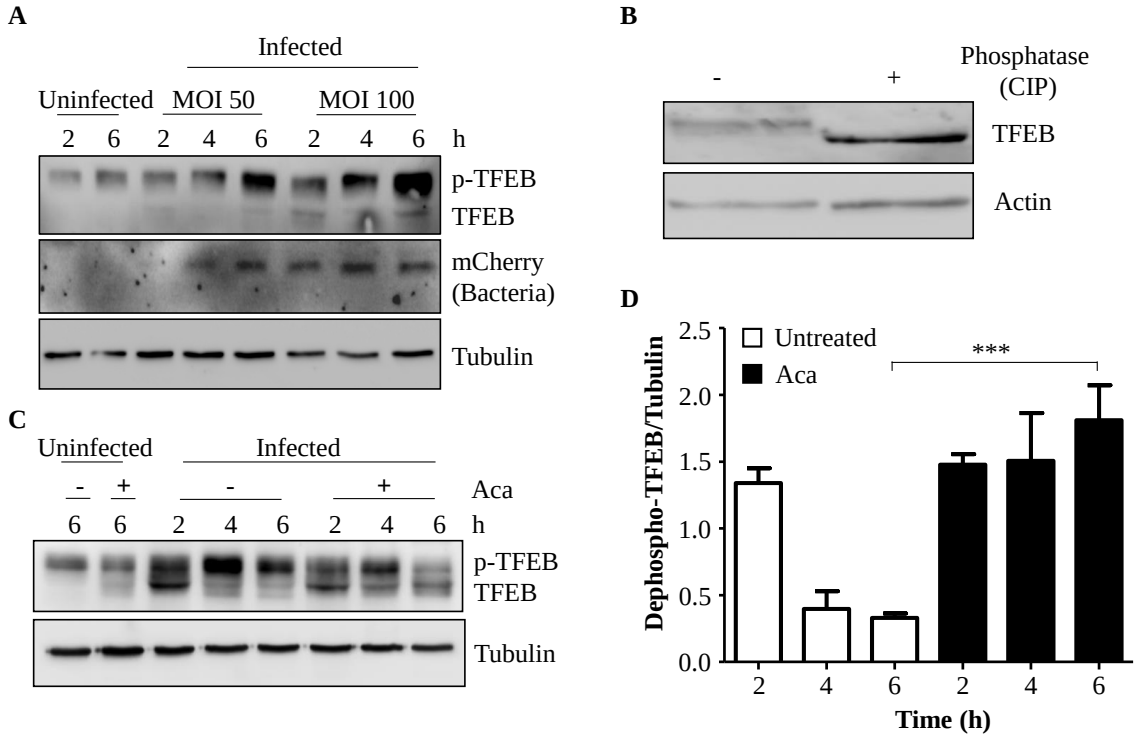


Figure 4.4. Acacetin enhances the dephosphorylation of TFEB during *S. typhimurium*. (**A**) Representative immunoblot indicating the phosphorylation status of TFEB post *S. typhimurium* infection across different time points and MOI. The lower molecular weight TFEB band corresponds to dephosphorylated TFEB. (**B**) Representative immunoblot for infected HeLa cell lysates treated with phosphatase (CIP) and probed for TFEB. β-actin was used as a loading control. (**C**) Representative immunoblot indicating the phosphorylation status of TFEB post *S. typhimurium* infection and acacetin treatment in a time-dependent manner. (**D**) Graph representing the dephosphorylated TFEB induced by acacetin post *S. typhimurium* infection (N=3). β-tubulin was used as a loading control. Statistical analyses on three independent experiments were performed using Two-way ANOVA with Bonferroni test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

In order to identify if acacetin mediates its autophagy and xenophagy induction through dephosphorylation of TFEB, we silenced TFEB in HeLa cells. Acacetin treatment post TFEB silencing did not show accumulation of autolysosomes. This reduction was quantitated by overexpressing RFP-GFP-LC3 construct in TFEB silenced HeLa cells treated with acacetin (**Figure 4.5A and 4.5B**). Similarly, LC3-II accumulation was not observed in immunoblotting post TFEB silencing in acacetin treated samples in comparison to enhanced LC3-II accumulation as seen in scramble control with acacetin treatment (**Figure 4.5C**). Taken together, these results suggest that for acacetin mediated autophagy induction, TFEB is necessary. Furthermore, infection of TFEB silenced HeLa cells showed a significant decrease in the capture of intracellular *Salmonella* post acacetin treatment (**Figure 4.6A and 4.6B**). Results cumulatively suggest that during infection, acacetin increases the level of dephosphorylated TFEB in the host cells leading to increased capture and degradation of *Salmonella*.

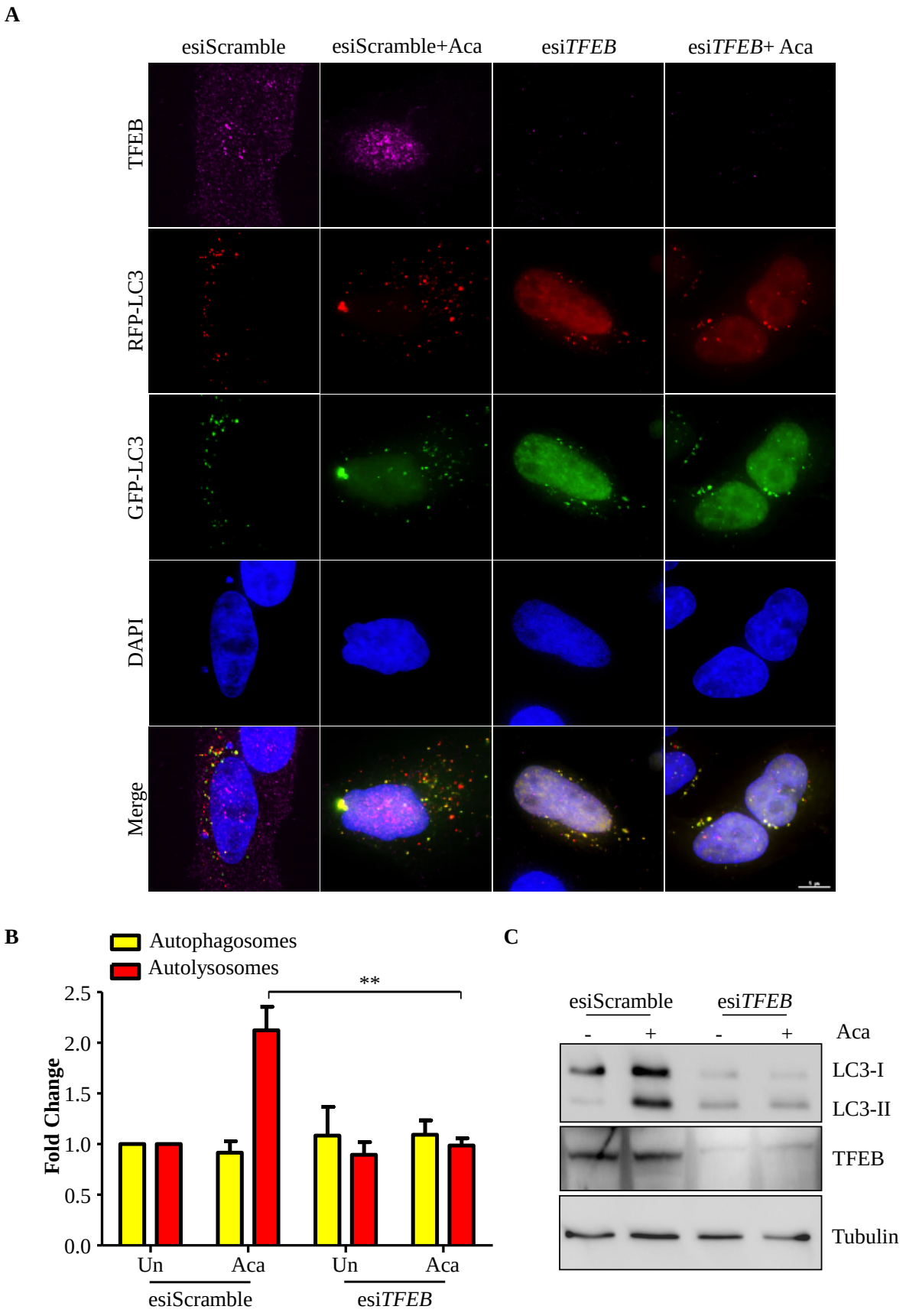
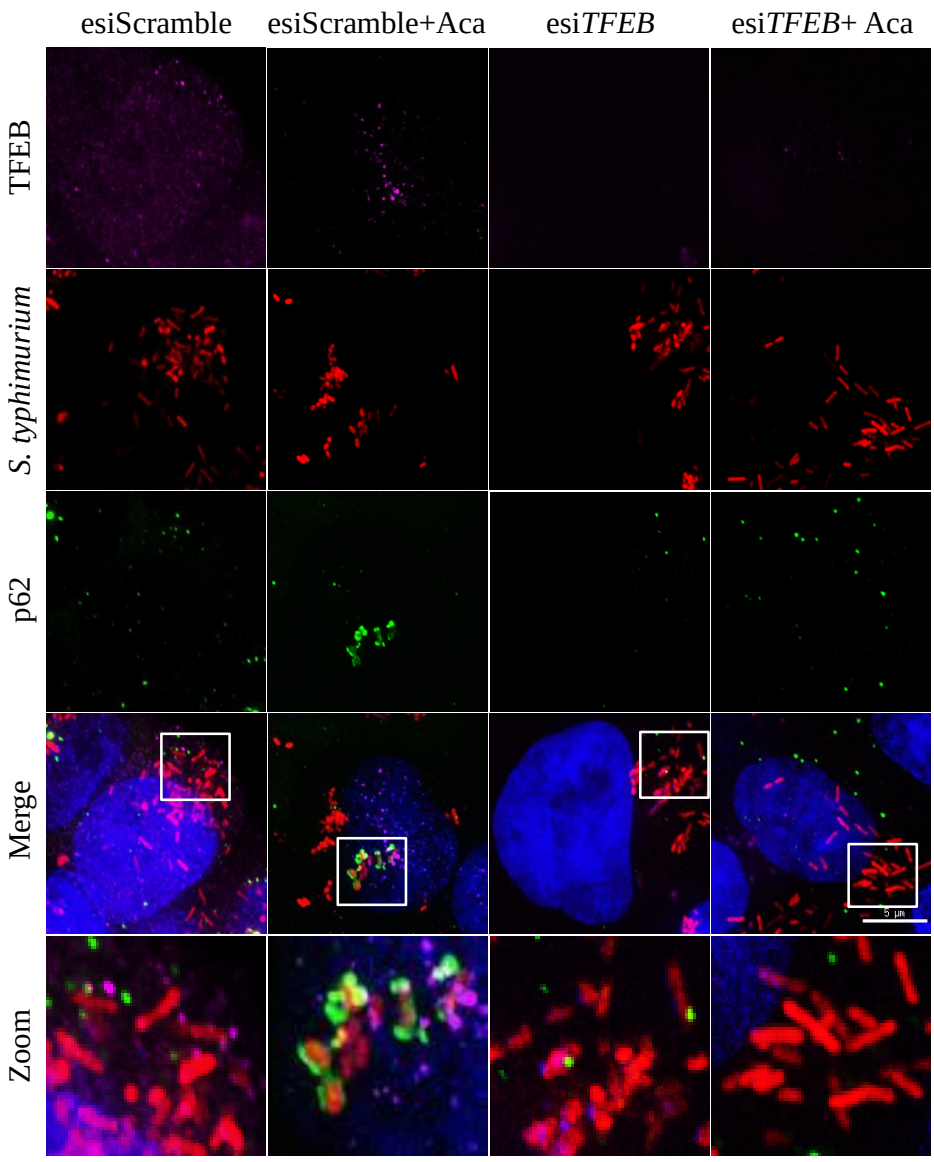

A
esiScramble
esiScramble+Aca
esiTFEB
esiTFEB+ Aca
TFEB
RFP-LC3
GFP-LC3
DAPI
Merge
B
Autophagosomes
Autolysosomes
**
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
Un
Aca
Un
Aca
esiScramble
esiTFEB
C
esiScramble
esiTFEB
-
+
-
+
Aca
LC3-I
LC3-II
TFEB
Tubulin

Figure 4.5. Acacetin mediated autophagy induction is TFEB-dependent. (**A**) Representative microscopy images of HeLa cells transfected with either scrambled or *TFEB* esiRNA for 48 h along with tandem RFP-GFP-LC3. Cells were either left untreated or treated with acacetin. Scale bar: 5 µm. (**B**) The fold change in the number of autophagosomes and autolysosomes post acacetin treatment were quantified. Quantification of microscopy images was performed on projected images. (**C**) Representative immunoblot for TFEB levels and LC3-II accumulation in control and *TFEB* silenced HeLa cells treated with acacetin for 2 h. β tubulin was used as loading control. Statistical analyses on three independent experiments were performed using Two-way ANOVA with Bonferroni test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

A


esiScramble
esiScramble+Aca
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esi*TFEB*+ Aca
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S. typhimurium
p62
Merge
Zoom

B

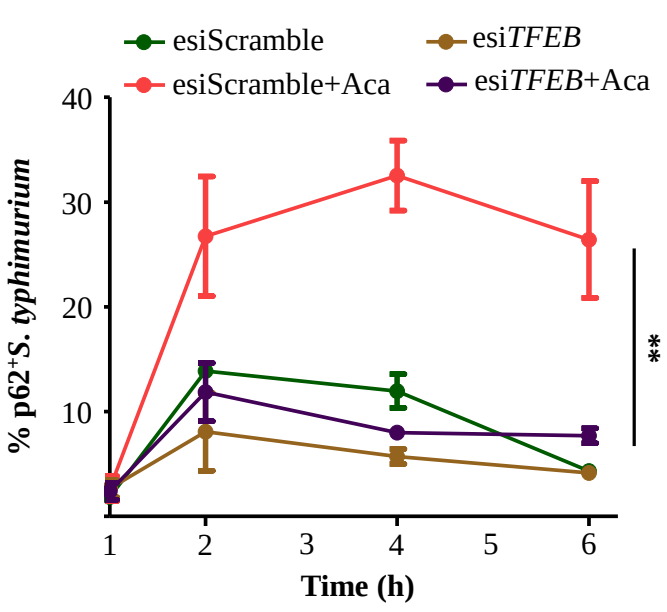
esiScramble
esiScramble+Aca
esi*TFEB*
esi*TFEB*+Aca
% p62+*S. typhimurium*
40
30
20
10
1
2
3
4
5
6
Time (h)
**

Figure 4.6. Acacetin treatment results in enhanced capture of *S. typhimurium* in a TFEB-dependent manner. (**A**) Representative microscopy images of control and *TFEB* silenced HeLa cells after *S. typhimurium* infection for 6 h and immunostained for p62 and TFEB. Cells were either left untreated or treated with acacetin. Scale bar: 5 µm. (**B**) The graph represents the time course recruitment of p62 to *S. typhimurium* induced by acacetin treatment (n=25, N=3). Quantification of microscopy images was performed on individual Z slices. Statistical analyses on three independent experiments were performed using Two-way ANOVA with Bonferroni test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

4.3.3 Acacetin induces TFEB dephosphorylation independent of mTOR

mTOR is the well-studied kinase for its role in phosphorylating TFEB. It is shown that mTOR phosphorylates specific serine residues of TFEB such as Ser122, Ser211 and Ser142. The study by Martina *et al* showed that cytoplasmic TFEB shows enhanced affinity for another cytoplasmic protein, 14-3-3. Interestingly, this interaction with 14-3-3 is phosphorylation-dependent. Phosphorylation of Ser211 by mTOR is necessary and sufficient for TFEB to bind 14-3-3 (Martina et al., 2012). This binding leads to masking of the nearby NLS sequence in TFEB thereby preventing its nuclear translocation (Martina et al., 2012; Roczniak-Ferguson et al., 2012). Further, the mechanism by which Ser122 and Ser142 mediate TFEB cytoplasmic retention is not clear. However, a study by Sha *et al* revealed that phosphorylation of Ser122 and Ser142 increases its binding to an E3 ubiquitin ligase, STUB1 thereby increasing the proteasome degradation. This study suggests a possible mechanism of regulation by protein stability (Sha et al., 2017).

Most of the known chemical modulators that modulate TFEB phosphorylation status function by inactivating mTOR. To study the effect of acacetin on mTOR kinase activity (**Figure 4.7A**), various strategies were employed. Immunoblotting analysis of mTOR substrates revealed phosphorylation of its downstream targets such as p70S6K and 4EBP1 was not affected (**Figure 4.7B**). Additionally, the effect of acacetin on mTOR was confirmed after keeping the

kinase constitutively active by overexpressing RHEB^{N153T} mutant construct. It was observed that acacetin treatment did not affect nuclear TFEB translocation even in the background of constitutive mTOR activation (**Figure 4.8A-C**) going to show that acacetin mediates TFEB translocation independent of mTOR kinase activity. Furthermore, mTOR is located on the surface of lysosomes in its active form and acacetin treatment did not affect this localization as seen by immunostaining with LAMP1 and mTOR (**Figure 4.8D and 4.8E**).

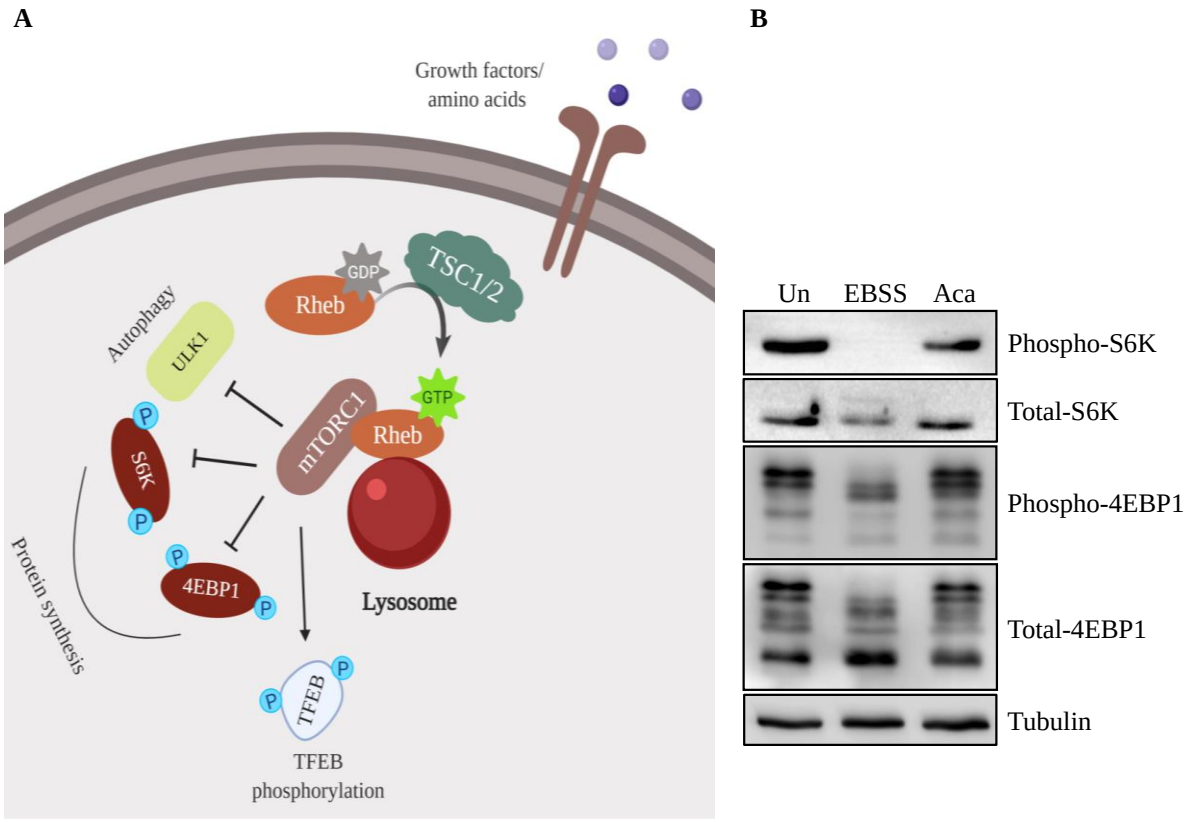

Figure 4.7. Acacetin functions in an mTOR-independent manner. (**A**) Schematic of mTOR kinase signalling pathway. The activity of mTOR which is located on the lysosomal membrane is regulated by Rheb and Rag GTPases. The downstream substrates of mTOR include S6K and 4EBP1. The image was created using BioRender.com (**B**) Representative immunoblot indicating the phosphorylation status of mTOR substrates, S6K and 4EBP1 caused by acacetin and Earle's Balanced Salt Solution (EBSS) treatments. β-tubulin was used as a loading control.

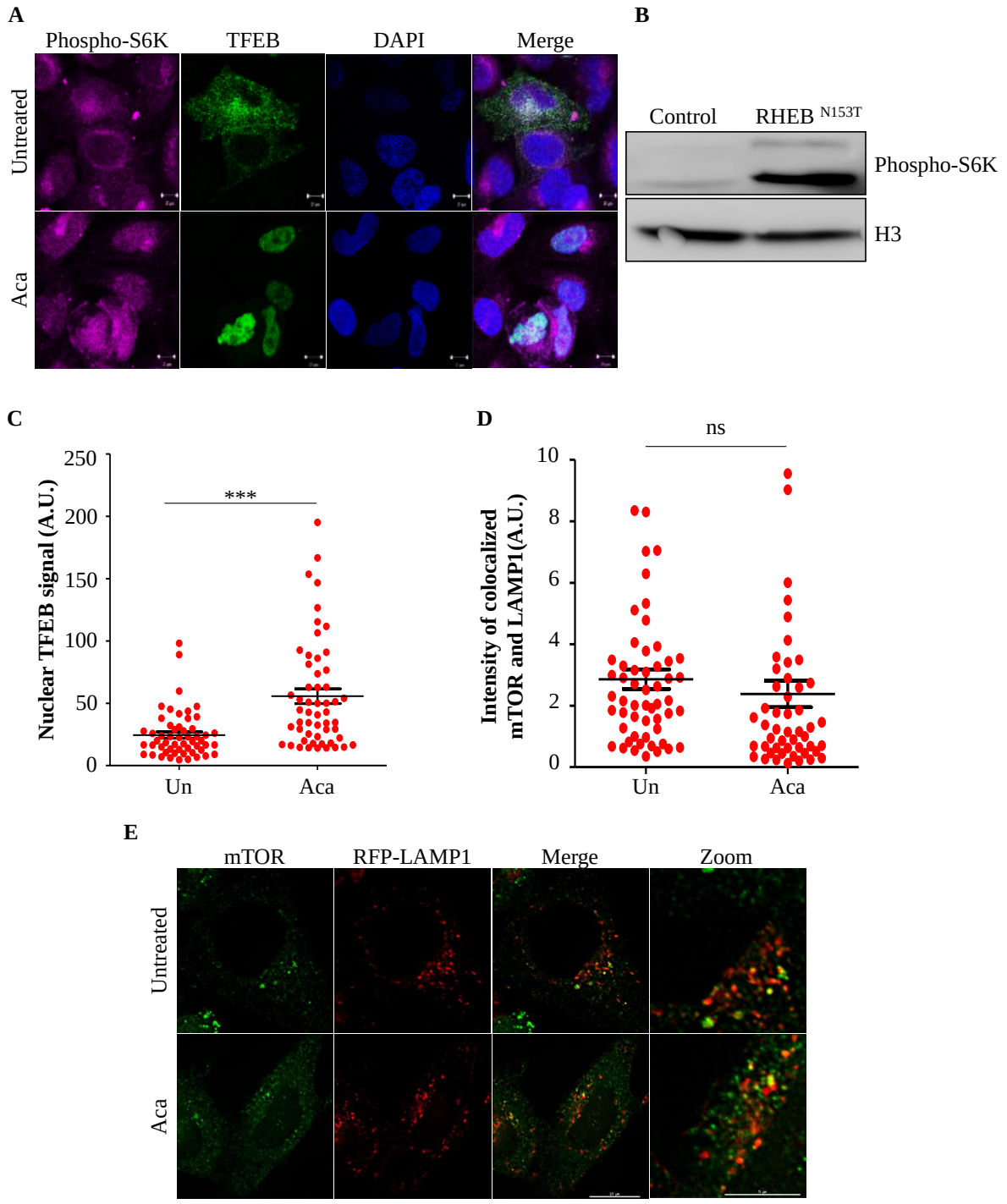

Figure 4.8. Acacetin induces nucleus translocation of TFEB in an mTOR-independent manner. (**A**) Representative microscopy images of HeLa cells transfected with GFP-TFEB and FLAG-RHEB^{N153T} constructs. The cells were treated with acacetin for 2 h and immunostained for p-RPS6KB1. Scale bar: 10 µm. (**B**) Immunoblotting of HeLa cells transfected with FLAG-RHEB^{N153T} construct and probed for p-RPS6KB1 and compared with untransfected control. (**C**) The graph represents the difference in the intensity of nuclear TFEB signal (N=3). A.U. represents arbitrary units. Quantification of microscopy images was performed on individual Z slices. Each red dot represents a quantified cell.

(**D**) The graph represents the intensity of colocalization between mTOR and LAMP1 in the presence or absence of acacetin (N=3). (**E**) Representative microscopy images of HeLa cells transfected with a plasmid expressing RFP-LAMP1 and treated with acacetin for 2 h and immunostained with mTOR. Scale bar: 10 µm. Quantification of microscopy images was performed on individual Z slices. A.U. represents arbitrary units. Statistical analysis was performed using unpaired student's two-tailed t-test; ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

4.3.4 Acacetin treatment increases the proteolytic activity of *Salmonella*-containing vacuoles

Acacetin induces an increase in the number of lysosomes and autolysosomes in the host cell. To verify if this increase also concomitantly increase the lysosomal activity of the lysosomes, dye quenched-bovine serum albumin (DQ-BSA) was used. DQ-BSA is a BODIPY conjugated dye which acts as a protease substrate and is cleaved by lysosomal cysteine and aspartyl proteases present in functionally active enzymes (**Figure 4.9A**). As seen in **figure 4.9B** and **4.9D** acacetin treatment on HeLa cells showed an increase in DQ-BSA intensity by immunofluorescence analysis. This increase corresponds to lysosomal mediated processing resulting in brighter mono-conjugates of DQ-BSA.

During intracellular *Salmonella* infection, the pathogen replicates in modified *Salmonella* containing endosomes-like structures referred to as *Salmonella* containing vacuoles (SCVs). SCVs resemble lysosomes by membrane composition but do not contain proteases similar to functional lysosomes. These protease-deficient vesicles provide an ideal niche for *S. typhimurium* to replicate within cells. In order to verify if acacetin treatment during infection can induce functionally active lysosomes, DQ-BSA intensity from SCVs was measured. As seen in **figure 4.9C and 4.9E**, SCVs of acacetin treated cells showed increased DQ-BSA processing. DQ-BSA processing to monoconjugates is a sign of increased proteolytic activity in the contained vesicles. These results suggest that apart from enhanced acacetin-mediated *S. typhimurium* capture by xenophagy proteins, there is an increase in functional lysosomal

numbers which also target SCVs to enhance their proteolytic activity and thus reduce *S. typhimurium* replication.

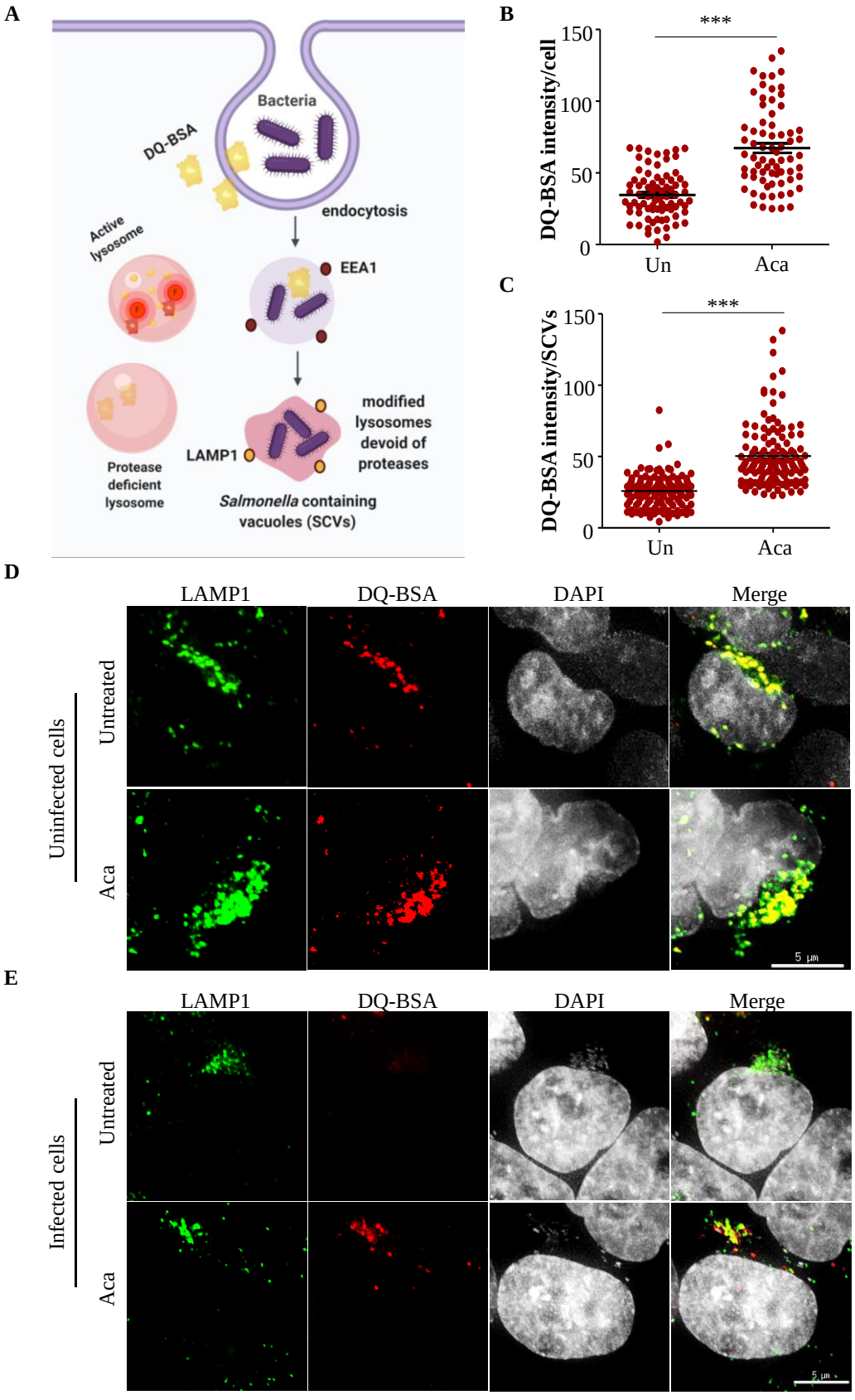

Figure 4.9. Acacetin treatment increases the proteolytic activity of *Salmonella*-containing vacuoles. (**A**) Schematic of intracellular DQ-BSA processing principle. DQ-BSA is tagged to BODIPY dye which is self-quenched due to near proximity. When DQ-BSA reaches lysosomes that are protease rich, BSA gets degraded and BODIPY fluoresces. Image created using BioRender.com (**B** and **C**) The DQ-BSA intensity per cell or SCVs induced by acacetin treatment were quantified (n=25, N=3). Quantification of microscopy images was performed on projected images. (**D**) Representative microscopy images of HeLa cells treated with DQ-BSA for 2 h followed by 4 h incubation of DQ-BSA along with acacetin treatment. Cells were immunostained for LAMP1 (n=25, N=3). Scale bar: 5 µm. (**E**) Representative microscopy images of mCherry *S. typhimurium* infected HeLa cells treated with DQ-BSA for 2 h followed by 4 h incubation of DQ-BSA along with acacetin treatment. Cells were immunostained for LAMP1. Scale bar: 5 µm. Statistical analyses on three independent experiments were performed using unpaired student's two-tailed t-test; ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

4.3.5 Acacetin induces xenophagy in a mouse model of infection

Salmonella typhimurium is a gastrointestinal infection that typically occurs by ingestion of contaminated food or water. Although the majority of the ingested bacteria gets killed in the stomach, some may survive and traverse the intestinal epithelium. The primary site of infection in the intestine is distal ileum. *Salmonella* utilizes SPI1 T3SS to drive its entry. This leads to infection of both enterocytes and M cells of payer's patch. Subsequently, there is the dissemination of bacteria into distant organs such as liver, spleen and kidney via the lymphatics and bloodstream. There are also alternative routes for dissemination through CD+18 mononuclear phagocytes (Watson and Holden, 2010).

Previous studies have reported that the xenophagy potential of the organs during *Salmonella* infection is short-lived, returning to basal levels at around 72 hours p.i. To test the efficacy of acacetin in inducing xenophagy-mediated bacterial clearance, an *in vivo* mouse model of infection was established. Based on our preliminary studies, we found that administering 10^{10} bacteria through oral gavage, disseminates bacteria across key organs involved during *Salmonella* infection like intestine, liver, spleen and kidney at 7 days p.i. Three groups of mice

were tested namely uninfected, infected and infected along with acacetin treatment. In this infection model, acacetin (20 mg/kg body weight) was administered intraperitoneally starting from 1 day before *S. typhimurium* infection in order to maintain an induced autophagy status in the organs. Acacetin treatment continued until 7 days p.i. At the end of 7 days p.i, mice from all groups were sacrificed and the number of intracellular *S. typhimurium* was determined by quantitating CFU and autophagy induction was visualized in liver using immunohistochemistry (Figure 4.10A). As seen in **figure 4.10B**, there is a reduction in the number of intracellular bacteria in various organs of infected mice like the liver, spleen, and intestine in the acacetin treated group. In addition, immunohistochemical analysis revealed induction of autophagy as seen by increased LC3-II puncta in the infected group that received acacetin (**Figure 4.10C and 4.10D**).

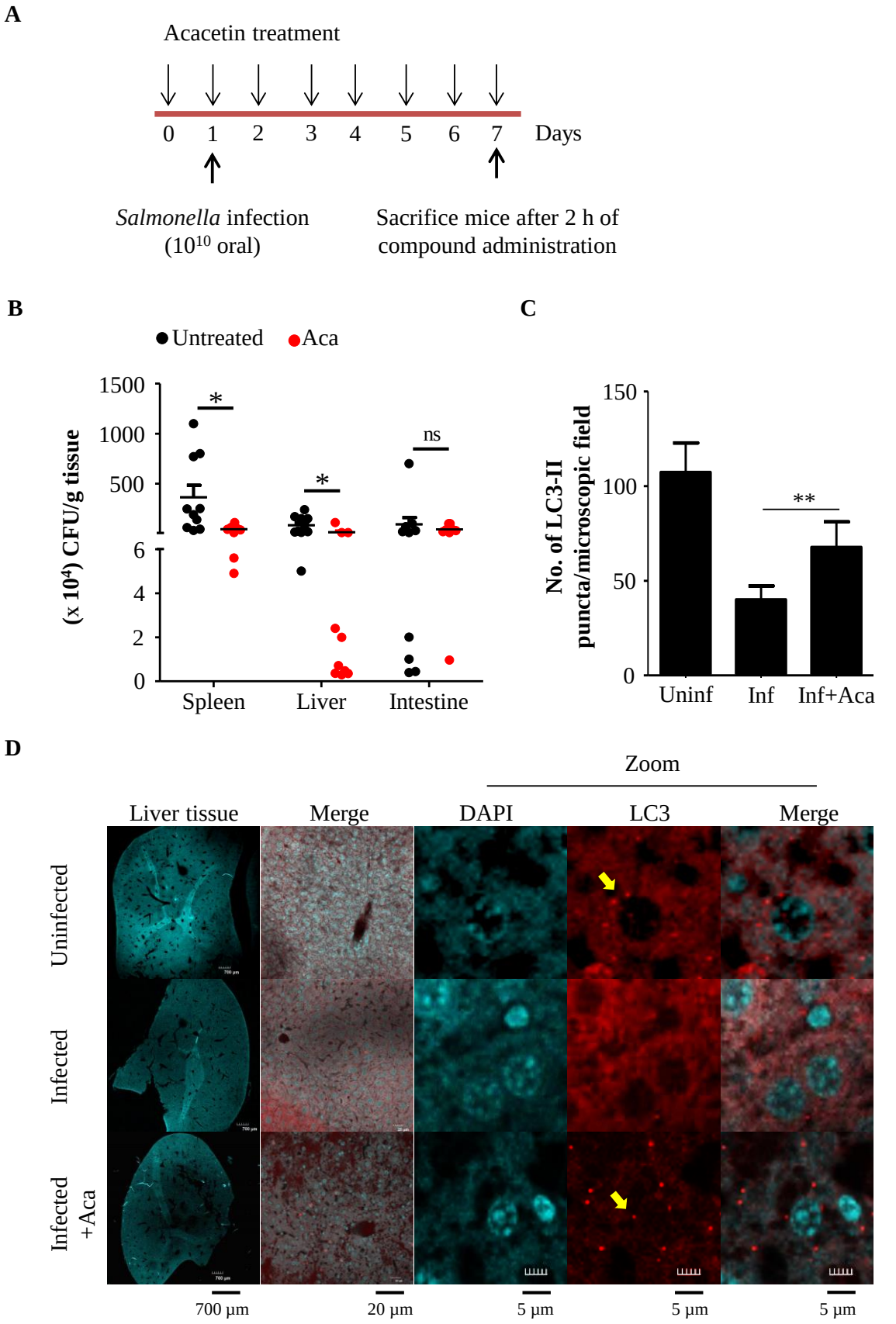
A
Acacetin treatment
0 1 2 3 4 5 6 7 Days
Salmonella infection (10^10 oral)
Sacrifice mice after 2 h of compound administration
B
Untreated
Aca
(x 10^4) CFU/g tissue
1500
1000
500
6
4
2
0
*
*
ns
Spleen
Liver
Intestine
C
No. of LC3-II puncta/microscopic field
150
100
50
0
**
Uninf
Inf
Inf+Aca
D
Zoom
Liver tissue
Merge
DAPI
LC3
Merge
Uninfected
Infected
Infected +Aca
700 µm
20 µm
5 µm
5 µm
5 µm

Figure 4.10. Acacetin induces xenophagy in a mouse model of infection. (**A**) Scheme for infection assay. (**B**) Graph representing the reduction in intracellular *S. typhimurium* burden in various organs of acacetin treated mice (N=10). Statistical analyses of three independent experiments was performed using unpaired student's two-tailed t-test; ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM. (**C**) Graph representing the difference in the number of LC3 puncta per microscopy field (1X1 binning and 1024X1024 pixel area) between different groups of mice. Statistical analyses on three independent experiments were performed using One-way ANOVA with Bonferroni test: ns- non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM. (**D**) Representative immunohistochemistry images of liver cryosections stained for autophagosome membrane marker, LC3 (Olympus FV3000 1.25X objective was used for imaging entire DAPI stained liver section, 20X objective was used to choose a region of interest stained for LC3 in red and DAPI. 40X objective was used for observing LC3 puncta, indicated by yellow arrows).

4.4 Discussion

TFEB plays an important role in regulating the expression of lysosomal biogenesis and autophagy pathway genes (Napolitano and Ballabio, 2016; Zhao and Czaja, 2012). The transcriptional regulation of TFEB is achieved by its cellular localization. This subcellular localization is controlled by its phosphorylation-dephosphorylation status.

In this chapter, we show that acacetin induces nuclear translocation of TFEB and concomitant increase in lysosomal biogenesis and autophagy pathways. Further, silencing of TFEB abrogates the ability of acacetin to induce autophagy and xenophagy going to show that acacetin functions in a TFEB dependent manner. In our study, we observed that during intracellular *S. typhimurium* infection, there is an accumulation of phosphorylated TFEB. Whereas, during acacetin treatment, there is increased dephosphorylated TFEB even in the presence of infection.

Given the importance of autophagy during infection, it is not surprising that TFEB, which is considered as the master transcriptional regulator of autophagy is modulated in response to intracellular *S. typhimurium* infection. Previously published literature has shown that

Caenorhabditis elegans TFEB homologue, HLH30, induces expression of antimicrobial and autophagy genes in response to *Staphylococcus aureus* and *S. typhimurium*. There is a nuclear accumulation of HLH30 during infection owing to transcriptional expression (Visvikis et al., 2014). Additionally, another study from the same group identified the role of the kinase, PKD to induce TFEB during *S. typhimurium* infection (Najibi et al., 2016). However, these studies monitored TFEB activation only during early time points (2 hours) after intracellular *S. typhimurium* infection. In the current study, we temporally monitored the TFEB status and observed an accumulation of phosphorylated TFEB with increasing time points.

Furthermore, we also showed that acacetin treatment induces proteolytically active lysosomes in the host cells. A study by McGourty *et al* showed that *S. typhimurium* perturbs intracellular trafficking of proteases from ER to lysosomes. The lysosomes of *S. typhimurium* infection are therefore protease deficient (McGourty et al., 2012). In our study, we observed that acacetin mediated induction of proteolytically active lysosomes fuses with SCVs. This observation indicates that SCVs of acacetin treated cells are proteolytically active thereby disrupting the ideal niche of SCVs for *S. typhimurium* replication. Additionally, we show that acacetin is effective in inducing xenophagy during *S. typhimurium* infection in an *in vivo* mouse model. We observed increase in LC3B-II levels and concomitant reduction in the intracellular bacteria in organs of infected mice such as liver and spleen.

4.5 References

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Chapter 5

Identification of upstream regulators of acacetin-mediated TFEB activation

Chapter 5

Identification of upstream regulators of acacetin-mediated TFEB activation

5.1 Overview

The previous chapters explained in detail the molecular mechanisms of acacetin action, to induce the xenophagy of intracellular *S. typhimurium*. The key observations include (a) Increase in lysosomal population and autophagy-related pathways after acacetin treatment, (b) Activation of a key transcriptional regulator of the process, TFEB that regulates the gene network of both lysosomal biogenesis and autophagy pathway, and, (c) TFEB activation is beneficial during intracellular infection by increasing the proteolytically active lysosomes of the host cells.

Based on our observations, we next wanted to explore the regulation of TFEB. As elaborately discussed earlier, TFEB is post-translationally modified by kinases or phosphatases which dictates its cellular localization needed for the activation. Studies by multiple groups have identified kinases such as mTOR, ERK, GSK3β, Akt and phosphatase calcineurin to modulate the phosphorylation status of TFEB. However, acacetin treatment did not modulate the known kinases or calcineurin to activate TFEB. Additionally, we hypothesised that crosstalk with upstream immune signalling pathways is involved in regulation of xenophagy. To understand these unanswered questions gene expression analyses were carried out.

The specific objectives of gene expression analyses include

1. Identifying the upstream players/pathways that mediate TFEB activation during acacetin treatment.
2. Identifying the molecular players involved in the accumulation of phosphorylated TFEB during intracellular *S. typhimurium* infection.
3. Studying the crosstalk of immune pathways in inducing xenophagy.

5.2 Results

5.2.1 Gene expression analyses of acacetin treated HeLa cells

To study the changes in gene expression induced by acacetin, an unbiased approach of microarray analysis was performed (**Figure 5.1A**). The total RNA isolated from HeLa cells with and without acacetin treatment for 2 hours was studied. The quality of the isolated RNA was measured by calculating the RNA integrity number (RIN) before proceeding to microarray analysis. RIN is an algorithm generated by Agilent technologies to measure the degree of RNA degradation by electrophoretic separations based on size (Schroeder et al., 2006). The value is generated by considering various characteristics of RNA electropherogram such as area under 28S and 18S rRNA peaks to the total area under the graph, the height of the peaks and the distance between 5S and 18S peaks. A higher value of 28S rRNA (which is prone to easy degradation by ubiquitous RNases) would indicate a good RNA integrity. A value between 1 to 10 is assigned with 10 indicating the least degradation. The RIN values of all the samples used for microarray analysis were above 8 (**Table 5.1**).

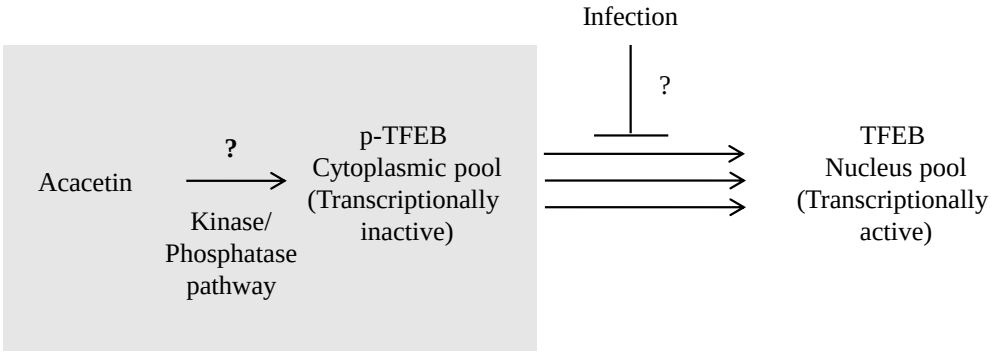
Table 5.1: The RIN values of the samples used in the microarray analysis

Sample		RIN value	RNA concentration (ng/µl)	rRNA ratio (28s/18s)
Experiment 1	Untreated 1	8.4	410	1.5
	Untreated 2	8.9	336	2.0
	Acacetin 1	8.8	329	2.0
	Acacetin 2	8.7	187	2.1
Experiment 2	Untreated 1	8	1521.4	2.08
	Untreated 2	8.1	1681.7	2.07
	Acacetin 1	8	926	2.12
	Acacetin 2	8.1	1401	2.1

Following this, Clariom D Affymetrix microarray technology (ThermoScientific) was used for the gene expression analysis of acacetin treated HeLa cells. Clariom D is a next-generation transcriptome-profiling tool that provides information regarding gene and exon-level expression including alternative splicing events of coding and long non-coding RNA (Zhang et al., 2020). It is also aimed at detecting rare and low expressing genes. The data analysis post-run was carried out using the Applied Biosystems supported Transcriptome Analysis Console (TAC) Software 4.0.

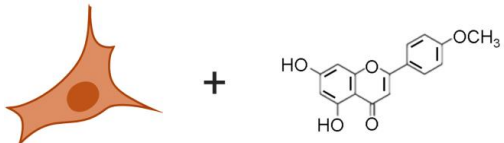
Genes whose expression showed a fold change of 2 and above post acacetin treatment, with a p-value less than 0.05 were shortlisted for further analysis. Based on this threshold assigned, 139 genes were upregulated, and 238 genes were downregulated in the acacetin treated samples (**Figure 5.1B**). The selected genes were further taken forward for gene enrichment analysis. It is a bioinformatics approach to designate genes/gene based on the biological processes it is known to participate. The software also allows classification based on the model organisms such as *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*. This method of understanding of gene lists could be helpful especially while analysing gene expression studies such as microarray or RNA seq data as it highlights the pattern or enrichment of genes under certain conditions.

A



B

Gene expression analysis: Microarray analysis
+/- Acacetin (n=2; N=2); Fold change> 2.0 p<0.05

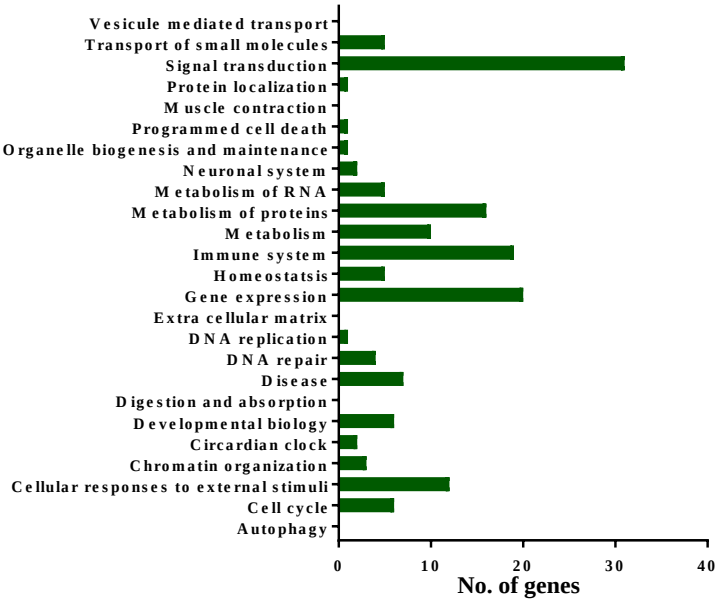


↑ 139 genes	↓ 238 genes

Pathway analysis (Reactome browser)

↑ 59 genes	↓ 162 genes

C



D

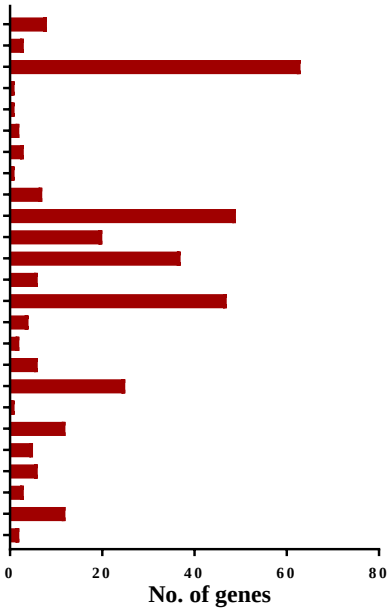


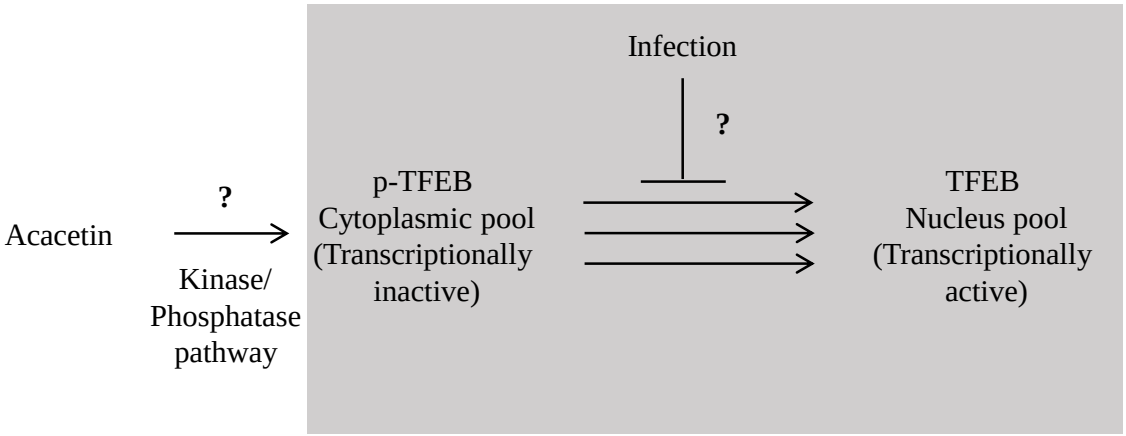
Figure 5.1: **Gene expression analyses of acacetin treated HeLa cells** (A) Schematic of acacetin mediated TFEB activation during intracellular *S. typhimurium*: During intracellular *S. typhimurium* infection there is an increase in phosphorylated TFEB levels. In the presence of acacetin, there is an increase in dephosphorylated TFEB levels. The molecular player involved in shifting the phosphorylation-dephosphorylation balance during infection as well as by acacetin is currently not known. The shaded region indicates the question to be addressed using microarray analysis. (B) Results of gene expression analysis. Green arrows indicate upregulated genes whereas red arrows indicate down-regulated genes. The threshold for shortlisting genes was fold change in gene expression >= 2 and $p < 0.05$. (C-D) Pathway analysis using the Reactome pathway browser of shortlisted upregulated genes (C) and down-regulated genes (D) from microarray analysis.

Further, in order to understand the pathways enriched post acacetin treatment, we used the Reactome pathway browser (Haw et al., 2011). Reactome is a manually curated database of biological pathways that are currently modelled for *Homo sapiens* and 17 other non-human species. The software detects the pathways that are enriched in the submitted data. To eliminate the pathways that are enriched by chance; a probability score is generated using Benjamani-Hochberg method which is represented as "false-discovery rate" (FDR). The browser lists the pathways that are enriched in the submitted list of genes along with the FDR. Additionally, the genes that are not found in the Reactome browser are listed as "not found". Using this pathway browser, we analysed the shortlisted genes from microarray of acacetin treated samples. We have considered only those genes that are well-annotated with known function curated in the Reactome browser for further analysis. Based on this, the number of genes was further narrowed to 59 upregulated and 162 downregulated genes. The pathways that are enriched post acacetin treatment were analysed (**Figure 5.1C and 5.1D**).

5.2.2 Gene expression analyses of acacetin treated HeLa cells in the presence of *S. typhimurium* infection

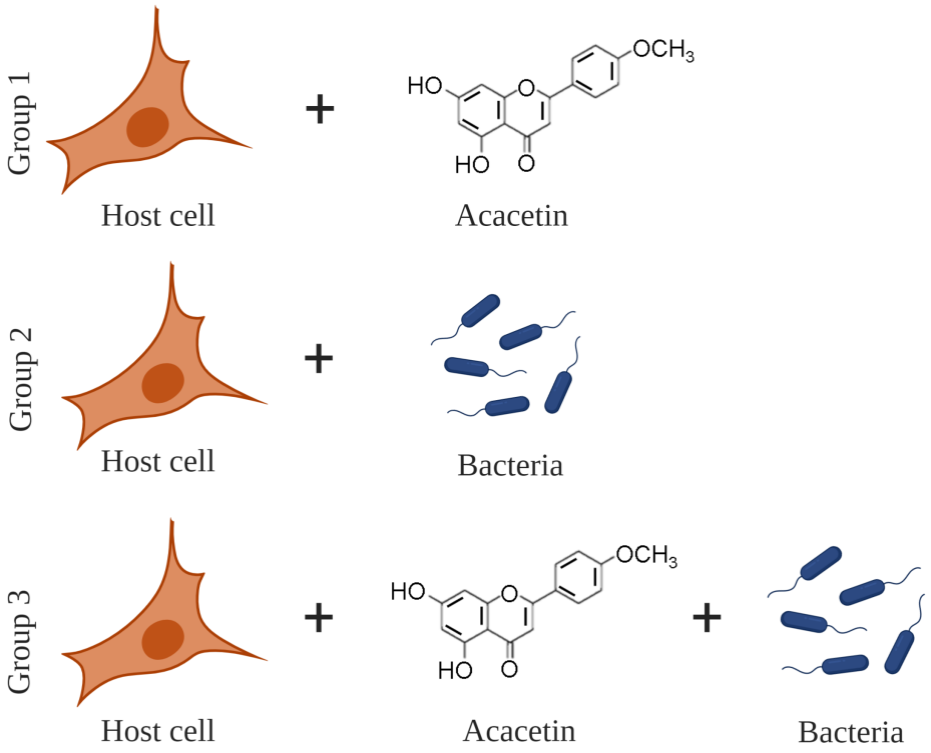
Another aspect that we were interested in studying is the identification of the molecular player(s) involved in the accumulation of phosphorylated TFEB during *S. typhimurium* infection. (**Figure 5.2A**) To analyse this, gene expression analysis of an array of immunity-related genes (540 genes) were tested during intracellular *S. typhimurium* infection for 6 hours in HeLa cells (Kulkarni, 2011; Warren, 2018). We employed NanoString nCounter gene expression system which quantitates individual mRNA transcript count (Geiss et al., 2008). Additionally, acacetin treatment after the infection was also compared to analyse the immunity-related genes that are exclusively modulated by acacetin during infection (**Figure 5.2B**). The raw data was analysed using NanoString software, nSolver 4.0. The threshold to select significantly modulated genes was set as mRNA counts of 40 and above with p-value less than 0.05. The assay enabled investigation of genes induced by acacetin on host with and without infection and compared with genes induced by infection on host cells. Additionally, the molecular pathways enriched in each group was assessed using the Reactome pathway browser to differentiate pathways induced by acacetin with and without infection (**Figure 5.2C**). NanoString gene expression assay was performed along with Dr. Keerti Rawat, Post-doctoral fellow, Autophagy laboratory, JNCASR.

A



B

Gene expression analysis: Nanostring analysis
+/- Infection +/- Acacetin (n=2; N=2); No. of counts > 40 p<0.05



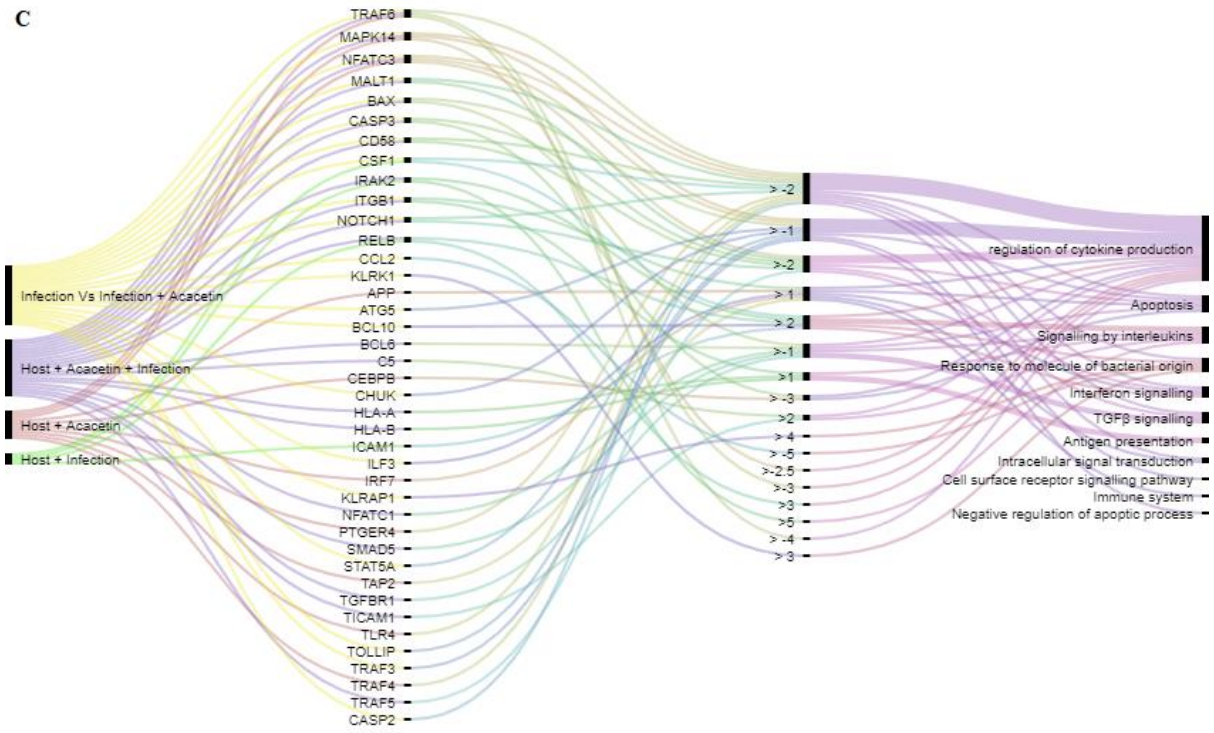
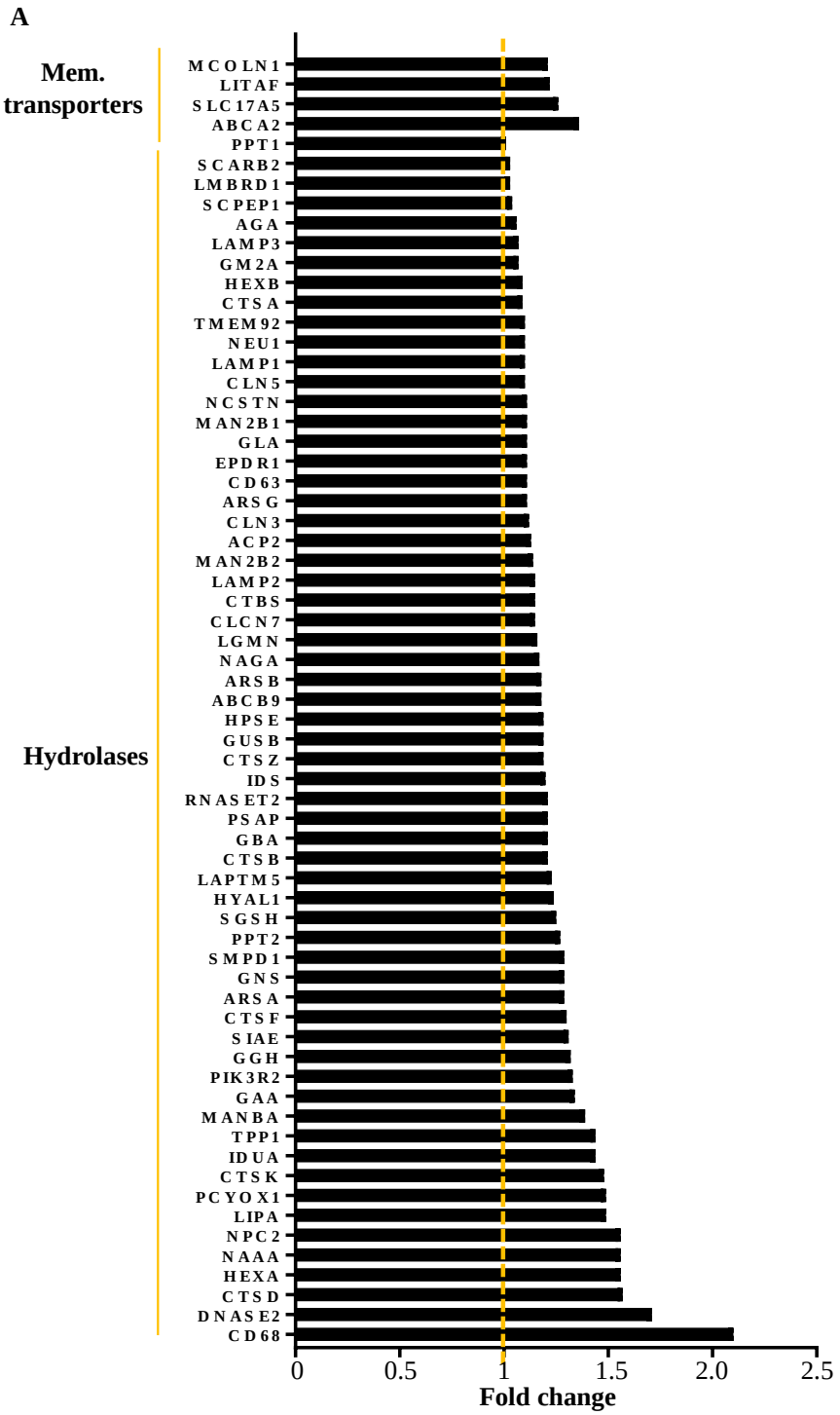


Figure 5.2: Gene expression analyses of acacetin treated HeLa cells in the presence of *S. typhimurium* infection (A) Schematic of acacetin mediated TFEB activation during *S. typhimurium* infection. As described in figure 5.1A, the infection causes phosphorylation of TFEB. The shaded region indicates the question to be addressed using the NanoString nCounter platform. (B) Schematic of the three groups used in the gene expression analysis using NanoString nCounter system viz. Group1: Hela cells treated with acacetin, Group 2: Hela cells infected with *S. typhimurium* and Group 3:Hela cells infected with *S. typhimurium* and treated with acacetin. (C) Alluvial diagram representing the changes in gene expression of immune-related genes induced by *S. typhimurium* infection and acacetin treatment. Comparison of results from different groups is represented in the diagram, indicating the immune-related genes identified by the analysis, fold change in gene expression and the pathways enriched by each group. The threshold for shortlisting genes was no. of mRNA transcript counts >= 40 and $p < 0.05$.

The results obtained from the microarray analysis showed induction in an array of TFEB target genes after acacetin treatment (**Figure 5.3A**). Additionally, the gene expression changes obtained from both the gene expression platforms showed a similar trend, validating the microarray results (**Figure 5.3B**). Further, to study the role of shortlisted genes in TFEB activation, we commenced with the genes modulated in immune-related pathways. In the

current work, we have analysed the role of NFkB and TGFβ (Transforming Growth Factor- β) signalling pathways to regulate TFEB activation, as it was highly enriched in the immune system network post acacetin treatment in both the gene expression platforms.



B

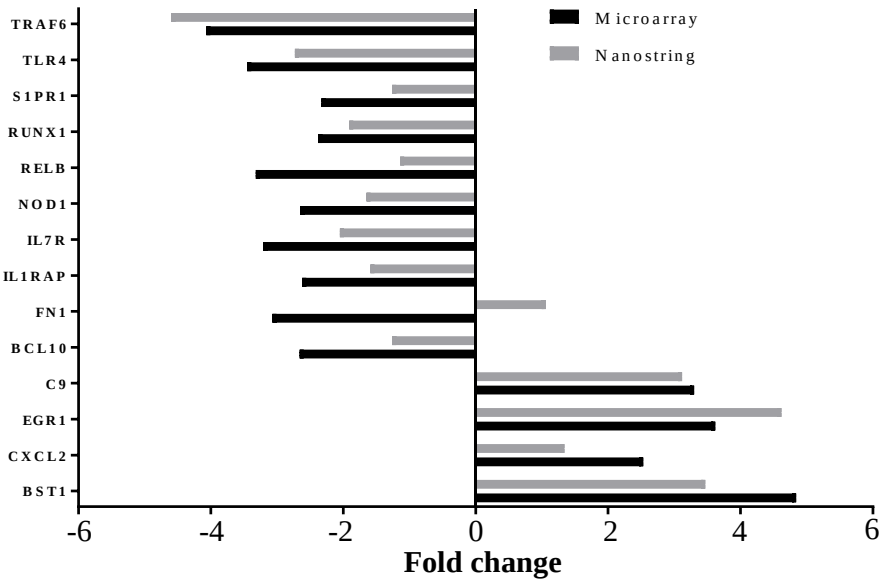


Figure 5.3: Validation of microarray analyses of acacetin treated HeLa cells (A) Gene expression changes of TFEB target genes induced by acacetin in HeLa cells. (B) Graph comparing the expression changes of selected immunity-related genes from microarray and NanoString gene expression platforms. Negative value of fold change indicates extent of downregulation while positive value of fold change indicates extent of upregulation of the selected genes.

5.2.3 Image-based screening for genes modulating TFEB nuclear translocation revealed the involvement of NFkB signalling pathway.

Both NFkB and TGFβ signalling pathways are well studied for their involvement in immune response and its dysregulation are implied in inflammatory diseases (Batlle and Massague, 2019). However, their role in modulating TFEB activation is not explored. In the current study, genes were transiently overexpressed in HeLa cells and immunostained with TFEB for image-based analysis. The metrics for the genes to be classified as putative inhibitor or inducer depend upon the TFEB nuclear translocation. Untreated HeLa cells were used as control (**Figure 5.4A**). Based on the study, we identified that 4 out of 7 genes tested from NFkB signalling cascade that showed decreased nuclear translocation of TFEB compared to the control cells,

whereas there were no significant changes in TFEB nuclear accumulation after overexpressing genes related to TGFβ signalling **(Figure 5.4B)**.

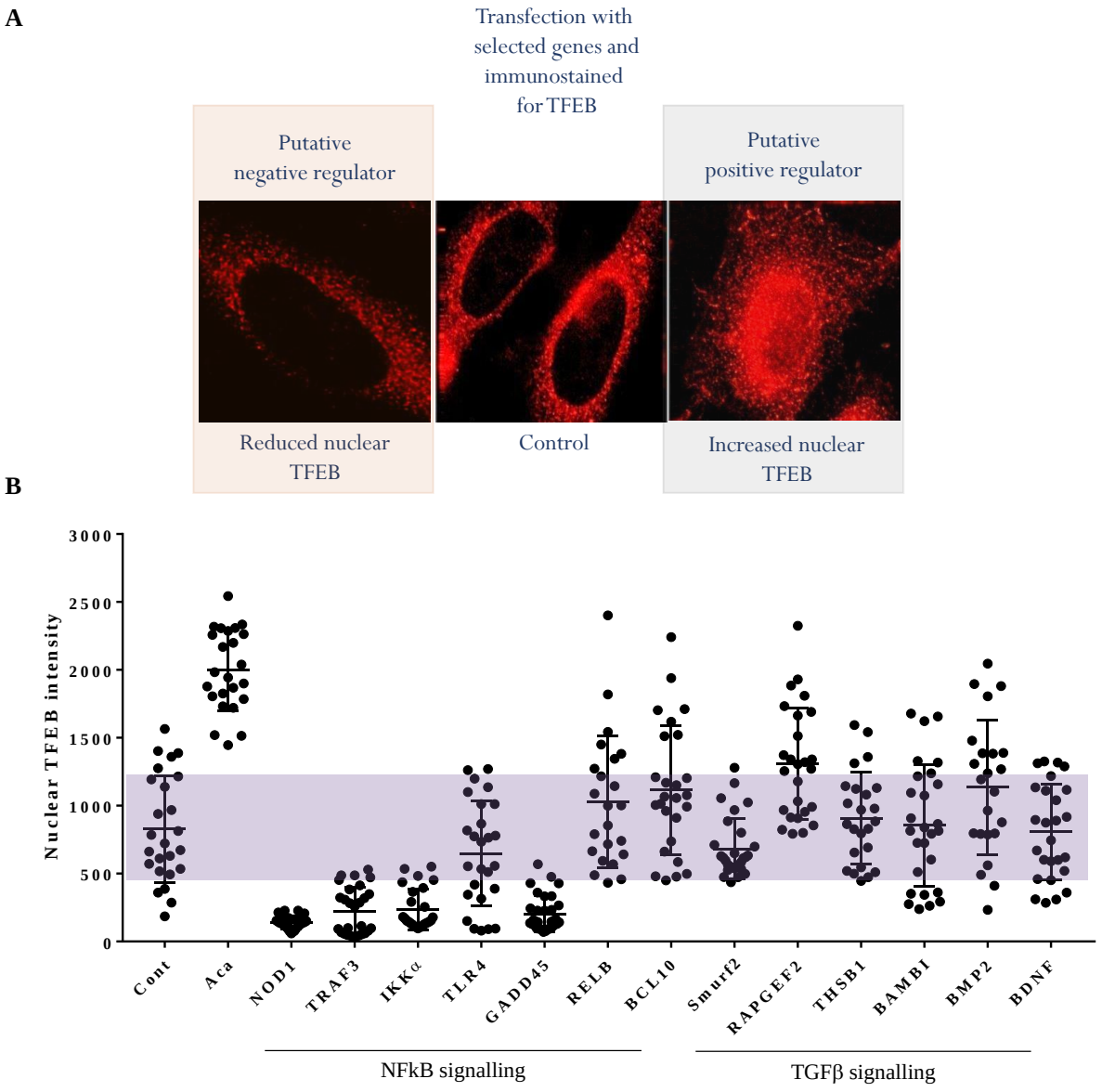
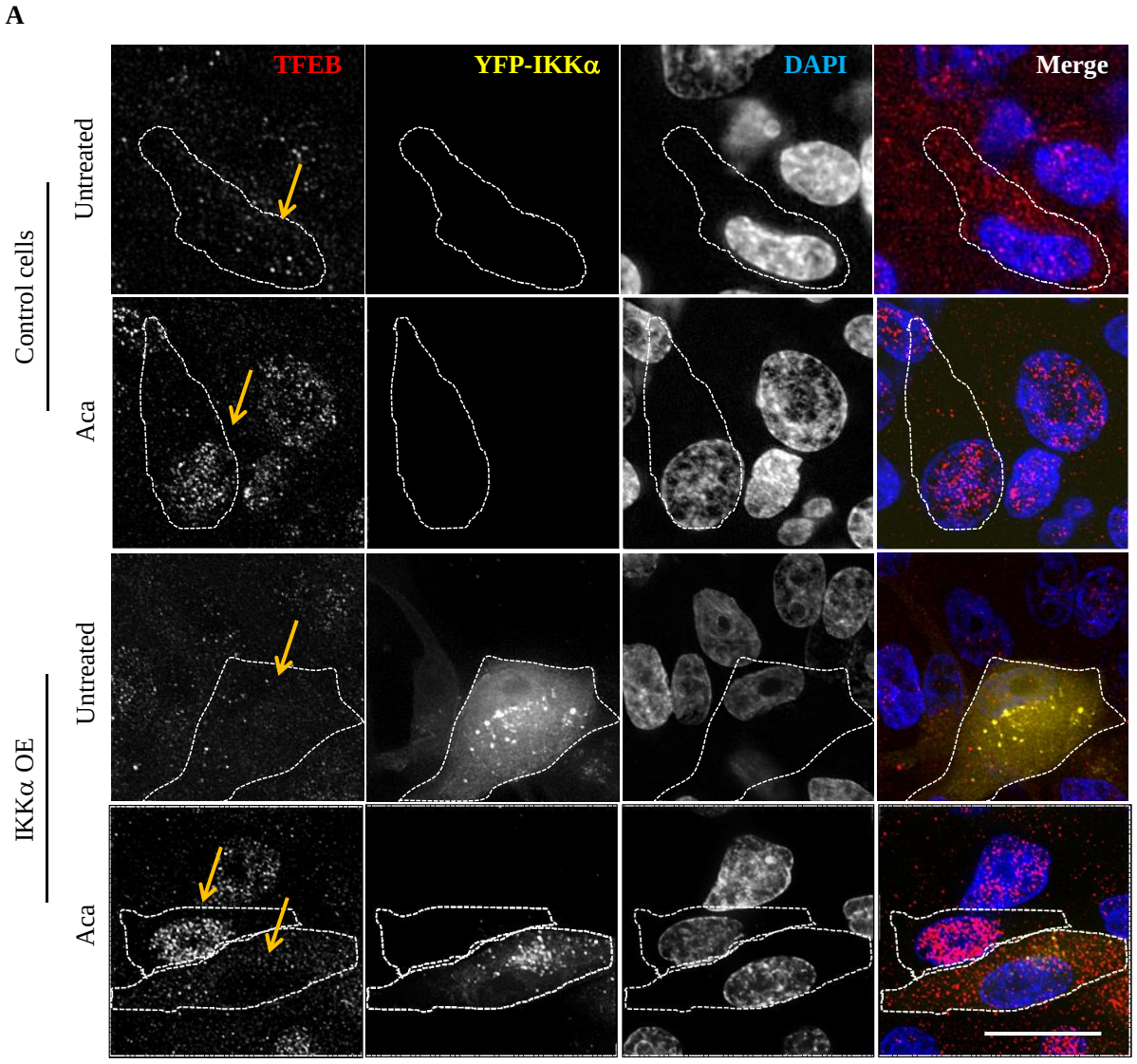


Figure 5.4: Image-based screening for genes modulating TFEB nuclear translocation revealed the involvement of NFkB signalling pathway (A) Schematic for an image-based screening of selected genes for TFEB activation. HeLa cells overexpressing the selected genes and immunostained for TFEB will be scored for TFEB nuclear translocation. (B) Graph indicating the nuclear TFEB intensity after overexpression of NFkB and TGFβ signalling pathway genes. The shaded region indicates the standard deviation of control cells.

5.2.4 Overexpression of IKKα abrogates the acacetin mediated TFEB nuclear translocation

To further access the role of NFkB signalling in acacetin-mediated TFEB activation, we performed immunostaining analysis in the presence of compound treatment. As phosphorylation status of TFEB regulates the subcellular localization of TFEB needed for activation, we prioritized kinases or phosphatases of the pathway. One out of the four NFkB signalling related genes that showed a reduction in TFEB nuclear accumulation is the kinase, IKKα (inhibitor of nuclear factor-κB (IκB) kinase). To study the involvement of IKKα in acacetin-mediated TFEB translocation, HeLa cells transiently overexpressed with YFP-IKKα were either left untreated or treated with acacetin for 2 hours (**Figure 5.5A**). Acacetin treated samples showed reduced nuclear TFEB accumulation after IKKα overexpression, going to show that IKKα inhibits acacetin-mediated nuclear translocation of TFEB. As expected, cells that do not overexpress IKKα respond to acacetin treatment by inducing nuclear TFEB accumulation as indicated by yellow arrows in the representative images (**Figure 5.5B**).

Hence, we conclude that IKKα which was obtained as down-regulated hit after acacetin treatment in gene expression analysis, could prevent the acacetin-mediated nuclear translocation of TFEB. Further experiments to confirm the role of IKKα in TFEB phosphorylation is underway.


A
TFEB
YFP-IKKα
DAPI
Merge
Control cells
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Aca
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B
1*10³ Nuclear TFEB intensity
20
15
10
5
0
****
Un
Aca
Un
Aca
Control cells
IKKα OE

Figure 5.5: **Overexpression of IKKα abrogates the acacetin mediated TFEB nuclear translocation** (A) Representative microscopy images of HeLa cells overexpressed with YFP-IKKα and immunostained for TFEB. The yellow arrows point the nucleus of the representative cells. (B) Graph showing nuclear TFEB intensity after overexpression of YFP-IKKα. (n=50, N=3). Statistical analyses on three independent experiments were performed using One-way ANOVA with Bonferroni test: ns-non-significant, *p<0.05, **p<0.01, ***p<0.001. Error bars represent mean +/- SEM.

5.3 Discussion

There is a constant battle between host and pathogen during intracellular infections (Lengeling et al., 2001; Monack and Hultgren, 2013). Hence, it is essential to identify the mechanisms by which the pathogen evade host immune responses so as to identify potential therapeutic targets (Rana et al., 2015). As mentioned earlier, a study by McGourty *et al* identified that SifA protein of *S. typhimurium* perturbs the functional activity of lysosomes (McGourty et al., 2012). In our study we observed an accumulation of transcriptionally inactive TFEB during intracellular *S. typhimurium* infection, indicating another mechanism by which the pathogen affects lysosomal biogenesis in addition to autophagy induction. Additionally, in the presence of a xenophagy inducer like acacetin, the proteolytic activity of lysosomes was restored. These observations posed two questions- 1) What are the molecular players that are modulated to cause accumulation of phosphorylated-TFEB during *S. typhimurium* infection? and 2) What is the regulator of TFEB that modulates its subcellular localization upon acacetin treatment?

To answer these questions, we performed gene expression analysis in two different platforms both having their own advantages. For example, microarray analysis is an unbiased approach that measured the global transcription changes induced by acacetin. In contrast, the NanoString nCounter system is more sensitive than microarray and in comparison, similar in terms of sensitivity to TaqMan PCR with the capability to multiplex (Geiss et al., 2008). We, therefore, used this system to study an array of more than 500 immunity-related genes. As seen from our gene expression analysis, acacetin treatment significantly modulated pathways related to

immune response. Additionally, the immune pathways modulated by acacetin during intracellular *S. typhimurium* infection were also identified. By analysing the results from NanoString platform, we observed upregulation in genes related to host signalling pathways such as MAPK, Ras, TNF and NFkB during infection. Interestingly, acacetin treatment either on host cells alone or during infection showed downregulation of genes related to MAPK, TNF and NFkB signalling pathways. Further experimentation to confirm the role of these pathways on acacetin-mediated xenophagy is needed. Based on an image-based analysis, putative modulators of TFEB related to immune signalling pathways were identified. Among the genes of NFkB signalling studied, we chose to further work on IKKα, as TFEB translocation is dependent on phosphorylation-dephosphorylation balance. Additionally, acacetin-mediated TFEB nuclear translocation was perturbed when IKKα is overexpressed. Further characterization of these signalling pathways will help in dissecting the novel upstream regulatory pathways of TFEB.

The NFkB family of transcription factors play a pivotal role in regulating the expression of genes related to immune response, cell proliferation and differentiation (Hayden and Ghosh, 2011). The interplay of the NFkB pathway and autophagy is complex having many facets. Briefly, autophagy upon heat shock protein 90 (Hsp90) induction is shown to degrade the components of the IKK complex (IKKα,β,γ) (Trocoli and Djavaheri-Mergny, 2011). Additionally, NFkB is a transcription factor known to positively regulate genes involved in the autophagy process. This regulation of autophagy by NFkB is mediated in more than one mechanism. There is a competitive binding of NFkB with E2F to upregulate BNIP3 expression essential for autophagy induction. Additionally, Beclin1 harbours multiple NFkB binding consensus sequences suggesting its regulation by p65, a member of NFkB family (Copetti et al., 2009). However, the crosstalk role of NFkB in regulating the master transcription factor of autophagy, TFEB is not studied so far. Conversely, a study by Song *et al*, suggests that

TFEB inhibits NFkB signalling to attenuate inflammation in a diabetic mouse model (Song et al., 2019). Hence, further investigations to understand the interaction of IKKα/NFkB pathway and TFEB are essential. Future studies in this regard will be focused on identifying if TFEB is a potential substrate of IKKα/NFkB pathway genes for phosphorylation status and studying the importance of NFkB pathway in inducing xenophagy.

Overall, our study so far has identified an evasion strategy employed by intracellular *S. typhimurium*, by accumulating phosphorylated TFEB and a concomitant decrease in proteolytic active lysosomes of the host. This provides an ideal niche for *S. typhimurium* to replicate intracellularly in SCVs. This observation was made by using a novel small molecule inducer of xenophagy, acacetin which reduces intracellular *S. typhimurium* replication by activating TFEB. This leads to an increase in recognition of intracellular *S. typhimurium* by xenophagy adaptors such as p62 and enhanced fusion of proteolytically active lysosomes with SCVs. Further investigations into finding the upstream regulator of acacetin-mediated TFEB activation revealed putative regulators such as IKKα (**Figure 5.6**). In summary, this study utilized a chemical-genetic approach where a chemical xenophagy modulator, acacetin was used to understand the molecular pathways regulating xenophagy to restrict replication of *S. typhimurium*. Similarly, as a next step in this line of investigation, results will also be verified in other relevant cell lines such as intestinal cell types (CaCo2) and macrophages (Thp1).

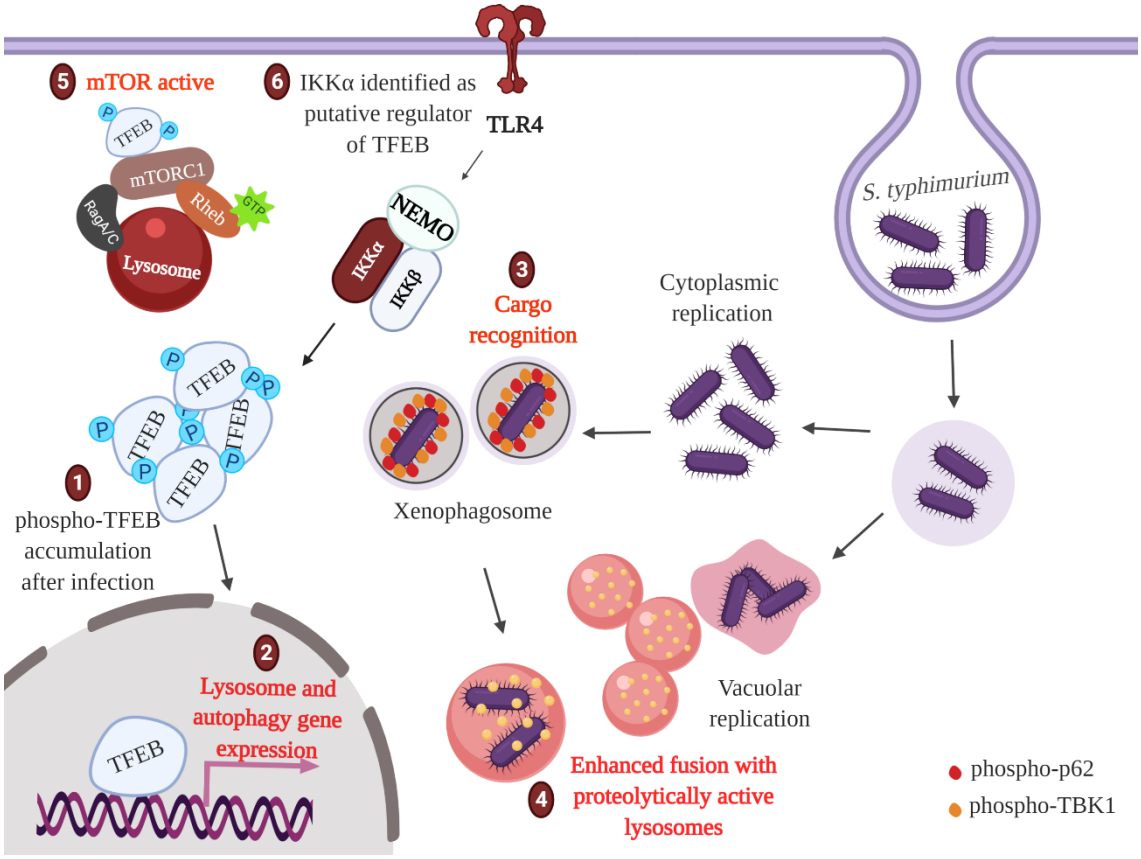

Figure 5.6: *In cellulo* molecular mechanisms of acacetin to induce xenophagy. (1) Intracellular *S. typhimurium* infection leads to accumulation of phosphorylated TFEB. The molecular events induced by acacetin is labelled in red. Briefly, in the presence of acacetin, (2) there is increased translocation of TFEB to nucleus inducing genes involved in lysosomal biogenesis and autophagy, (3) there is enhanced recruitment of phospho-p62 and phospho-TBK1 to capture *S. typhimurium*, (4) there is enhanced fusion of proteolytically active lysosomes with SCVs, (5) the acacetin mediated TFEB translocation is mTOR independent, (6) Gene expression analysis of acacetin treated host cells with and without *S. typhimurium* identified putative molecular players such as IKKα to regulate TFEB nuclear translocation. Image created using BioRender.com.

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Chapter 6

Development of high throughput screening to identify novel xenophagy inducers

Chapter 6

Development of high throughput screening to identify novel xenophagy inducers

6.1 Overview

As described in the previous chapters, small molecules can be used as tools to modulate xenophagy and gain mechanistic insights into the process. It is essential to develop a robust and sensitive screening platform to identify novel chemical modulators of the process. Previously reported pharmacological screens monitor autophagy reporters like LC3 (Kuo et al., 2015; Zhang et al., 2007) or alternatively quantitate the bacterial load by techniques such as high content imaging (Nagy et al., 2019).

The conventional xenophagy assay used in our study involves meticulous serial dilution and plating of intracellular bacteria followed by counting of CFU. This assay is not amenable to screen a large number of small molecules and therefore the development of a high throughput screening format that can be used to test several chemical modulators in a single experiment was indispensable. *S. typhimurium* expressing the *Photorhabdus luminescens* lux operon has been used in studies to monitor its growth in real-time. For example, study by Karsi *et al* applied this bioluminescent system to study the *Salmonella* contamination in food products. The study also tested the dependence of bioluminescence activity to bacterial density and observed that bioluminescence activity correlates positively with bacterial cell number (Karsi et al., BMC Microbiol, 2008). Bioluminescence-based assays have gained importance HTS due

to the sensitivity and robustness of the system (Fan F and Wood KV., Assay Drug Dev Techno, 2007). Bioluminescence reporters are used in both cell-free systems (enzyme activity) as well as cell based assays measuring biological response (Kent et al., J Biomol Screen, 2005).

In this chapter, the development and standardization of screening assay that is based on quantitating degradation of bioluminescently tagged bacterial cargo (Brodl et al., 2018) will be discussed. Bioluminescence is a process for visible light emission that can be used in the living organisms. This is a powerful methodology to monitor the biological processes in real-time and more importantly is a non-invasive technique (Fleiss and Sarkisyan, 2019; Wilson and Hastings, 1998). We developed a bioluminescence-based assay that as mentioned provides the added advantage of real-time and sensitive assessment of intracellular bacterial replication. The assay was found to be more time and cost-effective than the conventional CFU assay. After successful standardization of the assay, a custom-made small molecule chemical library comprising of 410 compounds were screened. The library consists of potent compounds with known targets that are not previously reported for their involvement in autophagy.

6.2 Results

6.2.1 Generation of bioluminescent *S. typhimurium* strain

The principle of the assay involves measuring the intracellular bacterial burden after treatment with compounds. To achieve this, we generated a bioluminescent *S. typhimurium* strain that can be used to monitor bacterial growth in real-time **(Figure 6.1A)**. Bioluminescence has the added advantage that there is no need for exogenous addition of substrates. The bacterial vector expressing bioluminescent operon was electroporated into *S. typhimurium*.

Bioluminescence plasmid, pAKlux1 is derived from the pBBR1 vector that supports broad host range (Karsi et al., 2006). The vector contains luxCDABE operon taken from *Photorhabdus*

luminescens conjugated to lacZ promoter. Bacteria expressing this vector catalyze the oxidation reaction of long-chain aldehydes and $FMNH_2$ causing the emission of light **(Figure 6.1B)**. The stability of the pAKlux1 plasmid was previously evaluated by Karsi *et al* in a variety of Gram-negative bacterial strain. The study showed that the plasmid is stable for 5 days with continuous subculturing under selection (ampicillin) and for 2 days without antibiotic selection in *S. typhimurium* (Karsi et al., 2008). Further characterization has shown that there are no alterations in terms of biochemical and structural phenotypes due to constitutive bacterial luciferase. It is therefore considered that luminescence obtained is directly proportional to the number of bacteria.

The plasmid expressing strains were compared for its growth kinetics with the wild type S. typhimurium. As seen in **figure 6.2A**, the plasmid expression caused no alterations in the bacterial doubling rate. Additionally, the transformed bacterial strain showed detectable luminescence whereas untransformed wildtype *S. typhimurium* did not show any background reading indicating that the reading obtained is exclusively from the expression of luminescent vector in the transformed strain **(Figure 6.2B)**.

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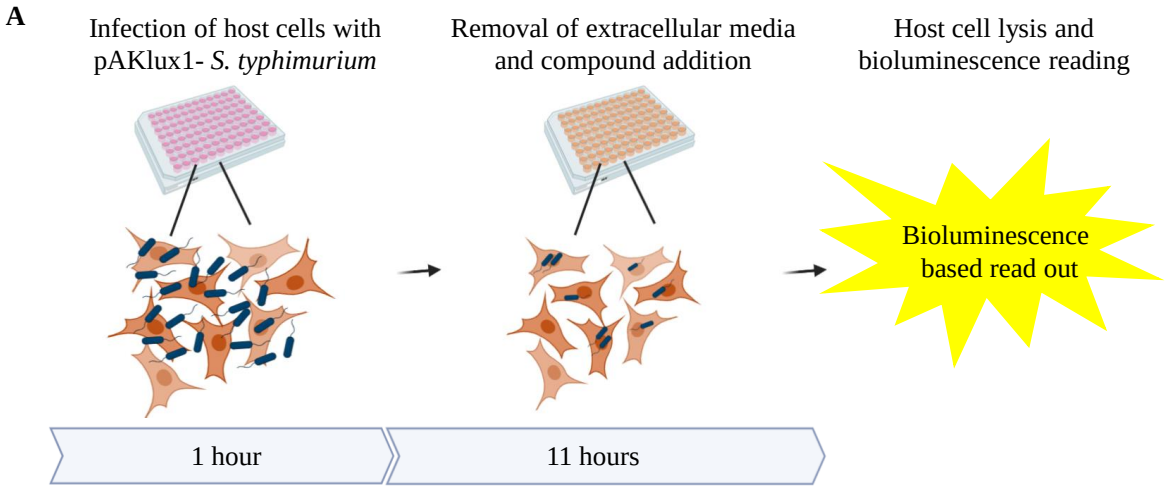

Figure 6.1: Development of a bioluminescence-based assay to monitor intracellular *S. typhimurium* in real-time. (A) Bioluminescent *S. typhimurium* was constructed by electroporating pAKlux1 vector consisting of lux CDABE operon under lacZ promoter. The pAKlux1 expressing bacterial strain was used to infect the HeLa cells for one hour. Following this, extracellular bacteria were removed and incubated with compounds. After 11 hours of incubation, the host cells were lysed to measure the bioluminescence. Monitoring luciferase activity is directly proportional to the number of intracellular *S. typhimurium*.

6.2.2 Standardization of intracellular infection assay using bioluminescent *S. typhimurium* strain

The assay involves the infection of host cells using bioluminescent *S. typhimurium* for one hour. Following this, the extracellular bacteria is removed and further incubated in the presence of compounds to study the effect of the small molecules on intracellular bacterial survival. To

set up the bioluminescent based readout, it was essential to standardize the infectivity rate and duration of the assay to sensitively monitor the bioluminescence. Firstly, we compared the bioluminescence obtained from different multiplicity of infections (MOI) on HeLa cells. It was observed that luciferase activity correlates well with the infectivity rate. Further, the assay was standardized for the duration that can optimally represent the intracellular bacterial replication. An MOI of 200 with an incubation time of 12 hours was finalized **(Figure 6.2C)**.

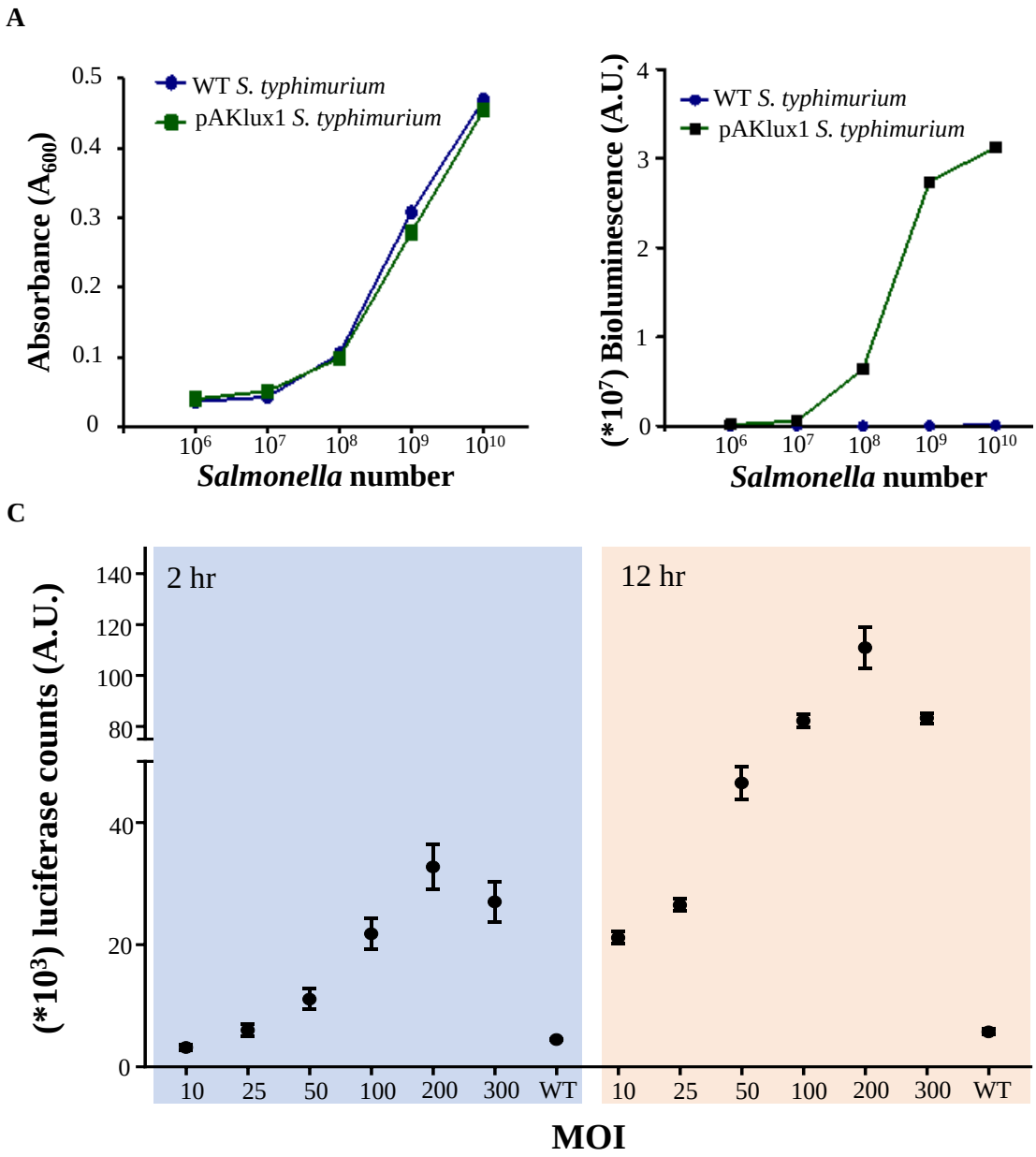

Figure 6.2: Standardization of bioluminescent *S. typhimurium* for a high throughput format. (A) The growth kinetics of wildtype and bioluminescent *S. typhimurium* by measuring absorbance at 600 nm. (B) The intracellular bioluminescence reading measured from wildtype and bioluminescent *S. typhimurium* by measuring luminescence at 490 nm. (C) Intracellular infection of pAKlux1 expressing bacterial strain at different MOI in HeLa cells. The bioluminescence was observed after 2 hours and 12 hours post-infection. The reading corresponds to the number of intracellular *S. typhimurium*.

6.2.3 High throughput chemical screening for xenophagy inducers

After standardizations, the intracellular infection assay was performed in a 96-well format to screen for small molecule chemical modulators of xenophagy. The assay included rapamycin (8µM), a known xenophagy inducer and chloramphenicol (antibiotic) as positive controls. The results observed for positive controls were in concordance with the conventional CFU assay. Rapamycin and chloramphenicol treatments showed a significant decrease in the luciferase activity indicating reduced intracellular *S. typhimurium* survival/growth **(Figure 6.3A)**. Further, the proof-of-principle HTS was performed using a custom-made library comprising of 410 compounds. The bioluminescent readings post-infection with pAKlux1 expressing *S. typhimurium* and incubation with compounds were collected **(Figure 6.3B)**.

6.2.4 HTS data correction using HTS corrector

HTS has emerged as a valuable strategy in modern drug discovery research. However, HTS imposes the threat for random and systemic errors occurring in the results. Such errors occur majorly because HTS is carried out for a large number of compounds that cannot be accommodated in a single plate. Hence the across-plate variations that emerge must be normalized to reduce the false-positive rate. It is therefore imperative to imply quality control measures to reduce the impact of such errors during HTD data analysis. In this study, we employed more than twenty-five 96-well plates to screen the compound library where each compound was used in triplicates and two different concentrations (12.5µM and 25µM) **(Figure 6.3B)**. Hence, we have used HTS corrector, a software application to analyse the HTS data and have applied the necessary correction to the readings (Makarenkov et al., 2006). The software was used to perform well-correction by calculating the background subtraction.

Finally, sigma distribution to set well-specific standard deviation was applied. The normalized data obtained from HTS corrector was further plotted using R Studio for better data representation (Refer chapter 2 Materials and methods section Rstudio packages used for analysis).

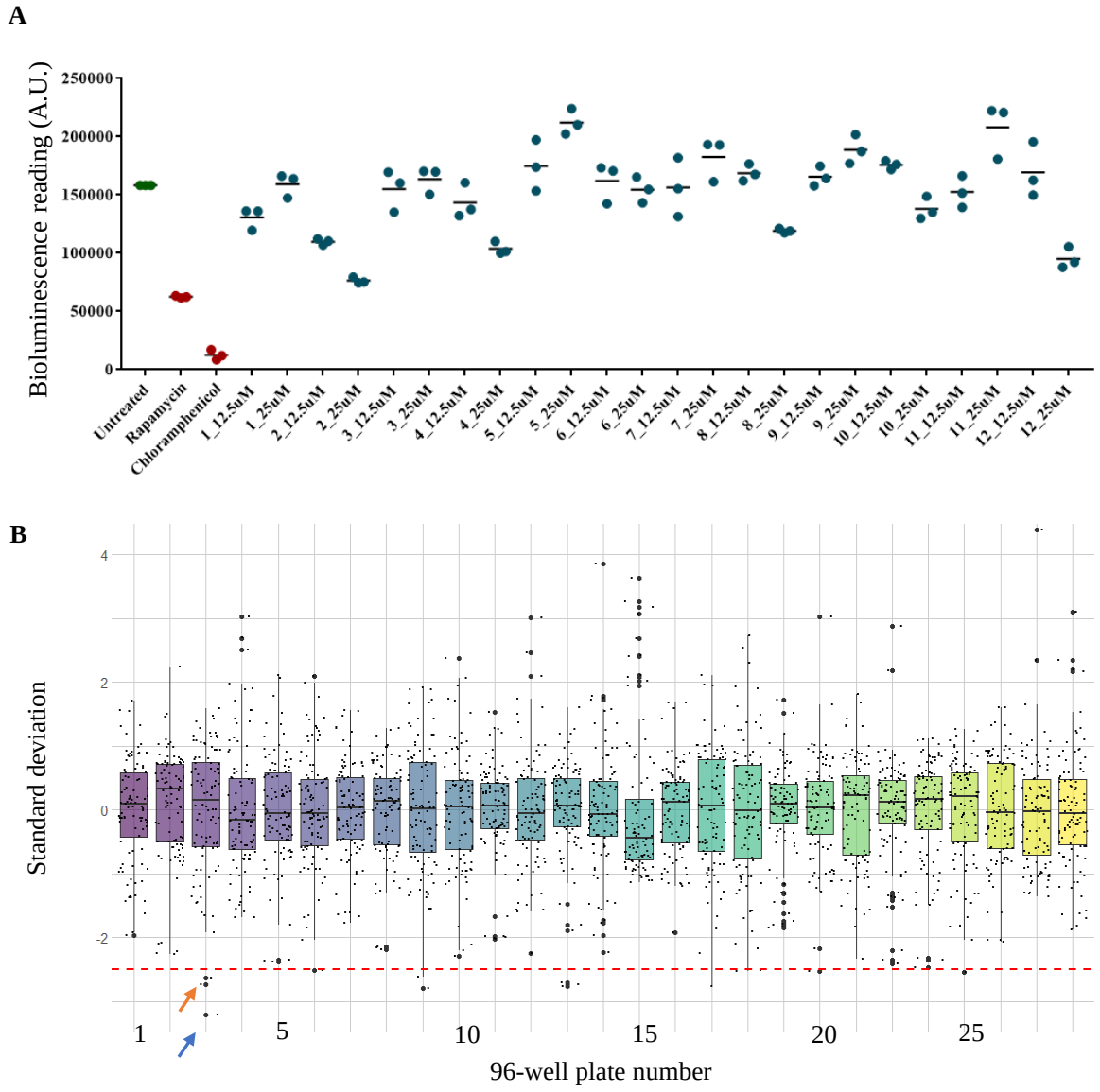

Figure 6.3: Intracellular infection assay using bioluminescent *S. typhimurium* in a 96-well format. (A) The bioluminescence readings obtained from intracellular infection assay. Representative values from one plate used in the screening. (B) Normalized bioluminescence readings obtained from the HTS of a custom-made compound library consisting of 410 compounds. Each box plot represents the readings from a single 96-well plate used in HTS and each dot represent a compound. The red dotted line marks the threshold (> - 2.5 S.D) above which the compounds are shortlisted for further studies.

The blue arrow points the bioluminescence reading obtained from chloramphenicol control and orange arrow points the reading obtained from rapamycin treatment.

6.3 Discussion

Xenophagy has emerged as an innate immune mechanism of the host to prevent establishment of intracellular infection. Studies have shown that modulating xenophagy either genetically or pharmacologically have a positive impact on controlling intracellular pathogen survival. Apart from identifying modulators, these interventions can also be used as tools for effective understanding of the process.

In the past, high throughput screens with diverse methodologies for studying xenophagy has been employed. Existing screening approaches for the process include genetic screens like siRNA screen to identify host or pathogen factors that modulate xenophagy as well as pharmacological screens to identify novel or repurposed drug-like molecules. Of the two approaches, pharmacological small molecule compounds not only give a handle to modulate xenophagy but also provide a therapeutic potential.

In this study, we developed a bioluminescence-based assay to effectively monitor the intracellular replication of *S. typhimurium*. Briefly, we generated bioluminescently labelled *S. typhimurium* to robustly quantitate the bacterial burden in real-time. Following this, standardization tests to measure the sensitivity of the assay was carried out. We observed that luminescent tagged bacteria showed significantly higher values in comparison with untagged *S. typhimurium* indicating negligible background luminescence. Besides, the growth kinetics of both bacterial strains was comparable indicating that presence of operon expressing the luminescent protein in bacteria did not affect the growth rate.

Our results show that bioluminescent *S. typhimurium* can be effectively used as a tool in high throughput screening because of the sensitivity and ability to monitor in real-time. However,

in the current study, end-point luminescent readings were collected due to the temperature, humidity, and CO_2 requirements for maintaining HeLa cells during intracellular infection assay.

Although the primary screening was not aimed at monitoring autophagy or xenophagy specific reporter, it can be coupled with a secondary assay that monitors xenophagy potential of the high confident hits obtained from the primary screen. For instance, the primary screen hits can be further tested on autophagy-deficient cell lines to study its dependency on autophagy. This strategy has the added advantage of highlighting compounds that functions solely by inducing xenophagy as well as those compounds that couples xenophagy effect with other antibacterial strategies like antibiotic effect. Further studies are currently focussed on validating the putative hits obtained from the HTS using secondary screening with a xenophagy-specific reporter. Furthermore, the short-listed hits for their xenophagy effect can also be verified in other cell types such as intestinal epithelial (CaCo2) and macrophages (Thp1) cell types.

Finally, another interesting aspect emerging in the field that can be coupled with the existing platform is bioluminescence imaging (BLI). Applications of BLI during *in vivo* studies are gaining importance as the emission wavelength of luciferase can be imaged from tissues several centimetres deep. This technique can be exploited to organismal level studies to monitor *in vivo* bacterial replication from organs of interest (Benaron et al., 1997; Sadikot and Blackwell, 2005; Tang et al., 2003).

6.4 References

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Chapter 7

Discussion and future directions

Chapter 7

Discussion and future directions

7.1 Discussion

To establish infection, pathogens evade host immune system and parallelly, the host employ various defence mechanisms thereby imposing a biological arms race (Kwon and Song, 2018; Pao and Rape, 2019). Xenophagy is one of the defence mechanisms employed by the host in parallel with other strategies such as antimicrobial peptides and reactive oxygen species. As expected, pathogens breach these by a plethora of diverse tactics to overcome host defences including xenophagy. Therefore, enhancing host xenophagy to mitigate infection progression has been reported by many studies (Chiang et al., 2018).

Although both genetic and pharmacological interventions restore xenophagy, chemical modulators provide a distinct advantage wherein apart from revealing insights into the process, they can also serve as a potential therapeutic tool. Compounds such as trifluoperazine and BRD5631 are such examples - both of them were not only identified for their ability to induce xenophagy but showed therapeutic potential by engaging novel intracellular targets (Conway et al., 2013; Kuo et al., 2015).

Recent studies have expanded the horizon of xenophagy encompassing its close nexus with inflammation and immunity-related pathways (Deretic et al., 2013; Zhong et al., 2016). This has fuelled the search for identifying novel “drug-like” small molecule xenophagy modulators to tackle intracellular infections. The main objective of the project was to find such novel

compounds that would enhance host immunity through xenophagy and additionally use it as a tool to understand the mechanisms of xenophagic flux.

Although it is ideal to have a strategy that directly measures xenophagic flux to identify chemical modulators, we utilised a previously established yeast-based high throughput screening (HTS) carried out in our laboratory to identify novel chemical modulators of autophagy. It was a luminescent readout based HTS for pexophagy, a selective autophagic degradation of peroxisomes (Mishra et al., 2017a; Mishra et al., 2017b). Two compound libraries, LOPAC (comprising of 1280 compounds) and ENZO (comprising of 502 compounds) were screened using this assay. The screening yielded close to 35 primary enhancer hits. Owing to the conserved nature of autophagy, the hits obtained from yeast-based screening were further tested for their ability to induce xenophagy of intracellular *Salmonella typhimurium* in mammalian cell lines. The colony forming unit (CFU) was quantitated to account for the selective degradation of intracellular bacteria. One of the promising candidate compounds, acacetin showed a consistent reduction in intracellular *S. typhimurium* burden in both epithelial cell lines (HeLa and U1752) and macrophages (RAW 264.7). Nevertheless, later in the study we employed strategies to identify other xenophagy inducers directly by monitoring xenophagic flux. Firstly, we developed a bioluminescent based screening assay that measures the degradation of intracellular *S. typhimurium*. By using this assay, we screened a custom-made compound library comprising of 410 compounds whose intracellular targets are known but not tested previously for their autophagy potential. Apart from screening for novel xenophagy inducers, we also invested in another recent approach of measuring the xenophagy potential of existing antibiotics. Extrapolating from this rationale, we tested known antibiotics and its derivatives to induce xenophagy. In this respect, we identified a vancomycin derivative, VanQAmC10 that showed increased autophagic potential compared to conventional vancomycin and also higher efficacy against drug-resistant *Acinetobacter baumannii* (Sarkar

et al., 2020). This work was carried out in collaboration with Antimicrobial research laboratory at JNCASR.

The major part of this study is focussed on using the identified xenophagy inducer, acacetin as a tool to modulate the process and study the mechanism of xenophagic flux. Investigations revealed that acacetin did not affect *in vitro Salmonella* growth *per se* in Luria Bertani Broth. Similarly, the expression of virulence genes of the pathogen, specifically of *Salmonella* Pathogenicity Island-2 (SPI-2) required for intracellular replication was also not affected by acacetin (Veena, MS Thesis, 2016). Further investigations revealed that both genetic ($ATG5^{-/-}$ HeLa cell line) or pharmacological inhibition (3-MA and wortmannin) of autophagy abrogated the acacetin mediated decrease in intracellular *S. typhimurium*. Taken together, these results suggested that acacetin mediates the reduction in intracellular *S. typhimurium* burden by inducing host-mediated xenophagy. We further showed that acacetin does not affect other trafficking pathways like endocytosis which functions in concert with autophagy sharing certain molecular effectors and both pathways culminating in the lysosome.

In terms of autophagy induction, acacetin treatment on HeLa cells resulted in the accumulation of LC3-II which further increased when cotreated with BafA1- both of these observations together is considered as a gold standard phenotype for autophagy induction. Further, microscopic analysis revealed an enhanced fusion of autophagosomes with lysosomes leading to accumulation of autolysosomes in the cell post acacetin treatment.

Further by studying the mechanism of xenophagy induction by acacetin revealed the following:-

1) Acacetin enhances recruitment of xenophagy mediators to efficiently capture the pathogen by enhancing post-transcriptional mechanisms

As detailed earlier, pathogens exert their virulence to overcome the host defence mechanisms such as xenophagy. Studies in the past have identified multiple ways by which *S. typhimurium* prevents xenophagy mediated degradation. One of the evasion strategies is to prevent the recognition of *S. typhimurium* by xenophagic proteins after few hours post-infection (p.i.). The recognition by xenophagic adaptor proteins such as p62 and NDP52 is elevated immediately after infection (1 to 2 hours p.i.). There is a significant reduction in the recruitment of xenophagy adaptor proteins after 2 to 3 hours p.i. which correlates with the expression of pathogen virulence genes. Upon acacetin treatment, temporal microscopic studies showed increased recruitment of xenophagy machinery proteins such as p62 and LC3 to intracellular bacteria. Additionally, we observed that mRNA expression of p62 was not enhanced by acacetin treatment and immunoblotting analysis revealed temporal p62 degradation as commonly seen during autophagy induced state. We, therefore, wanted to check the reason for increased p62 recognition during acacetin treatment. Further mechanistic analysis revealed that enhanced recruitment of p62 after acacetin treatment was possibly due to its phosphorylation at S403 position in its UBA domain. This post-translational modification is previously reported to increase the affinity of p62 to bind ubiquitinated cargoes. Similarly, we also observed increased recruitment of TBK1, which phosphorylates p62 at S403 to *S. typhimurium* after acacetin treatment. A similar observation was previously reported where the occurrence of enzymatically active TBK1 was shown in the bacterial vicinity and more recently Ravenhill *et al* identified TBK1 bound to ULK1 complex getting recruited to intracellular *S. typhimurium* (Ravenhill et al., 2019; Thurston et al., 2016). This hierarchal cargo recognition is not unique to xenophagy but is also reported in other selective autophagy mechanisms such as mitophagy to capture damaged mitochondria (Matsumoto et al., 2015).

2) Functions by activating transcriptional regulator of autophagy and lysosomal biogenesis, TFEB

As mentioned earlier, acacetin treatment increased both autolysosomes and lysosomes of the cell. Studies by Ballabio's group identified TFEB, one of the members of MITF family to control the gene network involved in lysosome biogenesis and autophagy pathways. TFEB binds and regulates expression of a larger subset of genes involved in lysosomal biogenesis and is therefore referred to as master regulator of lysosomal biogenesis and autophagy. As acacetin treatment induces both pathways we investigated the involvement of TFEB in inducing acacetin mediated autophagy and xenophagy induction. We observed enhanced nuclear translocation of TFEB and concomitant increase in selected target genes of TFEB. Activation is mediated by modulating the phosphorylation-dephosphorylation status of TFEB. Phosphorylated TFEB is transcriptionally inactive and is localized on the lysosomal surface or bound to cytosolic 14-3-3 protein. However, upon dephosphorylation, TFEB translocates to the nucleus and induces the expression of its target genes participating in lysosome biogenesis and autophagy. Further investigation is needed to identify the role of acacetin in inducing other family members of MITF transcription factors such as TFE3. However, we observed that silencing of TFEB abrogates the ability of acacetin to induce autophagy and xenophagy. It is, therefore, possible that acacetin mediated induction is predominantly mediated by TFEB.

Studies have shown that activation of TFEB is beneficial during intracellular infections (Visvikis et al., 2014). However, the role of TFEB during intracellular *Salmonella* infection was not clear. An existing study by Najibi *et al* on TFEB and *S. typhimurium* show that there is an induction of TFEB after two hours of *Salmonella* infection (Najibi et al., 2016). However, our study showed that there is a temporal accumulation of phosphorylated-TFEB post *S. typhimurium* infection. This transcriptionally inactive TFEB was observed to be dephosphorylated after acacetin treatment. Concomitantly, the number of lysosomes and autolysosomes of the host cells was increased after acacetin treatment. Furthermore, there was

an enhanced fusion of lysosomes with *Salmonella* containing vacuoles making them more proteolytically active.

3) Acacetin is effective in an *in vivo* mouse model of bacterial infection.

In vivo studies during *Salmonella* infection emphasized on the importance of xenophagy-mediated immune response (Benjamin et al., 2013; Conway et al., 2013). These studies highlighted that the basal level of autophagy is very low in tissues such as intestine which is the major site of gastrointestinal infection like Salmonellosis. Although intestinal epithelial cells show initial induction in xenophagy 24 hours post Salmonella infection, it reverts to low basal levels by 72 hours. However, it is not clear if the reversal of xenophagy induction at later stages of infection occurs due to the expression of *Salmonella* virulence. Additionally, studies on restoring the *in vivo* xenophagic block imposed by *Salmonella* infection is also limiting. In the current study, we show that compound treatment *in vivo* led to an increase in LC3B-II with a concomitant reduction in the bacterial burden in the various organs of infected animals. The *in vivo* study was standardized to study at a time where the basal level of xenophagy is low (7 days after infection). This also provides sufficient time for the bacteria to disseminate to various organs such as intestine, liver and spleen. Although acacetin treatment after 7 days of infection reduced the bacterial burden in the liver and spleen, the bacterial load from intestinal tissues had no significant changes. This is possibly due to the known complex microbial-gut interactions. Intestinal epithelial cells accommodate highest microbial flora exhibiting symbiotic or commensal relationship with the microbes. Hence more investigations are required to understand the intestinal xenophagy regulation (Mahida, 2004). However, due to technical limitations (incompatible antibody specificity), the *in vivo* status of TFEB after acacetin treatment was not studied. However, TFEB is shown to regulate the expression of host defence genes during *in vivo S. typhimurium* infection in *C. elegans* (Visvikis et al., 2014).

The effect of acacetin in reducing intracellular infection phenotype in both *in cellulo* and *in vivo* models encouraged us to probe further on the regulatory mechanisms of xenophagy using acacetin as a tool to induce the process. We first resorted to identifying the upstream molecular players involved in activating TFEB. As mentioned earlier, activation leads to its nuclear translocation. Dynamic changes in TFEB localization is mainly regulated by its phosphorylation status. Multiple conserved serine residues are known to be phosphorylated and play a crucial role in determining the subcellular localization (Puertollano et al., 2018). The changes in this post-translational modification occur in response to various cellular stress conditions such as nutrient starvation. The major kinases implicated in this process include mTOR, ERK, Akt, GSK3β. Furthermore, calcineurin dephosphorylates TFEB in response to lysosomal calcium release (Medina et al., 2015; Parr et al., 2012; Puertollano et al., 2018; Roczniak-Ferguson et al., 2012; Settembre et al., 2012). However, acacetin did not activate TFEB through the reported kinases or phosphatase, calcineurin. To identify the novel phosphorylation modulator involved in acacetin action, an unbiased gene expression analysis was carried out post acacetin treatment. Additionally, an array of key immunity-related genes was also studied for its expression after *S. typhimurium* infection in the presence of acacetin. Analysis of the gene expression studies identified putative molecular players that regulate TFEB translocation.

Overall, the results from this study identified a novel xenophagy inducer, acacetin that functions by activating a key transcriptional regulator, TFEB. We exploited the ability of the acacetin-mediated xenophagy to study the xenophagic flux during intracellular *S. typhimurium* infection. Further characterization revealed putative regulators of TFEB in modulating its subcellular localization (**Figure 7.1**).

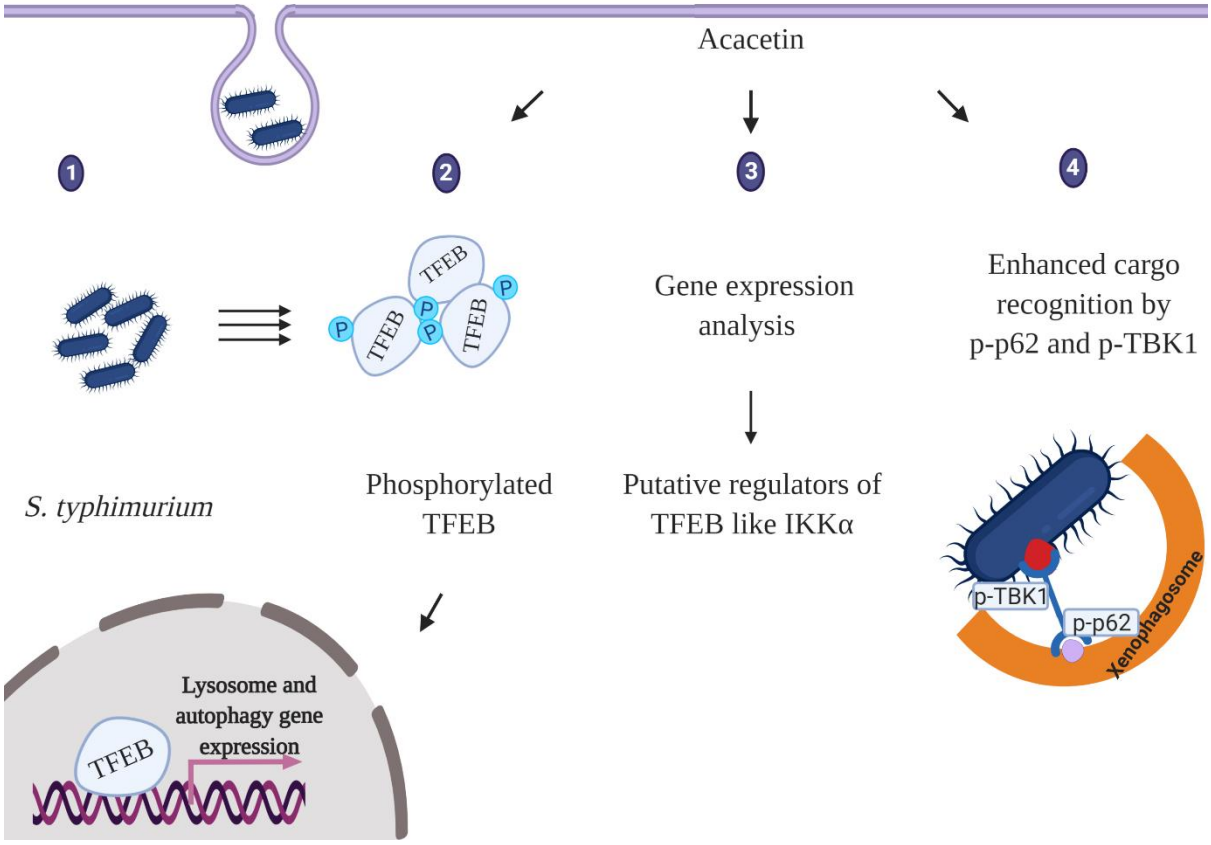

Figure 7.1: **Insights into xenophagy-pathogen interactions revealed by the study.** (1) Intracellular *S. typhimurium* infection leads to accumulation of phosphorylated TFEB. (2) In the presence of acacetin, there is increased translocation of TFEB to nucleus inducing genes involved in lysosomal biogenesis and autophagy. (3) Gene expression analysis of acacetin treated host cells with and without *S. typhimurium* identified putative pathways and molecular players that regulate TFEB nuclear translocation. (4) Acacetin treated host cells recruit phosphorylated p62 and phosphorylated TBK1 to capture intracellular *S. typhimurium*.

The critical insights obtained by characterizing one compound gave us confidence is scaling up the chemical screening assay. The key to identifying such potent molecules is to develop an effective HTS that evades the time consuming plating methods. To do so, we shifted from the conventional CFU based readout to luminescent based assay and by using this new strategy, we performed a proof-of-principle screening of a custom-made library comprising of 410 compounds. Those compounds that decreased the intracellular *S. typhimurium* load will be screened in autophagy-deficient cell lines in a secondary screen to shortlist putative xenophagy inducers.

7.2 Future directions

In this study, we explored the regulatory aspects of xenophagic flux using chemical genetics approach. Briefly, we an employed chemical small molecule, acacetin to modulate xenophagy and identify the molecular players that regulate the process. Based on the previously explained observations, the study has opened further avenues to explore and understand the intricate host-pathogen relationship.

Some of the future aspects of the study include:

1. Context-dependent regulators of TFEB during intracellular bacterial infection.

As mentioned earlier, various cellular stress conditions induce TFEB activation. Although multiple modulators are known, context-dependent regulators of TFEB is identified in the recent past. For example, a recent study by Yin *et al* identified the role of cyclin-dependent kinases, CDK4/6 in modulating TFEB activation during the cell cycle (Yin et al., 2020). Similarly, Vojo Deretic's group reported the role of immunity-related GTPase M (IRGM) in regulating TFEB during *Mycobacterium* infection (Kumar et al., 2020).

Our study has identified putative regulators of TFEB during intracellular infection of *S. typhimurium*. The verification of the shortlisted pathways to modulate TFEB during acacetin treatment and infection is underway. The kinases and/or phosphatases in the verified pathways will be studied for their physical interaction with TFEB and the residues that could be post-translationally modified.

2. The repertoire of TFEB target genes during bacterial infection.

Additionally, gene expression analysis has identified genes harbouring CLEAR motif in the promoter of some of the immunity-related genes. We would like to further test if those genes are putative TFEB target genes. It is also possible that the repertoire of TFEB target genes is

context-dependent, and hence the genes identified in the study could be either due to acacetin mediated TFEB activation or specifically induced during infection and acacetin treatment.

3. Broad-spectrum xenophagy induction.

Mechanistically, xenophagy is a process that is active against a broad spectrum of microbes, unlike antibiotics which are specific against a subset of pathogens depending upon the mechanism of action of the drug. Hence, we would like to test the ability of acacetin in inducing xenophagy against other bacterial infections. We presume that the pathogens that replicate in the cytosol, as well as those that modulate the lysosomal activity, could possibly be affected by acacetin.

4. Crosstalk of xenophagy with other immune signalling pathways.

The extensive gene expression analysis studied in the project during compound treatment and infection has identified modulation in immune-related pathways. Apart from studying the regulators of TFEB, we would also explore the role of upstream immune signalling pathways to induce xenophagy. Additionally, xenophagy is active in both epithelial and immune-related cell types. In the current study, most of the observations were made in epithelial cell type. We would further extend the analysis in infection relevant cell types such as human macrophages.

5. Identifying the intracellular physical target of acacetin.

Additionally, the most challenging aspect of the study will be to identify the intracellular physical target of the compound. Techniques such as affinity-based target identification will be employed to study the target. Finally, to increase the potency of the compound, structure-activity relationship studies can be performed to chemically modify the compound to function with higher potency at lower concentrations.

6. Decoupling xenophagy from homeostatic autophagy.

Although autophagy and selective autophagy shares the core machinery proteins, there are exclusive upstream regulatory molecules and cargo recognition proteins reported (Chandra and Kumar, 2016). In our study, the gene expression analysis of acacetin treated HeLa cells with infection identified pathways that are different from acacetin treated on HeLa cells in the absence of infection. We would in future explore pathways that can specifically modulate xenophagy without affecting global autophagy status. Similarly, previous studies from our laboratory identified pharmacological compounds that induced only in certain selective autophagy types but failed in others. On the contrary, one compound XCT-790, identified in our laboratory induced both aggrephagy and xenophagy (Suresh et al., 2018). These studies reinforce that depending on the stage of autophagy modulation, either general autophagy or selective autophagy can be modulated.

7. Host-mediated therapies against bacterial infections.

Antibiotics directly target bacteria and are currently the most effective strategy for infection. However, their efficacy *in vivo* is dependent on two major factors.

I. The ability of antibiotics to cross hydrophobic membranes of host cells. For example, gentamycin and streptomycin do not cross the cell membrane and is effective only on bacteria in tissue fluids (Kadurugamuwa and Beveridge, 1998).
II. Bacteria most commonly replicate in intracellular phagosomal reservoirs entrapped in multivesicular membranes. Antibiotics such as aminoglycosides and macrolides are less active in the low pH of phagosomes (Schlessinger, 1988).

Another major hurdle is antibiotic resistance, where antibiotics overtime becomes ineffective due to the emergence of resistant strains. Hence there is a limitation of "antibiotic-overuse". In agreement with this, increasing evidence highlight the importance of host-mediated strategies during infection and warrants the need for modulators that can effectively tackle these issues. Taken together we believe that modulation of xenophagy could be explored as a new paradigm for clearance of intracellular pathogens as a combinatorial approach with antibiotics.

7.3 References

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Publications, patent

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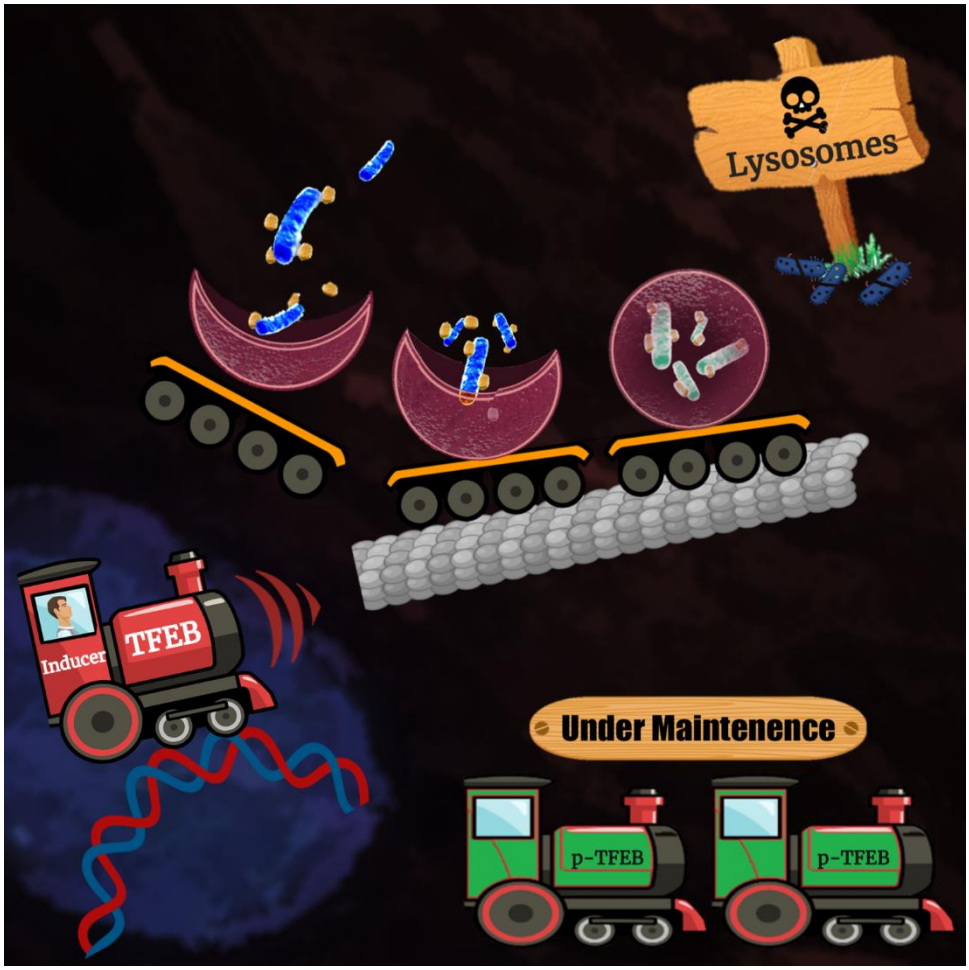

DST Vigyan Samachar press release: JNCASR scientists find a way to boost innate immune system against intracellular infection- Jan30th 2020

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Vigyan Samachar is a news agency maintained by Department of Science and Technology, Govt. of India that aggregates and provides news and related articles in the domain of Science and Technology.

The publication arised from the current study "Restriction of intracellular *Salmonella* replication by restoring TFEB-mediated xenophagy by Ammanathan *et al*, 2019" was highlighted in the press release on 30th January 2020 under the title "JNCASR scientists find a way to boost innate immune system against intracellular infection".

Publications arising from this study:

Research articles:

1. Restriction of intracellular Salmonella replication by restoring TFEB-mediated xenophagy.

   Ammanathan V, Mishra P, Chavalmane AK, Muthusamy S, Jadhav V, Siddamadappa C, Manjithaya R. Autophagy. 2019 Nov 19:1-14. doi: 10.1080/15548627.2019.1689770.

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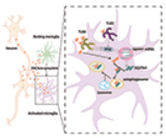
Patents:

1) Indian Patent Application No. 6596/CHE/2015 Title: Modulator and modulation of autophagy and applications thereof. Indian patent application filed.

Autophagy



Autophagy

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/kaup20

Restriction of intracellular *Salmonella* replication by restoring TFEB-mediated xenophagy

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To cite this article: Veena Ammanathan , Piyush Mishra , Aravinda K. Chavalmane , Sasikumar Muthusamy , Vidya Jadhav , Chandrashekaran Siddamadappa & Ravi Manjithaya (2020) Restriction of intracellular *Salmonella* replication by restoring TFEB-mediated xenophagy, Autophagy, 16:9, 1584-1597, DOI: 10.1080/15548627.2019.1689770

To link to this article: https://doi.org/10.1080/15548627.2019.1689770

AUTOPHAGY
2020, VOL. 16, NO. 9, 1584–1597
https://doi.org/10.1080/15548627.2019.1689770

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RESEARCH ARTICLE

Restriction of intracellular *Salmonella* replication by restoring TFEB-mediated xenophagy

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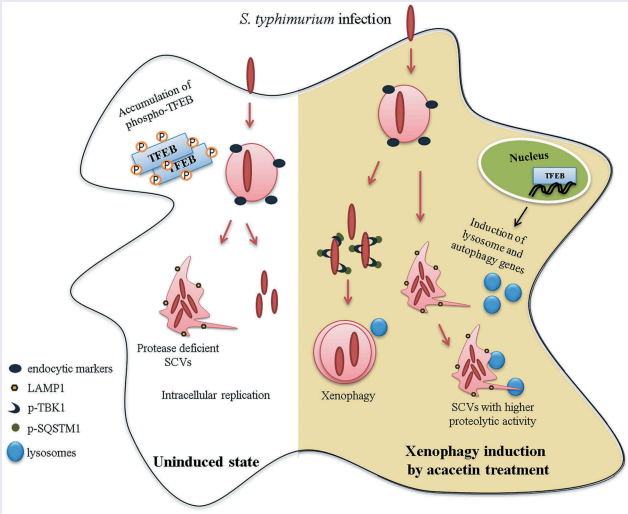
ABSTRACT

Macroautophagy/autophagy functions as a part of the innate immune system in clearing intracellular pathogens. Although this process is well known, the mechanisms that control antibacterial autophagy are not clear. In this study we show that during intracellular *Salmonella typhimurium* infection, the activity of TFEB (transcription factor EB), a master regulator of autophagy and lysosome biogenesis, is suppressed by maintaining it in a phosphorylated state on the lysosomes. Furthermore, we have identified a novel, antibacterial small molecule autophagy (xenophagy) modulator, acacetin. The xenophagy effect exerted by acacetin occurs in an MTOR (mechanistic target of rapamycin kinase)-independent, TFEB-dependent manner. Acacetin treatment results in persistently maintaining active TFEB in the nucleus and also in TFEB mediated induction of functional lysosomes that target *Salmonella*-containing vacuoles (SCVs). The enhanced proteolytic activity due to deployment of lysosomes results in clamping down *Salmonella* replication in SCVs. Acacetin is effective as a xenophagy compound in an *in vivo* mouse model of infection and reduces intracellular *Salmonella* burden.

Abbreviations: 3-MA: 3-methyladenine; BafA1: bafilomycin A_1; CFU: colony-forming units; DQ-BSA: dye quenched-bovine serum albumin; EEA1: early endosome antigen 1; FITC: fluorescein isothiocyanate; FM 4-64: pyridinium,4-(6-[4-{diethylamino}phenyl]-1,3,5-hexatrienyl)-1-(3[triethylammonio] propyl)-dibromide; GFP: green fluorescent protein; LAMP1: lysosomal associated membrane protein 1; MAPILC3/LC3: microtubule associated protein 1 light chain 3; MOI: multiplicity of infection; MTOR: mechanistic target of rapamycin kinase; RFP: red fluorescent protein; SCVs: *Salmonella*-containing vacuoles; SD: standard deviation; SDS: sodium dodecyl sulfate; SEM: standard mean error; SQSTM1: sequestosome 1; TBK1: TANK binding kinase 1; TFEB: transcription factor EB.



ARTICLE HISTORY
Received 1 October 2018
Revised 4 October 2019
Accepted 21 October 2019

KEYWORDS
Acacetin; autolysosomes; lysosomes; MTOR-independent; *Salmonella typhimurium*; *Salmonella*-containing vacuoles; TFEB; xenophagy

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Supplementary data for this article can be accessed here.

Introduction

Intracellular pathogenic bacteria invade mammalian host cells in membrane bound vesicles called phagosomes (or endosomes). Among the various anti-microbial strategies, the host exerts its defense against intracellular bacteria by directing the phagosomes to fuse with the lysosomes for degradation. But the pathogens have evolved several survival mechanisms to prevent this fusion event. Prominent ploys include causing phagosome arrest or escaping to the cytoplasm by damaging phagosomes [1]. Both these outcomes allow unrestricted access to the nutrient-rich cytoplasm and provide an ideal environment for the replication of bacteria. As a countermeasure, the host strategies include a selective macroautophagy (hereafter autophagy) process known as xenophagy to capture such intracellular pathogens by encapsulating them within a double-membrane vesicle known as xenophagosome and subject them for lysosomal clearance [2–4]. Several studies employing diverse bacterial species have highlighted the tactical interplay between the pathogen and host with respect to xenophagy [5,6].

Salmonella typhimurium is a well-studied bacterium species in xenophagy. They enter eukaryotic cells in phagosomes (or endosomes) mediated by virulence gene island, SPI-I that codes for type III secretion system. *Salmonella* can damage the endosomes and enter the nutrient-rich cytosol. Host response in capturing such cytosolic bacteria involves tagging them with ubiquitin [7]. The ubiquitinated bacteria are cargoes that are recognized by a variety of xenophagy receptor proteins such as SQSTM1/p62 (sequestosome 1), CALCOCO2/NDP52 (calcium binding and coiled-coil domain 2/nuclear domain 10 protein 52), OPTN (optineurin), NBR1 (NBR1 autophagy cargo receptor) [8]. These receptor proteins then bind to MAPILC3/LC3 (microtubule associated protein 1 light chain 3), a phagophore and autophagosomal membrane-associated protein [9]. Alternatively, *Salmonella* containing endosomes-like structures mature into SCVs. These SCVs are spacious vacuoles that get acidified slowly as compared to phagosomes containing dead bacteria [10]. Although SCVs resemble lysosomes due to their membrane composition, they are devoid of hydrolytic enzymes [11].

Various studies have shown that immediately after *Salmonella* invasion, host cells undergo amino acid starvation and this energy stress leads to the activation of autophagy. However, this activation is reported to be transient and at later time points of infection, *Salmonella* prevents xenophagy-mediated capture [12,13].

Salmonella invasion *in vivo* occurs via gastrointestinal tract by infecting the intestinal epithelium followed by dissemination to other epithelial tissues such as liver, kidney, spleen and mesenteric lymph nodes. Recent studies have highlighted the role of xenophagy in restricting *Salmonella* infection in various organs of mice. However, akin to *in cellulo* models, elevated levels of xenophagy as seen during early stages of infection, drops to basal levels after 2 to 3 d post infection [14].

Several studies have suggested that restoring the xenophagic potential by intervention through genetic or pharmacological means results in effective subjugation of intracellular pathogen survival [15–18]. Although the capture and degradation mediated processes are well studied, the host mechanisms that recognize the intracellular pathogens to induce xenophagy remain unclear. Moreover, the relevance of xenophagy *in vivo*, in mammalian immunity is also less explored. Recent studies have shown activation of TFEB ortholog in *Caenorhabditis elegans*, HLH30 (helix loop helix 30) during *Staphylococcus aureus* or *Salmonella typhimurium* infections [19,20]. TFEB is considered as a master regulator of autophagy and lysosomal genes [21,22]. The activity of TFEB is determined by its phosphorylation status. Phosphorylated TFEB remains in the cytoplasm on the lysosomal surface whereas the dephosphorylated TFEB translocate to the nucleus where it can activate its target genes which include lysosomal and autophagy genes [23]. Some of the regulators reported to control TFEB activation include kinases like MTOR, MAPK/ERK (mitogen-activated protein kinase), GSK3B (glycogen synthase kinase 3 beta) and AKT1/PKB (AKT serine/threonine kinase 1) and a phosphatase, PPP3/calcineurin [23–27]. Inhibition of these negative regulator kinases or activation of calcineurin results in dephosphorylation of TFEB.

Thus, modulation of autophagy and lysosomal pathways can curb intracellular infection. We reasoned that a previously identified set of small molecule autophagy inducers from our laboratory may also contain potent xenophagy modulators. In this study we identify and characterize a xenophagy inducer, acacetin, which reduces intracellular infection of *S. typhimurium* in both *in vitro* and *in vivo* models. Acacetin induces TFEB dephosphorylation thereby increasing lysosomal and autolysosomal populations in the cell. This effect, during *Salmonella* infection, helps in restoring the acidification of SCVs thus restricting the replication of intracellular *Salmonella*. We also report that TFEB is kept inactivated during later time points of infection and prolonged activation by pharmacological means is beneficial. This compound-mediated restriction of *S. typhimurium* is also seen in a mouse model of infection by translocating TFEB to the nucleus and inducing xenophagy.

Results

Acacetin induces autophagy in yeast and mammalian cell lines

Acacetin was previously identified in our laboratory as an autophagy inducer in yeast based high throughput screening of ENZO library containing 502 pharmacologically active compounds [28]. Briefly, the rates of degradation of luciferase containing peroxisomes in yeast were tested in the presence of the compounds [29]. Those compounds that decreased the time taken for 50% peroxisome degradation compared to the untreated cells were selected as putative autophagy inducers. Acacetin in a dose-dependent manner increased the autophagic flux (**Fig. S1A and B**). Because autophagy is an evolutionarily conserved process, we next examined the effect of this compound on mammalian cells. In HeLa cells, as even 100 μM of acacetin did not appear to be toxic after 15 h of treatment (**Fig. S1C**), we used 50 μM of acacetin in our further experiments. HeLa cells transiently expressing tandem

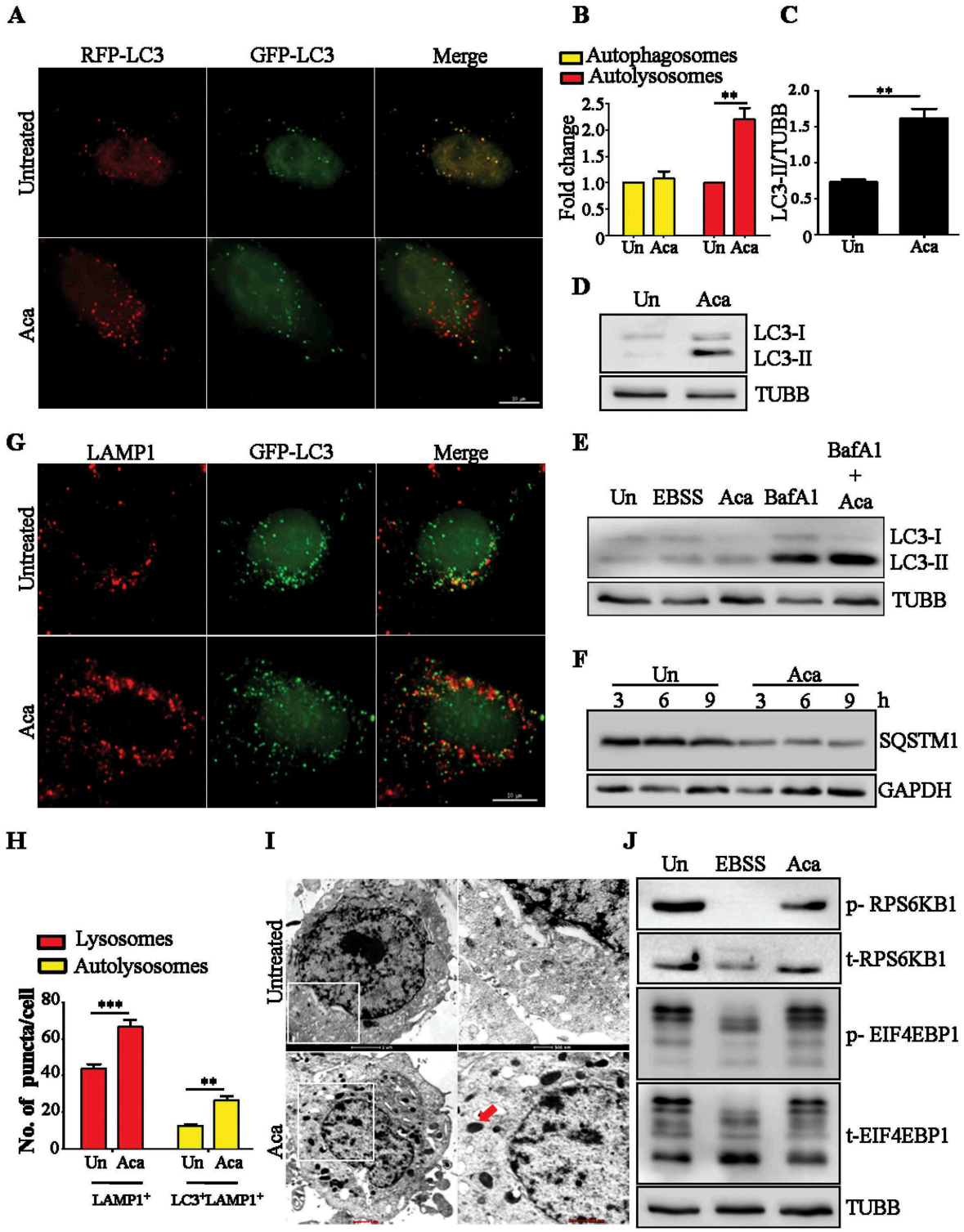

Figure 1. Acacetin induces autophagy and increases lysosomal population. (A) Representative microscopy images for tandem RFP-GFP-LC3 transfected HeLa cells treated with acacetin (50 μM) for 2 h. Yellow puncta correspond to autophagosomes whereas red puncta correspond to autolysosomes. Scale bar: 10 μm. (B) Fold change in autophagosomes and autolysosomes induced by acacetin were quantified (n = 25, three independent experiments N = 3). (C) Fold change in normalized LC3-II levels between growth condition and acacetin treatment were quantified (N = 3). (D) Representative immunoblot for LC3-I to LC3-II conversion in HeLa cells in the presence of the compound for 2 h. (E) Representative immunoblot for LC3-II accumulation in the presence of acacetin only and acacetin with BafA1 (100 nM). (F) Representative immunoblot for SQSTM1 degradation post acacetin treatment. (G) Representative immunofluorescence microscopy images of HeLa cells stained for LAMP1 and LC3 after 2 h of acacetin treatment (n = 25, N = 3). Scale bar: 10 μm. (H) Fold change in lysosomes and autolysosomes induced by acacetin were quantified (n = 25, N = 3). (I) Representative electron micrographs of acacetin treated HeLa cells. Electron dense structures in the zoomed-in panel represent lysosomes (red arrow). (J) Representative immunoblot indicating the phosphorylation status of MTOR substrates, RPS6KB1/p70S6K and EIF4EBP1 caused by acacetin and Earle's Balanced Salt Solution (EBSS) treatments. TUBB/β-tubulin was used as a loading control. Quantification of microscopy images was performed on projected images. Statistical analyses were performed using unpaired two-tailed student's t-test; ns- non-significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars represent mean ± SEM.

RFP-EGFP-LC3, when treated with the compound for 2 h in growth medium, showed a significant increase in autolysosome population suggesting an increase in fusion flux (Figure 1A,B). Immunoblotting also revealed a concomitant increase in LC3-II levels (Figure 1C,D). Although LC3-II accumulation is the most common indicator to check autophagy modulation, it could be either due to autophagy induction or a block in degradation. To verify if the compound is a *bona fide* autophagy inducer, cells were treated with the compound in the presence or absence of fusion inhibitor, bafilomycin A_1 (BafA1). As shown in Figure 1E, immunoblotting with LC3 antibody revealed that the combination of BafA1 and the compound resulted in further increase in LC3-II levels over and above that of BafA1 treatment alone, indicating that the compound indeed induced autophagy. Similarly, autophagy receptor protein SQSTM1 was degraded post compound treatment also indicating autophagy induction (Figure 1F).

In addition to stimulating basal autophagy, acacetin treatment also resulted in an increase in total lysosomal population as seen by LAMP1 (lysosomal associated membrane protein 1)-positive vesicles by fluorescence microscopy (Figure 1G,H). The same observation was also validated by electron microscopy where an increase in electron dense structures indicative of lysosomes was seen (Figure 1I and S1D). The compound treatment did not inhibit MTOR activity as revealed by the phosphorylation status of its downstream targets such as EIF4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) and RPS6KB1/p70S6K (ribosomal protein S6 kinase B1) (Figure 1J). Additionally, p-MTOR and MTOR localization on lysosomes also did not change upon acacetin treatment, suggesting that MTOR was active in presence of acacetin (**Fig. S2A to S2C**). Collectively, these results suggest that acacetin acts in an MTOR-independent manner to induce autophagy.

Acacetin induces xenophagy of Salmonella typhimurium

Apart from inducing general autophagy, we tested whether acacetin also induces selective autophagy pathways such as xenophagy. For this approach, we employed an intracellular *S. typhimurium* infection model. The ability of the compound to clear intracellular bacteria through xenophagy was examined by adding acacetin to HeLa cells. The compound treatment showed a reduction in the replication of intracellular *S. typhimurium* as quantified by colony forming unit assay and also exhibited a dose-dependent effect (Figure 2A, **S3A, and SVideo 1–3**). Similarly, we also validated these results in RAW 264.7 macrophages (Figure 2A).

Furthermore, to understand whether the compound works through autophagy, we examined the bacterial burden from autophagy deficient HeLa cells (*ATG5*$^{-/-}$) treated with acacetin. The compound was ineffective in lowering the intracellular bacterial burden which was similar to that of untreated samples (Figure 2B). Similar results were obtained when autophagy inhibitors such as 3-methyladenine (3-MA) and wortmannin were used (Figure 2C, **S3B and C**). These results suggest that autophagy is essential for the compound-mediated xenophagy action. In addition, the decrease in bacterial burden could be due to a potential antibacterial property of the compound along with autophagy, as seen for certain antibiotics such as Isoniazid and Pyrazinamide [30]. We therefore examined the growth of extracellular *S. typhimurium* in the presence of acacetin. As seen in Figure 2D, there was no apparent lag in the bacterial growth between the untreated and compound-treated samples.

Xenophagy induction involves capture of pathogens by autophagy related receptor proteins (SQSTM1, NDP52, OPTN and NBR1) and xenophagosome membrane proteins (LC3 and its isoforms). To elucidate the xenophagy-dependent action of acacetin, recruitment of SQSTM1 and LC3 to intracellular *S. typhimurium* was studied post compound treatment. As seen in Figure 2E-H acacetin treatment for 6 h resulted in increased recruitment of autophagic proteins to bacteria in a temporal manner. As opposed to untreated infected cells where *S. typhimurium* is known to evade xenophagy capture, acacetin treatment resulted in increased recruitment of SQSTM1 and LC3. In order to understand the mechanism of SQSTM1 recruitment to *S. typhimurium* post acacetin treatment, we specifically studied the S403 phosphorylation in the ubiquitin-associated domain (UBA) of SQSTM1 that has been associated with enhanced binding to ubiquitinated cargoes (**Fig. S4A**). Furthermore, TBK1 (TANK-binding kinase 1) phosphorylates SQSTM1 at S403. Active TBK1 itself is phosphorylated at S172 within its activation loop. Thus, monitoring the levels and recruitment of p-SQSTM1 and p-TBK1 can be used to assess effectiveness of cargo capture [31]. As seen in Figure 2I,J, there was an increased recruitment of p-TBK1 and p-SQSTM1 to *Salmonella* post acacetin treatment until 6 h tested. Immunoblotting analysis revealed an increase in p-TBK1 levels of HeLa cells post acacetin treatment (**Fig. S3D**). Furthermore, to differentiate the cytosolic *S. typhimurium* from those contained in SCVs, we resorted to electron microscopy. Electron micrographs of infected HeLa cells exhibited both vacuolar and cytosolic intracellular *S. typhimurium* populations (Figure 2K,L). Upon compound treatment, host-induced capture of *S. typhimurium* was observed with reduced bacterial numbers and increased electron dense lysosomal structures 6 h post infection (Figure 2M, **S3E**). These observations suggest that acacetin increased the xenophagic potential of cells.

Acacetin treatment results in enhanced capture of S. typhimurium in a TFEB-dependent manner

Given the ability of the compound to induce lysosomal biogenesis along with increased autophagy flux, we suspected a potential role for TFEB, which upon dephosphorylation, translocate to the nucleus to transcriptionally upregulate its target genes. Such increased nuclear translocation of TFEB was seen by immunofluorescence staining of HeLa epithelial cells treated with acacetin for 2 h (Figure 3A,B). In addition, immunoblotting analysis showed a distinct dephosphorylated TFEB band (Figure 3C,D) and increased TFEB in nuclear fraction of acacetin-treated cell lysates (Figure 3E). Similarly, 2 h of acacetin treatment also led to transcriptional induction of TFEB target genes related to autophagy and lysosomal pathways (Figure 3F). Finally, to study the involvement of MTOR in acacetin-mediated TFEB dephosphorylation, MTOR was constitutively activated by over expressing the $RHEB^{N153T}$ (Ras homolog enriched in brain) mutant [32].

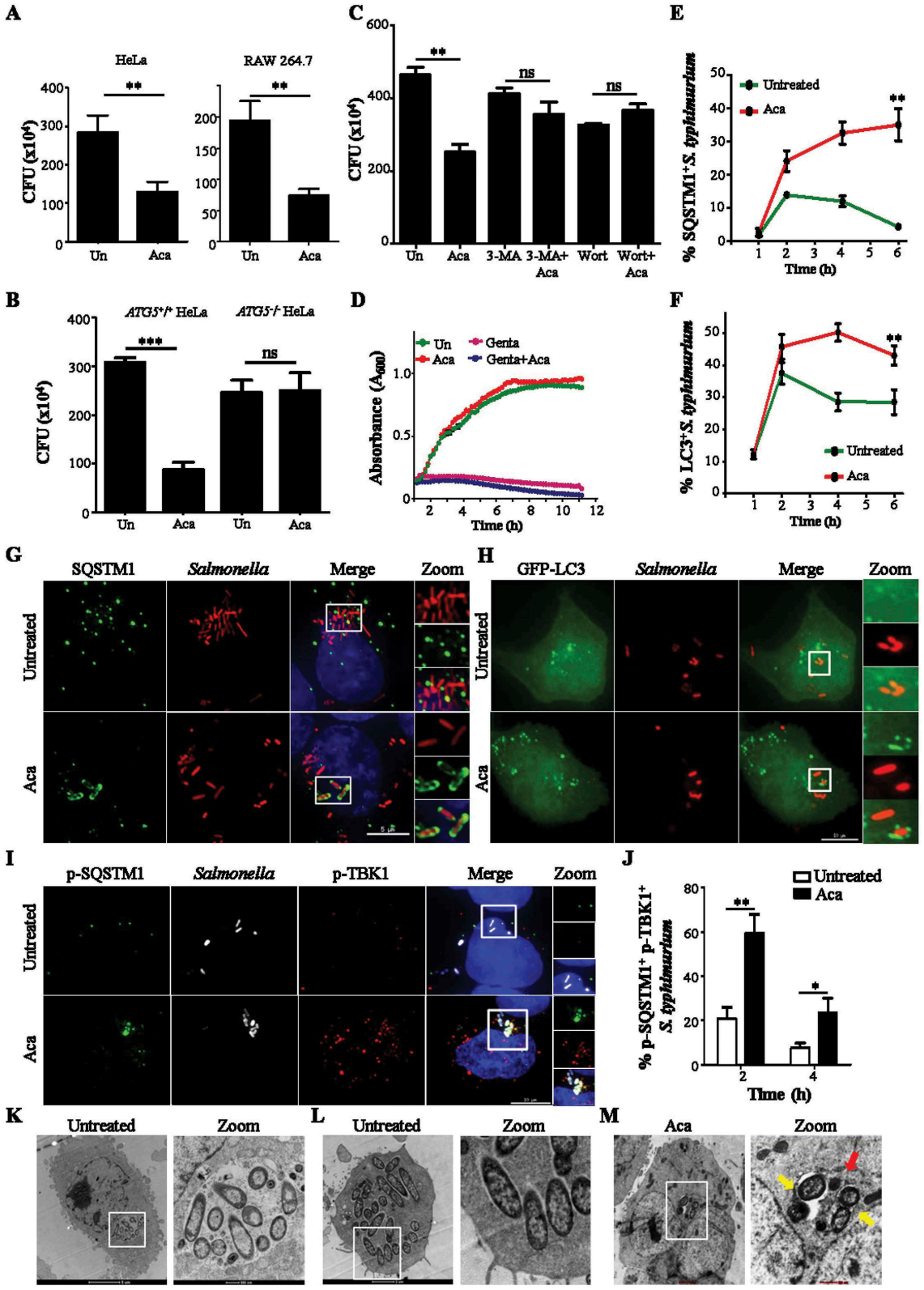

Figure 2. Acacetin induces xenophagy of *S. typhimurium*. (A) Graph showing CFU indicating intracellular *S. typhimurium* in HeLa cells and RAW 264.7 after acacetin treatment (N = 3). (B) Graph showing CFU indicating intracellular *S. typhimurium* in HeLa cells and *ATG5*$^{-}$/$^{-}$ HeLa cells after acacetin treatment (N = 3). (**C**) Graph showing CFU indicating intracellular *S. typhimurium* in HeLa cells after various treatments like acacetin, wortmannin and 3-MA (N=3). (D) Growth curve of *S. typhimurium* in cell free Luria Broth containing acacetin. (E) Graph representing the percentage of time course recruitment of SQSTM1 to *S. typhimurium* induced by acacetin. (F) Graph representing the percentage of time course recruitment of LC3 to *S. typhimurium* induced by acacetin. (G) Representative microscopy images of HeLa cells infected with mCherry expressing *S. typhimurium* and immunostained for SQSTM1 at 6 h post infection (n = 25, N = 3). Scale bar: 5 µm. (H) Representative microscopy images of GFP-LC3 transfected HeLa cells and infected with mCherry expressing *S. typhimurium* at 6 h post infection. Scale bar: 10 µm. (I) Representative microscopy images of HeLa cells infected with mCherry expressing *S. typhimurium* and immunostained for p-TBK1 and p-SQSTM1 at 6 h post infection (n = 25, N = 3). Scale bar: 10 µm. (J) Graph representing the percentage recruitment of p-TBK1 and p-SQSTM1 to *S. typhimurium* induced by acacetin. Quantification of microscopy images was performed on individual Z slices. (K-M) Representative electron micrographs of *S. typhimurium* infected HeLa cells (showing (K) Vacuolar and (L) cytoplasmic *Salmonella* population) with and without acacetin. Red arrows indicate electron dense lysosomes and yellow arrows indicate host mediated capture of *S. typhimurium*. Statistical analyses on three independent experiments were performed using unpaired two-tailed student's t-test; ns- non-significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars represent mean ± SEM. The concentrations of 3-MA, wortmannin and acacetin used were 5 mM, 100 nM and 50 µM respectively.

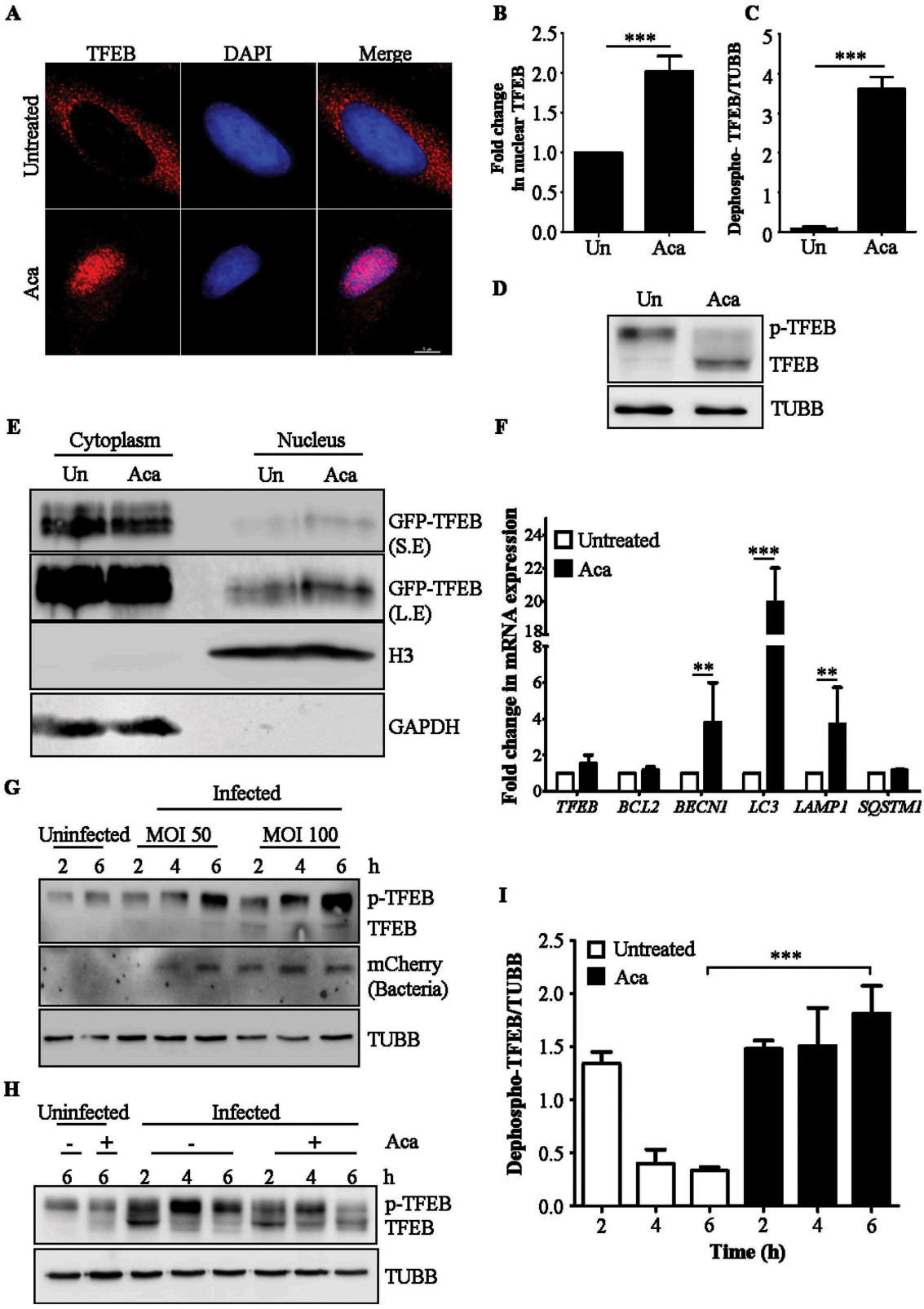

Figure 3. Acacetin enhances nuclear translocation of TFEB. (A) Representative microscopy images of HeLa cells treated with acacetin for 2 h and immunostained for TFEB. Scale bar: 5 µm. (B) Fold change in nuclear TFEB intensity induced by acacetin were quantified (n = 50, N = 3). Quantification of microscopy images were performed on individual Z slices. (C) The graph represents the fold change in dephosphorylated form of TFEB caused by acacetin (N = 3). (D) Representative immunoblot for HeLa cells treated with acacetin and probed for TFEB. Molecular weight shift in TFEB band corresponds to dephosphorylated TFEB. TUBB/β-tubulin was used as a loading control. (E) Representative immunoblot of cytoplasmic-nuclear fractionation indicating TFEB levels in nucleus and cytoplasm. 2X concentration of nuclear fraction was loaded compared to cytoplasmic fraction. S.E and L.E represents short and long exposure respectively. (F) Fold change in mRNA levels of indicated TFEB target genes related to autophagy and lysosomal pathways post 2 h of acacetin treatment (N = 3). (G) Representative immunoblot indicating the phosphorylation status of TFEB post *S. typhimurium* infection across different time points and MOI. The lower molecular weight TFEB band corresponds to dephosphorylated TFEB. (H) Representative immunoblot indicating the phosphorylation status of TFEB post *S. typhimurium* infection and acacetin treatment in a time dependent manner. (I) Graph representing the difference in dephosphorylated TFEB induced by acacetin post *S. typhimurium* infection (N = 3). TUBB/β-tubulin was used as a loading control. Statistical analyses on three independent experiments were performed using unpaired student's two-tailed t-test; ns- non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Error bars represent mean ± SEM.

Upon expression of this mutant, phosphorylation of RPS6KB1 increased (as shown by both immunoblotting and fluorescence microscopy analysis) suggesting that overexpression of this dominant mutant resulted in constitutive activation of MTOR (**Fig. S5C**). In addition, cells transfected with this construct (as represented by enhanced p-RPS6KB1 staining) showed translocation of TFEB to the nucleus upon acacetin treatment (**Fig. S5A and B**). These results suggest that acacetin-mediated TFEB translocation into the nucleus occurs in an MTOR-independent manner.

We further investigated the levels and phosphorylation status of TFEB during *S. typhimurium* infection. We noticed that the overall levels of TFEB increased during infection, especially the higher molecular weight band (Figure 3G). To test if this band was indeed p-TFEB, we treated the lysates with calf intestine phosphatase (CIP). CIP treated samples showed accelerated migrating bands of TFEB (dephosphorylated) suggesting that infection results in maintaining TFEB in a phosphorylated state (**Fig. S5D**). Contrastingly, acacetin treatment during infection resulted in increase in dephosphorylation status of TFEB (Figure 3H,I) thus reversing the *Salmonella*-mediated block in TFEB dephosphorylation.

In order to access the dependency of acacetin on TFEB, we silenced TFEB in HeLa cells. Acacetin-mediated autophagy induction was significantly decreased upon TFEB silencing as seen by reduced LC3-II puncta in microscopy and absence of LC3-II accumulation by immunoblotting analysis (**Fig. S6A to S6C**). Additionally, to selectively evaluate if dephosphorylation of TFEB is also important for xenophagy, we tested the bacterial capture upon TFEB silencing. In cells silenced for TFEB, the acacetin-mediated SQSTM1 recruitment was significantly reduced (Figure 4A,B) confirming the role of TFEB in acacetin action. Results thus far suggested that acacetin exerts its effect by mediating dephosphorylation of TFEB and subsequent autophagy and xenophagy induction.

Acacetin treatment increases the proteolytic activity of Salmonella-containing vacuoles

To verify if the acacetin induced increase in the number of lysosomes also corresponds to a concomitant increase in the lysosomal activity, cells were treated with dye quenched-bovine serum albumin (DQ-BSA). It is a derivative of bovine serum albumin that is heavily conjugated to BODIPY dye that acts as a protease substrate and gets cleaved by lysosomal cysteine and aspartyl proteases such as CTSB and CTSD. As shown in Figure 5A,C, acacetin treatment resulted in increased fluorescence of DQ-BSA due to lysosomal-mediated processing resulting in brighter mono-conjugates of DQ-BSA. In addition, to study the effect of acacetin on fluid phase endocytosis, we temporally followed reporter cargoes such as fluorescein isothiocyanate (FITC) labeled Dextran beads and pyridinium,4-(6-[4-{diethylamino}phenyl]-1,3,5-hexatrienyl)-1-(3[triethylammonio] propyl)-,dibromide (FM 4–64) dye. Furthermore, FITC-Dextran was colocalized with early endosomal marker EEA1 (early endosome antigen 1) and the acidified late endosomes or lysosomes stain, LysoTracker Deep Red. As seen in **Fig. S7**, there was neither any significant difference between the rate of uptake (**Fig. S7A to S7D**) nor in the temporal colocalization between the endocytic and lysosomal markers (**Fig. S8A and B**). These results suggest that acacetin treatment, for the time assessed, did not perturb the uptake and trafficking of endocytic cargoes. Taken together, acacetin specifically increases the number of functional lysosomes in addition to enhancing autophagy flux.

SCVs are modified *Salmonella* containing endosomes-like structures that resemble lysosomes by membrane composition but do not contain proteases similar to functional lysosomes [11]. These protease-deficient vesicles provide a niche for *S. typhimurium* to replicate within cells. In addition to increasing the number of active lysosomes in cells, acacetin treatment also increases the proteolytic activity of SCVs preventing bacterial replication, as seen by DQ-BSA processing in the SCVs (Figure 5B,D). DQ-BSA processing to monoconjugates is a sign of increased proteolytic activity in the contained vesicles. These results suggest that apart from enhanced acacetin-mediated *S. typhimurium* capture by xenophagy proteins, there is an increase in functional lysosomal numbers which also target SCVs to enhance their proteolytic activity and thus reduce *S. typhimurium* replication.

Acacetin induces xenophagy in mouse model of infection

Salmonella invasion via gastrointestinal route establishes infection initially in the intestine followed by dissemination to other organs like liver, kidney, spleen and lymph nodes. It is reported that the xenophagy potential of the organs during *Salmonella* infection is short lived, returning to basal levels at around 72 h post infection [14]. In order to test the efficacy of acacetin in inducing xenophagy-mediated bacterial clearance, an *in vivo* mouse model of infection was established. Based on our preliminary studies, we found that administering 10^{10} bacteria through oral gavage, disseminates bacteria across key organs involved during *Salmonella* infection like intestine, liver, spleen and kidney at 7 d post infection. Additionally, the concentration of acacetin to be tested was decided based on previously available reports that did not show any change in body weight or mortality. Three groups of mice were tested namely uninfected, infected and infected along with acacetin treatment. In this infection model, acacetin (20 mg/kg body weight) was administered intraperitoneally starting from 1 d prior to *Salmonella* infection in order to maintain an induced autophagy status in the organs. Acacetin treatment continued until 7 d post infection. At the end of 7 d post infection, mice from all groups were sacrificed and the number of intracellular *Salmonella* was determined by counting the number of colony-forming units (CFU) and autophagy induction was visualized in liver using immunohistochemistry (Figure 6A). Results obtained are comparable to the study done by Benjamin *et al* highlighting the role of *in vivo* xenophagy [14]. As seen in Figure 6B, there is a reduction in the number of intracellular bacteria in various organs of infected mice like liver, spleen, and intestine in the acacetin treated group. In addition, immunohistochemical analysis revealed induction of autophagy as seen by increased LC3-II puncta (Figure 6C,D) in the infected group that received acacetin.

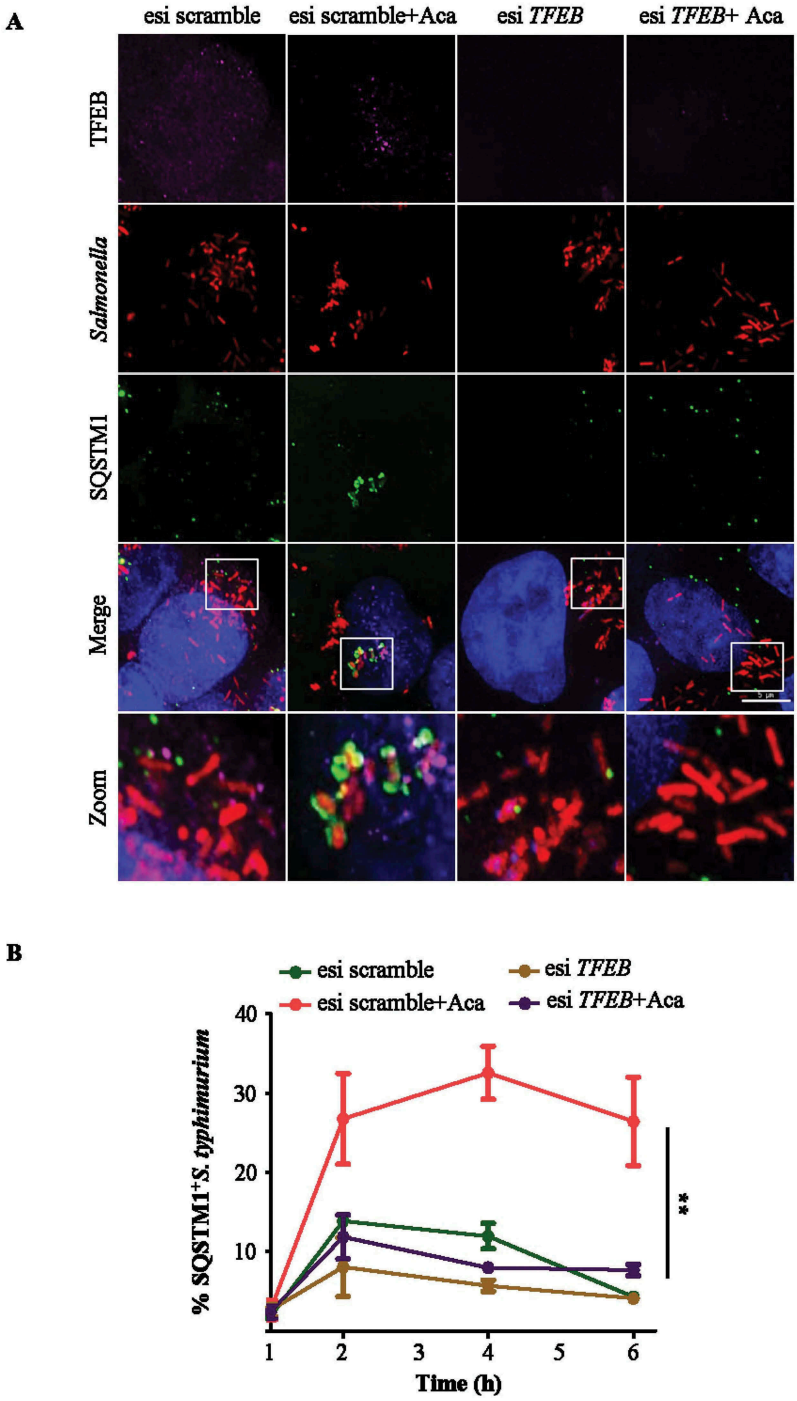

Figure 4. Acacetin treatment results in enhanced capture of *S. typhimurium* in a TFEB-dependent manner. (A) Representative microscopy images of control and *TFEB* silenced HeLa cells post *S. typhimurium* infection for 6 h and immunostained for SQSTM1 and TFEB. Scale bar: 5 μm. (B) Graph represents the time course recruitment of SQSTM1 to *S. typhimurium* induced by acacetin treatment (n = 25, N = 3). Quantification of microscopy images were performed on individual Z slices. Statistical analysis was performed using unpaired student's two-tailed t-test; ns- non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Error bars represent mean ± SEM.

Discussion

In the constant battle between the host and pathogens for one upmanship, both sides employ several strategies and counter strategies. One powerful mechanism the host cells employ is xenophagy that functions in concert with other antimicrobial strategies of the host cell and is implicated in a number of intracellular bacterial infections [33]. However, in order to successfully establish infection, bacteria deploy diverse tactics to down regulate xenophagy. Host capabilities for counteracting this subversion resulting in enhanced clearance of intracellular bacteria and hence mitigate infection pathology has been reported by several studies [34].

Chemical biology approaches to understand xenophagy has two-fold benefits. Characterization of xenophagy "hits" not only provides insights into this process, their specificity can also reveal therapeutic potential. For example, chemical modulators

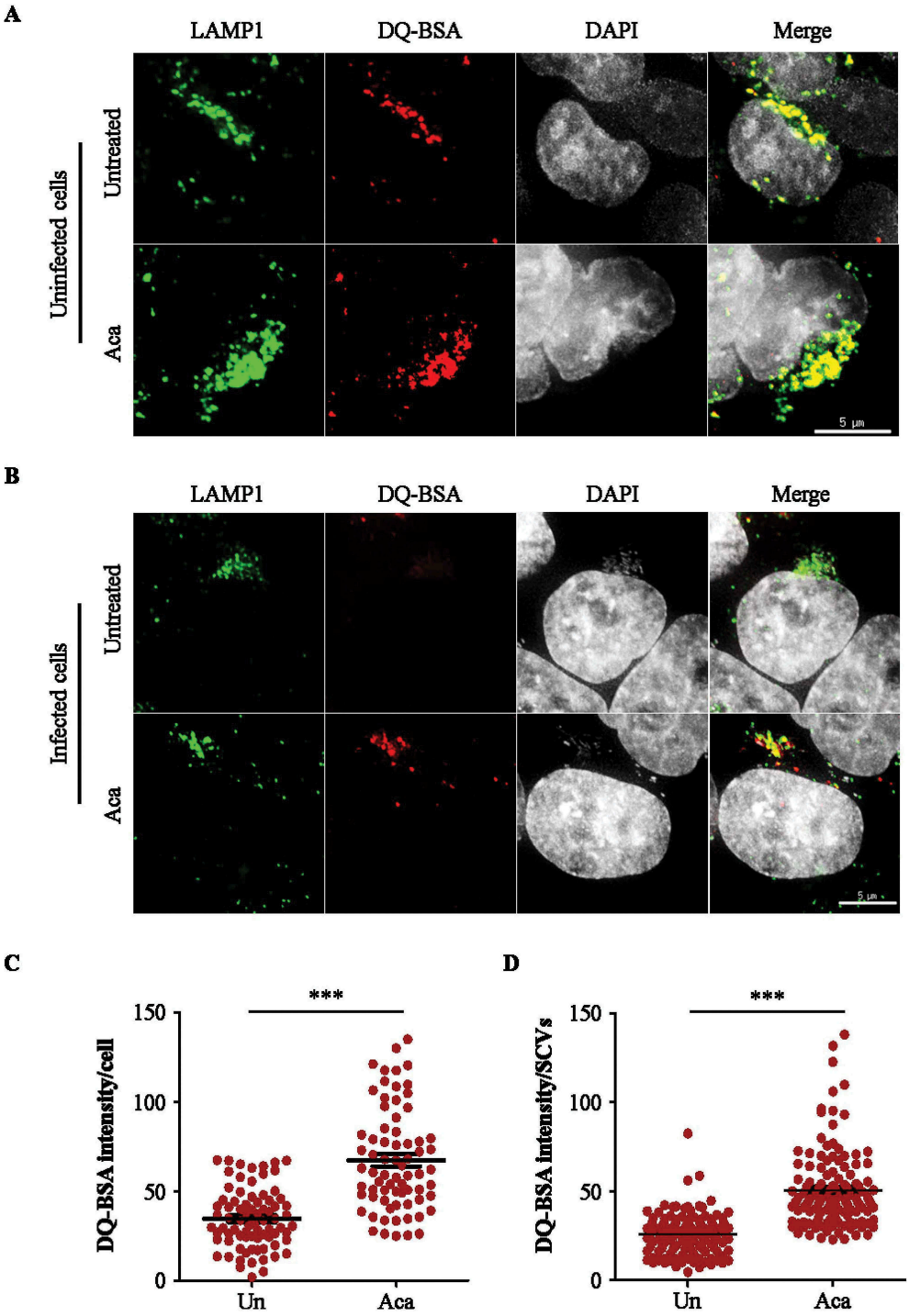

Figure 5. Acacetin treatment increases the proteolytic activity of *Salmonella*-containing vacuoles. (A) Representative microscopy images of HeLa cells treated with DQ-BSA for 2 h followed by 4 h incubation of DQ-BSA along with acacetin treatment. Cells were immunostained for LAMP1 (n = 25, N = 3). Scale bar: 5 µm. (B) Representative microscopy images of mCherry *S. typhimurium* infected HeLa cells treated with DQ-BSA for 2 h followed by 4 h incubation of DQ-BSA along with acacetin treatment. Cells were immunostained for LAMP1. Scale bar: 5 µm. (C and D) The differences in DQ-BSA intensity per cell or SCVs induced by acacetin treatment were quantified (n = 25, N = 3). Quantification of microscopy images were performed on projected images. Statistical analyses on three independent experiments were performed using unpaired student's two-tailed t-test; ns- non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Error bars represent mean ± SEM.

of xenophagy like trifluoperazine, BRD5631 act through distinct but diverse mechanisms suggesting therapeutically novel strategies to induce xenophagy [18]. In this study, we identify and characterize a novel xenophagy modulator acacetin that clears intracellular *S. typhimurium* infection in an MTOR-independent but TFEB-dependent manner. Although prolonged exposure of high concentration of acacetin has been shown to induce apoptosis in cancer cells recently [35], we show that at the lower concentrations used in our study, acacetin robustly induces autophagy and is effective as a xenophagy inducer in curbing *S. typhimurium* infection both in cell lines and in an *in vivo* model.

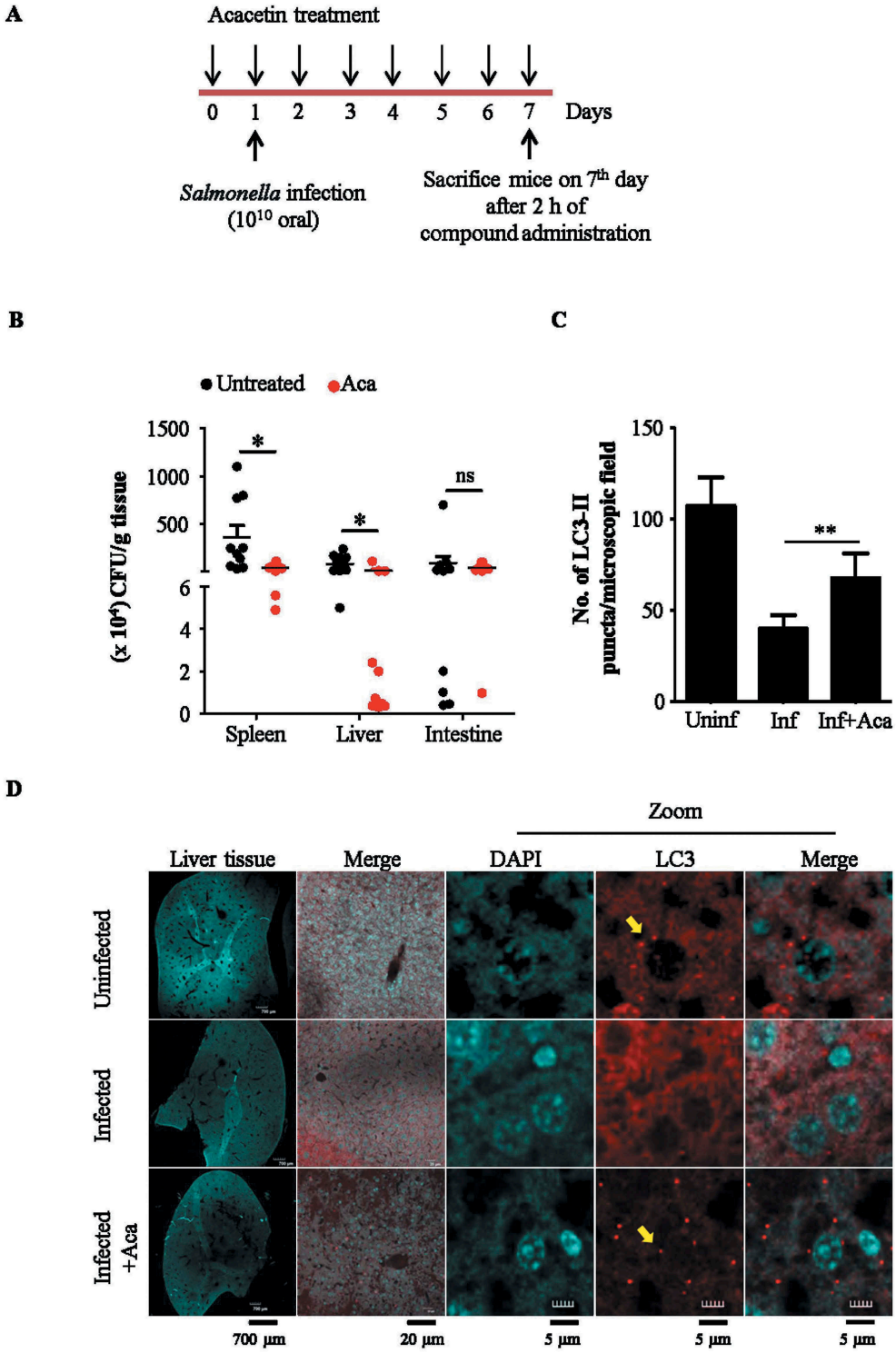

Figure 6. Acacetin induces xenophagy in mouse model of infection. (A) Scheme for infection assay. (B) Graph representing the reduction in intracellular *S. typhimurium* burden in various organs of acacetin treated mice (N = 10). (C) Graph representing the difference in number of LC3-II puncta per microscopy field (1X1 binning and 1024 × 1024 pixel) between different groups of mice (N=3). (D) Representative immunohistochemistry images of liver cryosections stained for autophagosome membrane marker, LC3 (Olympus FV3000 1.25X objective was used for imaging entire DAPI stained liver section, 20X objective was used to choose a region of interest stained for LC3 in red and DAPI. 40X objective was used for observing LC3 puncta, indicated by yellow arrows). Quantification of microscopy images were performed on projected images. Statistical analyses of three independent experiments was performed using unpaired student's two-tailed t-test; ns-non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Error bars represent mean ± SEM.

Acacetin treatment resulted in an overall increase in the number of autolysosomes. Further, increased formation of autolysosomes suggests that acacetin treatment results in enhanced fusion of autophagosomes and lysosomes thus leading to an increased number of autolysosomes. In spite of this increase in autolysosome number, autophagosomes number did not decline upon acacetin treatment, further suggesting that the overall autophagy flux is upregulated in presence of acacetin. When

tested for its xenophagy potential, acacetin was able to inhibit intracellular *S. typhimurium* multiplication. Detailed mechanistic analysis revealed the following: Firstly, the compound treatment induced enhanced recruitment of autophagy receptor proteins such as SQSTM1 to *Salmonella*. This increased recruitment of SQSTM1 after acacetin treatment is possibly facilitated by phosphorylation of SQSTM1 (S403) in its UBA domain thereby enhancing its binding affinity to ubiquitinated cargoes rather than transcriptional induction of SQSTM1. Additionally, phosphorylation of TBK1 and its recruitment to *S. typhimurium* both were increased. These observations are in agreement with the published literature where p-TBK1 has been shown to be actively phosphorylating SQSTM1 at S403 in its UBA domain. Such a mechanism has been shown for capturing damaged mitochondria by the selective autophagy process known as mitophagy [31]. Secondly, acacetin functions by activating the transcription factor TFEB, a master regulator of lysosome biogenesis and autophagy pathways [36]. Phosphorylated TFEB is localized on the lysosomes, but upon dephosphorylation, it migrates to the nucleus and activates the expression of several genes involved in lysosomal biogenesis and autophagy related pathways [37]. However, TFEB-mediated mechanisms are complex with multiple phosphorylation sites and co-ordinated regulation of this phosphorylation-dephosphorylation balance by a number of kinases (MTOR, ERK, GSK3B, Akt) and the phosphatase, calcineurin governs the metabolic state of the cell [38]. Apart from increasing autophagy flux in presence of acacetin, TFEB also promoted autolysosome formation. Thirdly, acacetin treatment promoted TFEB translocation into the nucleus. Previous studies have shown that activation and translocation of MiT-TFE family of transcription factors (TFEB, TFE3) that regulate lysosome biogenesis and autophagy, increase the innate immune response of LPS-activated macrophages [39]. In fact, phagocytosis of bacteria and IgG opsonized beads activate TFEB. A study by Gray *et al* showed that depletion of TFEB lead to a loss in pathogen restriction by downregulating lysosomal degradation during infection [40]. In addition, the TFEB ortholog in *C. elegans*, HLH30 has been reported to be activated during *S. typhimurium* and *S. aureus* infection and provide cytoprotection [20,41]. Our results show that upon *Salmonella* infection, although TFEB levels increase, it is maintained in a transcriptionally inactive, phosphorylated form. However, acacetin treatment resulted in increased levels of dephosphorylated form of TFEB with a concomitant induction of autolysosome numbers and enhanced xenophagy flux.

A recent study on xenophagy in mice during *Salmonella* infection highlighted the role of ATG5, an essential autophagy protein needed for autophagosome formation [14]. Intestinal knockdown of *Atg5* increased the bacterial load in mice, suggesting the importance of xenophagy-mediated host immune response. Similarly, a previous study by Conway *et al* reported the requirement of another autophagy protein, ATG16L1 for xenophagy during *Salmonella* infection [42]. Although the above studies emphasized the role of xenophagy process, the molecular mechanisms that induce xenophagy *in vivo* are not well known. Here, we show that consistent acacetin treatment *in vivo* led to increase in LC3B-II levels, with a concomitant restriction of bacterial infection. Together, our data suggests a mechanism to overcome the reported host xenophagy inhibition mediated by intracellular pathogens such as *S. typhimurium* [14].

Furthermore, some of the well-known potent TFEB inducers like torin1, work by inhibiting MTOR, a critical kinase that regulates cellular growth and survival. It is therefore essential to identify TFEB inducers that function in an MTOR-independent manner such as acacetin. Similar studies exploring the mechanisms of MTOR-independent TFEB activation are reported [24,26] and in particular, a small molecule compound trehalose, activates TFEB by inhibiting Akt and enhances clearance of protein aggregates [27].

Thus, we show that identifying potent xenophagy inducers can strengthen the host response against pathogens. As a potential translational application, such molecules can be envisaged as a host mediated therapy which may be especially effective when dealing with multidrug resistant pathogens.

Materials and methods

Chemicals and antibodies

3-MA (M9281), wortmannin (W1628), acacetin (00017), anti-LC3B antibody (L7543), Atto 663 (41176), trypsin EDTA (59418C), EBSS (E7510) and FITC-Dextran (46945) were purchased from Sigma-Aldrich. Anti-TFEB antibody (4240), anti-RPS6KB1/p70S6K antibody (9208), anti-p-RPS6KB1/p70S6K (9202) antibody, anti-p-EIF4EBP1 antibody (2855), anti-EIF4EBP1 antibody (9452), anti-LAMP1 antibody (9091), anti-TBK1/NAK antibody (3504), anti-p-TBK1/NAK Ser172 antibody (5483), anti-EEA1 antibody (3288), anti-ACTB/β-actin antibody (4970), anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP; 7074) were purchased from Cell Signaling Technology. Anti-SQSTM1/p62 (ab56416) was purchased from Abcam. Anti-p-SQSTM1/p62 Ser403 (D343-3) was purchased from MBL. Anti-TUBB/β-tubulin antibody (DSHB-C1-377) was purchased from Developmental Studies Hybridoma Bank. Anti H3 was a kind gift from Prof. Tapas Kundu, JNCASR. DQ-BSA Red BSA (D12051), FM 4–64 (F34653), LysoTracker Deep Red (L12492) were purchased from Thermo Fisher Scientific. Bafilomycin A_1 (11038) was purchased from Cayman chemical. CIP (M0290S) was purchased from New England Biolabs.

Plasmid constructs, esiRNA and bacterial strains

Plasmids used in mammalian cell culture include ptfLC3 (21074, deposited by Tamotsu Yoshimori), pEGFP-N1-TFEB (38119, deposited by Shawn Ferguson), LAMP1-RFP (1817, deposited by Walther Mothes), $RHEB^{N153T}$ (19997, deposited by Fuyuhiko Tamanoi) were purchased from Addgene. Bacterial strains used for infectious studies include untagged and mCherry expressing *S. typhimurium* (kind gift from Dr. C.V Srikanth, RCB, India). For silencing experiments, *TFEB* esiRNA (EHU059261-20UG) and scramble esiRNA (SIC001-10NMOL) were purchased from Sigma-Aldrich.

Cell culture

RAW 264.7 and HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, 5648) supplemented

with 3.7 g/L sodium bicarbonate (Sigma-Aldrich, S5761), 10% fetal bovine serum (FBS; Gibco, 10270–106) and 100 units/ml of penicillin and streptomycin (Gibco, 15140–122) and maintained in 5% CO_2 at 37°C. *ATG5*$^{-/-}$ HeLa cells was a kind gift from Prof. Richard Youle, NIH.

Immunofluorescence microscopy

Cells were seeded on coverslips and allowed to attach overnight. Cells were transfected with plasmid constructs (ptf-LC3/GFP-LC3) using lipofectamine 2000 (Invitrogen, 11668019) as per the manufacturer's instructions. After 48 h post transfection, compound treatment was carried out for indicated time points and cells were fixed using 4% paraformaldehyde (Sigma-Aldrich, P6148). For antibody staining, permeabilization with 0.25% Triton X-100 (HiMedia Laboratories, MB031) was followed by primary antibody incubation overnight at 4°C and appropriate secondary antibody incubation for 1 h at room temperature. The cover slips were mounted using Vectashield antifade reagent with or without DAPI (Vector laboratories, H-1000/H-1200).

DQ-BSA processing assay

HeLa cells were seeded on cover slips and allowed to attach overnight. DQ-BSA was added to HeLa cells or HeLa cells post *S. typhimurium* infection for 2 h. The DQ-BSA processing was allowed for 4 h and fixed using 4% paraformaldehyde.

Immunoblotting

Cells were seeded in 6-well plates and allowed to attach overnight. After 2 h of compound treatment, cells were collected in Laemmli buffer (10% SDS (HiMedia Laboratories, GRM886), 10 mM DTT (ThermoFisher Scientific, R0862), 20% glycerol (Merck, 1.07051.0521), 0.2M Tris-HCl (Merck, 1.93315.0521), 0.05% bromophenol blue (ThermoFisher Scientific, 115-39-9), pH 6.8) and boiled for 10 min at 95°C. Samples were electrophoresed on SDS-PAGE and transferred onto PVDF membrane (BioRad, 1,620,177). After incubation with primary antibody overnight at 4°C and HRP conjugated secondary antibody for 1 h at room temperature, signals were obtained using enhanced chemiluminescence substrate (Clarity Bio-Rad, 170–5061) and image was acquired using gel documentation system (G-box, Chemi XT 4, Syngene, USA). The bands were quantified using ImageJ software (NIH).

Infection assay

Single colony of *S. typhimurium* (kind gift from Dr. C.V. Srikanth, RCB, India) was grown for 6 h at 37°C in a shaking incubator. Secondary culture (0.2% inoculum) was grown overnight in micro-aerophilic conditions. HeLa (WT and *ATG5*$^{-/-}$) or RAW264.7 cell lines were infected at a multiplicity of infection (MOI) of 200 for 1 h. The cells were treated with media containing 40 µg/ml gentamycin (Abbott, EAI03089) for 1 h to kill the extracellular bacteria. The cells were then treated with the compound and incubated for 4 h. Finally, the mammalian cells were lysed using lysis buffer (0.1% SDS, 1% Triton X-100, 1X PBS (Sigma-Aldrich, D6773)) and the intracellular *S. typhimurium* was plated and the CFU was counted.

Fluorescence microscopy and analysis

Images were acquired on the widefield Delta Vision microscope (API, GE, USA, 29,065,728) using DAPI, FITC, TRITC, Cy5 filters with Olympus 60X/1.42 NA objective. Post-acquisition, the images were deconvolved using Delta Vision SoftWorx software. For Fig. S3A and Figure 6D, confocal microscopy (Zeiss, LSM880) was used without deconvolution. For analyzes that involved colocalization (Figure 2E,F,J, 3B, 4B, S2C, S3B, S5B, S8B), individual Z-stacks were analyzed using Colocalization plugin with "colocalization highlighter" option in ImageJ (NIH) and the number of colocalized events were counted using cell counter plugin of ImageJ . Whereas, for images that required counting total puncta inside cells or measuring total intensities of cells (Figure 1B,H, 5C,D, 6C, S3B, S6B, S7C, S7D), projected image (collapsed Z-stacks) were used for quantification. Graphs were plotted and significance levels were tested using unpaired two-tailed student's t-test in GraphPad Prism software. However, all representative images are projected images for better clarity.

Live cell microscopy

GFP-LC3 transfected HeLa cells were infected with mCherry-*S. typhimurium* for 15 min (MOI 400) and were treated with gentamycin for 15 min. The cells were then washed with 1X PBS and were treated with the compound and imaged on FV10i- Olympus confocal live cell imaging microscope using 60X water immersion lens with confocality aperture set to 1.0. Images were taken at an interval of 15 or 20 min (Five Z sections of 1 micron each). The intensity of the Red channel was analyzed using Image J – Stacks T function- intensity vs. time plot plugin.

RNA extraction and quantitative PCR

Total RNA from samples was isolated using TRIzol (Ambion, 15596–026). Reverse transcription was carried out using Taqman reverse transcription kit (Applied Biosystems, N8080234). Autophagy and lysosomal specific gene primers which were previously reported [21,43,44] were purchased from Sigma-Aldrich. The housekeeping gene *ACTB* was used as normalizing control to calculate the fold change.

Primers used

Gene	Forward	Reverse
ACTB	CATCATGAAGTGTGACGTGGAC	CTTGATCTTCATTGTGCTGGGTG
LC3B	ACCGTGTGATCAGTAAGATTCC	GTGACCACTCACATGGGATATAG
BECN1	CCCGTGGAATGGAATGAGATTA	CCGTAAGGAACAAGTCGGTATC
BCL2	AGATGGAGCATGAATGGTACTG	TCTGTGCTCAGCTTGGTATG
TFEB	CCAGAAGCGAGAGCTCACAGAT	TGTGATTGTCTTTCTTCTGCCG
LAMP1	ACGTTACAGCGTCCAGCTCAT	TCTTTGGAGCTCGCATTGG
SQSTM1	GCACCCCAATGTGATCTGC	CGCTACACAAGTCGTAGTCTGG

Electron microscopy

Sample processing for Transmission Electron Microscopy was carried out in Christian Medical College, Vellore, India. Briefly, HeLa cells were trypsinized and washed with 1X PBS. Cells were fixed using 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 h at 4°C. Fixed cells were dehydrated in ethanol series and embedded in epoxy resin (TAAB laboratory and microscopy, CY212 KIT, E009). Ultra-thin sections were stained and observed using Tecnai, G2 F-30 with a point resolution of 2.2 Å.

Cell viability assay

HeLa cells were seeded on a 384-well plate and treated with different concentrations of acacetin. After 15 h of compound treatment, cell viability was measured using luminescence-based Cell Titer Glo cell viability assay kit (Promega, G7572) using a micro-titer plate reader (Varioskan Flash, Thermo Fisher Scientific, USA).

Immunohistochemistry

Liver cryosections of 40 µm thickness were collected on gelatine coated slides. Every eighth section was used for immunostaining. Equilibration of the sections was done using 0.1 M PBS (pH 7.4) for 10 min. This was followed by blocking using 3% bovine serum albumin (Sigma-Aldrich, A7906) for 4 h. Then, the sections were incubated for 36 h in primary antibody. After washes the sections were incubated in fluorescent secondary antibody for 4 h. The sections were mounted using Vectashield mounting media after required washing. Images were acquired using Olympus FV 3000 (1.25X objective was used for imaging entire liver section, 40X objective was used for observing LC3-II puncta).

Animal studies

All procedures carried out in the study were approved by JNCASR Institutional Animal Ethics Committee. BALB/c mice (6–8 weeks of age) were distributed into three groups namely- uninfected, infected and infected along with acacetin treatment. Acacetin (20 mg/kg) was administered intraperitoneally to the infected with acacetin treatment group. The compound treatment continued for next 7 d with one injection a day. Other two groups were injected with vehicle solvent, dimethyl sulfoxide (Sigma-Aldrich, D8418). Infection (10^{10}) was done through oral gavage for the second and third group on the first day. All animals were sacrificed on the seventh day post infection after 2 h of compound injection and the organs (liver, spleen and intestine) were processed for plating and immunohistochemistry.

Statistical analysis

For all observations made in this study, a minimum of three independent experiments were performed. For fluorescence microscopy based analysis, at least 25 cells per experiment (so across three experiments, a minimum of 75 cells) were considered for quantification. Immunoblotting and microscopy image quantifications were performed using ImageJ software with appropriate plugins (NIH) mentioned elsewhere. The significance levels between the control and test groups were tested using unpaired two-tailed student's t-test where ns-non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Error bars represent mean ± SEM. All statistical tests and graphs were plotted using GraphPad prism.

Video 1: HeLa cells infected with *S. typhimurium* SL1344 at MOI of 400 for 15 min followed by gentamycin treatment (40 µg/ml) for 15 min. Fresh medium was added to the cells and imaged every 20 min for 5 h 40 min. Scale bar: 10 µm.

Videos 2&3: HeLa cells infected with *S. typhimurium* SL1344 at MOI of 400 for 15 min followed by gentamycin treatment (40 µg/ml) for 15 min. Fresh medium containing 50 µM of acacetin was added to the cells and imaged every 15 min for 5 h 45 min. Scale bar: 10 µm.

Acknowledgments

We thank Dr. C V Srikanth, RCB, for his support, help in *Salmonella* infection assays and for providing several reagents, Prof. Deepak Saini and his student Vignesh, MRDG, IISc for help with live cell microscopy, Prof. Jonathan Sexton, North Carolina for the kind gift of Enzo library of compounds, Prof. Udaykumar Ranga and Prof. Tapas Kundu, MBGU, JNCASR, for reagents, Prof. Subba Rao MCBL, IISc for reagents, Prof. Richard Youle, NINDS, for WT and *ATG5*$^{-/-}$ HeLa cells. We thank Mr. Gaurav Barve, Ms. Aparna Hebbar and Dr. Shanmuga Sundari for critical reading of the manuscript. We thank Mr. Ganesh Kadasoor from Olympus for helping in live cell microscopy video processing. This work was supported by Wellcome Trust/DBT India Alliance Intermediate Fellowship (509159-Z-09-Z), LSRB-DRDO grant (LSRB-310/BTB/2017) and JNCASR intramural funds to RM. Use of acacetin in autophagy related therapeutic applications such as xenophagy is patent pending.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Wellcome Trust/DBT India Alliance Intermediate Fellowship [500159-Z-09-Z]; JNCASR intramural funds [JNCASR/MBGU/RM]; Life Sciences Research Board-DRDO [LSRB-310/BTB/2017].

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RESEARCH ARTICLE

SPECIAL ISSUE: CELL BIOLOGY OF HOST–PATHOGEN INTERACTIONS

Foot-and-mouth disease virus induces PERK-mediated autophagy to suppress the antiviral interferon response

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ABSTRACT

Foot-and-mouth disease virus (FMDV) is a picornavirus that causes contagious acute infection in cloven-hoofed animals. FMDV replication-associated viral protein expression induces endoplasmic reticulum (ER) stress and the unfolded protein response (UPR), in turn inducing autophagy to restore cellular homeostasis. We observed that inhibition of BiP (also known as HSPA5 and GRP78), a master regulator of ER stress and UPR, decreased FMDV infection confirming their involvement. Further, we show that the FMDV infection induces UPR mainly through the PKR-like ER kinase (PERK; also known as EIF2AK3)-mediated pathway. Knockdown of PERK and chemical inhibition of PERK activation resulted in decreased expression of FMDV proteins along with the reduction of autophagy marker protein LC3B-II [the lipidated form of LC3B (also known as MAP1LC3B)]. There are conflicting reports on the role of autophagy in FMDV multiplication. Our study systematically demonstrates that during FMDV infection, PERK-mediated UPR stimulated an increased level of endogenous LC3B-II and turnover of SQSTM1, thus confirming the activation of functional autophagy. Modulation of the UPR and autophagy by pharmacological and genetic approaches resulted in reduced numbers of viral progeny, by enhancing the antiviral interferon response. Taken together, this study underscores the prospect of exploring PERK-mediated autophagy as an antiviral target.

KEY WORDS: Autophagy, Foot-and-mouth disease virus, Interferon, LC3, p-eIF2α, PERK, Unfolded protein response

INTRODUCTION

Foot-and-mouth disease virus (FMDV), is a positive-sense non-enveloped RNA virus of the *Picornaviridae* family. It is a major cause of contagious acute viral infection in cloven-hoofed animals, though asymptomatic persistent infection is also reported (Knowles and Samuel, 2003). FMDV proliferates rapidly and causes vesicular lesions on oro-nasal mucosa and the interdigital cleft. Worldwide, there exist seven serotypes – O, A, C, Asia1, South African Territories (SAT) 1, SAT2 and SAT3. In addition, numerous variants and subtypes have also been reported (Bachrach, 1968). The infection causes high morbidity in adult animals and high mortality in young animals, leading to reduced animal productivity and economic loss (Grubman and Baxt, 2004). Also, FMD is considered as the most important constraint for international trade of animals and animal products (Leforban, 1999).

FMDV derives membranes from the endoplasmic reticulum (ER) and pre-Golgi membranes of the early secretory pathway for its replication (Midgley et al., 2013; Moffat et al., 2005). FMDV 2B protein localizes mainly in the ER exerting viroporin-like activity and increases Ca^{2+} levels in the cytosol. Also, the 2BC protein inhibits host protein secretion (Moffat et al., 2005), therefore it is likely that FMDV infection induces ER stress. The induction of ER stress results in the activation of the unfolded protein response (UPR). During this process, BiP (also known as HSPA5 and GRP78), a major ER chaperone is released from ER transmembrane signal transducers, including PKR-like ER kinase (PERK; also known as EIF2AK3), inositol-requiring enzyme 1 (IRE1; also known as ERN1), and activating transcription factor 6 (ATF6), leading to their activation (Lee, 2005). The UPR promotes cell survival either by attenuating translation mediated by phosphorylation of eIF2α to reduce the load of unfolded proteins (Malhotra and Kaufman, 2007; Ron and Walter, 2007) or by enhancing the ER protein folding capacity via ER chaperone expression through the PERK–ATF6–IRE1α pathway (Harding et al., 2000b; Hetz, 2012). The UPR also targets misfolded proteins for degradation (Harding et al., 2002). UPR activation consequently induces an adaptive cellular homeostasis response – autophagy (Jheng et al., 2014; Senft and Ronai, 2015). Autophagy is responsible for sequestering damaged organelles and cytoplasmic protein aggregates within double-membrane vesicles for degradation and recycling (Klionsky and Emr, 2000). While some viruses suppress the autophagy pathway, a few others appear to utilize this pathway to benefit their replication by avoiding the host immune response. The study of the intrinsic link between ER stress and autophagy is gaining research importance in RNA virus infections for the purpose of developing antiviral strategies (Jheng et al., 2014).

Although the involvement of autophagy in FMDV infection has been reported earlier, the mechanism of its induction and implication in FMDV multiplication is not clear. There exist ambiguous reports on the role of autophagy during FMDV infection (Medina et al., 2018; Rodriguez Pulido and Saiz, 2017). Earlier studies showed that FMDV induces and utilizes autophagosomes at an early stage of the replication (Berryman et al., 2012; O'Donnell et al., 2011). It has also been reported that the 2B protein of FMDV disturbs cellular Ca^{2+} homeostasis and induces autophagy (Ao et al., 2015), in contrast to an earlier report showing that the interaction of

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Handling Editor: Derek Walsh
Received 16 October 2019; Accepted 19 May 2020

Journal of Cell Science (2021) 134, jcs240622. doi:10.1242/jcs.240622

FMDV 2C protein with Beclin1 prevented the fusion of lysosomes to autophagosomes, allowing virus survival (Gladue et al., 2012). Another study showed that FMDV induces autophagy only at the early stage of infection and viral 3C protease degrades the Atg5–Atg12 complex, suppressing autophagy at a later time point of infection (Fan et al., 2017). While our study was underway, a recent work reported that FMDV induces complete autophagy, with the fusion of autophagosomes and lysosomes for subsequent degradation (Sun et al., 2018). Although a very recent study showed the activation of PERK and ATF6 mediated UPR during FMDV infection, however, they reported that these pathways did not influence FMDV replication (Han et al., 2019). Even more recently, Yang et al. (2020) showed that FMDV 3A protein upregulates the autophagy-related protein leucine-rich repeat containing protein 25 (LRRC25), which negatively regulates RLR-mediated type I interferon (IFN) signaling by interacting with Ras-GAP SH3-binding protein 1 (G3BP1). However, the role of ER stress-mediated UPR and autophagy, and their function in modulating innate immunity during FMDV infection mostly remains unclear. Therefore in this study, we aimed to systematically investigate FMDV-induced ER stress, UPR and autophagy, and their role in innate immune responses.

Our results demonstrate that FMDV infection induces ER stress and the UPR response through the PERK-mediated pathway. The induction of the autophagy marker lipidated LC3B (LC3B-II; LC3B is also known as MAP1LC3B) is influenced by PERK activation during the virus infection. This work also provides insights into the fact that the FMDV replication in cells is dependent on the expression of PERK and the autophagy marker LC3B-II. Furthermore, the inhibition of PERK pathway and autophagy significantly restricted FMDV replication by enhancing the levels of antiviral IFN-β and IFN-λ3 levels. Overall, these results help in the understanding of FMDV pathogenesis and host cellular antiviral mechanisms for the future development of antiviral strategies.

RESULTS

FMDV infection induces ER stress and activates the PERK-mediated pathway of UPR

To study the impact of viral infection on cellular ER, we observed for ER morphology at 4 h post infection (hpi) [1 multiplicity of infection (MOI)] by transmission electron microscopy (TEM) in LFBK cells. FMDV-induced ER stress caused a dilation of the ER lumen (Fig. 1A, right panel). SYBR green based quantitative real-time RT-PCR (RT-qPCR) in relation to that of the housekeeping β-actin gene was carried out to monitor the levels of ER stress and UPR marker genes. The genes encoding BiP, PERK, ATF4 and IRE1 were upregulated in the range of 1.8–2.5-fold at 6 hpi (1 MOI). However, there was no significant upregulation of the genes encoding ATF6 and CHOP (C/EBP homologous protein; also known as DDIT3) (Fig. 1B). ATF6 transcript level remained unchanged, suggesting that the FMDV-induced UPR signaling may not utilize the ATF6-mediated pathway. Although IRE1 was significantly (P<0.05) upregulated at 6 hpi, there was no apparent splicing of its downstream XBP1 mRNA up to 12 hpi with FMDV, while the positive control, treatment with tunicamycin, led to splicing of XBP1 mRNA with the appearance of both spliced (269 bp) and unspliced (295 bp) forms (Fig. 1C), suggesting that the FMDV-induced UPR response is also independent of the IRE1–XBP1 pathway. Western blot analysis of the cells infected with FMDV (1 MOI) showed distinct enhancement in the BiP protein level and the PERK protein expression from 4 hpi (Fig. 1D), correlating with the data of real-time quantification of increased mRNA levels of the genes encoding BiP and PERK. To further investigate whether activation of the PERK pathway leads to phosphorylation of the downstream eIF2α transcription factor, immunofluorescence was carried out using an anti-p-eIF2α antibody which showed an increase in the fluorescence of phosphorylated eIF2α (p-eIF2α) at 6 hpi (1 MOI) (Fig. 1E). Furthermore, the time course of phosphorylation of the PERK substrate eIF2α was assessed by immunofluorescence following FMDV infection. A time-dependent virus cytopathic effect associated with the p-eIF2α signal was evident (Fig. S1). In line with this, the mRNA level of ATF4 was increased at 6 hpi (Fig. 1B). Taken together, these results indicate that FMDV infection triggers ER stress and induces the UPR through the PERK-mediated pathway.

PERK-mediated UPR induces autophagy to promote FMDV replication

To understand the relationship between ER stress-induced UPR and autophagy, VER-155008 (20 µM) an inhibitor of BiP (Macias et al., 2011), which is a target of both the ER stress response and UPR, was used to treat LFBK cells 1 h prior to infection. Our results indicated a significant reduction in the viral titer at 12 hpi (1 MOI) upon VER-155008 treatment (Fig. 2A). Similarly, the PERK pathway was inhibited by treatment with ISRIB (Rabouw et al., 2019), which reverses the effects of eIF2α phosphorylation. ISRIB (200 nM) treated and infected cells showed a decrease in autophagy marker protein LC3B-II, an absence of SQSTM1 degradation and, correspondingly, there was a reduction in viral protein levels and the viral titer (Fig. 2B–D). The concentrations of the VER-155008 and ISRIB drugs used in this study did not affect cell viability (Fig. S2).

To preclude any non-specific effect caused by chemical inhibition, knockdown of the *PERK* gene was carried out. Two PERK-specific artificial microRNAs (amiRNAs) with different targets in 5′-UTR (miR-PERK-T1 and miR-PERK-T2) along with negative control (miR-Neg) were expressed in LFBK cells. The reduction in expression of PERK protein was more significant in cells expressing miR-PERK-T2 (data not shown); hence, further work was done using miR-PERK-T2. Upon FMDV infection (1 MOI) of PERK-specific amiRNA-expressing cells, expectedly PERK expression was reduced, resulting in decreased LC3B-II conversion and reduced SQSTM1 degradation. Also, the virus replication was affected, as seen by decreased viral protein expression (Fig. 3A). The knockdown of PERK also resulted in the reduction of extracellular progeny virus titer (P<0.05) at 6 and 12 hpi (Fig. 3B). Furthermore, the levels of IFN-β and IFN-λ3 proteins in the infected culture supernatant of cells expressing miR-PERK-T2, as determined by ELISA, showed a significant increase at 8 and 12 hpi (Fig. 3C,D). Therefore, these results suggest that PERK knockdown reduces FMDV replication by enhancing the levels of anti-viral IFNs. The data indicate that the ER stress is induced during FMDV infection, which in turn activates the PERK pathway of UPR-mediated autophagy.

FMDV infection induces double-membrane vesicles characteristic of autophagosomes

To confirm the induction of autophagy during FMDV infection, we investigated the cellular changes associated with the virus infection, using TEM. Double-membrane vesicles resembling autophagic vesicles were observed in FMDV-infected (1 MOI) LFBK cells at 6 hpi (Fig. 4B). However, similar vesicles were absent in the uninfected LFBK cells (Fig. 4A). A magnified view of the part highlighted in FMDV infected cell shows the presence of double-

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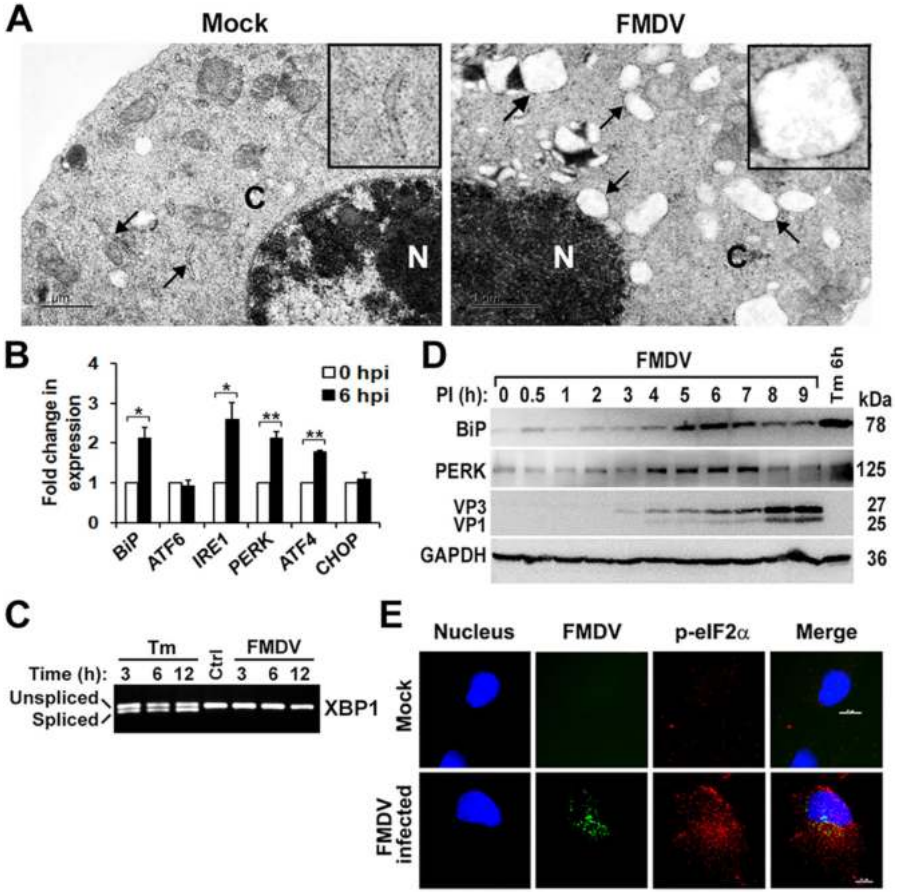

Fig. 1. FMDV causes ER stress and activates the PERK pathway-mediated UPR in LFBK cells. (A) TEM image showing the normal rough ER (left panel) and dilated rough ER in the FMDV-infected (1 MOI, 4 hpi) cells (right panel) indicated by arrows. The insets show magnified view of the normal rER (left panel) and dilated rER (right panel). N, nucleus; C, cytoplasm. Scale bars: 1 µm. (B) RT-qPCR-based analysis of transcripts involved in ER stress and UPR, at 6 hpi (1 MOI). Bar graph showing relative expression levels (fold change±s.d.) of *BiP*, *ATF6*, *IRE1*, *PERK*, *ATF4* and *CHOP*, calculated by the $2^{-\Delta\Delta Ct}$ method using β-actin as an endogenous control. The level of *BiP*, *IRE1*, *PERK* and *ATF4* were significantly increased at 6 hpi compared to uninfected state (**P*<0.05; ***P*<0.01). (C) Agarose gel (2%) electrophoresis of *XBP1* fragments (unspliced, 295 bp; spliced, 269 bp), obtained by RT-PCR of RNA isolated from LFBK cells treated with tunicamycin (Tm, 2.5 µg/ml) or infected with FMDV (1 MOI), for 3, 6 and 12 h. Splicing of XBP1 was detected with Tm treatment but not with FMDV infection. Ctrl, control cells. (D) Western blotting for BiP and PERK protein levels and viral protein expression in FMDV-infected (1 MOI) cells (from 0.5 to 9 hpi), with tunicamycin-treated (2.5 µg/ml) cells serving as a positive control for ER stress. GAPDH level served as loading control, while blotting for viral structural proteins (VP1 and VP3) served as infection control. The levels of BiP and PERK were enhanced from 4 hpi. (E) Widefield immunofluorescence microscopy of LFBK cells mock or FMDV infected (1 MOI) for 6 h, following fixing and immunostaining for phospho(Ser51)-eIF2α (using anti-p-eIF2α antibody) in red and FMDV (using anti-3AB antibody) in green. Nuclei (blue) are stained with DAPI. The p-eIF2α expression is prominently observed in cells infected with FMDV. Scale bars: 10 µm.

membrane vesicles, characteristic of autophagosomes (Fig. 4C). A quantification showed that the number of autophagosome vesicles per cell was significantly increased in FMDV-infected cells (Fig. 4D).

FMDV infection upregulates the gene transcripts for proteins involved in the initiation of autophagy

To further investigate the effect of FMDV infection on autophagy, the expression of candidate genes involved in autophagy activation was studied. There was clear evidence of upregulation of *ATG9A* and *LC3B* by 2.6-fold, and *ULK1* by 3.3-fold in 6 h infected (1 MOI) samples ($P<0.05$), but there was no significant change in *SQSTM1* level (Fig. 5A). These data suggest that there is an upregulation of autophagy initiation genes during FMDV infection.

FMDV infection induces functional autophagy

The Atg12–Atg5–Atg16L complex assists in the conjugation of LC3B-I with phosphatidylethanolamine (PE), which is essential for the formation of the autophagosomal membrane. The conversion of cytosolic LC3B-I into PE-conjugated LC3B-II is indicative of increased autophagic activity (Kabeya et al., 2000; Klionsky et al., 2008). Western blotting results showed an increase in the levels of Atg5–Atg12 conjugate together with an increase in LC3B-II in cells infected with FMDV (1 MOI) from 4 hpi onwards and that further maintained for more than 9 hpi (data not shown). Rapamycin-treated cells were used as autophagy-positive control (Fig. 5B,C). The adaptor protein SQSTM1, mediating selective autophagy, gets degraded by the autophagolysosome pathway. It accumulates when autophagy is inhibited and its level decreases when functional autophagy is induced, thus serving as a marker for autophagic flux (Klionsky et al., 2008). Western blot analysis showed a gradual decline in the levels of SQSTM1 protein from 4 hpi (Fig. 5B,D), suggesting the activation of functional autophagy with the fusion of autophagosomes with lysosomes for its degradation.

The induction of autophagy brings about the association of LC3B-II to the autophagosomal membrane, revealing a

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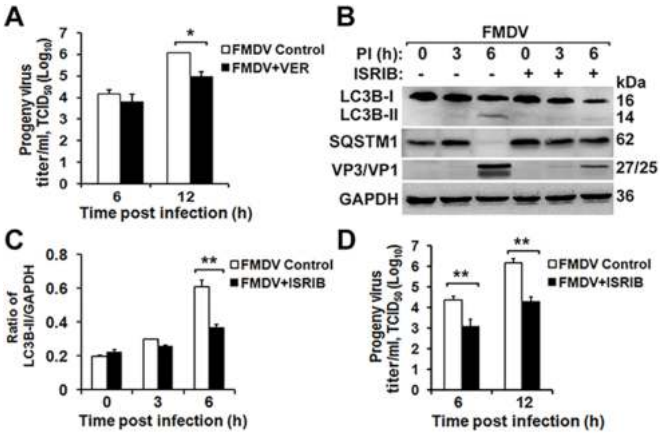

Fig. 2. Pharmacological inhibition of ER stress and PERK–eIF2α signaling reduces FMDV titer and LC3B-II levels. (A) Bar graph showing the progeny virus titer (log_{10}) at 6 and 12 h in the supernatant of cells infected with FMDV (1 MOI) with or without treatment of BiP inhibitor VER-155008 (20 µM). (B) Western blotting for autophagy marker LC3B-II and SQSTM1 protein expression following FMDV infection (1 MOI) for 3 and 6 h, without or with treatment of PERK inhibitor, ISRIB (200 nM). ISRIB treatment significantly reduced LC3B-II level and viral protein expression. (C) Bar graph showing quantitative determination of band intensity ratio of LC3B-II to GAPDH in the FMDV-infected LFBK cells without or with ISRIB treatment. (D) Bar graph showing the progeny virus titer (log_{10}) at 6 and 12 h in the supernatant of cells infected with FMDV (1 MOI) with or without treatment of ISRIB. Results in A, C and D are mean±s.d. of three independent experiments. *P<0.05, **P<0.01.

characteristic punctate pattern (Klionsky et al., 2008). The widefield immunofluorescence microscopy revealed a significant increase (P<0.01) in characteristic endogenous LC3B puncta in FMDV-infected LFBK cells (1 MOI at 6 hpi) (Fig. 5E,F). These puncta were similar to those seen in cells treated with the autophagy inducer rapamycin (5 µM) (Fig. S3). This confirmed the activation of autophagy.

Suppression of autophagy reduces the FMDV titer by enhancing the antiviral IFN levels

The inhibition of autophagic flux by bafilomycin A1, a specific inhibitor of the V-ATPase (Yamamoto et al., 1998) prevented the degradation of LC3B-II (Fig. 6A, right panel) compared to no BafA1 treatment (Fig. 6A, left panel). Consequently increased accumulation of SQSTM1 was observed in BafA1-treated cells (Fig. 6A, right panel, B). This resulted in reduced virus replication, as evidenced by considerably low viral protein expression in BafA1-treated cells (Fig. 6A, right panel, C), confirming the positive correlation between LC3B, SQSTM1 turnover and FMDV replication.

In addition, the extracellular viral titer was significantly reduced in cells treated with autophagic flux inhibitor BafA1 at 6 and 12 hpi (P<0.01). Furthermore, treatment with spautin-1, which acts by blocking the initiation of autophagy (Liu et al., 2011), also caused a significant reduction (P<0.01) in the viral titer at 12 hpi. However, the virus titer increased significantly in FMDV-infected cells treated with the autophagy inducer rapamycin compared to control FMDV-infected cells at 12 h (P<0.05) (Fig. 6D). To understand the antiviral mechanism of autophagy inhibition, RT-qPCR for IFN-β and IFN-λ3 transcript levels were analyzed in the BafA1- and spautin-1-treated and FMDV-infected (1 MOI) LFBK cells. The IFN-β and IFN-λ3 transcripts were upregulated in the range of 4.5–6-fold at 8 hpi in the presence of autophagy inhibitors (Fig. 6E).

Knockdown of LC3B reduces FMDV replication by enhancing antiviral IFN levels

Two of LC3B-specific amiRNAs (miR-LC3B-ORF and miR-LC3B-5′UTR) along with negative control (miR-Neg) were expressed in LFBK cells. Western blotting showed that there was a reduction in the expression of LC3B in cells expressing miR-LC3B-ORF and miR-LC3B-5′UTR, but it was more significant in the latter (data not shown). Therefore, miR-LC3B-5′UTR was used for our study. Upon FMDV infection (1 MOI) of LC3B-specific amiRNA-expressing cells, expectedly LC3B expression was decreased and SQSTM1 turnover was impeded, and the virus replication was affected as seen by decreased viral protein expression (Fig. 7A). The knockdown of LC3B also resulted in reduction of extracellular progeny-virus titer (P<0.05) at 6 and 12 hpi (Fig. 7B).

In addition, cells expressing miR-LC3B-5′UTR leading to knockdown of LC3B had enhanced levels of IFN-β and IFN-λ3 transcript levels of 8- and ∼11-fold respectively at 8 hpi (1 MOI) as determined by RT-qPCR (Fig. 7C). Furthermore, the levels of IFN-β and IFN-λ3 proteins in the infected culture supernatant of cells expressing miR-LC3B-5′UTR, as determined by ELISA, showed a significant increase at 8 and 12 hpi (Fig. 7D,E). These data reveal that suppression of autophagy affects the replication ability of FMDV by enhancing IFN levels.

DISCUSSION

During infection by cytoplasmic viruses, host organelles like ER, are utilized for virus replication. It is known that FMDV derives replication site membranes from ER and pre-Golgi membranes (Midgley et al., 2013; Moffat et al., 2005). In the process, induced ER stress initiates the UPR to restore cellular homeostasis (Pincus et al., 2010). Here, we have investigated the role of ER stress, the UPR and autophagy in FMDV replication. We demonstrated by TEM that the FMDV infection induces volume expansion and dilation of the ER due to stress. During ER stress, pronounced dilation of the ER lumen occurs in pancreatic β cells and also in yeast cells under UPR-inducing conditions (Bernales et al., 2006; Despa, 2009). This suggests that FMDV infection-associated stress causes a dilation of ER. This, in turn, activates UPR signaling for cell survival. Analysis of mRNA transcripts of ER stress-associated UPR genes during FMDV infection showed that the levels of BiP, PERK, ATF4 and IRE1α were significantly upregulated at 6 hpi. However, the levels of CHOP and ATF6 remained unchanged (P>0.05). Furthermore, the protein levels of BiP and PERK were enhanced from 4 h post-FMDV infection and the phosphorylation of eIF2α was also observed from 6 hpi, indicating the involvement of ER stress-associated UPR.

Among the three arms of the UPR, the level of ATF6 mRNA did not alter. Therefore, we then studied the downstream effect of IRE1α and PERK activation. Activation of IRE1 is generally determined by measuring the splicing of mRNA encoding the XBP1 (Chen et al., 2014; Hetz et al., 2011). We observed that during FMDV infection, the splicing event was not apparent, suggesting that FMDV did not activate the IRE1–XBP1 pathway. However, an enhanced level of PERK protein and Ser51 phosphorylation of eIF2α were evident. The transcript level of ATF4 was increased significantly (P<0.01) at 6 hpi, the time point at which enhanced phosphorylation of eIF2α was observed. This is in line with the fact that eIF2α phosphorylation at Ser51 results in the repression of global protein synthesis and preferential translation of selected genes, such as *ATF4* to reduce ER stress (Rajesh et al., 2015). It was reported previously that *ATF4* mRNA transcript levels

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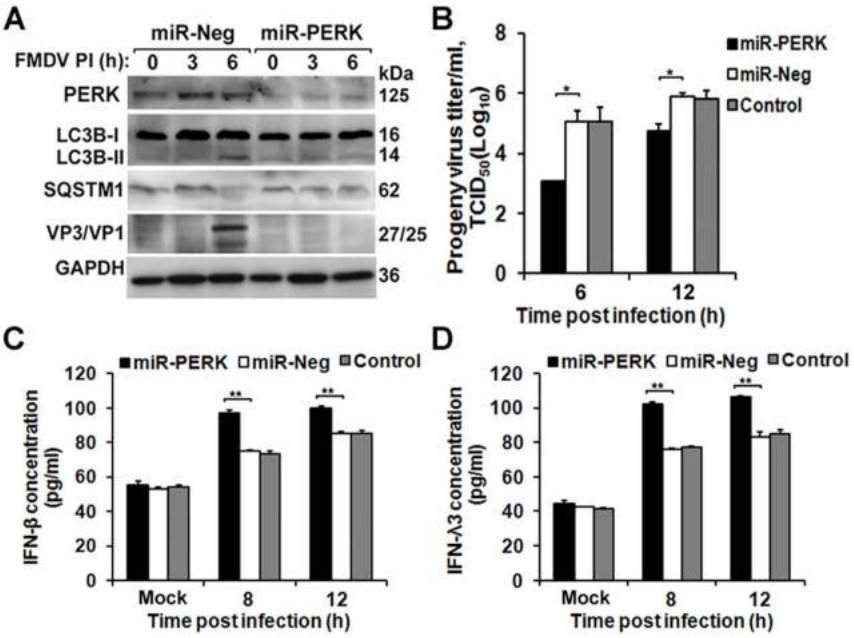

Fig. 3. Knockdown of PERK results in reduction of LC3B-II levels, decreased FMDV replication and increased antiviral IFN response. (A) Western blot showing levels of PERK, LC3B-II and SQSTM1 proteins upon FMDV infection (1 MOI) in LFBK cells at 3 and 6 hpi with knockdown of *PERK*. The miR-Neg was used as negative control. The blot shows significant reduction in the level of PERK and LC3B-II, absence of SQSTM1 degradation and reduced viral protein expression in cells expressing miR-PERK. A significant increase in the level of PERK, LC3B-II and SQSTM1 degradation and viral protein expression was seen at 6 hpi in miR-Neg expressing cells. (B) Bar graph showing the extracellular virus yield (log_{10}) at 6 and 12 hpi (1 MOI) of LFBK cells expressing the indicated amiRNA, as determined by $TCID_{50}$ method in BHK-21 cells and expressed as titer/ml. The data show that knockdown of *PERK* reduces the viral titer. (C) Bar graph showing the extracellular IFN-β protein yield following FMDV infection (1 MOI) in LFBK cells following knockdown of PERK signaling. The data show that knockdown of *PERK* enhances IFN-β level during FMDV infection. (D) Bar graph showing the extracellular IFN-λ3 protein yield following FMDV infection (1 MOI) in LFBK cells following knockdown of *PERK*. The data show that knockdown of *PERK* enhances IFN-λ3 level during FMDV infection. All data in B–D represent the mean±s.d. of three independent experiments. **P*<0.05, ***P*<0.01.

are elevated in response to ER stress (Harding et al., 2000a; Dey et al., 2010), as well as in response to amino acid starvation (Siu et al., 2002). This could possibly be because the expression of ATF4 is subject to both transcriptional regulation and translational control. On the other hand, the mRNA level of CHOP, which plays a role in the induction of apoptosis (Nishitoh, 2012; Oyadomari and Mori, 2004), was not significantly altered during FMDV replication. Therefore, upregulated ATF4 likely influences the induction of autophagy (Matsumoto et al., 2013; Rzymski et al., 2010). Furthermore, FMDV-infected cells treated with VER-155008, a novel small-molecule inhibitor of BiP, a master regulator of UPR (Macias et al., 2011), caused a reduction in virus titer (P<0.05), suggesting that mitigation of UPR negatively influenced FMDV replication. Our results are partly in agreement with the very recent study that showed activation of PERK–eIF2α and ATF6 branches of UPR signaling while inhibiting the IRE1α pathway during FMDV infection (Han et al., 2019). However, they reported that either overexpression or knockdown of PERK or ATF6 did not affect FMDV multiplication (Han et al., 2019). By contrast, we found that treatment with ISRIB, a potent inhibitor of the PERK pathway (Rabouw et al., 2019), and knockdown of PERK, achieved by transient expression of specific amiRNA, significantly reduced the viral protein expression. Thus, we find that the PERK pathway is important for FMDV multiplication. The UPR and autophagy occur simultaneously following ER stress (Schröder, 2008). Furthermore, ER stress-mediated PERK and eIF2α phosphorylation is known to induce LC3 conversion (Kouroku et al., 2007). Therefore, we investigated the role of the PERK–p-eIF2α–ATF4 pathway on autophagy. We found that the PERK pathway inhibitor ISRIB, as well as gene silencing of PERK by amiRNA significantly reduced the FMDV induced LC3B-II levels and associated degradation of SQSTM1, thus suggesting that PERK and p-eIF2α play an important role in FMDV replication by

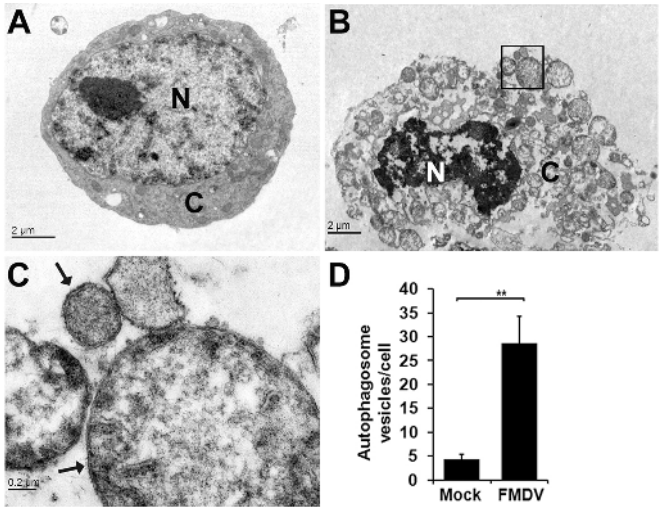

Fig. 4. TEM of FMDV-infected LFBK cells reveals presence of autophagosomes. (A) Mock-infected cells. (B) FMDV infection (1 MOI) for 6 h. FMDV-infected cells showed the presence of vesicles. (C) Magnified view of the part highlighted by the square in B, showing the double membranous nature of the vesicles (indicated by arrows). N, nucleus; C, cytoplasm. (D) Quantification (mean±s.d.) of the number of autophagosome vesicles per cell in mock and FMDV-infected LFBK cells. Counting was obtained from 10 each of mock and FMDV-infected cells. ***P*<0.01.

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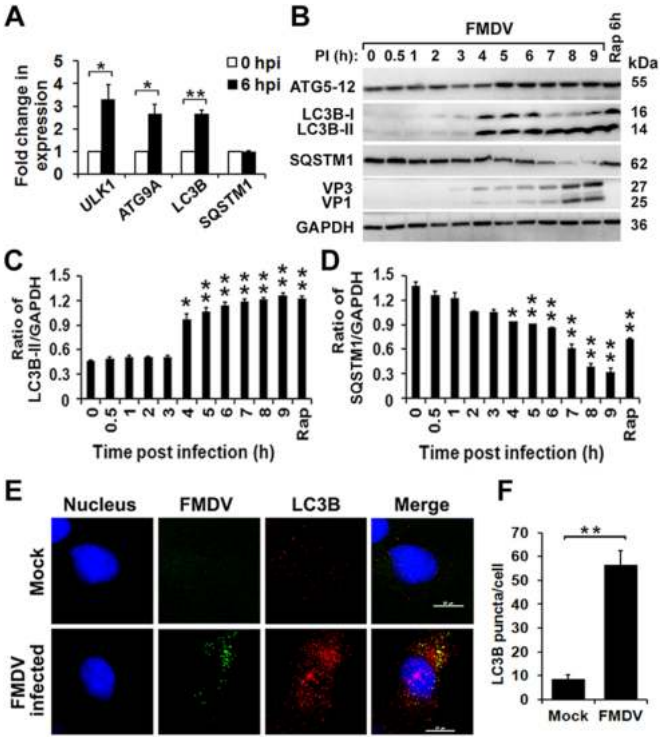

Fig. 5. FMDV infection induces autophagy in LFBK cells. (A) RT-qPCR analysis for *ULK1, ATG9A, LC3B* and *SQSTM1* performed on cDNA prepared from RNA isolated from LFBK cells infected with FMDV (1 MOI) for 6 h, by SYBR green RT-qPCR. The mRNA level of the β-actin was used as an internal control. Results are mean±s.d. for three independent experiments. (B) Western blot analysis for Atg5–Atg12 levels, the turnover of LC3B-I to LC3B-II, SQSTM1 degradation and viral protein expression in FMDV-infected (1 MOI) cells. Rapamycin-treated (5 µM) cells were used as a positive control of autophagy. Level of GAPDH was used as a loading control, while blotting for viral structural proteins (VP1 and VP3) served as infection control. (C) The band intensity ratio of LC3B-II to GAPDH as measured with densitometry, representing level of autophagy in FMDV-infected cells. Compared to mock control, significant increase was observed from 4 hpi onwards, similar to rapamycin, positive control for autophagy. (D) The band intensity ratio of SQSTM1 to GAPDH, representing level of autophagic flux as measured with densitometry. Compared to mock control, significant decrease was observed from 4 hpi onwards, as seen with rapamycin. Results in C and D represent the mean±s.d. of three independent experiments. (E) Widefield immunofluorescence microscopy of LFBK cells mock infected or FMDV infected (1 MOI) for 6 h, following fixing and immunostaining for endogenous LC3B (using anti LC3B antibody) in red and FMDV (using anti 3AB antibody) in green. The nucleus (blue) is stained with DAPI. LC3B expression is prominently observed in cells infected with FMDV. Scale bars: 10 µm. (F) Graph showing the mean±s.d. number of endogenous LC3B-II puncta in FMDV-infected (1 MOI) and control cells, counted from at least 100 cells using ImageJ quantification tool. **P*<0.05; ***P*<0.01.

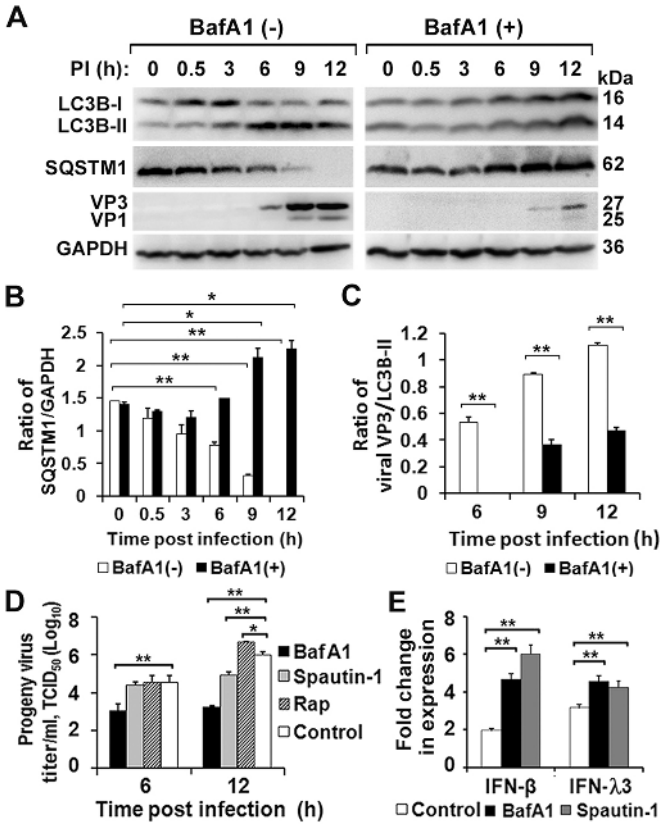

Fig. 6. Pharmacological inhibition of autophagy reduces viral titer by increasing the antiviral IFN response. (A) Western blot analysis for the turnover of LC3B-I to LC3B-II, SQSTM1 degradation and viral protein expression in FMDV-infected (1 MOI) cells, in the absence (left panel) or presence (right panel) of BafA1 (100 nM). Cells were harvested at indicated time points and extracts analyzed with anti-LC3B antibody, anti-SQSTM1 antibody and polyclonal antibody against purified serotype O FMDV, respectively. The level of GAPDH was used as a loading control. (B) Bar graph showing quantitative determination of band intensity ratio of SQSTM1 to GAPDH in the FMDV-infected LFBK cells without or with BafA1 treatment. (C) Bar graph showing quantitative determination of band intensity ratio of viral protein VP3 to LC3B-II, both normalized to GAPDH, without or with treatment of BafA1, at indicated time points post infection. Viral proteins were significantly reduced in BafA1-treated cells. (D) Bar graph showing the extracellular virus yields following FMDV infection (1 MOI) in presence of autophagy inhibitors BafA1 (100 nM) and spautin 1 (20 µM) or autophagy inducer rapamycin (5 µM), determined by the $TCID_{50}$ method in BHK-21 cells and expressed as titer/ml. The data show that inhibition of autophagy reduces extracellular viral yield. However, cells treated with the autophagy inducer rapamycin showed an increase in the virus titer at 12 h (*P*<0.05) compared to control-infected cells. (E) SYBR green based RT-qPCR analysis for antiviral genes IFN-β and IFN-λ3, performed on cDNA prepared from the RNA isolated from LFBK cells infected with FMDV (1 MOI) for 8 h with or without autophagy inhibitor (BafA1 or spautin-1) treatment. The mRNA level of the β-actin was used as an internal control. The data show that inhibition of autophagy enhances IFN response to FMDV infection. Results in B–E are mean±s.d. of three independent experiments. **P*<0.05, ***P*<0.01.

prompting activation of functional autophagy. Since there are ambiguous reports on the role of autophagy in FMDV replication, we examined the FMDV-infected cells under a transmission electron microscope for autophagosome-like vesicles, as this is one of the valid methodologies to monitor autophagy (Klionsky et al., 2008). We observed that FMDV infection induced an increased accumulation of double-membrane structures resembling autophagosomes. These structures were similar to the autophagy vesicles induced by rapamycin, a positive control for autophagy (Lee et al., 2013). Thus, our data suggest that induction of ER stress via the PERK pathway of UPR prompts autophagy during FMDV infection.

Furthermore, widefield immunofluorescence microscopy of FMDV-infected LFBK cells revealed an increase in the level of endogenous LC3B. The colocalization of FMDV with LC3B is indicated by yellow puncta, which shows the association of FMDV with autophagosomes in LFBK cells. A western blot analysis of FMDV-infected cell lysates showed a gradual increase in the conversion of endogenous LC3B-I to LC3B-II and associated degradation of SQSTM1, suggesting a positive correlation between

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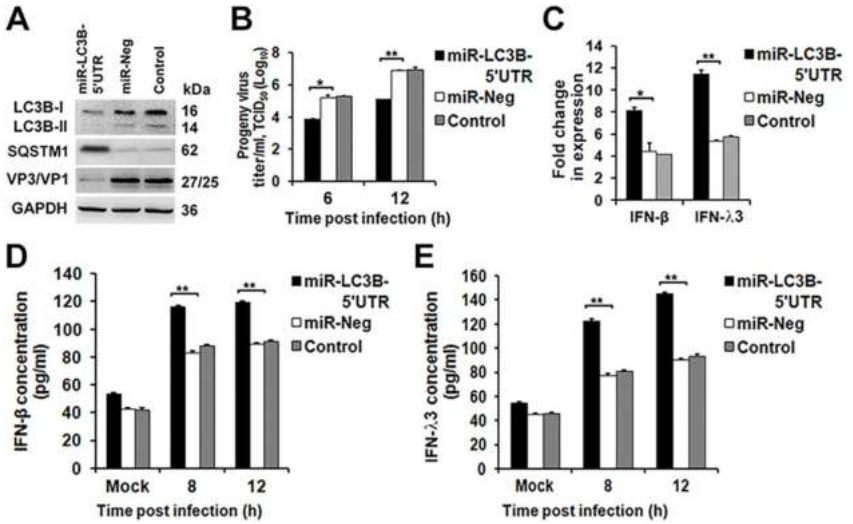

Fig. 7. Knockdown of the autophagy marker LC3B results in reduction of FMDV replication by increasing the antiviral IFN response. (A) Western blot showing levels of LC3B and SQSTM1 proteins upon FMDV infection (1 MOI) in LFBK cells with knockdown of LC3B using miR-LC3B-5′UTR. The miR-Neg was used as negative control. The blot shows significant reduction in the level of LC3B and absence of SQSTM1 degradation and reduced viral protein expression in cells expressing the LC3B gene-specific amiRNAs. (B) Bar graph showing the extracellular virus yield (log_{10}) at 6 and 12 hpi (1 MOI) of LFBK cells expressing the indicated amiRNA, as determined by the $TCID_{50}$ method in BHK-21 cells and expressed as titer/ml. The data show that knockdown of LC3B reduces the viral titer. (C) RT-qPCR-based analysis of IFN-β and IFN-λ3 transcripts performed on cDNA prepared from the RNA isolated from amiRNA miR-LC3B-5′UTR-expressing LFBK cells infected with FMDV (1 MOI) for 8 h. The miR-Neg was used as negative control. The mRNA level of the β-actin was used as an internal control. (D) Bar graph showing the extracellular IFN-β protein yield following FMDV infection in LFBK cells following knockdown of the LC3B gene using amiRNA miR-LC3B-5′UTR. miR-Neg was used as negative control. The data show that knockdown of LC3B enhances IFN-β level during FMDV infection. (E) Bar graph showing the extracellular IFN-λ3 protein yield following FMDV infection in LFBK cells following knockdown of LC3B gene using specific amiRNA miR-LC3B-5′UTR. The miR-Neg was used as negative control. The data show that knockdown of LC3B enhances IFN-λ3 level during FMDV infection. Data in B–E represent the mean±s.d. of three independent experiments, **P*<0.05; ***P*<0.01.

virus replication, LC3B lipidation and SQSTM1 turnover. This also suggested the induction of autophagic flux (Klionsky et al., 2008), involving a fusion of the autophagosome with lysosome, during the course of FMDV infection. A previous study had shown that Atg5–Atg12 conjugate, a ubiquitin-protein ligase (E3)-like enzyme for LC3–PE conjugation, decreased through degradation mediated by the viral protease 3Cpro (Fan et al., 2017). However, in contrast, our study clearly showed that the level of Atg5–Atg12 complex increased during the course of FMDV infection, indicating that the Atg5–Atg12 conjugate helps in the continuous formation of autophagosomes. Upon assessing the effect of autophagy-modulating drugs on FMDV replication, rapamycin (a known autophagy inducer) treatment caused an increase in the viral titer in the infected cells. The viral titer considerably reduced in the presence of autophagy-initiation blocker spautin-1 (Liu et al., 2011). Furthermore, bafilomycin A1, an inhibitor of fusion between autophagosomes and lysosomes, also reduced the FMDV replication, which is in contrast to what was shown in a previous report where no effect of BafA1 was observed in MCF-10A cells (Gladue et al., 2012). We also observed that BafA1 treatment significantly increased LC3B levels and decreased SQSTM1 turnover together with the reduction in virus multiplication. Our data suggest that FMDV infection induces functional autophagy. The virus relies on the autophagic flux for its multiplication, as BafA1 treatment reduced the virus replication. Furthermore, to study the dependence of FMDV infection on autophagy, LFBK cells with knockdown of *LC3B*, a known autophagy marker, were infected with the virus. This affected SQSTM1 degradation and significantly reduced FMDV protein expression and the titer, suggesting that FMDV replication is dependent on autophagy.

Both type I and III IFNs are well recognized as anti-FMDV molecules (Ma et al., 2018; Perez-Martin et al., 2012). We, therefore, estimated their levels by blocking PERK pathway and autophagy. PERK knockdown by means of amiRNA enhanced the level of IFN-β and IFN-λ3 proteins and reduced viral replication. Earlier studies have shown that activation of PERK promotes ligand- and Jak-independent phosphorylation of IFNAR1, leading to its ubiquitylation and degradation. This enables efficient viral replication (Liu et al., 2009). However, our study indicated that PERK may as well be negatively controlling the production of antiviral IFNs. Furthermore, we noticed that inhibition of FMDV-associated autophagy upon knockdown of LC3B or chemical inhibition resulted in enhancement of the IFN-β- and IFN-λ3-encoding genes, over and above the levels induced by the virus infection alone. It was noted previously that the Atg5–Atg12 conjugate directly associates with the CARD domain of the RIG-I and IFN-β promoter impairing their interaction (Jounai et al., 2007). Also, it has been reported that autophagy diminishes the early IFN-β response to influenza A virus (Perot et al., 2018) and knockdown of autophagy-related genes increased the expression of IFNs and IFN signaling pathway in hepatitis C virus-infected hepatocytes (Shrivastava et al., 2011). These results, taken together, suggest that autophagy may interfere with the RIG-mediated IFN production during FMDV infection, and therefore inhibition of autophagy enhances the levels of the antiviral IFNs.

In summary, this study substantiated that FMDV infection induces ER stress and PERK-mediated UPR, and for the first time, demonstrated the link between PERK-mediated UPR and the activation of autophagic flux. The autophagy in turn, affected IFN production to promote virus multiplication (Fig. 8). Therefore, blocking of the PERK pathway and autophagic flux reduced FMDV multiplication, with a concomitant increase in the levels of antiviral molecules IFN-β and IFN-λ3. We, therefore, propose that this pathway offers potential targets to develop therapy against FMDV

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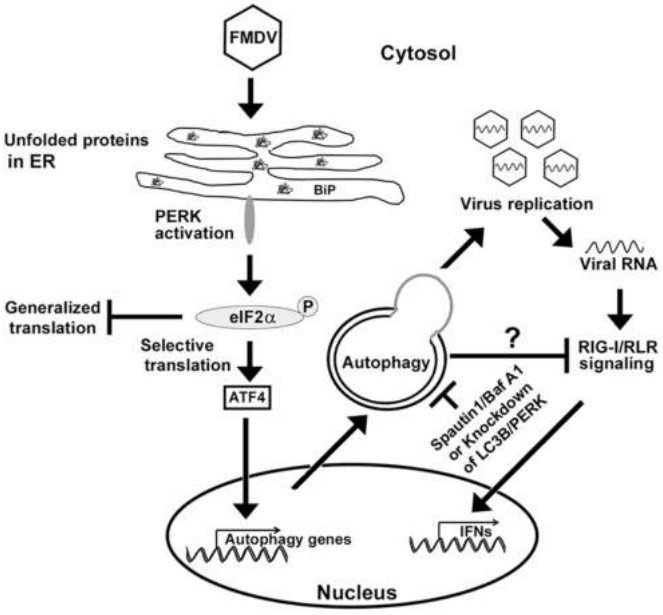

Fig. 8. Model representing autophagy activation and its effect on interferon production during FMDV infection. The virus replication induces ER stress and the PERK–eIF2α–ATF4 pathway of the UPR, triggering autophagy. The induced autophagy promotes FMDV replication. However, autophagy by unknown mechanism negatively affects RIG-I/RLR signaling involved in antiviral IFN production. Therefore, inhibition of autophagy enhances the IFN levels.

infection. These findings could form the basis for further investigations to explore the interactions of non-enveloped RNA viruses with the host cell and may help elucidate molecular targets for the development of novel antiviral strategies in the future.

MATERIALS AND METHODS

Cells and virus

LFBK, a porcine origin cell line (Swaney, 1988), provided by PIADC (ARS), and the baby hamster kidney 21 (BHK-21) cell line (clone 13, ATCC) were used in this study. The cells were maintained in Glasgow's minimum essential medium (GMEM) (Himedia, India) supplemented with 10% fetal bovine serum (FBS; USA origin), 60 µg/ml penicillin (Sigma, P3032), 100 µg/ml streptomycin (Sigma, S9137) and 100 µg/ml kanamycin (Sigma, K1377), in a humidified incubator with 5% CO_2 at 37°C.

FMDV serotype O/IND/R2/75, a vaccine strain (passage level 5–8), was used in this study. The virus titer was determined by a median tissue culture infective dose ($TCID_{50}$) assay (Reed and Muench, 1938) in BHK-21 cells. Uniformly, 1 MOI was used in the study.

Chemicals, antibodies and other reagents

Oligonucleotide primers for PCR (Table S1) were designed using Primer 3 plus software, and were commercially synthesized (Shrimpex Biotech, India). Bafilomycin A1 (B1793), Rapamycin (R0395), Spautin-1 (SML0440), Tunicamycin (T7765), ISRIB (SML0843) and VER-155008 (SML 0271) were obtained from Sigma-Aldrich. Anti-LC3B antibody (3868S), anti-SQSTM1 antibody (5114S), anti-Atg5 antibody (A0731), anti-PERK antibody (3192S), anti-phospho-eIF2α, Ser51 (3398S) and anti-BiP antibody (3183S) were purchased from Cell Signaling Technology. Anti-GAPDH antibody (SC-59540) was from Santa Cruz Biotechnology. The monoclonal antibody against FMDV 3AB protein (10H9D8) and rabbit hyperimmune serum raised against 146S mature virus antigen of FMDV serotype O were from our laboratory stocks. Atto-633 anti-rabbit-IgG (Sigma, 41176), Alexa-Fluor-488 anti-mouse-IgG (ThermoFisher Scientific, A-11001), Alexa-Fluor 488 anti-rabbit-IgG (ThermoFisher Scientific, A-11008), anti-mouse-IgG HRPO (Dako, P0260) and anti-rabbit-IgG HRPO (Dako, P0448) conjugates were also used. Porcine IFN-β ELISA kit (E0086Po) and porcine IL28B (IFN-λ3) ELISA kit (E0471Po) were procured from Bioassay Technology Laboratory.

Cell culture and infection of virus

LFBK cells seeded either in six-well plates (1.5×10^6 cells/well) or in 24-well plates (2.0×10^5 cells/well) were infected with FMDV serotype O, at a multiplicity of infection (MOI) of 1. After 1 h adsorption, the cells were washed twice in GMEM (without FBS), pH 6.5, and cultured in GMEM, pH 7.4 (with 5% FBS).

Construction of plasmids expressing PERK- and LC3B-specific pre-miRNA

Pre-miRNA sequences were designed using the guidelines of Block-it RNAi designer tool (Invitrogen Inc.), for the porcine PERK and LC3B gene. For PERK, two targets (T1 and T2) within the 5′UTR were analyzed, while for LC3B, one targeting 5′UTR and other targeting ORF regions were analyzed. The effective artificial miRNAs that gave better knockdown were chosen for the study. The sequences of these miRNAs are given in Table S2. The required sense and antisense oligonucleotides for desired miRNAs were synthesized (Eurofins Genomics), annealed and were cloned into pcDNA™6.2-GW/miR vector, as described previously (Basagoudanavar et al., 2018) to generate pcDNA6.2-miR-PERK-T1, pcDNA6.2-miR-PERK-T2, pcDNA6.2-miR-LC3B-5′UTR and pcDNA6.2-miR-LC3B-ORF. As a negative control, pcDNA™6.2-GW/miR-neg control plasmid (Invitrogen) was used.

Knockdown of PERK and LC3B in cell lines using gene-specific amiRNA and evaluation of the effect on FMDV replication

LFBK cells were transfected with recombinant plasmids expressing pre-miRNAs targeting PERK and LC3B and negative control miRNA. After 24 h of plasmid delivery, the selection was carried out using blasticidin 6 µg/ml. The cells were maintained under the selection pressure.

To evaluate the effect of the amiRNAs targeting PERK and LC3B on FMDV replication, LFBK cell lines expressing PERK- and LC3B-specific amiRNA were infected with FMDV at a MOI of 1. The infected cell lysate was analyzed by western blotting. Extracellular progeny-virus titer was assayed by using the $TCID_{50}$ method (Reed and Muench, 1938). The protein levels in the supernatant of infected cells were determined by using the porcine IFN-β and IFN-λ3 ELISA kit as per the manufacturer's protocol and analyzed using four parameter logistic curve-fit software (www.elisaanalysis.com).

Drug treatment and infection of virus

LFBK cells (1.5×10^6 cells/well) were seeded in a six-well dish and incubated overnight. The cells were pre-treated with drugs – bafilomycin A1 (100 nM), rapamycin (5 µM), spautin-1 (20 µM), tunicamycin (2.5 µg/ml), VER-155008 (20 µM) or ISRIB (200 nM) – for 1 h prior to FMDV infection and also added after adsorption.

Transmission electron microscopy

Cells were fixed in 3% glutaraldehyde (TAAB UK, G002) in phosphate buffer (pH 7.2) for 24 h and post fixed in 1% osmium tetroxide for 1 h, followed by dehydration in grades of ethyl alcohol. Later, samples were cleared in propylene oxide, embedded in Araldite CY212 resin (TAAB UK, E009) and polymerized at 60°C for 48 h. The blocks were cut using a Leica EM UC7 ultramicrotome (Leica Mikrosysteme, Austria) and stained by using uranyl acetate and lead citrate (Frasca and Parks, 1965). The stained sections were scanned under JEM 1400 plus TEM (JEOL Japan) at 80 KVA and images captured using Gatan SC 1000B camera.

RNA isolation and RT-qPCR analysis

Total RNA from the samples was extracted using RNeasy mini kit (Qiagen, 74104). Reverse transcription was carried out with Genesure™ H Minus strand cDNA synthesis kit using oligo(Dt) primers (PureGene, PGK163). RT-qPCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, K0221) in Applied Biosystems 7500 real-time thermal cycler (Applied Biosystems, CA, USA). Each sample was run in duplicate. The fold change in expression levels of the genes was calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), relative to that of the housekeeping β-actin (*ACTB*) gene.

XBP1 splicing assay

An X-box-binding protein 1 (XBP1) fragment was PCR amplified from the cDNA isolated from FMDV-infected or tunicamycin-treated cells, using the specific primers pXBP1F, 5′-GGATGCCTTAGTTACTGAAG-3′, and pXBP1R, 5′-GTCCTTCTGGGTCGACTTCT-3′. The PCR amplicons were checked in 2% agarose gel and visualized using a UV transilluminator.

SDS-PAGE and western blot analysis

Lysates from the cells following drug treatment or infection or knockdown of LC3B, were prepared using RIPA lysis buffer (Amresco, N653) containing a protease inhibitor cocktail (Sigma, P2714) and 0.1 mM PMSF (SIGMA, P7626) for 10 min. Protein samples (10 µg per lane) were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane of either 0.22 or 0.45 µm pore size (Merck, ISEQ08100, BM7JA0926A). The membrane was incubated overnight at 4°C with a primary antibody (1:500) and for 1 h with species-specific HRP-conjugated secondary antibody (1:1000). Gels were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, RB231022) in a chemiluminescent imager (UVITEC Mini HD9). The protein band intensity was measured by densitometry (myImage Analysis, Thermo Scientific).

Immunofluorescence microscopy

LFBK cells (0.5×10^5 cells/well) were seeded on a coverslip (Genetix, 20012) in a 24-well-dish and incubated overnight. After infection with FMDV (1 MOI), for a required time, the cells were fixed with 4% paraformaldehyde (HiMedia, TCL119) for 15 min at 4°C and permeabilized using 0.1% Triton-X100 (Amresco, 0694). The cells were blocked with Tris-buffered saline (TBS) containing 3% bovine serum albumin (Alfa Aesar, J65788) and 0.02% Tween 20 (Merck, 8.22184) for 30 min at room temperature. Then the cells were incubated for 1.5 h with rabbit anti-LC3B (1:500), anti-peIF2α (1:100) or anti-3AB (1:100) mouse monoclonal antibody at 37°C, followed by probing with anti-species fluorescent dye conjugated secondary antibody for 1 h (1:500). The fluorescence signals were visualized using a widefield Delta Vision microscope (API, GE) with an Olympus 60×/1.42 NA objective. Post-acquisition, the images were deconvolved using DV softWoRX software. The number of LC3B-II punctae in the cells were quantified by using the projected image (collapsed *Z*-stacks). The colocalization of proteins was observed by analyzing the individual *Z*-stacks using the 'Colocalization' plugin in ImageJ (NIH).

Statistical analysis

All values are expressed as mean±s.d. Statistical analyses were performed using Student's *t*-test to identify statistical significance between groups. $P<0.05$ was considered significant.

Acknowledgements

We acknowledge the Director, ICAR-Indian Veterinary Research Institute (IVRI) Izatnagar, for facilitating this work, and also PIADC, ARS-USDA, USA, for providing LFBK cell line used in this study. H.B.R. acknowledges the fellowship received from IVRI, Izatnagar. We acknowledge the use of widefield Delta Vision microscope procured under the Wellcome Trust/DBT India Alliance Intermediate Fellowship (509159-Z-09-Z) and LSRB-DRDO grant (LSRB-310/BTB/2017) awarded to R.M. We thank Mr S. Ram Prasad for help in language editing.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Coneptualization: H.B.R., B.P.M., R.K.S., A.S., S.H.B.; Methodology: H.B.R., N.G., R.M., B.K.C.S., S.H.B.; Validation: H.B.R., N.G., S.H.B.; Formal analysis: H.B.R., N.G., M.H., S.H.B.; Investigation: H.B.R., V.A., N.G., M.H., B.P.S., V.V.D., R.S., B.K.C.S., S.H.B.; Resources: M.H., B.P.S., R.M., B.K.C.S., S.H.B.; Writing - original draft: H.B.R., N.G., S.H.B.; Writing - review & editing: V.A., M.H., B.P.S., V.V.D., R.S., B.K.C.S., B.P.M., R.K.S., A.S., R.M., S.H.B.; Visualization: H.B.R., V.A., S.H.B.; Supervision: B.P.S., S.H.B.; Project administration: B.P.M., R.K.S., A.S., S.H.B.; Funding acquisition: R.K.S., A.S., S.H.B.

Funding

The work was supported by the Institute funds of ICAR-Indian Veterinary Research Institute, Regional Center Bengaluru (IVRI/BANG/16-18/011).

Supplementary information

Supplementary information available online at
https://jcs.biologists.org/lookup/doi/10.1242/jcs.240622.supplemental

Peer review history

The peer review history is available online at
https://jcs.biologists.org/lookup/doi/10.1242/jcs.240622.reviewer-comments.pdf

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Seminars in Cancer Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Seminars in Cancer Biology

journal homepage: www.elsevier.com/locate/semcancer

Xenophagy in cancer

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ARTICLE INFO

Keywords:
Cancer
Carcinogenesis
Inflammation
Infection
Xenophagy

ABSTRACT

Macroautophagy (herein autophagy) is an intracellular pathway in which cytoplasmic components are captured by double-membrane vesicles (autophagosomes) that eventually fuse with lysosomes to degrade the cargo. Basal levels of autophagy in all eukaryotic cells maintain cellular homeostasis and under conditions of stress, organelles and proteins not essential for survival are degraded. Apart from these functions, cargoes like aggregated proteins, damaged organelles and intracellular pathogens, which are otherwise harmful to cells, are also selectively captured by autophagy and are destined for degradation. In terms of infectious diseases, pathogens are cleared by a specific form of autophagy known as xenophagy. This lysosomal mediated degradation of pathogens also increases the antigen presentation of cells thereby inducing a further immune response. The process of xenophagy provides a broad spectrum of defense mechanism to capture bacterial, viral and protozoan pathogens. However, pathogens have developed ingenious mechanisms to modulate xenophagy to enhance their intracellular survival. Meanwhile, certain pathogens also induce deleterious effects such as chronic inflammation and overexpression of oncogenes in the host system. This over time can increase the susceptibility of the host for tumorigenesis. Hence targeting tumor through anti-microbial mechanisms like xenophagy could be a novel strategy for combinatorial anti-cancer therapy. The recent developments in understanding the role of xenophagy in combating cancer causing pathogens will be discussed in this review.

1. Introduction

Autophagy is an evolutionarily conserved intracellular degradation process occurring in eukaryotic cells. During autophagy, a part of the cytoplasm is sequestered in double-membrane vesicles called autophagosomes that eventually fuse with lysosomes leading to the breakdown of its contents. This process occurs at basal levels to maintain the cellular homeostasis by turnover of long-lived proteins and damaged organelles [1]. It is also well known as a cellular stress response pathway that is up-regulated during conditions of nutrient deprivation, intracellular infections and when aggregated/misfolded proteins accumulate [2,3]. The degraded products of autophagy fuel the anabolic pathways that are important for cell survival especially during stress.

Christian de Duve, well known for his discovery of lysosomes, coined the term “Autophagy” meaning “Self-eating”. It was named after he observed electron micrographs containing damaged mitochondria enclosed in electron-dense structures in the rat liver sections [4]. Further studies showed that autophagy is a lysosomal mediated degradation process involving the capture of cytoplasmic cargoes in double-membrane vesicles.

The process of autophagy involves multiple steps that include phagophore induction, elongation, autophagosome completion and fusion with lysosomes [5]. This is followed by breakdown of cargo in lysosomes and efflux of the end products such as amino acids back to the cytoplasm (Fig. 1).

Initial studies projected autophagy as a non-selective process capturing the cytoplasmic components into autophagosomes for degradation. Later studies provided evidence that apart from the bulk degradation of cytoplasmic contents, there are also cargoes that are recognized selectively by means of autophagy receptors. Non-selective autophagy serves as an energy source during starvation whereas, in the case of selective autophagy, unwanted cytoplasmic cargoes such as damaged organelles and aggregated proteins are cleared so as to maintain cellular homeostasis. This selective autophagy is achieved by adaptor proteins that are not only involved in cargo recognition but also recruit autophagy machinery proteins such as Atg8/LC3 (Light Chain 3) through motifs known as Atg8-Interacting Motif (AIM) or LC3 Interacting Region (LIR). The various known selective autophagy pathways include mitophagy (autophagy of mitochondria) [6], aggrephagy (autophagy of aggregated proteins) [7], xenophagy (autophagy of pathogens), pexophagy (autophagy of peroxisomes) [8], ribophagy (autophagy of ribosomes) [9], nucleophagy (autophagy of nucleus) [10], and lipophagy (autophagy of lipid bodies) [11].

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https://doi.org/10.1016/j.semcancer.2020.02.015
Received 14 February 2020; Accepted 17 February 2020
1044-579X/

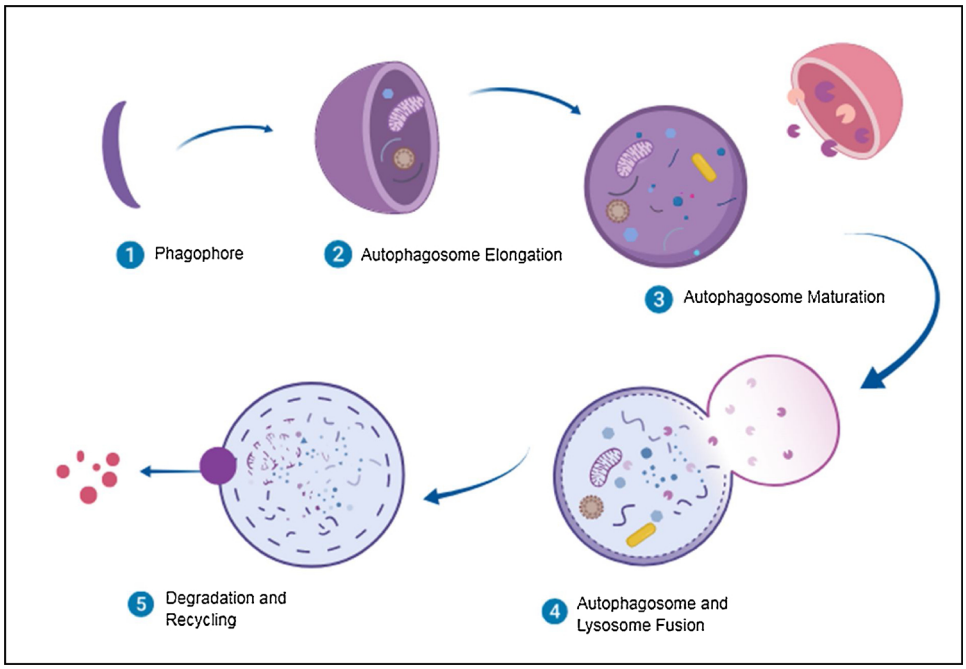

Fig. 1. Steps of autophagy process. Upon induction of autophagy, a double membrane precursor structure, the phagophore (1)forms which elongates forming double-membrane vesicles called autophagosomes (2 and 3). These vesicles fuse with lysosomes forming autolysosomes (4) wherein degradation of the captured cytosolic cargo occurs. The degraded products are subsequently recycled back into the cytoplasm for anabolic processes (5).

1.1. Xenophagy

Autophagy of intracellular micro-organisms is termed as xenophagy (Xeno- foreign; phagy- eating). The host cell (phagocyte/non-phagocyte) employ a variety of defense mechanisms to prevent pathogens from establishing infection. Xenophagy is one of the innate defense systems of the cells against multiple intracellular pathogens. It mediates direct capture of the pathogens for lysosomal mediated degradation as well as in augmenting the adaptive immunity by increasing the antigen presentation (Fig. 2).

1.2. Discovery of xenophagy

Although the process of general autophagy was discovered in 1963, xenophagy was first observed in a study by Rikisha in 1984, where guinea pig polymorphonuclear leukocytes (PMNs) incubated with *Rickettsia* (a gram-negative bacteria), showed autophagosome-like structures containing bacteria [12]. These structures were also positive for acid phosphatase, a component of lysosomes, showing that it is a degradative compartment for the entrapped bacteria. Xenophagy serves as a defense mechanism against various pathogen types like bacteria, virus, and parasites. The role of xenophagy in medically relevant pathogens such as Group A Streptococcus [13], Mycobacterium [14], Salmonella [15], Shigella [16,17]; HIV [18], Sindbis virus [19], and Toxoplasma [20] are well studied (Table 1).

1.3. Mechanism of xenophagy

Bacteria enter cells by phagocytosis (in the case of phagocytic cells) or by endocytosis (non-phagocytic cells). The conventional pathway for

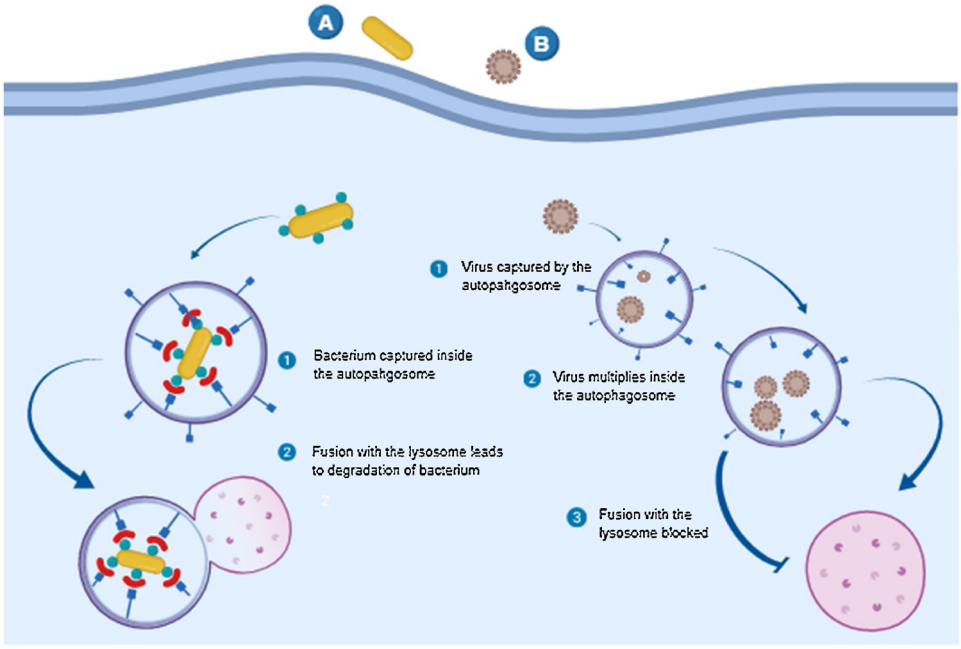

Fig. 2. Modes of pathogen capture by xenophagy. A- Bacterial capture by xenophagy. 1. Recognition of cytosolic bacteria by ubiquitin (blue dots) followed by binding of xenophagy adaptor proteins (red structures) and autophagosome membrane protein, LC3 (blue lines). 2. The fusion of bacteria containing autophagosomes to lysosomes for degradation. B- Virus capture by xenophagy. 1. Recognition of intact virus or viral particles by autophagosomes. 2. The captured virus replicates in autophagosomes and prevents fusion with lysosomes.

Table 1
Role of xenophagy during intracellular infections.

Pathogen	Disease	Host/pathogen effectors
Streptococcus pyogenes	Pharyngitis, Rheumatic fevers, Skin infections.	Streptolysin causes the escape of bacteria to cytosol, where it is captured by xenophagosomes [36].
Staphylococcus aureus	Skin infection, sinusitis, food poisoning	Xenophagy enhances the survival of *S. aureus* due to inflammasome degradation [37].
Salmonella typhimurium	Gastroenteritis	*Salmonella* escaping from endosomes into cytosol are captured by xenophagy [15].
Mycobacterium tuberculosis	Tuberculosis	Xenophagy induction leads to INF production which is shown to inhibit *M. tuberculosis* replication [38].
Legionella pneumophila	Legionellosis- fever, muscle pain and shortness of breath	Induces xenophagy in type IV system secretion (T4SS, also known as the secretion system Dot/Icm)-dependent manner [39].
Shigella flexneri	Shigellosis- diarrhoea and abdominal pain	Xenophagy is activated by the recognition of IcsA (surface protein) by ATG5 [17].
Helicobacter pylori	Peptic ulcers and gastritis	Xenophagy block caused by *H. pylori* facilitates in its intracellular survival [40].
Listeria monocytogenes	Listeriosis- gastroenteritis (immuno-compromised patients can get meningitis)	Listeriolysin causes the escape of bacteria to cytosol, where it is captured by xenophagosomes. At later time points of infection, *Listeria* escapes xenophagy by expressing ActA protein that forms actin tail and mediates bacterial movement within cells. [41].
Coxiella burnetii	Q fever	*C. burnetti* can survive and replicate in the harsh environment of large, acidified phagolysosome-like vacuoles [42].
Porphyromonas gingivalis	Periodontitis and infection of gastrointestinal tract and colon.	Bacteria get sequestered in xenophagosomes, but it evades the fusion with lysosomes [43].
Parvovirus B19	Erythema infectiosum	Increase in LC3 II levels during virus replication is observed and inhibition of autophagy led to more virus-induced cell death [44].
Poliovirus	Poliomyelitis	Inhibition of autophagy decreased poliovirus yield [45].
Hepatitis C	Jaundice	HCV induces autophagic vacuoles and recruitment of LC3 and Apg5 [46].
Influenza A	Influenza	The virus causes accumulation of autophagosomes through preventing fusion to lysosomes [47].
Human immunodeficiency virus (HIV)	Acquired Immune Deficiency Syndrome (AIDS)	HIV Gag-derived proteins co-localizes and interacts with the autophagy factor LC3 and is seen to be degraded [18].
Varicella zoster virus (VZV)	Varicella (Chicken pox)	VZV induces xenophagy at later time points of infection [48].
Epstein-Barr virus(EBV)	Infectious mononucleosis	EBV blocks autophagic flux during re-activation from latency [49].
Herpes Simplex Virus (HSV)	Herpes encephalitis	HSV protein γ34.5 inhibits xenophagy by binding to Beclin1 [50].
Sindbis virus	Sindbis fever	Binds to Beclin1 inhibiting xenophagy [51].

phagosome/endosome is to fuse with lysosomes for degradation. However, pathogens have developed several ways by which they can avoid fusion with lysosomes. One strategy they employ is to prevent fusion with lysosomes and reside in their modified endosomes as seen in *Legionella, Mycobacterium,* and *Salmonella*. Apart from endosomal entrapment, bacteria can also lyse the endosomes to escape into the cytosol. The best-studied examples of bacteria that enter cytosol include *Listeria*, *Shigella,* and *Salmonella*. Xenophagy induction during bacterial infection occurs by interaction of bacterial cell wall components such as LPS with PAMPs of the host cell. One of the well-known PAMPs, TLR4, in TRIF dependent pathway induces downstream pathways such as NFkB, MAPK and autophagy [21]. Subsequent studies have expanded the repertoire of TLRs involved in xenophagy induction [22]. Additionally, intracellular bacteria exposed to host cytoplasm gets ubiquitinated [23]. Ubiquitination leads to recognition by various xenophagy adaptors such as p62, NDP52 leading to capture of pathogens in xenophagosomes [24] (p62/Sequestosome (SQSTM1), Nuclear Dot like Protein (NDP52), Neighbor of BRCA1 (NBR1), Optineurin(OPTN)) capturing them in xenophagosomes (pathogen-containing autophagosomes). The adaptor proteins contain domains that bind to ubiquitin as well as recruit LC3 through their LIR domain. The ubiquitinated bacteria in cytosol get coated with such adaptor proteins and LC3, thereby inducing autophagosomes formation around them [25,26].

In addition to the cytosolic bacteria, the pathogens often reside in self-styled vacuolar compartments such as Salmonella Containing Vacuoles (SCVs) for *Salmonella*. These pathogen specific compartments are also targeted by the autophagic machinery. This capture could be either intact phagosomes or remnants of damaged phagosomes. For example, vacuolar capture of *Salmonella* residing in SCVs is an ubiquitin-independent xenophagy process, wherein galectin8, a lectin that marks damaged membranes recruits the autophagy adaptor protein NDP52. It, in turn, engages the autophagy machinery by binding to LC3 [27]. However, Pathogens also evade xenophagy by modulating the process in more than one way. In the case of *Salmonella*, one of the virulence factors, namely SifA, sequesters host Rab9 impairing the retrograde transport of mannose-6-phosphate pathway. This causes impairment in proteases loading in lysosomes leading to accumulation of lysosome-like structures characterized by membrane composition but devoid of proteases to actively degrade the cargo [28]. This also provides conditions optimum for *Salmonella* to replicate in SCVs by growing tubular structures known as *Salmonella*-induced filaments which exhibit higher metabolic activity rate to combat the nutritional stress in the intra-vacuolar environment [29]. *Salmonella* also utilizes host factors such as HOPS (HOmotypic fusion and Protein Sorting), to facilitate fusion with endosomal structures for their intracellular survival [30]. Recent screening for host factors by Krebich et al. identified Atg5 to be important to maintain the integrity of SCVs [31].

Viruses being obligate parasites require significant interaction with the host for its survival. Similar to bacterial infection, viral components are also reported to induce host immune sensors. Involvement of cell surface TLRs (TLR2 and TLR4) recognize viral envelope components leading to downstream cascade activation including autophagy [32]. Furthermore, after entry into host cells, viral uncoating occurs exposing viral nucleic acid products such as DNA, single/double-stranded RNA. These components are recognized by endosomal TLRs and RNA helicases (RIG1 and MDA5) [33]. Autophagy is shown to capture virions or viral proteins in autophagosomes to mediate degradation in autolysosomes [34,35]. Besides mediating lysosomal degradation, autophagy can also deliver viral components to endosomes that contain toll-like receptors enhancing viral recognition. This further initiates type-I interferon signalling. Interferons are cytokines which have anti-viral properties. Interferon response, especially IFNα and IFNβ, are considered as frontline defense mechanisms during viral infection. Additionally, studies have also shown that autophagy targets viral proteins to MHC class II loading compartments to generate CD4T-cell response. Thus autophagy acts as a part of both innate and adaptive systems to mount anti-viral responses [34].

1.4. Cancer and autophagy

Cancer refers to a group of genetic diseases involving accumulation of DNA mutational changes that leads to rapid cell division and tumor formation. Cancer initiation and progression requires some physiological changes in cellular properties. These changes contribute towards oncogenesis and are collectively termed as 'hallmarks of cancer'. The dual role of autophagy in cancer is well reported [52,53]. Apart from invasion and metastasis, autophagy also has a role in modulating other hallmarks of cancer. Cytoprotective autophagy prevents cancer initiation. In the absence of autophagy, cells accumulate ubiquitylated protein aggregates, misfolded proteins, and damaged organelles like mitochondria. Such an environment in the cell interior is conducive to the production of reactive oxygen species (ROS), causing metabolic insufficiency and increased proteotoxicity. This is a threat to the survival of the cell as well as the integrity of the genome. Autophagy acts as the guardian of the genome by preventing the accumulation of random DNA mutations which can in turn initiate tumours. On the other hand, high levels of autophagy in cancers with Kras or Braf mutations allow them to achieve sustained proliferation [54]. One of the major tumor-protective roles of autophagy in cancer is to help in the survival of tumor cells before they become vascularized. High levels of autophagy combat oxidative stress in tumor cells and aids in their survival prior to vascularization [55]. On the other hand, solid tumors battle severe hypoxic conditions and in this context, they switch to aerobic glycolysis for ATP production (rather than oxidative phosphorylation) to minimise reliance on oxygen through the blood supply. In addition, autophagy also scavenges biomolecules within the cell to provide new substrates for TCA cycle. This sustains ATP production in solid tumors even in the absence of oxygen [56].

1.5. Pathogen associated cancers

For a long time now, infections are known to be a major cause of sporadic cancer initiation by causing DNA damage or genomic instability (Table 2). This is achieved by pathogen-mediated chronic inflammation and release of toxic metabolites. Bacterial effector proteins interact and activate host oncoproteins that cause cell-cycle dysregulation triggering carcinogenesis progression [57,58].

2. Mechanisms of pathogen mediated oncogenesis

2.1. Inflammation

Inflammation as a physiological process is a double-edged sword. It is an adaptive host response against invading pathogens but excess inflammatory response, especially against endogenous aggressors, can be harmful. In a typical inflammatory response against infection, phagocytes recruited to the site of infection secrete pro-inflammatory cytokines such as Tumor Necrosis Factor alpha (TNFα) and chemokines which further amplify the inflammatory response by engaging more cells of the immune system at the site of infection [59]. Finally, this cascade primes dendritic cells which in turn activates adaptive immune response.

In a normal physiological state, inflammation is highly regulated by a balance between pro and anti-inflammatory cytokines. Perturbations in this fine-tuned balance of inflammation regulating chemicals or a long persistence of infectious agents can lead to chronic inflammation and ultimately neoplastic transformation. Xenophagy acts as a part of innate immune system in controlling intracellular infection. Recognition of pathogens by xenophagy leads to their capture in xenophagosomes and eventual degradation in lysosomes, thus reducing the intracellular pathogen load. Studies have shown that xenophagy mediated clearance of intracellular pathogens could possibly control inflammation. Elegant studies have identified several bacterial strains which can infect human tissues causing inflammation [60,61]. Some of them are discussed in detail.

2.2. Helicobacter pylori

Helicobacter pylori is a common gram-negative bacterium that infects approximately 50 % of the world population. *H. pylori* infection has a strong correlation with the occurrence of peptic ulcer, gastritis, and gastric adenocarcinoma. *H. pylori* virulence factors namely, cytotoxin associated gene-A (CagA) and vacuolating cytotoxin (VacA) can modulate host cellular pathways. CagA, which is secreted by Type IV secretion system, can activate a series of signalling cascades inside the cell, finally resulting in activation of NFκB (nuclear factor kappa light chain enhancer of activated B cells) pathway and production of interleukin-8. This results in severe and chronic inflammation [62,63]. Prolonged inflammation of gastric epithelium is a major factor in the development of peptic ulcer and peptic adenocarcinoma due to

Table 2
Modulation of xenophagy by carcinogenic pathogens.

Pathogen	Cancer Association	Xenophagy
Borrelia burgdorferi	B cell lymphoma	Xenophagy controls *Borrelia* infection as well as reduce cytokine production [82].
Chlamydia trachomatis	increases the risk for invasive squamous-cell carcinoma of the uterine cervix	Captured by xenophagosomes but are not degraded in lysosomes due to reduced lysosomal enzyme activity [83].
Helicobacter pylori	predisposal to gastric cancer or MALT lymphoma	Prolonged exposure to the vacuolating cytotoxin (VacA), produced by *H. pylori*, prevents maturation of the autolysosome [40].
Mycoplasma	prostate cancer	Genital *mycoplasmaUreaplasmaparvum* evade xenophagy by inducing significant endosomal–lysosomal damage [84,85].
Neisseria gonorrhoeae	Bladder cancer	Captured by xenophagosomes but avoid degradation by secreting proteases such as IgAP that cleaves lysosomal proteins [86,87].
Salmonella typhi and *Salmonella typhimurium*	Gallbladder carcinoma	Cytoplasmic *Salmonella* gets recognized by xenophagy proteins [88].
Hepatitis C virus (HCV)	Liver cancer	Replicates in xenophagosomes. Autophagy inhibition reduces HCV replication. The release of virions also requires autophagy [89].
Hepatitis B virus (HBV)	Hepatocellular cancer	Replicates in xenophagosomes. The release of HBV through exosomes requires autophagy [90,91].
Human Papilloma Virus (HPV)	Cervical cancer	E5 downregulates xenophagy proteins and E6 prevents fusion of xenophagosomes to lysosomes [92], [93].
Human T Lymphocyte Virus Type 1 (HTLV-1)	T-cell leukemia	Tax protein mediates replication of HTLV in LC3 positive xenophagosomes [94].
Epstein-Barr Virus (EBV)	B and T cell lymphomas, nasopharyngeal carcinomas	Latent Membrane Protein 1 (LMP1) induces xenophagy. The xenophagosome membrane is used for viral replication [95].
Kaposi Sarcoma Herpesvirus (KSHV)	Skin cancer (Kaposi Sarcoma)	Expresses Bcl2 that binds with BECN1 preventing xenophagosomes formation [96].

generation of oxidative stress and accumulation of mutations.

2.3. Salmonella typhimurium

Salmonella infects epithelial cells using type III secretion system (T3SS) encoded by its pathogenicity genes called *Salmonella* pathogenicity islands 1/2 (SPI1/SPI2). It can modify the host endosomes into replicating vacuoles called *Salmonella* containing vacuoles (SCVs). Gall bladder epithelium acts as a niche for persistent intracellular *Salmonella* along with *Salmonella* extrusion from infected cells. This leads to release of *Salmonella* into gall bladder lumen causing infiltration of macrophages and neutrophils, leading to a strong inflammatory response. Patients with gall stones exhibit biofilm formation on the gall stone surfaces and demonstrate very high *Salmonella* replication that is clinically related to the initiation of gall bladder cancer [64,65].

2.4. Activation of oncoproteins

Oncoproteins are coded by host oncogenes and are defined by their ability to transform and promote cancer/tumor growth. Interestingly, certain pathogens express proteins that are capable of interacting with the host oncoproteins and cause cell transformation leading to tumorigenesis.

As previously described, *H. pylori* induce severe inflammation mediated by its virulence factor CagA. Additionally, CagA can directly bind to oncoprotein, SHP-2 (Src homology region containing protein -2) and affect the functioning of cell cycle kinases such as MAPK pathways [66]. This CagA mediated activation of MAPK pathway also induces the expression of c-jun and c-fos proto-oncogenes that are also implicated in cell transformation [67]. Other examples include p37, a *Mycoplasma* lipoprotein that is expressed on the bacterial membrane which is also shown to modulate properties like cell adhesion, migration and invasion [68].*in vitro* studies have shown *M. fermentas* and *M. penetrans* mediated transformation to malignancy on cell lines mainly caused by overexpression of c-ras and c-myc oncoproteins [69].

Viral-derived oncoproteins play a major role in cellular transformation and contribute to tumor initiation. Transformed host cells continuously undergo cell division synthesizing the viral genome along with its own cellular genome - a desirable trait for the viral propagation. The well-studied oncoproteins of DNA viruses include T antigen of Simian Vacuolating virus 40 (SV40), E6 and E7 of papillomavirus, and E1A of adenovirus. All three oncoproteins function in a similar manner by inactivating a particular cellular protein known as retinoblastoma that is essential for regulating cell replication [70,71]. On the contrary, oncoproteins of retroviruses function either by expressing viral proto-oncogenes such as v-src of Rous sarcoma virus or insert provirus into host cellular genome, both activating multiple proto-oncogenes expression leading to neoplastic transformation [72].

2.5. Toxic secondary metabolites

2.5.1. Reactive oxygen species (ROS)

ROS is a defense weapon used by phagocytic cells to kill intracellular pathogens. It is generated by host NADPH oxidase in response to microbial recognition and inflammation. ROS is directed to pathogen-containing phagosomes where they cause oxidative damages such as DNA strand breakage, deamination, and base oxidation on pathogens. However, there are pathogens that survive the oxidative environment created by ROS. This is seen in the case of *S. typhimurium*, which is capable of rapidly fixing the DNA strand damage created by ROS and replicate normally in oxidative conditions [73]. Similarly, in the case of KSHV, by a mechanism still not identified, ROS can reactivate virus from latency and increase the viral load [74]. The generated ROS can dysregulate multiple downstream signalling pathways like MAPK, Akt, NFκB among others. These pathways are directly involved in cancer-promoting cellular properties such as survival, motility, and adhesion [75].

2.6. Nitrosamines

N-nitrosamines are well known potent carcinogens. Inflammation-mediated production of reactive nitrogen oxide species can be further processed in the stomach to nitrosamines by acid reactions that are catalyzed by bacteria. Additionally, activated macrophages show increased production of nitrites and nitrates. Patients with bladder infections rapidly absorb nitrates, secondary amines, and nitrosamines leading to cellular DNA damage of the host [76].

3. Impact of xenophagy on carcinogenesis

As mentioned in the earlier section, excessive inflammatory responses can have adverse effects leading to tumor initiation. Inflammatory responses during infection are mediated by a multiprotein complex called inflammasome, to release pro-inflammatory cytokines, particularly interleukin-1β (IL-1β) [77]. Autophagy, being a quality control mechanism of the cell keeps a check on the excessive and unwarranted inflammatory response. The process controls inflammation indirectly by removing damaged organelles of the cell. Furthermore, autophagy can also have a direct effect by degrading components of inflammasome. Inflammasomes are assembled multiprotein complexes that lead to secretion of cytokine precursors such as pro-IL1β and pro-IL-18. Inflammasome activation is inhibited by preventing accumulation of inflammasome agonists such as mitochondrial ROS and oxidized mitochondrial DNA. Individual components of inflammasome can also be directly degraded by autophagy in a SQSTM1/p62 dependent manner [78].

Altered levels of autophagy are a hallmark of several inflammatory disorders. Genome-Wide Association Studies (GWAS) have identified links between genetic polymorphisms in autophagy loci with a predisposition to inflammatory diseases. The most well-studied link is for Crohn's Disease (CD), an inflammatory bowel disease with polymorphisms in genes encoding ATG16L1, NOD2 and IRGM [79]. Analysis of ATG16L1 and IRGM polymorphisms during *H. pylori* infection revealed downregulation of xenophagy. In addition, polymorphism in these genes makes the individual more susceptible to bacterial infections including *H. pylori*. Finally, the combined effect of these mutation (s) and *H. pylori* infection has been shown to significantly increase the risk of gastric cancer [80]. These studies highlight the possibility of tumor initiation due to dysfunctional xenophagy.

In another example, infection with *Salmonella* at tumor sites induced a strong xenophagic response by the cancer cells and surprisingly, led to an eventual reduction in tumor growth. Although the exact mechanism is not known, xenophagy mediated cell death through inactivation of Akt/mTOR/p70S6K has been shown to contribute to the decline in tumor size [81].

4. Modulation of xenophagy by carcinogenic pathogens

4.1. Bacteria

In order to establish infections, pathogens overcome the host induced defense mechanisms including xenophagy by targeting different stages of the process (Table 1 and 2). A study by Raju et al. showed that during *H. pylori* infection, while limited exposure of its virulence factor, VacA induced xenophagy, sustained exposure made the cell unresponsive to the toxin, resulting in reduced xenophagy [40,80]. Similarly, patients infected with $CagA^+$ *H. pylori* showed decreased autophagic flux with an accumulation of autophagy adaptor protein, p62 compared to patients infected with $CagA^-$ *H. pylori* although the mechanism by which these factors inhibit the process is not clear [63].

Another carcinogenic pathogen, *Chlamydia trachomatis* that causes cervical and ovarian cancer, although gets captured by

xenophagosomes, it interestingly, decreases contents of lysosomal enzymes post-infection and thereby preventing its lysosomal mediated killing [83].

4.2. Viruses

Viruses play a significant role contributing close to 15 percent in human cancer development. Elaborate studies on viral pathogenesis revealed mechanisms that viruses adapt to overcome immune responses including xenophagy.

Host immune system detects the presence of cytoplasmic DNA, which is a sign of viral infection and initiates anti-viral interferon response by an enzyme called cGAS (cyclic guanosine monophosphate-adenosine monophosphate synthase) along with its adaptor protein STING, (stimulator of interferon genes) shortly referred to as cGAS-STING pathway. The viral oncoproteins E7 and E1A are known to antagonize this immune response by direct binding to STING [97]. This abrogates the function of STING protein that apart from being involved in interferon production also induces autophagy [98].

Viruses can also directly target the autophagy process and thereby prevent degradation in lysosomes. Examples of such mechanisms include HPV, which causes cervical cancer, anogenital cancer, head and neck cancer, and skin cancers. Oncoproteins of HPV, E5, and E6 are involved in inhibiting autophagy in more than one way. E5 down-regulates key autophagy proteins like BECN1, LC3, ULK1 whereas E6 decreases the fusion of autophagosomes to lysosomes [99]. Similarly, herpesviruses express proteins such as ICP34.5 and viral Bcl2 both of which bind to Beclin1 and prevent autophagosome formation.

Viruses like poliovirus and hepatitis C can also utilize autophagy for its survival by converting the double membrane autophagosomes to its own replicating niche and in addition prevent fusion of such vesicles with lysosomes.

5. Antimicrobial agents for cancer treatment

There is increasing evidence for using antibiotics in combination with other anti-cancer drugs for the targeted treatment of particular cancer types. To mention a few, Nifuroxazide, a nitrofuran antibiotic, selectively targets aldehyde dehydrogenase1 (ALDH1) which is a detoxifying enzyme that protects cells from alkylating agents. Studies have shown that using this drug on melanoma cells which generally has high ALDH1 levels, show a regression in tumor initiating cells [100]. Similarly, ionophore antibiotics like salinomycin and its multiple derivatives have shown anti-cancer properties in various cancer types including those on multidrug-resistant tumors. Although over-expression of Bcl2 and BAX proteins are seen upon salinomycin treatment, the exact mechanisms by which salinomycin induce apoptosis of cancer cells is not clear [101].

While using antibiotics for tumor treatment has been well addressed, there are limited studies on antibiotics inducing autophagy for anti-cancer treatment. Clarithromycin, a commonly used macrolide antibiotic against various bacterial infections including *Helicobacter,* is also shown to have an effect on mucosa-associated lymphoid tissue lymphomas (MALT) and incidentally, *Helicobacter* infection can lead to initiation and development of MALT [102,103]. Further studies by other groups observed autophagy inhibition by clarithromycin on myeloma cells leading to apoptosis. In another example, violacetin, a bis-indole pigment produced by select bacterial species, was shown to induce autophagy and apoptosis of head and neck cancer cells [104,105]. Similarly, tigecycline, a tetracycline antibiotic induced autophagy and prevented gastric cancer cell proliferation [106].

Recent studies have identified pharmacological methods to modulate xenophagy during intracellular bacterial infections. Rapamycin, a well-known autophagy inducer also induces xenophagy [107]. Similarly, studies from various groups have screened for compounds that could induce xenophagy. To mention a few, BRD5631 was identified to have antibacterial effect against *Mycobacterial* species [22]. Recently, we reported identification of plant derived flavonoid, acacetin to induce xenophagy and reduce intracellular *Salmonella* replication [108]. Additionally, certain antibiotics like isoniazide and pyrazinamide apart from having direct antibiotic effect also induce xenophagy to bring about an effective clearance mechanism [109].

6. Discussion

The selective form of autophagy, i.e. xenophagy is an evolutionary conserved innate immunity pathway that plays a key role in the pathogenesis of several clinically important infections. Xenophagy is known to directly capture pathogens or indirectly mediate immune responses to control intracellular infection. Genetic or pharmacological induction of xenophagy has shown promising results in controlling infection and thereby exerting its secondary effects in reducing inflammation [22,110]. Given the enormous scale of cancer research, a cure(s) for cancer still appear only in the horizon. There have been diverse approaches towards cancer therapies, including some out of the box ideas, and perhaps connecting the possibility of xenophagy as a possible tool to fight cancer falls in this category. There are emerging evidences of how xenophagy manipulates tumor microenvironment. As both carcinogenic pathogens and inflammation are shown to be drivers of carcinogenesis, these studies provide confidence in employing xenophagy as a possible strategy for anti-cancer combinatorial therapy. Employing anti-microbial agents for anti-cancer treatment is also beneficial because presence of pathogenic (viral) products in the tumor cells, help in developing targeted therapies to cancer cells and distinguishing the normal cells.

However, the prolonged or excessive use of antibiotics through the combination of antibiotics and anticancer agents might increase antibiotic resistance to pathogens. Therefore, as an alternative to antibiotics, compounds that induce host anti-microbial mechanisms like xenophagy can be tested. Few compounds such as rapamycin, BRD5631, acacetin are known to induce xenophagy and do not have antibacterial properties. However, their efficiency in treating carcinogenic bacteria is yet to be elucidated [22]. Inducing host immune response also has an additional advantage of tackling multiple pathogens irrespective of pathogen type.

Although modulating xenophagy can be beneficial in combinatorial therapy, before employing it as a treatment, it is essential to study in detail the evasive mechanisms employed by varied pathogens against xenophagy. Apart from preventing self-degradation, some pathogens have also evolved strategies to make use of autophagy for their increased replication and survival converting autophagosomes as an intracellular niche protected from other cellular immune mechanisms. Hence it is critical to know the type of pathogen and the specific stage of xenophagy that has to be modulated.

Similarly, it is also important to be cautious while employing autophagy/xenophagy as a strategy for cancer treatment. It is well proven by multiple studies that autophagy plays a dual role in tumor initiation and development. It contributes to tumor suppression during initial stages by suppressing inflammation and reactive oxygen species. However, during later stages of tumor, autophagy is known to support cancer cells survive nutrient starvation and hypoxic microenvironment of the solid tumors. This can be a double-edged sword. In this regard, this field is still in its infancy and extensive research is hence imperative to understand the nexus between cancer and xenophagy. This would help in deciphering effective combinatorial therapeutic approaches to treat pathogen mediated cancer.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Letters

Vancomycin Derivative Inactivates Carbapenem-Resistant *Acinetobacter baumannii* and Induces Autophagy

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Cite This: *ACS Chem. Biol.* 2020, 15, 884–889

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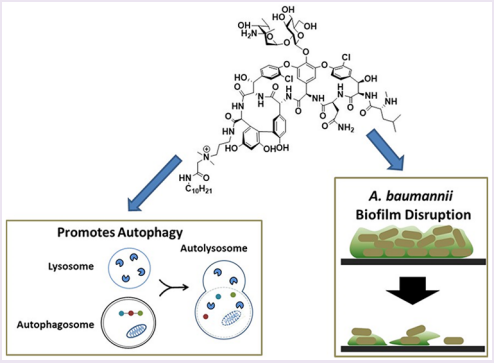
ABSTRACT: Vancomycin is a standard drug for the treatment of multidrug-resistant Gram-positive bacterial infections. Albeit, development of resistance (VRE, VRSA) and its inefficacy against persistent infections is a demerit. It is also intrinsically inactive against Gram-negative bacteria. Herein, we report a vancomycin derivative, $VanQAmC_{10}$, that addresses these challenges. $VanQAmC_{10}$ was rapidly bactericidal against carbapenem-resistant *A. baumannii* (6 $\log_{10}$ CFU/mL reduction in 6 h), disrupted *A. baumannii* biofilms, and eradicated their stationary phase cells. In MRSA infected macrophages, the compound reduced the bacterial burden by 1.3 $\log_{10}$ CFU/mL while vancomycin exhibited a static effect. Further investigation indicated that the compound, unlike vancomycin, promoted the intracellular degradative mechanism, autophagy, in mammalian cells, which may have contributed to its intracellular activity. The findings of the work provide new perspectives on the field of glycopeptide antibiotics.

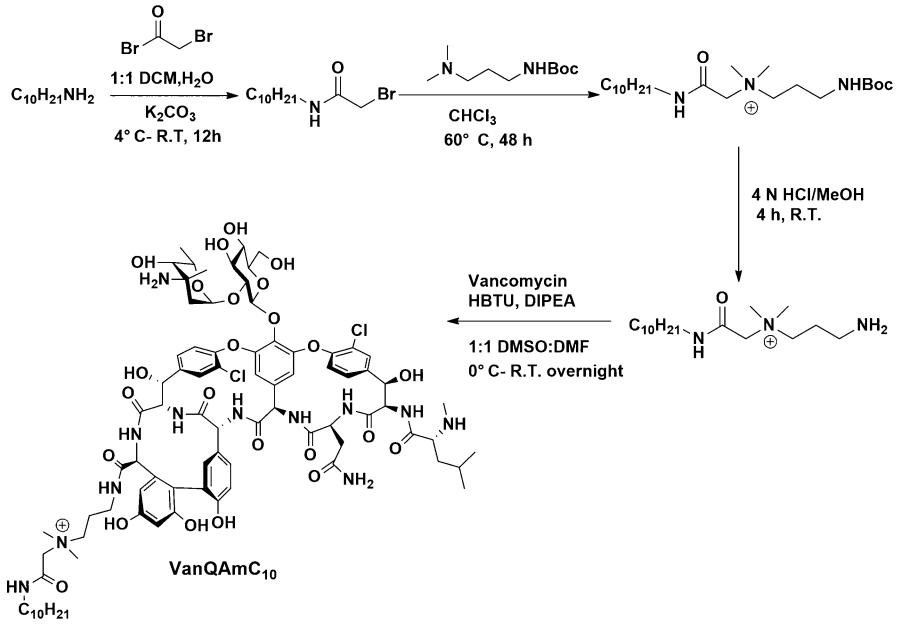


The threat of antimicrobial resistance (AMR) has been reiterated several times by the World Health Organization (WHO) and other international healthcare organizations.[1] The upsurge in incidences of multidrug resistance and pan-drug resistance in Gram-negative bacteria (MDR-GNB and PDR-GNB) has become a severe health challenge.[2] Additionally, bacterial pathogens often evade the host immune system and antibiotic treatment, thereby persisting within the host. These infections usually result from the ability of bacteria to form biofilms or survive within mammalian cells and are difficult to treat.[1,3] This necessitates the need for development of drugs for the treatment of complicated bacterial infections. Semisynthetic approaches are an attractive and clinically successful strategy toward achieving this goal. Although vancomycin has been widely used in the clinic for the treatment of MRSA infections, resistance has been reported in *Enterococci* and *Staphylococci* (vancomycin-resistant *Staphylococcus aureus*, VRSA; and vancomycin-resistant *Enterococci*, VRE).[4,5] Further, its inefficacy against Gram-negative bacteria and intracellular Gram-positive bacteria limits broad clinical usage.[6,7] Efforts have been directed to this end, which involve semisynthesis and nanoparticle-based approaches through encapsulation of vancomycin.[8−16] Previously, we had reported a cationic lipophilic vancomycin derivative that could eradicate Gram-negative bacteria both *in vitro* and *in vivo*. Although the derivative exhibited good activity, its LD_{50} was 28 mg/kg.[17] This warranted an improvement in molecular design to reduce toxicity while maintaining similar antibacterial activity. Morover, the multifaceted antibacterial activity of a semisynthetic glycopeptide against Gram-negative biofilms and intracellular bacteria remains underexplored.[4,18] In this repect, we report the design of a nontoxic vancomycin derivative, $VanQAmC_{10}$ (Scheme 1), and its efficacy against acquired resistance in Gram-positive bacteria and intrinsic resistance in Gram-negative bacteria to vancomycin. Further, the effect of the compound on biofilms of *A. baumannii* and the mammalian cellular degradative pathway, autophagy, was investigated.

Design and Synthesis. In various biophysical studies and molecular dynamic simulations against polymeric and small molecular systems, our group reported that the inclusion of an amide bond between the cationic moiety and the hydrophobic moiety imparts additional hydrogen bonding capability to the bacterial lipids.[19,20] This enhances the selective toxicity toward bacterial cells over mammalian cells. Therefore, the lipophilic moiety conjugated to vancomycin here contained an amide group between the cationic center and lipophilic moiety. A simple three step synthetic scheme was followed to obtain

Received: February 3, 2020
Accepted: March 20, 2020
Published: March 20, 2020

Scheme 1. Synthesis of Semi-Synthetic Vancomycin Derivative



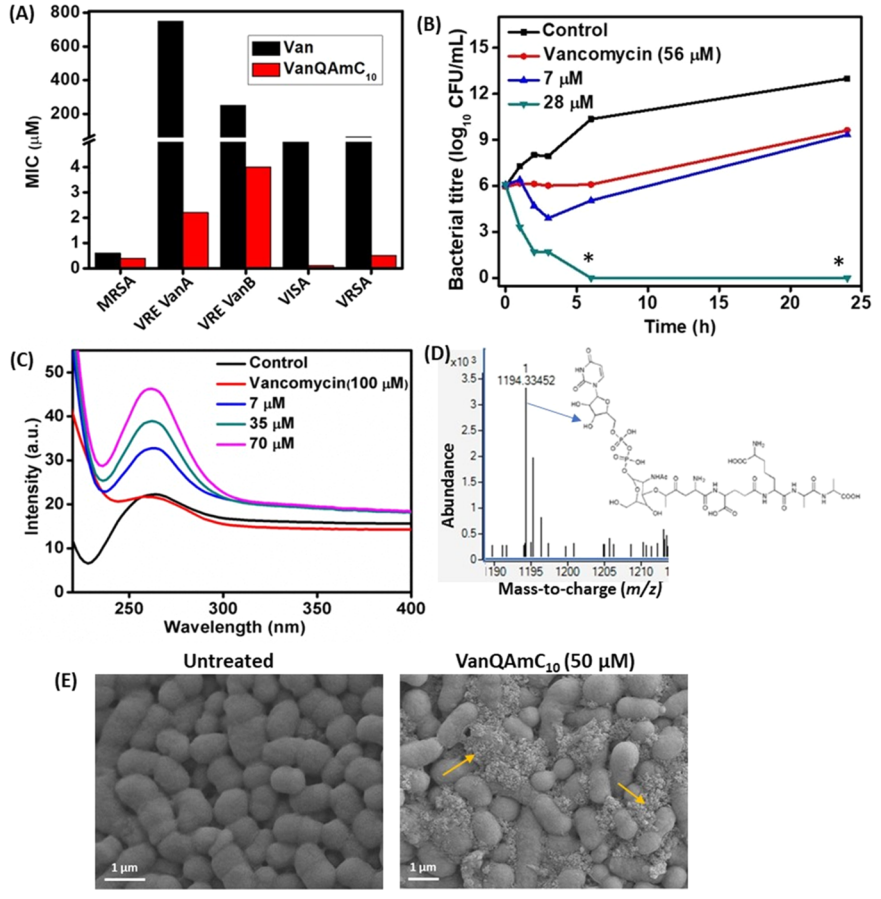


Figure 1. Antibacterial activity and mechanism of action of VanQAmC$_{10}$. (A) MIC against Gram-positive bacteria. (B) Time-kill kinetics against *A. baumannii* R674. Inhibition of cell wall biosynthesis in *A. baumannii* upon treatment with test compounds investigated through (C) accumulation of UDP-MurNAc-L-Lys-γ-Glu-m-DAP-D-Ala-D-Ala observed by absorbance at 260 nm. (D) Mass spectrum of UDP-MurNAc-PP (structure inset) identified at *m*/*z* 1194.334. (E) SEM micrographs of *A. baumannii* treated with VanQAmC$_{10}$ against exponentially growing bacteria (scale bar, 1 μm). *<50 cfu/mL.

VanQAmC$_{10}$ (Scheme 1). In the first step, decylamine was conjugated to bromoacetyl bromide to obtain the brominated amide (**1a**). Nucleophilic substitution of the bromide in **1a** with Boc-protected *N*,*N*-dimethyl propanediamine yielded **1b**,

https://dx.doi.org/10.1021/acschembio.0c00091
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which was deprotected to obtain the amphiphilic precursor **1c** with a primary amine group. Then the compound, **1c**, was reacted with the carboxylic acid group of vancomycin using *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1H-benzotriazol-1-yl)-uraniumhexafluoro-phosphate as a coupling agent to obtain the lipophlic cationic vancomycin derivative, VanQAmC$_{10}$. The final product was purified through reverse-phase HPLC with around 70% yield and was characterized using HPLC, ^{1}HNMR, ^{13}CNMR, and HR-MS.

***In Vitro* Antibacterial Activity.** VanQAmC$_{10}$ exhibited activity comparable to that of vancomycin against vancomycin-sensitive bacteria (MIC 0.4 μM against MRSA, 0.5 μM against *E. faecium*, Figure 1A). Against vancomycin-resistant bacteria, a 100−330-fold enhancement in activity as compared to vancomycin was observed. It showed a minimum inhibitory concentration (MIC) of 0.1 μM and 0.2 μM against VISA and VRSA, repectively, and 2.2 μM and 4 μM against VRE VanA and VanB phenotypes, respectively. Vancomycin showed a MIC of 250 μM (VRE VanA) and 750 μM (VRE VanB; Figure 1A). The activity of the compound was also tested against various Gram-negative pathogens such as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* (Table 1 and Table S1).

Table 1. Antibacterial Activity of VanQAmC$_{10}$ against Clinical Isolates of Gram-Negative WHO Priority Pathogen *A. baumannii*[a]

	MIC (μM)					
A. baumannii	PMB	Mero	Levo	Tige	Ceft	VanQAmC$_{10}$
NR-13374	<0.4	83.4	88.6	6.8	>85.9	7.7
NR-13375	<0.4	>167	177	6.8	>85.9	7.7
NR-13376	<0.4	167	177	3.4	>85.9	3.9
NR-13377	<0.4	167	177	6.8	>85.9	3.9
NR-13378	<0.4	>167	22	6.8	>85.9	3.9
NR-13379	<0.4	>167	11	3.4	>85.9	3.9
NR-13380	<0.4	10.4	22	6.8	>85.9	7.7
NR-13381	<0.4	>167	1	6.8	>85.9	7.7
NR-13382	<0.4	>167	22	3.4	>85.9	7.7
NR-13383	<0.4	83.4	11	3.4	>85.9	3.9
NR-13384	<0.4	>167	177	6.8	>85.9	7.7
NR-13385	<0.4	>167	88.5	6.8	>85.9	7.7
NR-17777	<0.4	2.6	22	1.7	21.5	15.5
NR-17786	<0.4	10.4	1.3	3.4	>85.9	3.9
NR-19299	<0.4	167	44.3	3.4	>85.9	3.9
BAA-1605	<0.4	167	22	6.8	>85.9	7.7
ATCC 17978	<0.4	<1.3	22	<0.8	21.5	7.7
MTCC 1425	ND	ND	ND	ND	ND	6.25
ABR 674	ND	>64	ND	ND	ND	10

[a]Levo, levofloxacin; Mero, Meropenem; Tige, tigecycline, PMB, Polymixin B, Ceft, ceftaroline; N.D. Not determined.

While the compound showed some activity against *E. coli* and *P. aeruginosa*, the highlight of the work was its potency against *A. baumannii* (Table 1). *A. baumannii* is notorious due to its unprecedented ability to acquire resistance to most antibiotics, which led to the World Health Organization categorizing it as the number one critical priority pathogen for drug discovery. Given the challenge presented by infections caused by this pathogen, this finding is promising toward extending the use of vancomycin against this pathogen.[21] VanQAmC$_{10}$ showed an MIC in the range 3.9−15.5 μM against various multidrug-resistant clinical isolates of *A. baumannii*. The bacteria tested were resistant to the currently used antibiotics, levofloxacin, meropenem, ceftaroline and only susceptible to polymyxin B and tigecycline. Its bactericidal activity was rapid; 4 log$_{10}$ CFU/mL reduction was observed within 2 h at around 3 × MIC (28 μM), while complete killing (6 log$_{10}$ CFU/mL reduction) was observed in 6 h (Figure 1B). At 7 μM (just below MIC), the compound initially showed a 2.5 log$_{10}$ CFU/mL reduction at 4 h and then subsequent growth up to 24 h. The incorporation of the lipophilic cationic moiety permitted interaction with the negatively charged bacterial membrane and resulted in depolarization and permeabilization of both the outer and inner membranes of the bacteria (Figure S1). The outer membrane permeabilization caused by the lipophilic cationic vancomycin derivative permits the entry of the compound to the cell wall region and thereby inhibits cell wall biosynthesis (Figure 1C and D). The membrane disruptive mode of action was further supported by the SEM images of VanQAmC$_{10}$ treated cells where cell debris and lysis are visible (Figure 1E).

Disruption of Biofilms of Carbapanem-Resistant *A. baumannii*. *A. baumannii* is notorious for biofilm related infections.[22] Most of the conventional antibiotics are rendered ineffective due to the permeability barrier created by the extracellular polymeric matrix (EPS).[3] Therefore, the activity of VanQAmC$_{10}$ against *A. baumannii* biofilms was of immense interest. Confocal laser scanning microscopy showed that VanQAmC$_{10}$ treated biofilms were disrupted and their thickness reduced to 2.2 μm at 50 μM, while untreated and vancomycin treated biofilms were 11 and 8.8 μm thick (Figure 2A). This reduction was comparable to that of colistin taken at the same concentration. The conjugation of the cationic lipophilic moiety onto vancomycin facilitated interaction with the negatively charged components of the EPS, resulting in disintegration of the biofilms. To further investigate biofilm eradication, the viability of bacteria within these disrupted biofilms upon treatment at various concentrations was determined. VanQAmC$_{10}$ reduced the number of viable bacteria in a concentration dependent manner. At 10 μM, it reduced cell viability by 1.8 log$_{10}$ CFU/mL. At higher concentrations of 25 μM and 50 μM, 2.8 log$_{10}$ CFU/mL and 3.8 log$_{10}$ CFU/mL reduction was obtained, respectively. Colistin, on the other hand, could completely eliminate the viable cells (6 log$_{10}$ CFU/mL reduction). Tobramycin was found to be ineffective, while vancomycin caused a 1 log$_{10}$ CFU/mL reduction of viability of bacteria within the biofilm at 50 μM (Figure 2B).

Eradication of Stationary Phase Cells of *A. baumannii*. Biofilms consist of bacteria in various metabolic phases; the reduction in viable cells in biofilms indicated activity against stationary phase bacteria as well. While vancomycin did not show any significant reduction in viability (0.6 log$_{10}$ CFU/mL), VanQAmC$_{10}$ reduced cell viability by 3 log$_{10}$ CFU/mL and 5.5 log$_{10}$ CFU/mL at 5 μM and 10 μM respectively within 2 h (Figure 2C). Since the integrity of the membrane is crucial for bacterial survival irrespective of its metabolic state, the membrane-disruptive nature of the compound resulted in this rapid bactericidal activity (Figure S2). The currently used antibiotic, tobramycin, showed a lower activity with a 2.7 log$_{10}$ CFU/mL reduction, while colistin completely killed the stationary-phase bacteria (Figure 2C). However, the advantage of VanQAmC$_{10}$ offered over colistin was that it did not induce any observable resistance in *A. baumannii* (Figure S3). Up to 19 passages, the compound showed no increase in MIC, while colistin induced resistance from the sixth passage itself. The

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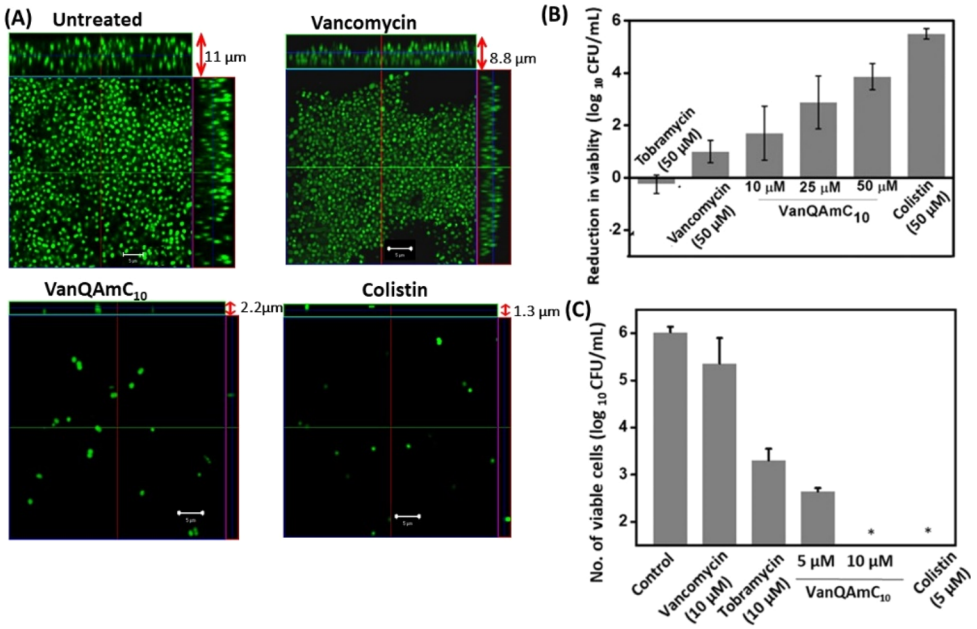

Figure 2. Activity of VanQAmC$_{10}$ against biofilms and stationary phase of *A. baumannii*. (A) Confocal laser scanning images of biofilms of a multidrug resistant strain when mature biofilms were left untreated, treated with vancomycin, VanQAmC$_{10}$, and colistin at a concentration of 50 μM each (scale bar, 5 μm). (B) Viability of bacterial cells in biofilms post-treatment with test compounds. (C) Viability of stationary phase cells 2 h post-treatment with test compound. *<50 cfu/mL.

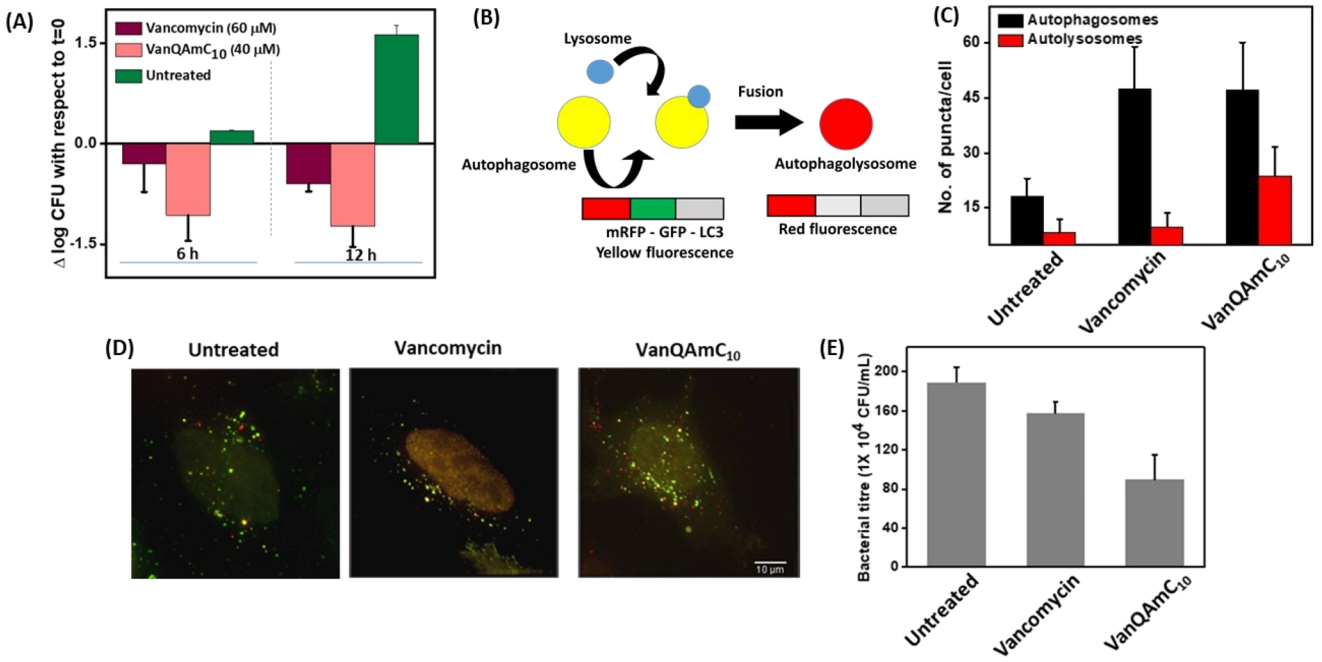

Figure 3. (A) Intracellular killing kinetics of VanQAmC$_{10}$ against MRSA. (B) Schematic of the tandem mRFP-GFP-LC3 assay. (C) Representative microscopic images of merged GRP and RFP channels of RFP−GFP−LC3 expressing HeLa cells treated with indicated compounds at 100 μM for 2 h (scale bar 10 μm). (D) Comparison of the number of puncta per cell of autolysosomes (red) and autophagosomes (black) in treated and untreated cells. (E) Activity of compounds against intracellular *Salmonella typhimurium*.

membrane-disrupting, nonspecific targeting by the vancomycin derivative may have contributed to this lack of propensity to induce resistance.

Toxicity. The *in vitro* and *in vivo* toxicity of the compound was then tested. The HC$_{50}$ of the compound was found to be greater than 1000 μM as opposed to 100 μM for a vancomycin derivative of comparable activity lacking an amide between the cationic center and lipophilic moiety (Figure S4).[17] The LD$_{50}$ in mice, when administered intravenously, was found to be 70 mg/kg as opposed to 28 mg/kg for the derivative lacking the amide spacer.[17] The H-bonding ability of the amide moiety has been reported to result in better interaction with the polar head groups of the bacterial membrane than with the mammalian cell membrane. This increased interaction due to H-bonding ability with the bacterial membrane makes up for the lower chain length of the hydrophobic moiety which results

https://dx.doi.org/10.1021/acschembio.0c00091
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in reduced interaction with the lipid membranes. Both the factors together probably contributed to the enhancement in the selectivity of the compound toward bacteria.

Intracellular Activity and Autophagy. Intracellular survival of *S. aureus* is a critical contributor to persistence, and vancomycin is known to be intracellularly ineffective.[23] On treatment with $VanQAmC_{10}$, mouse macrophages infected with MRSA resulted in a lower intracellular bacterial burden. The untreated intracellular bacterial burden increased by 0.5 $\log_{10}$ CFU/mL and 1.6 $\log_{10}$ CFU/mL in 6 and 12 h (Figure 3A). Upon treatment with vancomycin at 60 μM, around 0.5 $\log_{10}$ CFU/mL reduction in the intracellular bacterial burden was observed up to 12 h. $VanQAmC_{10}$ (at 40 μM) exhibited better intracellular activity than the parent drug. One $\log_{10}$ CFU/mL and 1.3 $\log_{10}$ CFU/mL reduction in bacterial titer was observed in 6 and 12 h, respectively. The morphology of the RAW 264.7 cells post incubation with $VanQAmC_{10}$ remained unaffected (Figure S5). Compounds that induce autophagy have been reported to reduce intracellular survival of pathogens.[24] The 1.3 log reduction of intracellular pathogen prompted us to explore if $VanQAmC_{10}$ exhibits such a property. Autophagy is a cellular degradative pathway that is crucial for defense against pathogen invasions. The process involves the fusion of the lysosome with the phagosome.[25] An upregulation in the process is associated with the clearance of intracellular pathogens. A tandem mRFP-GFP-LC3 assay was performed to study the effect of $VanQAmC_{10}$ on autophagy (Figure 3B). The induction of autophagy was monitored by quantitating the autophagic vesicles by tagging the LC3 protein, which is a biomarker for autophagosomes and autolysosomes. HeLa cells were transfected with a tandem red fluorescence protein−green fluorescence protein−LC3 expressing plasmid (mRFP-GFP-LC3). The GFP signal is sensitive to the acidic and proteolytic conditions of the lysosome lumen, and hence, its fluorescence is quenched, while that of RFP is stable.[26] The combination of an acid-sensitive GFP with an acid-insensitive RFP allows the visualization of change from autophagosomes (neutral pH) to autolysosomes (with an acidic pH). Upon induction of autophagy, the GFP-RFP labeled LC3 proteins are employed in the formation of autophagosomes. These are positive for both GFP and RFP and, hence, appear yellow. Fusion with the lysosome results in a drop of pH leading to quenching of GFP fluorescence, making the autophagolysosomes appear red.[27] Both vancomycin and $VanQAmC_{10}$ showed a higher number of autophagosomes as compared to the untreated cells (Figure 3C and D). However, interestingly, $VanQAmC_{10}$ promoted the fusion of autophagosomes with lysosomes. A 2-fold increase in the number of red puncta as compared to vancomycin-treated and untreated cases was observed. Vancomycin on the other hand showed no increase, which is in accordance with a literature report that vancomycin blocks autophagy. *S. aureus* is known to prevent phagosomal maturation during infection in order to create a niche for replication.[28] The upregulation of autophagy may have therefore contributed to the reduction of intracellular bacteria.[29] This was further supported by the 2-fold reduction of intracellular *Salmonella typhimurium* exhibited by $VanQAmC_{10}$, although it was inactive against the exponentially growing bacteria (MIC > 200 μM, Figure 3E). Vancomycin, as expected, did not show any significant reduction. This finding indicates the potential for exploring the role of the host immune system as a combinatorial mechanism with an antibiotic effect to enhance the efficacy in battling intracellular pathogens.

With the severity of AMR on the rise, strategies to fight them are necessary. Bacterial infections are further complicated by the inherent ability of bacteria to form biofilms and reside within macrophages which protect them from the antimicrobial action of the host immune system and external agents. This is the first report of a vancomycin derivative effective against multiple aspects of bacterial infections. $VanQAmC_{10}$ could eradicate biofilms and associated cells of carbapenem resistant *A. baumannii* and does not induce resistance. Another important finding is the ability of the compound to promote autophagy, which is a contributing factor to its ability to clear intracellular pathogens. This multifaceted antibacterial activity of the compound offers a new perspective to the field of new glycopeptide antibiotics.

EXPERIMENTAL SECTION

Synthetic procedures, characterization data, and experimental details are included in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00091.

Data on evaluation of mechanism of action against exponential phase bacteria, graph of percentage hemolysis evaluation, resistance study against *A. baumannii*, representative images of cells treated with $VanQAmC_{10}$, synthetic procedures, characterization, protocols for microbiological assays (PDF)

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https://dx.doi.org/10.1021/acschembio.0c00091
ACS Chem. Biol. 2020, 15, 884−889

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank C. N. R. Rao, F. R. S. (JNCASR) for his constant support. We gratefully acknowledge BIRAC SRISTI PMU 2016/002 and JNCASR for the funding. J.H. acknowledges Sheikh Saqr Career Award Fellowship. S.S. is grateful to BIRAC-GYTI for postdoctoral fellowship. We thank R. G. Prakash (in-house animal facility) for the *in vivo* study. We acknowledge the NMR facilities at NCBS, India, and Technology Platform Services, C-CAMP, India.

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frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 09 April 2018
doi: 10.3389/fnmol.2018.00109

Modulation of Autophagy by a Small Molecule Inverse Agonist of ERRα Is Neuroprotective

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Mechanistic insights into aggrephagy, a selective basal autophagy process to clear misfolded protein aggregates, are lacking. Here, we report and describe the role of Estrogen Related Receptor α (ERRα, HUGO Gene Nomenclature ESRRA), new molecular player of aggrephagy, in keeping autophagy flux in check by inhibiting autophagosome formation. A screen for small molecule modulators for aggrephagy identified ERRα inverse agonist XCT 790, that cleared α-synuclein aggregates in an autophagy dependent, but mammalian target of rapamycin (MTOR) independent manner. XCT 790 modulates autophagosome formation in an ERRα dependent manner as validated by siRNA mediated knockdown and over expression approaches. We show that, in a basal state, ERRα is localized on to the autophagosomes and upon autophagy induction by XCT 790, this localization is lost and is accompanied with an increase in autophagosome biogenesis. In a preclinical mouse model of Parkinson's disease (PD), XCT 790 exerted neuroprotective effects in the dopaminergic neurons of nigra by inducing autophagy to clear toxic protein aggregates and, in addition, ameliorated motor co-ordination deficits. Using a chemical biology approach, we unrevealed the role of ERRα in regulating autophagy and can be therapeutic target for neurodegeneration.

Keywords: estrogen related receptor α, autophagy, XCT 790, small molecule screen, Parkinson's disease, mTOR independent modulator, neuroprotection

OPEN ACCESS

Edited by:
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Jožef Stefan Institute (IJS), Slovenia

Reviewed by:
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Received: *21 December 2017*
Accepted: *19 March 2018*
Published: *09 April 2018*

Citation:
Suresh SN, Chavalmane AK, Pillai M, Ammanathan V, Vidyadhara DJ, Yarreiphang H, Rai S, Paul A, Clement JP, Alladi PA and Manjithaya R (2018) Modulation of Autophagy by a Small Molecule Inverse Agonist of ERRα Is Neuroprotective. Front. Mol. Neurosci. 11:109. doi: 10.3389/fnmol.2018.00109

INTRODUCTION

Proteostasis machineries associated with the clearance of various cellular cargos including toxic proteins and damaged organelles in eukaryotic cells primarily include the chaperone, the Ubiquitin–Proteasome System (UPS), and the autophagy pathways (Hipp et al., 2014). UPS predominantly degrades short-lived proteins via tagging them with ubiquitin at specific amino acid residues (Hipp et al., 2014). The bulk degradation of long-lived proteins or organelles is mediated largely by the evolutionarily conserved cellular process referred to as macroautophagy (hereafter autophagy). A selective degradation mechanism called aggrephagy can help cells to clear the toxic, long-lived, aggregate-prone proteins. Misfolded and aggregate prone proteins are substrates for autophagy (Nixon, 2013). Intracellular accumulation of misfolded protein aggregates is an evident feature of several neurodegenerative diseases including Parkinson's

disease (PD). Owing to their hydrophobic nature, these aggregates sequester cellular proteins, thereby, perturbing cellular proteostasis machineries leading to neuronal death. Neurons are non-dividing cells and can't dilute out the aggregates and, thus, more sensitive to proteotoxicity (Nixon, 2013). This condition is further exasperated upon aging as proteostasis efficiency decline. Recent studies highlight the importance of autophagy in curbing cellular cytotoxicity as a consequence of impaired clearance of aggregate prone proteins. Brain specific autophagy knockout mice (Atg5) display age related accumulation of protein aggregates, which eventually leads to neurodegenerative phenotypes, indicating that basal autophagy is vital for clearing protein aggregates (Hara et al., 2006). Additionally, tissue-specific knockdown of Atg7 in central nervous system of mice resulted in accumulation of inclusion bodies in autophagy-deficient neurons (Komatsu et al., 2006). Autophagy is shown to be dysfunctional during the neurodegenerative disease pathology (Nixon, 2013). Thus, restoration of autophagy through pharmacological approaches using small molecules has been reported to be neuroprotective (Sarkar et al., 2007; Khurana and Lindquist, 2010; Rajasekhar et al., 2015; Suresh et al., 2017). Small molecule that induces or restores defunct autophagy could aid in toxic aggregate clearance and essential for maintaining the cellular and organismal homeostasis (Rajasekhar et al., 2014, 2015). Broadly, small molecule autophagy modulators can be classified into mammalian target of rapamycin (MTOR)-dependent or -independent types, depending on its mechanism of action. Since MTOR has autophagy independent functions, targeting MTOR could have adverse side effects in patients with immunosuppression and impaired wound healing processes. Hence, this warrants for identifying new small molecules that are MTOR-independent with potent aggrephagy function/induction capabilities. More importantly, identifying the new molecular players that helps to decipher mechanistic interplay of autophagy and neuroprotective basic mechanisms remain a challenge.

In this study, we discovered a novel autophagy inducer, XCT 790, that was identified previously in our laboratory from a high-throughput screening of library containing pharmacologically active compounds (LOPAC^{1280}) in yeast. XCT 790, a thiadiazoleacrylamide, is the most selective inverse agonist of the orphan nuclear receptor, Estrogen-Related Receptor α (ERRα; Busch et al., 2004) was identified as a "Hit". Due to lack of any known natural ligand, XCT 790 has been used as a tool to delineate the lesser known functions of ERRα in different biological processes (Ariazi and Jordan, 2006). XCT 790 cleared α-synuclein aggregates in an autophagy-dependent manner in human neuronal cells. It significantly induced autophagy through an MTOR-independent mechanism and ERRα-dependent manner. This neuroprotective compound uncovers the role of ERRα in a basic autophagy pathway. We found that ERRα inhibits autophagy in fed conditions, thus, helps in regulating the basal autophagic flux. Additionally, in a preclinical mouse model of PD, XCT 790 was found to have a neuroprotective role through clearing the toxic protein aggregates as evidenced by immunohistological and behavior analyses.

MATERIALS AND METHODS

Chemicals and Antibodies

XCT 790 (X4753), 1-methyl-4-phenyl-1,2,3,6-tetra hydropyridine (MPTP, M0896), anti LC3 antibody (L7543), anti-FLAG antibody (F3165), 3-Methyl Adenine (3-MA) (M9281), DMEM F-12 (D8900), Penicillin and Streptomycin (P4333), DMEM (D5648), 3,3′-Diaminobenzidine (DAB, D3939), Trypsin EDTA (59418C), and Atto 663 (41176) were purchased from Sigma-Aldrich. Anti-phospho 4E-BP1T37/46 antibody (2855) and total 4E-BP1 antibody (9452), Anti-phospho P70S6K T389 antibody (9239) and total P70S6K antibody (9202), Anti-phospho AMPK antibody (8359) and total AMPK antibody (8359), Anti-phospho ULK1 antibody (8359) and total ULK1 antibody (8359) and anti-rabbit IgG, HRP (7074) antibody were purchased from Cell Signaling Technology. Cell Titre Glo® kit (G 7571) was procured from Promega. Anti-GAPDH (MA5-15738) and anti-β-tubulin (MA5-16308) antibodies were purchased from Thermo Scientific. Anti-p62 (ab 56416), Anti-ERRα (ab 16363) and Anti-Pgk1 (ab 38007) antibody were purchased from Abcam. Anti-EGFP (11 814 460 001) antibody was purchased from Roche. Anti-mouse IgG, and HRP (172-1011) antibody were purchased from Bio-Rad. Anti-A11 (AB9234) was purchased from Merck Millipore. Anti-Tyrosine hydroxylase (TH; N196) antibody was purchased from Santa Cruz Biotechnology. FITC conjugated anti-rabbit secondary antibody (F7512), and Cy3 conjugated anti-rabbit secondary antibody (C2306) were purchased from Sigma-Aldrich. CMAC-Blue (C2110) was purchased from Life Technologies. Bafilomycin A1 (11038) was purchased from Cayman chemical. VECTASTAIN Elite ABC Kit (PK-6101) was purchased from VECTOR laboratories.

siRNA, Plasmid Constructs and Bacterial Strains

For mammalian cell culture studies, plasmids used were ptf LC3 (Kimura et al., 2007; gift from Tamotsu Yoshimori, Addgene #21,074), EGFP-synuclein (Furlong et al., 2000; gift from David Rubinzstein, Addgene number #40,822). For infection studies, strains used were untagged or mCherry plasmid (Addgene #36,084) expressing *S. typhimurium* SL1344 (gift from Prof. C. V. Srikanth, RCB, India). pCMV flag ERRα was a gift from Toren Finkel (Ichida et al., 2002; Addgene plasmid #10,975). ERRα siRNA (L-0, 03, 403–00) and scrambled siRNA (D-001810-10-05) were procured from Dharmacon.

Mammalian Cell Culture, Infection and Autophagy Assays

SH-SY5Y cells were cultured in DMEM-F12 containing 10% FBS (Life technologies). HeLa cells were cultured in DMEM containing 10% FBS (Pan-Biotech). Cell lines were maintained at 37°C and 5% CO_2. The autophagy assays were performed by seeding equal numbers of sub-confluent HeLa or SH-SY5Y cells in 6-well dishes and allowed to attach for 24 h, then treated with XCT 790 (5 μM, **Figure 1**, Supplementary Figures S1A,B) and/or 3-MA (5 mM) and/or lithium chloride (10 mM) in fed

condition for 2 h. After treatments, the cell lysates were analyzed by immunoblotting.

RFP-EGFP-LC3 assay: sub-confluent HeLa and/or SH-SY5Y cells were seeded into 60 mm cell culture dishes, then transfected with ptf LC3 construct and/or siRNA, and allowed to express for 48 h. Cells were trypsinized, seeded again on poly-D-lysine coated cover slips in a 12 or 24 well plates and allowed to attach. After appropriate treatments, the coverslips containing cells were processed for imaging. For immunofluorescent antibody staining, the cover slips were incubated in primary antibody at 4°C for overnight followed by secondary antibody incubation at room temperature.

Intracellular colony forming unit (CFU) assay: *S. typhimurium* SL1344 were grown overnight at 37°C under micro-aerophilic conditions. HeLa cells were infected at a multiplicity of infection (MOI) of 200 for 1 h. The cells were treated with media containing gentamycin (100 μg/ml) for 1 h to kill the extracellular bacteria. The cells were then treated with XCT 790 (10 μM) and incubated further for 4 h. The HeLa cells were lysed using lysis buffer (0.1% SDS, 1% Triton X-100, 1× PBS) and the intracellular *Salmonella* were plated on LB plates, incubated overnight at 37°C, and the CFU was counted.

Immunoblot Analysis

Mammalian cell lysates preparation: after treatments, cells were collected in Laemmli buffer to perform LC3 processing assay, P70S6K, AMPK, ULK1 and 4E-BP1 immunoblotting. Samples were electrophoresed onto SDS-PAGE (8%–15%) and then transferred onto PVDF (Bio-Rad) membrane through Transblot turbo (Bio-Rad). Blots were stained with Ponceau S, and then probed with appropriate primary antibodies at 4°C for overnight and subsequently HRP-conjugated secondary antibody. Signals were attained using enhanced chemiluminescence substrate (Clarity, Bio-Rad) and imaged using a gel documentation system (G-Box, Syngene) and then bands were quantitated using ImageJ software (NIH).

Microscopy

For imaging the mammalian cells, after appropriate treatments, coverslips containing cells were fixed using 4% paraformaldehyde (PFA; Sigma) and then permeabilized using Triton X-100 (0.2%, HiMedia). Coverslips were mounted on slide using antifade, Vectashield mounting medium (Vector Laboratories). For antibody staining, coverslips were blocked using 5% BSA for 1 h at room temperature, then incubated with primary antibody at 4°C, overnight and then subsequently probed with corresponding fluorescent dye conjugated secondary antibody.

Images were acquired using DeltaVision Elite widefield microscope (API, GE) with following filters: FITC (490/20 and 529/38), TRITC (542/27 and 594/45) and Cy5 (632/22 and 676/34). Acquired images were processed using DV softWoRX software.

Cell Viability Assay

SH-SY5Y cells were seeded onto tissue culture treated 96-well plate and then transfected with EGFP-α-synuclein only, and/or co-transfected with siRNA. To cells, appropriate drugs were added (24 h) after 48 h of transfection. Using luminescence-based CellTitre-Glo® (Promega) kit, the cell viability was assayed using automated microtitre plate reader Varioskan Flash (Thermo Scientific).

Animal Studies

All procedures in this study were approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and JNCASR Institutional Animal Ethics Committee and conducted as per their guidelines. Inbred male C57BL/6J mice (3–4 months old) were used for all experimental groups ($n = 6$). The animals were maintained under standard laboratory conditions i.e., temperature 25° ± 2°C, 12 h light: 12 h dark cycle and 50 ± 5% relative humidity with *ad libitum* access to food and water.

MPTP.HCl and XCT 790 Treatment

The mice were distributed into three groups: vehicle, MPTP and MPTP+XCT 790, and injections were administered intraperitoneally. The vehicle group was injected with dimethyl sulfoxide (DMSO) i.e., the solvent. The MPTP group received 23.4 mg/kg MPTP.HCl in 10 ml/kg body weight of saline, administered four times at 2 h interval (Jackson-Lewis and Przedborski, 2007). The MPTP+XCT 790 group mice were injected with 5 mg/kg body weight of XCT 790 dissolved in DMSO, alongside the first MPTP injection. The treatment was continued by administering XCT 790 in "an injection a day regime" for 6 days. All the mice were sacrificed 7 days after MPTP administration and the brains were processed for immunohistochemistry.

Tissue Processing for Immunohistochemistry

The mice were anesthetized using halothane inhalation and perfused intracardially with saline, followed by 4% buffered PFA (pH 7.4). The brains were removed quickly and post fixed in the same buffer for 24 h to 48 h at 4°C and cryoprotected in an increasing gradient of sucrose. Coronal midbrain cryosections of 40 μm thick were collected serially on gelatinized slides. Every sixth midbrain section was used for immunostaining.

Immunoperoxidase Staining of Tyrosine Hydroxylase (TH)

The immunoperoxidase labeling protocol was a slight modification of that reported earlier (Vidyadhara et al., 2016). Briefly, the endogenous expression of peroxidase was quenched using 0.1% H_2O_2 in 70% methanol, followed by blocking of non-specific staining by 3% buffered solution of bovine serum albumin for 4 h at room temperature. The sections were then incubated with the rabbit polyclonal anti-TH antibody (1:800, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by anti-rabbit secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA, USA). The tertiary labeling was performed using avidin–biotin complex solution (1:100, Elite ABC kits; Vector Laboratories, Burlingame, CA, USA).

The staining was visualized using 0.05% solution of DAB, in 0.1 M acetate imidazole buffer (pH 7.4) with 0.1% H_2O_2. Phosphate buffered saline (0.01 M) containing 0.3% Triton X-100 (0.01 M PBST, pH 7.4) was used as both diluent and washing buffer. Appropriate negative controls were processed identically.

Stereological Quantification of TH-Immunoreactive (TH-ir) Neurons at SNpc

Stereological quantification of TH-ir dopaminergic neurons was performed using optical fractionator probe (Vidyadhara et al., 2016, 2017). The SNpc was delineated on every sixth TH-ir midbrain section (Fu et al., 2012) using 4× objective of the Olympus BX61 Microscope (Olympus Microscopes, Japan) equipped with StereoInvestigator (Software Version 7.2, MicroBrightfield Inc., Colchester, VT, USA). The cells were counted using oil immersion lens (100×), with a regular grid interval of 22,500 μm^2 (x = 150 μm, y = 150 μm) and counting frame of 3600 μm^2 (x = 60 μm, y = 60 μm). The mounted thickness averaged to 25 μm. A guard zone of 4 μm was implied on either side, thus, providing 17 μm of z-dimension to the optical dissector. The quantification was performed starting with the first anterior appearance of TH-ir neurons in SNpc to the caudal most part in both hemispheres and added to arrive at the total number. The volume of SNpc was estimated by planimetry.

Densitometry Based Image Analysis

The offline evaluation of TH expression was performed on high magnification images of TH immunostained nigral dopaminergic neurons using Q Win V3 (Leica Systems, Germany); a "Windows" based image analysis system (Alladi et al., 2010; Vidyadhara et al., 2017). A cumulative mean was derived from the values obtained from sampling approximately 200 dopaminergic neurons per animal and expressed as gray values on a scale of 0–255, where "255" meant absence of staining and "0" equaled intense staining.

Immunofluorescence Based Double Staining of SNpc Dopaminergic Neurons

The sequential immunolabeling procedure was used to co-label the TH and LC3 and/or A11 (Alladi et al., 2010). First, the midbrain sections were equilibrated with 0.1 M PBS (pH 7.4) for 10 min and then incubated with buffered bovine serum albumin (3%) for 4 h to block non-specific epitopes. Then, the sections were incubated in rabbit anti-LC3 antibody (1:1000) and/or anti-oligomer antibody (A11, 1:1000) for 72 h at 4°C. After subsequent washes, the sections were incubated in corresponding fluorescent secondary antibody (1:200) overnight at 4°C. Co-labeling with TH was performed on the same sections using rabbit anti-TH antibody (1:500), followed by secondary labeling. PBST (0.01 M, pH 7.4) was used as both working and washing buffer. The sections were then mounted using Vectashield mounting medium.

Behavioral Studies

Open Field and Rotarod experiments were performed using 3–4-month-old C57BL/6J male mice. The experimental procedures were modified from references: Brooks and Dunnett (2009), Patil et al. (2014) and Liu et al. (2015). All the animals were handled for three consecutive days by experimenters prior to the start of training session. Mice were habituated to behavior room (light intensity maintained at 100 lux) for 15 min before the commencement of handling, training and tests. The health of each mouse was monitored every day before training or test sessions by recording their body weight. The behavioral experiments were scheduled in such a way that the fatigue of one behavioral test does not affect the other. Thus, Open Field trials (low stress activity) were done in forenoon, whereas, Rotarod trials (high stress activity) were performed in the afternoon. Double blind approach was taken in which the experimenters were unaware of the drug treatment given to the animals as well as the results of data analysis. Data were represented as bar diagram using GraphPad prism 5 software.

Open Field Trials

Open Field tests were done in a wooden box (Open Field arena) of dimensions: 50 cm × 50 cm × 45 cm in which the internal surface was coated with white polish. The Open Field arena was custom made in JNCASR. Mice were trained in an Open Field arena for two consecutive days prior to the day of actual tests. During training or tests, a single mouse was left in the Open Field arena for 5-min to explore. The activity of mouse was recorded with the help of a digital camera (SONY® color video camera, Model no. SSC-G118) supported by the software SMART v3.0.04 (Panlab Harvard Apparatus, Holliston, MA, USA). At the end of 5-min, the mouse was removed and returned to its home cage. The Open Field arena was cleaned with 70% ethanol and dried before placing the next mouse in it. The distance traveled in the zone periphery was calculated by SMART v3.0.04 software (Panlab Harvard Apparatus, Holliston, MA, USA). The data were compared, analyzed and represented as bar diagrams by using the software GraphPad prism5.

Rotarod Trials

Rotarod was custom made at the mechanical workshop, National Centre for Biological Sciences, Bengaluru, India. The instrument includes a textured horizontal rod (diameter 3.3 cm) made of Delrin. The rod was fixed at a height of 30 cm above a platform, which was cushioned for comfortable fall of mice from rotating rod. The rod was divided into three small areas (9.3 cm each) by fitting two circular discs (diameter 40 cm) made up of Teflon to allow three mice, completely shielded from each other, to be trained simultaneously. The speed of rotation was incremented from 5 rpm to 20 rpm manually by an electric motor fitted in the instrument. The mice were trained for five consecutive days prior to the day of injection. The training was given by gradually increasing the speed of the rod from lower to higher rpm by accelerating at the rate of 1 rpm/5 s. On 1st day, the mice were trained at 5–10 rpm, on 2nd day 7–12 rpm, on 3rd day 13–20 rpm and at 20 rpm (fixed) on 4th and 5th day. During training and tests, mice were placed on the non-rotating rod and

were allowed to balance themselves before increasing the speed manually. For every rpm, the mice were given a stabilization period of 5 s. Mice that failed to learn to stabilize on the rod were given two more chances. Once the stabilization period was completed successfully, the mice were allowed to run on the rotating rod for 60 s at the respective rpm. The entire Rotarod trial was recorded on a digital camera (SONY® HDR-CX405) and the latency to fall was calculated manually. The data obtained were compared, analyzed and plotted as mean latency to fall in the form of bar diagrams using GraphPad prism5.

Statistical Analysis

One-way or Two-way ANOVA followed by Bonferroni's *post hoc* test was applied to derive statistical significance. Values were expressed as mean ± SEM.

RESULTS

Modulation of Mammalian Aggrephagy and Xenophagy by XCT 790

Toxic protein aggregates and intracellular pathogens are known to be substrates of the autophagy pathway for their effective cellular degradation (Deretic and Levine, 2009; Nixon, 2013). Based on a previous small molecule screening for aggrephagy inducers from our laboratory (Suresh et al., 2017), we identified thiadiazoleacrylamide, XCT 790, as a drug-like molecule that abrogates α-synuclein cellular toxicity, and intracellular *Salmonella typhimurium*. We tested the potential of XCT 790 to clear toxic α-synuclein protein aggregates, and *Salmonella* burden through autophagy in mammalian cells such as human neuroblastoma SH-SY5Y and HeLa cell lines.

To test the modulation of mammalian autophagy and its flux by XCT 790, we used immunoblot analysis based LC3 (autophagosome marker) and microscopy-based tandem RFP-EGFP-LC3 assays. In tandem RFP-EGFP-LC3 assay, XCT 790 treatment significantly induced autophagosomes and autolysosomes formation in both SH-SY5Y (control vs. XCT 790 treated, autophagosomes, ∼2-fold, $P < 0.05$; autolysosomes, ∼4-fold, $P < 0.01$ Supplementary Figure S2A) and HeLa cells (control vs. XCT 790 treated, autophagosomes, ∼5-fold, $P < 0.001$; autolysosomes, ∼2-fold, $P < 0.001$ **Figure 1B**). Additionally, XCT 790 treatment enhanced accumulation of LC3-II levels indicating the induction of autophagy (∼2.5-fold, untreated vs. XCT 790, $P < 0.001$, **Figure 1A**). These results clearly demonstrated that XCT 790 modulates mammalian autophagy.

We then addressed whether XCT 790 protects SH-SY5Y cells from EGFP-α-synuclein mediated toxicity. Overexpression of EGFP-α-synuclein in SH-SY5Y cells was toxic leading to significant cell death as measured by cell viability assay (∼4-fold, vector control or untransfected vs. α-syn transfected, **Figure 1C**). Upon administration of XCT 790 to cells overexpressing EGFP-α-synuclein, the cell viability increased significantly than that of untreated cells (∼4-fold, α-syn over expressed cells, untreated vs. XCT 790 treated, $P < 0.001$, **Figure 1C**) and comparable to that of vector control (vector control vs. α-syn over expressed cells XCT 790 treated, ns, $P > 0.05$, **Figure 1C**). We observed that the potential of XCT 790 to protect cells from EGFP-α-synuclein toxicity is abrogated in presence of pharmacological autophagy inhibitor, 3-MA (α-syn over expressed cells, XCT 790 vs. XCT 790 + 3-MA, ∼4-fold, $P < 0.001$, **Figure 1C**) that is comparable to that of XCT 790 untreated cells (α-syn over expressed cells, untreated vs. XCT 790 + 3-MA, ns, $P > 0.05$, **Figure 1C**). These results clearly demonstrate that XCT 790 protects human neuroblastoma cells from EGFP-α-synuclein mediated toxicity in an autophagy dependent manner.

We also checked if XCT 790 clears the intracellular bacterial cargo. Upon XCT 790 treatment, we observed significant reduction in the intracellular *S. typhimurium* SL1344 compared to untreated (∼2-fold, untreated vs. XCT 790 treated, $P < 0.001$, **Figure 1D**). We also observed increased recruitment of autophagy adaptor protein p62 and autophagosome membrane marker LC3 to *S. typhimurium* SL1344 (**Figure 1E**).

This study identifies XCT 790 as an autophagy inducer with a potential to clear toxic protein aggregates. We demonstrate that XCT 790 exerts protection to the cells against EGFP-α-synuclein mediated toxicity by inducing autophagy which helps clear the toxic aggregates.

XCT 790 Modulates Autophagy Through an MTOR Independent Pathway

Autophagy is regulated by MTOR-dependent and MTOR-independent pathways that are amenable to chemical perturbations (Kim et al., 2011). To delineate the mechanism of autophagy modulation by XCT 790, we examined the activity of MTOR through monitoring its substrates such as P70S6K and 4EBP1. Upon XCT 790 treatment, MTOR activity was unaffected as revealed by its substrates such as phospho-P70S6K and phospho-4EBP1 protein levels which were comparable to that of nutrient rich condition (**Figure 1F**; Supplementary Figure S2B). In contrast, the levels of phospho-P70S6K and phospho-4EBP1 were attenuated under starvation conditions where autophagy was regulated in an MTOR-dependent manner. Lithium Chloride (10 mM) is known to induce autophagy through an MTOR independent mechanism served as a positive control (Sarkar et al., 2005; **Figure 1F**, Supplementary Figure S2B). These observations asserted that XCT 790 is MTOR independent autophagy modulator.

We further examined whether XCT 790 exerts its effect through AMPK pathway, one of the predominant MTOR-independent mechanisms known to regulate autophagy. It was observed that treatment of XCT 790 for 2 h did not affect the activity of AMPK, as evident by the unchanged T172 phosphorylation of AMPK (**Figure 1G**) when compared to nutrient rich conditions. AMPK promotes autophagy in an MTOR-independent manner by directly activating ULK1 through phosphorylation of Ser 555 (S555). Whereas, under nutrient sufficiency, high MTOR activity inhibits ULK1 activation by phosphorylating ULK1 at Ser 757 (S757) and disrupting the interaction between ULK1 and AMPK (Kim et al., 2011). Therefore, we further examined the regulation of

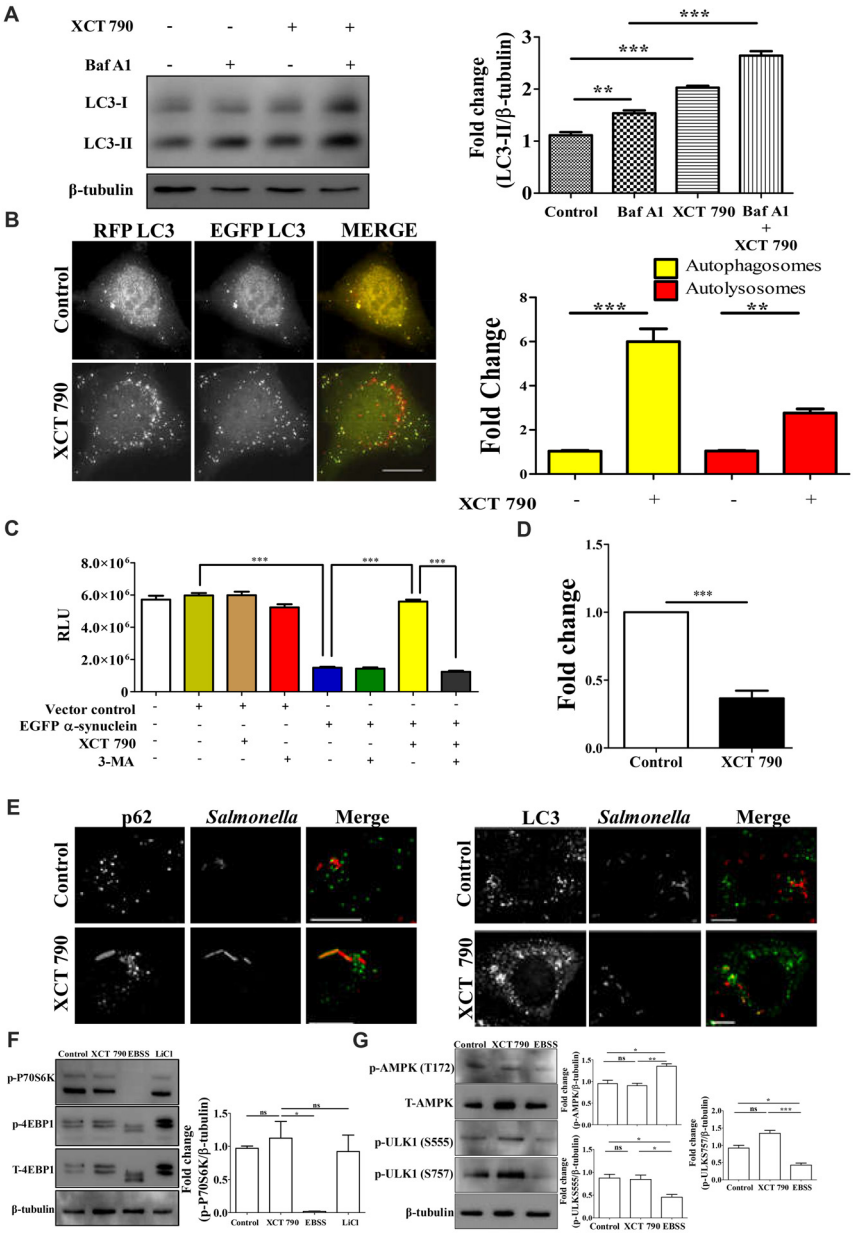

FIGURE 1 | XCT 790 modulates aggrephagy and xenophagy in mammalian cells. **(A)** Representative Western blot of LC3 processing assay in SH-SY5Y cells treated with XCT 790 (2 h) under growth condition and normalized LC3-II levels were quantified ($n = 3$). β-tubulin was used as a loading control. Statistical analysis was performed using one-way ANOVA and *post hoc* Bonferroni test. Error bars, mean ± SEM. ns-non significant, $^{**}P < 0.01$, $^{***}P < 0.001$. **(B)** Representative microscopy images of tandem RFP-EGFP-LC3 assay in HeLa cells treated with XCT 790 for 2 h. Yellow puncta was autophagosomes and red was autolysosomes. Fold change in autophagosomes and autolysosomes by XCT 790 were quantified ($n = 50$ cells and three independent experiments). Scale bar was 15 μm. Statistical analysis was performed using one-way ANOVA and *post hoc* Bonferroni test. Error bars, mean ± SEM. ns-non significant, $^{**}P < 0.01$, $^{***}P < 0.001$. **(C)** Graph indicating the cell viability read out of SH-SY5Y overexpressing EGFP-α-synuclein treated with XCT 790 in presence of pharmacological autophagy inhibitor 3-MA. Cell viability was analyzed using CellTitre Glo (Promega) assay. More RLU readout was indicative of more cell viability and vice-versa (three independent experiments). Statistical analysis was performed using one-way ANOVA and *post hoc* Bonferroni test. Error bars, mean ± SEM. $^{***}P < 0.001$. **(D)** Graph for colony forming unit (CFU)s indicating the intracellular burden of *S. typhimurium* treated with XCT 790 (10 μM) for 6 h. CFU represent survival of *S. typhimurium* within the host cells. Fold change between the untreated and XCT 790 treated samples were quantified (three independent experiments). Statistical analysis was performed using two-tailed paired *t*-test. Error bars, mean ± SEM. ns-non significant, $^{**}P < 0.01$, $^{***}P < 0.001$. **(E)** Representative microscopy images of HeLa cells infected with mCherry expressing *S. typhimurium* treated with XCT 790 for 6 h. Cells were immunostained for either p62 or LC3 in untreated and XCT 790 treated samples ($n = 25$ cells and three independent experiments). Scale bar 10 μm. **(F)** Representative Western blots of MTOR substrates—P70S6K (phospho and total form) and 4EBP1 (phospho and total form)—regulation by various treatments like XCT 790, EBSS and LiCl. β-tubulin was used as a loading control. Normalized p-P70S6K levels were quantified for three independent experiments. Statistical analysis was performed using one-way ANOVA and the *post hoc* Bonferroni test. Error bars,

(*Continued*)

FIGURE 1 | Continued
mean ± SEM. ns-non significant, $^{*}P < 0.05$. **(G)** Representative Western blots of signaling pathway proteins like AMPK (phospho and total form) and ULK1 (phospho and total form) regulation by XCT 790 and EBSS. Normalized p-AMPK, p-ULK1 (S555), p-ULK1 (757) levels were quantified for three independent experiments. β-tubulin was used as a loading control. Statistical analysis was performed using one-way ANOVA and the *post hoc* Bonferroni test. Error bars, mean ± SEM. ns-non significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.β-tubulin was used as a loading control. Concentrations of XCT 790, 3-MA and LiCl used were 5 μM, 100 nM and 10 mM.

levels of activating (S555) and inhibitory (S757) phosphorylation of ULK1 by XCT 790. Consistent with unchanged levels of phosphorylated AMPK after treatment with XCT 790 for 2 h, the downstream phosphorylation of ULK1 at S555 was unaffected and comparable to the nutrient rich conditions (**Figure 1G**). This suggests that XCT 790 does not exert its effects through AMPK pathway. Importantly, MTOR-dependent phosphorylation of ULK1 at S757 remained unaltered in XCT 790-treated cells unlike in starvation conditions, where a concomitant decrease in the phospho-ULK1 S757 protein levels is observed. These results further confirm that XCT 790 acts through an MTOR-independent mechanism but not through AMPK pathway.

XCT 790 Induces Autophagy Through Regulation of Estrogen-Related Receptor Alpha (ERRα)

XCT 790 was found to be the first potent and selective inverse agonist of ERRα (Busch et al., 2004). To elucidate the role of ERRα in contributing to the function of XCT 790 as autophagy inducer, we used the following two approaches: (a) siRNA-based silencing of ERRα; and (b) over expression of ERRα.

To evaluate the level at which the knockdown exerted its effect, cells were transfected with siRNAs targeting ERRα. A non-targeting pool was used as a control. The effects of knockdown on regulation of autophagy by ERRα 48 h post-transfection were monitored by microscopy-based tandem RFP-EGFP-LC3 assays. Knockdown efficiency was confirmed by Western blotting to be around 80% (Scrambled vs. ERRα siRNA, $P < 0.001$, **Figure 2A**). Consistent with the effect of XCT 790, knockdown of ERRα also resulted in a significant induction of autophagosomes (∼5-fold, Scrambled vs. ERRα siRNA treated, $P < 0.001$, **Figure 2B**) and autolysosomes (∼3-fold, Scrambled vs. ERRα siRNA treated, $P < 0.001$, **Figure 2B**). Autophagosome and autolysosome numbers in XCT 790 treated and ERRα downregulated cells were found to be comparable. These results suggested that XCT 790 modulated autophagy through ERRα.

We addressed this question through another approach to understand the autophagy modulation upon overexpression of ERRα. In ERRα over expressed cells, we found more autophagosomes (∼2-fold, $P < 0.01$, ERRα overexpressed vs. untreated) and less autolysosomes (∼2-fold, $P < 0.01$, ERRα overexpressed vs. untreated) than that of control (**Figures 2D,E**). From this, we could interpret that autophagy was inhibited at its autophagosome to lysosome fusion step upon over expression of ERRα. When XCT 790 was treated in ERRα over expressed cells, more autophagosomes (∼2-fold, $P < 0.01$, ERRα overexpressed vs. untreated) and less autolysosomes (∼2-fold, $P < 0.01$, ERRα overexpressed vs. untreated) than that of untreated were found (**Figures 2D,E**). This autophagic scenario was similar to that of only ERRα over expressed cells (Autophagosomes; ERRα over expressed + XCT 790 vs. ERRα overexpressed only, ns, $P > 0.05$ and Autolysosomes; ERRα over expressed + XCT 790 vs. ERRα overexpressed only, ns, $P > 0.05$, **Figures 2D,E**). When ERRα was over expressed, the autophagic modulating ability of XCT 790 was indeed abrogated.

Collectively, these results suggest that XCT 790 modulates autophagy through ERRα.

ERRα Regulates Autophagy by Localizing Onto Autophagosomes

From knock down and over expression of ERRα studies, there was a clear indication that ERRα could modulate autophagy pathway. Autophagy was induced upon ERRα downregulation (**Figures 2B,C**) but inhibited when over expressed (**Figures 2D,E**). We examined whether active transcription was required for autophagic function of XCT 790. Upon XCT 790 treatment in the presence of actinomycin D, the autophagosomes and autolysosomes were similar to that of only XCT 790 (XCT 790 + Act D vs. XCT 790 only, $P > 0.05$, Supplementary Figures S3A,B). This result indicates that autophagic activity of XCT 790 remained unaffected when active transcription was inhibited. Subsequently, we attempted to know whether ERRα localizes to autophagic related structures such as autophagosomes and autolysosomes. Pearson's Colocalization Coefficient (PCC) of ERRα with autophagosomes (∼0.85) were found to be significantly more than that with autolysosomes (∼0.3) under nutrient rich condition (∼2.5-fold, autophagosomes vs. autolysosomes, $P > 0.001$, **Figures 3A,B**). In basal autophagy conditions, colocalization of ERRα with autophagosomes was significantly reduced in ERRα silenced and XCT 790 treated cells (∼3.5-fold, untreated or scrambled siRNA vs. ERRα siRNA, $P < 0.001$, **Figures 3A,B**). Significantly more ERRα colocalized with autophagosomes when ERRα was over expressed (control or scrambled siRNA vs. ERRα over expressed, $P < 0.001$, **Figures 3A,B**). Colocalization of ERRα with autolysosomes was not regulated compared to that of control (control or scrambled siRNA vs. ERRα siRNA or XCT 790 or ERRα over expressed, $P > 0.05$, **Figures 3A,B**) suggesting that ERRα might not interact with the autolysosomes. ERRα could localize, most likely, to autophagosomes than autolysosomes through its non-canonical LIR motif that might interact with LC3 to localize onto autophagosomes.

These results suggest that, perhaps, ERRα might regulate autophagy through its localization with the autophagosomes.

XCT 790 Alleviates MPTP Induced Dopaminergic Neuronal Loss

A significant proportion of dopaminergic neurons in Substantia Nigra pars compacta (SNpc) were lost after MPTP treatment (∼68%, MPTP vs. Vehicle, $P < 0.001$, **Figures 4A,B**,

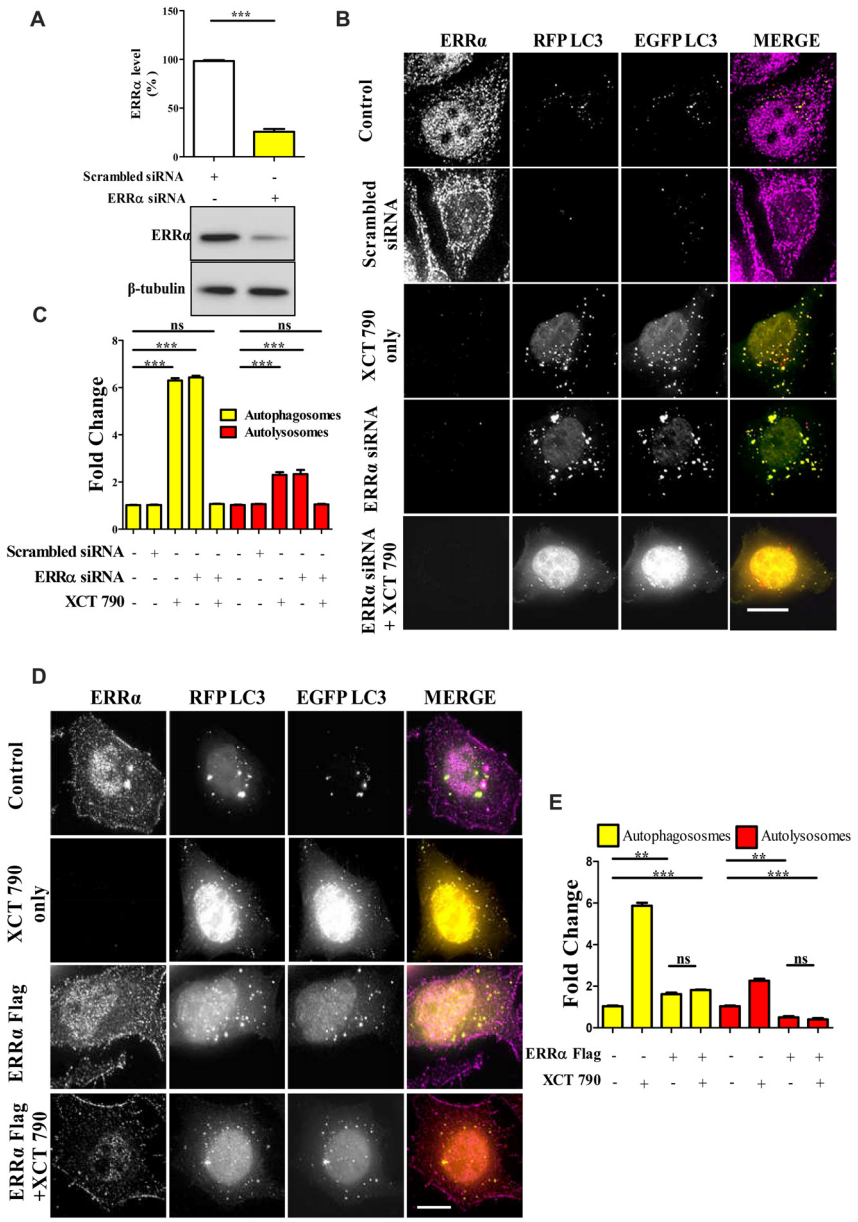

FIGURE 2 | XCT 790 modulates autophagy through estrogen related receptor α (ERRα). **(A)** ERRα protein levels after transfecting either scrambled siRNA (100 picomoles) or ERRα siRNA (100 picomoles) for 48 h in HeLa cells was analyzed by Western blotting and then quantified ($n = 3$). β-tubulin was used as a loading control. Statistical analysis was performed using two-tailed paired *t*-test. Error bars, mean ± SEM. $^{***}P < 0.001$. **(B,C)** Microscopy images **(B)** of tandem RFP-EGFP-LC3 assay in XCT 790 treated HeLa cells (2 h) post ERRα siRNA transfection (48 h). Cells were immunostained for ERRα in various treatments. Scale bar was 15 μm. Quantification **(C)** of autophagosomes (yellow puncta) and autolysosomes (red puncta) modulated by XCT 790 treatment in ERRα siRNA transfected cells ($n = 50$ cells and three independent experiments). Statistical analysis was performed using one-way ANOVA and the *post hoc* Bonferroni test. Error bars, mean ± SEM. ns-non significant, $^{***}P < 0.001$. **(D,E)** Microscopy images **(D)** of tandem RFP-EGFP-LC3 assay in XCT 790 treated HeLa cells (2 h) post ERRα Flag transfection (48 h). Cells were immunostained for ERRα in all treatment groups. Scale bar used was 15 μm. Quantification **(E)** of autophagosomes (yellow puncta) and autolysosomes (red puncta) modulated by XCT 790 treatment in ERRα Flag transfected cells ($n = 50$ cells and three independent experiments). Statistical analysis was performed using one-way ANOVA and the *post hoc* Bonferroni test. Error bars, mean ± SEM. ns-non significant, $^{**}P < 0.01$, $^{***}P < 0.001$. Concentration of XCT 790 used was 5 μM.

Supplementary Figure S4A) as previously described (Jackson-Lewis and Przedborski, 2007). Co-administration of XCT 790 with MPTP, however alleviated this loss by 80% (MPTP+Co vs. Vehicle, $P < 0.05$; XCT 790 vs. MPTP, $P < 0.01$, **Figures 4A,B**). In a congruent manner, volume of SNpc reduced significantly after MPTP injection (MPTP vs. Vehicle, $P < 0.01$, Supplementary Figure S4C), whereas the shrinkage was prevented by approximately 85% when MPTP and XCT

790 were administered together (MPTP+Co vs. MPTP, $P < 0.01$, **Figure 4B**, Supplementary Figure S4C).

Cellular Tyrosine Hydroxylase (TH) Expression Was Preserved in XCT 790 Co-treatment Group

The cellular TH expression of individual TH-immunoreactive (TH-ir) dopaminergic, as measured by densitometry, was significantly reduced in surviving neurons in MPTP group (MPTP vs. Vehicle, $P < 0.001$, Supplementary Figure S4B). TH expression in the nigral neurons of MPTP and XCT 790 co-treated mice was comparable to that of the vehicle control group. Thus, XCT 790 significantly alleviated the MPTP-induced depletion of cytoplasmic TH expression (MPTP+Co vs. MPTP, $P < 0.001$, Supplementary Figure S4B).

XCT 790 Enhances Autophagy and Clears Toxic Protein Aggregates in an *in Vivo* Mouse Model of PD

In neurons, the autophagy process is indispensable for clearing the misfolded toxic protein aggregates (Hara et al., 2006). During the neurodegenerative progression, autophagy would be defunct and becomes incompetent to maintain cellular proteostasis (Nixon, 2013). To delineate the mechanism of neuroprotective action of XCT 790, we examined the autophagy status in the various mice treatment cohorts. Our cell lines result strongly indicates that XCT 790 might exert neuroprotection through modulating autophagy. In MPTP toxicity model, the LC3 puncta per neuron was reduced significantly than that of vehicle treated (∼0.8-fold, vehicle vs. MPTP treated, $P < 0.01$, **Figures 4C,D**) indicating the dysfunctional autophagy during neurodegenerative disease progression. Interestingly, XCT 790 only cohort exhibited significantly increased LC3 puncta per cell compared to that of vehicle treated cohort (∼3-fold, vehicle vs. XCT 790 only, $P < 0.001$, **Figures 4C,D**). This demonstrated that XCT 790 is a strong autophagy inducer in the dopaminergic neurons of SNpc. We observed significantly increased LC3 puncta per cell in the MPTP and XCT 790 co-administered than that of vehicle treated cohort (∼3-fold, vehicle vs. MPTP+Co, $P < 0.001$, **Figures 4C,D**). These results demonstrate that XCT 790 could induce autophagy in the SNpc of brain and remarkably surpass the autophagic deficit caused due to pathogenesis.

During protein aggregation, the toxic misfolded protein oligomeric species is shown to be accumulated in the neurons (Luk et al., 2012). We examined whether autophagy induction by XCT 790 could clear the toxic oligomeric intermediates in the neurons. In a vehicle treated cohort, the occurrences of aggregates were significantly less compared to that of MPTP treated cohort (∼6.5-fold, vehicle vs. MPTP treated, $P < 0.001$, **Figures 4E,F**). These observations reaffirming that toxic misfolded protein aggregates are formed during disease pathology. Upon co-administration of MPTP along with XCT 790, we observed a significant reduction in the toxic aggregates compared to that of MPTP only treated cohort (∼6-fold, MPTP vs. MPTP+Co, $P < 0.001$, **Figures 4E,F**). We found that aggregate reduction in the MPTP+Co cohort were comparable to that of vehicle treated cohort (MPTP+Co vs. vehicle, ns, $P > 0.05$. **Figures 4E,F**) indicating its strong potential to clear

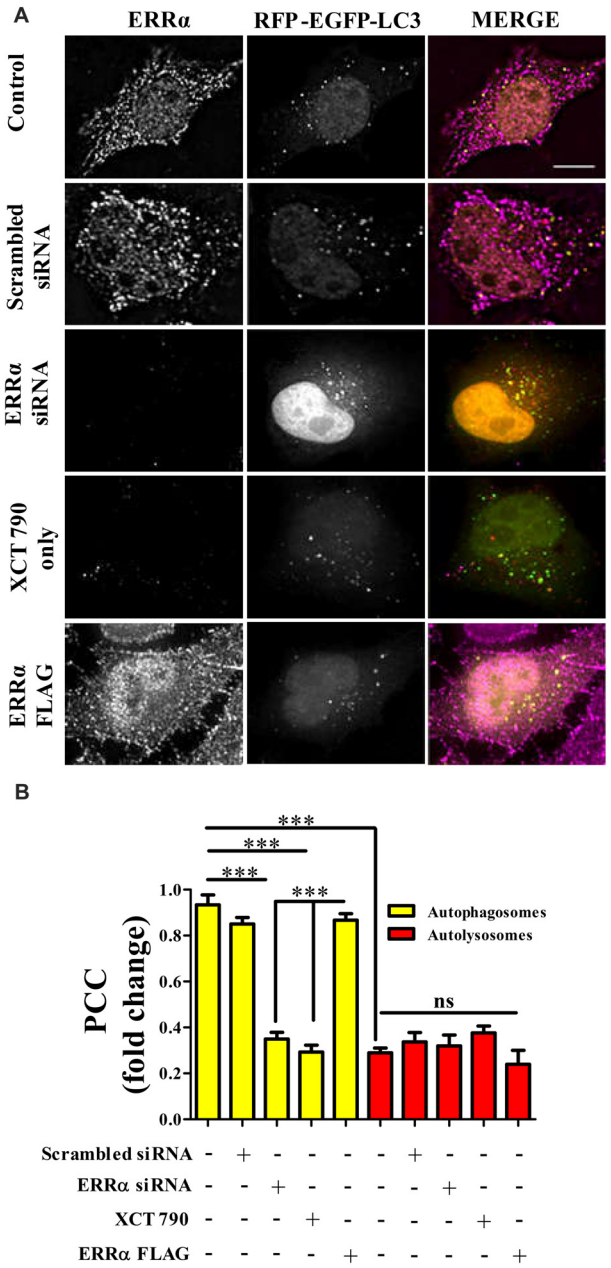

FIGURE 3 | ERRα localizes onto autophagosomes to modulate autophagy. **(A)** Microscopy images of tandem RFP-EGFP-LC3 assay in HeLa cells transfected (48 h) with either ERRα siRNA or ERRα Flag treated with XCT 790 for 2 h. Cells were immunostained for ERRα. $n = 50$ cells and three independent experiments were performed. Scale bar was 15 μm. **(B)** Pearson's colocalization coefficient (PCC) analyses of ERRα with either autophagosome (yellow) or autolysosomes (red) in HeLa cells transfected (48 h) with either ERRα siRNA or ERRα Flag treated with XCT 790 for 2 h were plotted. Statistical analysis was performed using one-way ANOVA and *post hoc* Bonferroni test. Error bars, mean ± SEM. ns-non significant, $^{***}P < 0.001$.

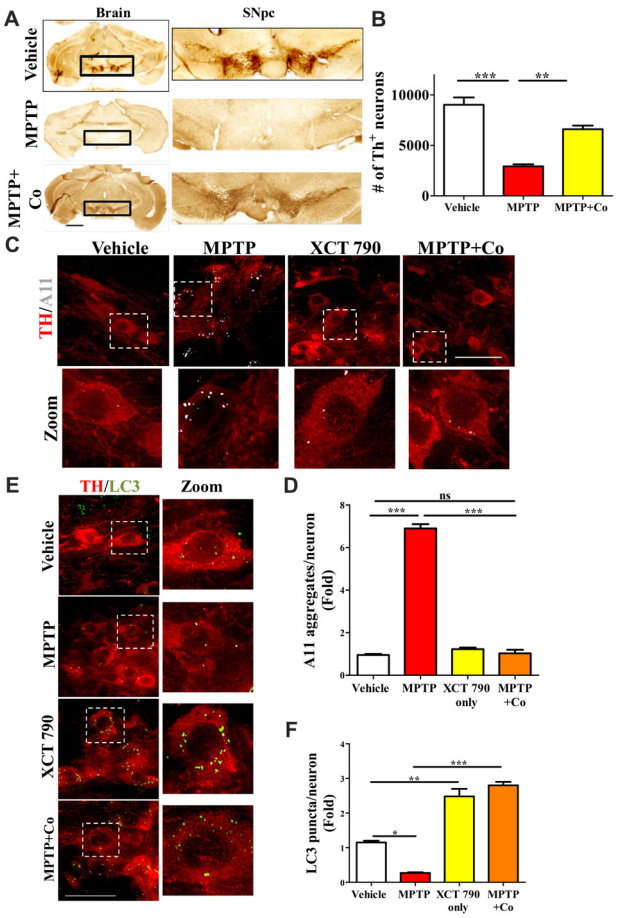

FIGURE 4 | XCT 790 is neuroprotective by degrading toxic protein aggregates through inducing autophagy in dopaminergic neurons of midbrain of mice. **(A)** Representative photomicrographs of whole brain and SNpc for various cohorts namely vehicle, MPTP (23.4 mg/kg of body weight) and MPTP+Co (Co-administration of MPTP and XCT 790: MPTP; 2 mg/kg of body weight and XCT 790; 5 mg/kg of body weight, $n = 4$ animals per cohort). Scale bar is 600 μm. **(B)** Graph representing the unbiased stereological quantification of tyrosine hydroxylase (TH)-ir dopaminergic neurons in SNpc for above mentioned cohorts. Statistical analysis was performed using one-way ANOVA and the *post hoc* Bonferroni test. Error bars, mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$. **(C)** Representative IHC photomicrographs of SNpc dopaminergic neurons double stained for A11 (toxic oligomer marker) and TH (dopaminergic marker) antibodies for the above-mentioned cohorts ($n = 4$ animals per cohort). Scale bar is 50 μm. **(D)** Plot indicating the A11 puncta per dopaminergic neuron in SNpc was quantitated for all cohorts. Statistical analysis was performed using one-way ANOVA and the *post hoc* Bonferroni test. Error bars, mean ± SEM. ns-non significant, $^{***}P < 0.001$. **(E)** Representative fluorescent IHC photomicrographs of dopaminergic neurons in SNpc double stained for LC3 (autophagy marker) and TH (SNpc marker) antibodies for various cohorts namely vehicle, MPTP, XCT only and MPTP+Co ($n = 4$ animals per cohort). Scale bar is 50 μm. **(F)** Graph representing the LC3 puncta per neuron for various cohorts. Statistical analysis was performed using one-way ANOVA and the *post hoc* Bonferroni test. Error bars, mean ± SEM. ns-non significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

misfolded toxic protein aggregates. In addition, the presence of aggregates in the steady state level of cell in the XCT 790 only was comparable to that of vehicle cohort (vehicle vs. XCT 790 only, ns, $P > 0.05$, **Figures 4E,F**). This result indicates that administrated dosage regimen was not exerting any proteotoxic stress to the neurons. We demonstrated that XCT 790 could clear the pathological toxic misfolded protein aggregates upon disease progression, one of the main causative of neurodegeneration.

Mechanistically, XCT 790 exerts neuroprotection by clearing misfolded protein aggregates through inducing autophagy as demonstrated in the *in vivo* preclinical mouse model of PD.

XCT 790 Ameliorated MPTP-Induced Behavioral Impairments

PD patients exert movement disorder symptoms such as motor co-ordination, exploration and locomotion disabilities that can be recapitulated in a MPTP mice toxicity model. As our data shows neuro-protective role of XCT 790 at both cellular and tissue level, we wished to test whether its effect can be translated up to behavioral level. To address this, we performed a set of well-known behavioral experiments—Rotarod and Open Field

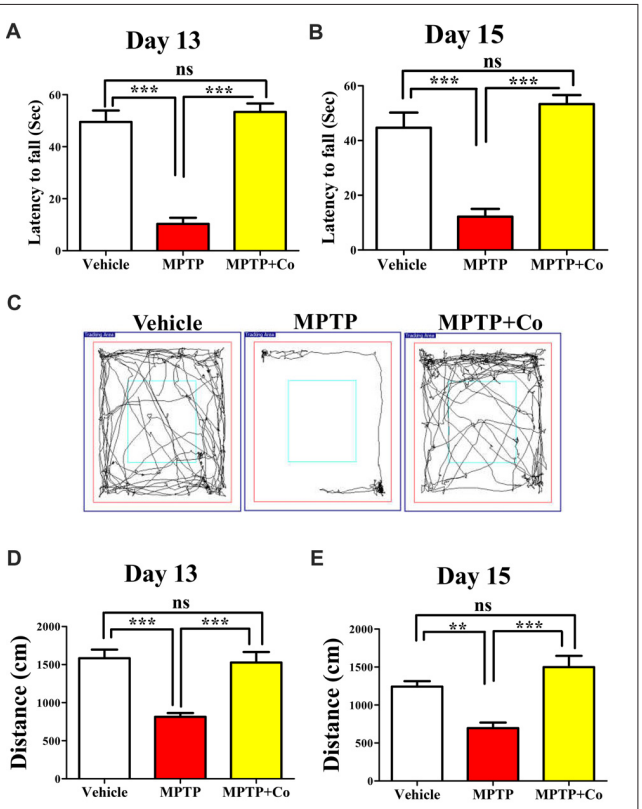

FIGURE 5 | XCT 790 ameliorates MPTP-induced behavioral impairments. Latency to fall for various cohorts such as vehicle, MPTP and MPTP+Co on both day 13 **(A)** and 15 **(B)** were monitored using Rotarod test ($n = 8$ animals per cohort). Statistical analysis was performed using one-way ANOVA and the *post hoc* Bonferroni test. Error bars, mean ± SEM. ns-non significant, $^{***}P < 0.001$. **(C)** Representative trajectory maps were indicated for all the mentioned cohorts. **(D,E)** Plots indicating the peripheral distance traveled by mice were assessed through Open Field test on both day 13 **(D)** and 15 (**E**; $n = 8$ animals per cohort). Statistical analysis was performed using one-way ANOVA and the *post hoc* Bonferroni test. Error bars, mean ± SEM. ns-non significant, $^{**}P < 0.01$, $^{***}P < 0.001$.

tests—specific for assaying the movement disorders. The scheme for behavior assay is illustrated in Supplementary Figure S5.

To test the exploratory ability of mice, the distance traveled in periphery zone of open field arena was compared across different cohorts. We observed that distance traveled in the zone periphery was drastically reduced in MPTP treated cohort compared to that of vehicle treated cohort (MPTP vs. vehicle control, $P < 0.001$, **Figures 5D,E**) on both day 13 and day 15, validating the MPTP's effect on exploratory ability. Upon co-administration of XCT 790 along with MPTP, the distance traveled were significantly longer than that of MPTP cohort (Co vs. MPTP cohort, $P < 0.001$, **Figures 5D,E**), and, more importantly, comparable to that of vehicle treated cohort on both day 13 and day 15 (Co vs. vehicle control, $P > 0.05$, **Figures 5D,E**). Importantly, exploratory behavior of various cohorts was evident in the represented trajectory maps (**Figure 5C**).

Rotarod test, another standard behavioral assay to test motor co-ordination, was also employed. In this test, the time spent by mice on a horizontal rotating rod (latency to fall) was used to assess the motor co-ordination ability across different cohorts. In parallel to the results observed in Open Field, the co-treated cohort showed improved latency to fall compared to that of MPTP treated cohort (Co vs. MPTP cohort, $P < 0.001$, **Figures 5A,B**), which showed reduced latency to fall against vehicle treated cohort on both day 13 and day 15 (MPTP vs. vehicle control, $P < 0.001$, **Figures 5A,B**). In addition, the results of vehicle treated, and co-treated cohorts were fairly comparable (Co vs. vehicle control, $P > 0.05$, **Figures 5A,B**) on both days.

Therefore, these results demonstrate restoration of exploratory and motor coordination abilities in MPTP toxicity model, upon administration of XCT 790. Therefore, XCT 790 ameliorates the behavioral disabilities of MPTP treated mouse model.

DISCUSSION

Currently, there are no therapeutic interventions available for neurodegenerative diseases. New strategies to curb neurodegeneration involve identification of druggable targets, and novel small molecules are necessary. In this study, we identified small molecule XCT 790 as a novel ERRα mediated autophagy modulator that ameliorates α-synuclein toxicity and exerts neuroprotection in a preclinical mouse model of PD.

Lewy bodies are primarily due to the aggregation of misfolded proteins such as α-synuclein that exert cellular toxicity leading to neuronal death (Wakabayashi et al., 2007). Such aggregates result in perturbation of cellular homeostasis due to exaggerated proteotoxicity that trigger apoptosis and eventual loss of neurons (Hara et al., 2006). In addition, cellular proteostasis efficacy and also compensatory action among protein quality control machineries decline with age; as a result the relatively non-dividing neuronal population are more susceptible to proteostatic insult (Anglade et al., 1997). Crucial protein quality control pathways like autophagy are impaired in neurodegenerative disease pathologies (Nixon, 2013). Neurons with defective autophagy hamper the turnover of proteins and harbor protein aggregates such as ubiquitin positive inclusions and Lewy bodies (Komatsu et al., 2006). Genetic ablation of neuronal autophagy function results in progressive accumulation of neuronal aggregates and such mice manifest neurodegenerative symptoms (Hara et al., 2006). Conversely, genetically enhancing autophagy flux results in, among other things, marked clearance of autophagy adaptors (p62) that are involved in ubiquitin aggregate capture and perhaps contributing to the extended life span of such mice (Pyo et al., 2013). Corroborating such observations, pharmacological studies have implied that autophagy inducing small molecules have therapeutic potential as they restore autophagy flux which eventually mitigates neuronal loss and improves motor co-ordination in models of PD (Sarkar et al., 2007; Williams et al., 2008; Khurana and Lindquist, 2010). In this context, our study reveals the small molecule XCT 790 as an autophagy enhancer that exerts neuroprotective action.

Autophagy, a tightly regulated process is maintained at low (basal) levels during fed condition and elevated in presence of stress such as starvation (Hara et al., 2006). This low level of autophagy is maintained by negative regulation by MTOR. We now show that, in addition to MTOR pathway, ERRα negatively regulates autophagy flux. When the ERRα inverse agonist, XCT 790, relieves this regulation, the number of autophagosomes and autolysosomes increase several folds. Furthermore, it is known that MTOR activity is not perturbed in the absence of ERRα (Chaveroux et al., 2013). We, therefore, investigated the interdependance of MTOR and ERRα in controlling autophagy and show that XCT 790 induces autophagy independent of MTOR signaling, a well-desired characteristic for further drug development to combat adverse side effects. In addition, MTOR regulates transcription of Ubiquitin Proteasome System (UPS) related genes leading to degradation of ERRα upon induction of autophagy by rapamycin (Chaveroux et al., 2013). Our results suggest that autophagy inhibiting activity of ERRα in nutrient rich conditions is through MTOR independent mechanism. In autophagy triggering conditions, where basal inhibitory activity needs to be removed, ERRα gets ubiquitinated and degraded by UPS (Chaveroux et al., 2013), that corroborates our findings.

Among the MTOR independent pathways, AMPK regulates autophagy flux. Previously, XCT 790 was reported to modulate AMPK pathway in ERRα independent fashion (Eskiocak et al., 2014). We observed XCT 790 modulates autophagy by not regulating the AMPK pathway, one of the reported MTOR independent pathways, but through subcellular ERRα localization dynamics.

XCT 790 is reported to be the most selective inverse agonist of ERRα (Eskiocak et al., 2014). Though it is known that ERRα localizes primarily in the cytoplasm (Sladek et al., 1997; Ju et al., 2009), its cytoplasmic function is not yet reported. Thus, apart from its transcriptional function in nucleus (Sladek et al., 1997), our findings reveal a cytoplasmic role for ERRα in autophagy. Recently, ERRα has been shown to up regulate autophagy related genes in the context of clearance of *Mycobacterium* in a transcriptional-dependent manner (Kim et al., 2018). Our results further demonstrate that XCT 790 not only induces aggrephagy but also clears intracellular *Salmonella* burden via

recruiting autophagy related proteins such as LC3 and p62. Thus, ERRα uniquely induces selective autophagy pathways by both transcriptional-dependent and -independent mechanism to clear misfolded aggregates and intracellular pathogens.

AUTHOR CONTRIBUTIONS

RM, SNS and AKC conceived the project. SNS, MP, AKC, DJV and HY carried out the experiments. PAA supervised the mice histology experiments. JPC and RM designed the behavior experiments. SR, AP and SNS performed the behavior experiments. SNS, JPC and RM analyzed the behavior results. VA conducted the xenophagy experiments. RM, SNS, MP, DJV, HY, JPC, SR and PAA analyzed the results and wrote the manuscript. All authors approved the manuscript.

FUNDING

This work was supported by Wellcome Trust/DBT India Alliance Intermediate Fellowship (500159-Z-09-Z), DST-Science and Engineering Research Board (SERB) grant (EMR/2015/001946) and JNCASR intramural funds to RM, and SERB, DST (SR/SO/HS/0121/2012) to PAA, and DST-SERB (SB/YS/LS-215/2013) to JPC. Application of XCT 790 in autophagy modulation and neurodegeneration is patent pending.

ACKNOWLEDGMENTS

We thank Y. Ohsumi, Paulo Ludovico, David Rubinzstein, Toren Finkel and Tamotsu Yoshimori for the kind gift of plasmids and Dr. K. Vijayachandra, Aparna Hebbar and members of the autophagy lab (JNCASR) for critical reading of the manuscript. We acknowledge Dr. Prakash's expertise at the animal facility of JNCASR.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00109/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 27 November 2018
doi: 10.3389/fcell.2018.00160

Chemical Biology Strategies to Study Autophagy

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Growing amount of evidence in the last two decades highlight that macroautophagy (generally referred to as autophagy) is not only indispensable for survival in yeast but also equally important to maintain cellular quality control in higher eukaryotes as well. Importantly, dysfunctional autophagy has been explicitly shown to be involved in various physiological and pathological conditions such as cell death, cancer, neurodegenerative, and other diseases. Therefore, modulation and regulation of the autophagy pathway has emerged as an alternative strategy for the treatment of various disease conditions in the recent years. Several studies have shown genetic or pharmacological modulation of autophagy to be effective in treating cancer, clearing intracellular aggregates and pathogens. Understanding and controlling the autophagic flux, either through a genetic or pharmacological approach is therefore a highly promising approach and of great scientific interest as spatiotemporal and cell-tissue-organ level autophagy regulation is not clearly understood. Indeed, chemical biology approaches that identify small molecule effectors of autophagy have thus a dual benefit: the modulators act as tools to study and understand the process of autophagy, and may also have therapeutic potential. In this review, we discuss different strategies that have appeared to screen and identify potent small molecule modulators of autophagy.

Keywords: autophagy, high throughput, chemical biology, luciferase, small molecule screening, fluorescence microscopy

OPEN ACCESS

Edited by:
Ioannis Nezis,
University of Warwick,
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Reviewed by:
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Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *30 July 2018*
Accepted: *06 November 2018*
Published: *27 November 2018*

Citation:
Mishra P, Ammanathan V and Manjithaya R (2018) Chemical Biology Strategies to Study Autophagy. Front. Cell Dev. Biol. 6:160. doi: 10.3389/fcell.2018.00160

INTRODUCTION

Macroautophagy (herein autophagy) is a major intracellular process that is critically crucial for maintaining cellular homeostasis. Autophagy has been reported in several organisms from different kingdoms ranging from yeast to humans suggesting that it is an evolutionarily conserved process. This process was first reported by Christian de Duve (Deter et al., 1967), when he observed organelles captured within the lysosomes with the help of electron microscopy (De Duve and Wattiaux, 1966). This entire phenomenon of cargo capture and ultimately its degradation in the lysosomes is called "autophagic flux" (Klionsky, 2007; Rabinowitz and White, 2010; Boya et al., 2013).

Basal levels of autophagy occur in all cells during nutrient rich conditions and help in housekeeping functions to maintain cellular quality control by clearance of damaged or surplus organelles and misfolded proteins, recycling and providing basic building blocks like amino acids for reuse (Mizushima and Klionsky, 2007; Musiwaro et al., 2013). However, the levels of autophagy are highly modulated in response to different stimulus, both intracellular and exogenous

such as starvation, pathogen invasion, organelle damage and protein aggregation in cytoplasm (Takeshige et al., 1992; Komatsu and Ichimura, 2010). Because autophagy is central to maintaining cellular homeostasis, defective autophagy has been attributed to a variety of disease conditions such as cardiovascular diseases, atherosclerosis, certain myopathies, innate and adaptive immune responses, neurodegeneration and cancer (Choy and Roy, 2013; Kroemer, 2015).

Dysfunction of autophagy leads to cell death, cancer, neurodegenerative, and other diseases. Therefore, studying the molecular aspects of autophagy is of current research interest for the treatment of various disease conditions. Genetic and pharmacological modulation of autophagy has been shown to be beneficial in many such situations (Rubinsztein et al., 2012). Modulation of autophagy has been shown to be beneficial in diseases such as diabetes, cancers, neurodegenerative disorders and some infectious diseases (Sarkar et al., 2007; Sarkar and Rubinsztein, 2008). Several studies in the recent years have discovered novel or repurposed drugs for restoring autophagic balance. For instance, Rapamycin, an autophagy inducer and its analogs were used by Ravikumar et al., to abrogate neurodegeneration in a *Drosophila* based model by enhancing the rates of autophagy (Ravikumar et al., 2004; Sarkar, 2013a). In some of these studies, distinct assays have been developed and used for a High Throughput Screening (HTS) to identify small molecules that modulate autophagy (**Table 1**). Several autophagy modulators have been discovered in the recent past but very few of them have led to potential candidate drug molecules. Many of these compounds are specific toward different targets in the autophagy pathway. For example, specific screens to identify novel candidate molecules such as ULK1 (Rosenberg et al., 2015), ATG4 (Ketteler and Seed, 2008), class III phosphatidylinositol 3-kinase (Farkas et al., 2011), and MTOR (Butcher et al., 2006), have been carried out. In addition, compounds with broad spectrum effects have also been identified as well (Sarkar, 2013b). The scope for the discovery of new autophagy modulators that can be later taken up to clinical trials is ever increasing. It has been postulated that deeper insights into autophagy through chemical modulation can lead to better understanding of various diseases. In addition, understanding of the mechanism of these molecules may provide deeper mechanistic insights and understanding of the finely regulated process of autophagy. Chemical biology approach to study autophagy can be compared to a genetic screen (Tsukada and Ohsumi, 1993; Thumm et al., 1994; Harding et al., 1995; Titorenko et al., 1995), where further studies on the hits reveal more about the mechanism of autophagy. For example, just as the identification of a gene and its function, a manner in which a small molecule modulates autophagy can also shed some light regarding the regulation of autophagy (Seglen and Gordon, 1982; Kunz et al., 1993). In search of potential candidate drugs

TABLE 1 | Autophagy modulators identified through High Throughput Screening of Chemical compound libraries.

Compound name	Autophagy modulation	General/Selective autophagy modulator	Mechanism of autophagy modulation	Reference
ARP101	Inducer	General	Induction of autophagosome biogenesis	Jo et al., 2011
Bay 11	Inhibitor	General	Inhibition of autophagosome biogenesis	Mishra et al., 2017a
BRD5631	Inducer	Aggrephagy/Xenophagy	–	Kuo et al., 2015
Carbamazepine	Inducer	Xenophagy	By myo-inositol depletion and AMPK activation	Schiebler et al., 2015
Cardiac glycosides, e.g., Digoxin, Helveticoside	Inducer	General	Inhibition of Na^+K^+ATPases leading to increase in Ca^{2+} levels	Hundeshagen et al., 2011
KU55933 and Gö6976	Inhibitor	General	Inhibition of PI3K	Farkas et al., 2011
Loperamide	Inducer	Aggrephagy	Regulation of intracellular Ca^{2+} levels	Zhang et al., 2007
P29A03	Inducer	General	Increase in Beclin levels	Lee et al., 2013
P23C07	Inhibitor	General	Inhibition of autophagosomes fusion with lysosomes	Lee et al., 2013
Rottlerin	Inducer	General	Inhibition of mTOR through TSC2 pathway	Balgi et al., 2009
6-Bio	Inducer	Aggrephagy	GSK-3 beta inhibitor	Suresh et al., 2017
Fasudil	Inducer	General	–	Iorio et al., 2010
Flubendazole	Inducer	Xenophagy	Microtubules destabiliser	Chauhan et al., 2015
Minoxidil and clonidine	Inducer	Aggrephagy	Modulation of cAMP levels	Williams et al., 2008
Niclosamide	Inducer	General	Inhibition of mTOR	Balgi et al., 2009
Perhexiline	Inducer	General	Inhibition of mTOR	Balgi et al., 2009
SEN177	Inducer	Aggrephagy	Inhibition of glutaminyl cyclase	Jimenez-Sanchez et al., 2015
SMER10, SMER18, SMER28	Inducer	Aggrephagy	–	Sarkar et al., 2007
Trifluoperazine	Inducer	Aggrephagy/Xenophagy	Increase in FYVE containing vesicles	Zhang et al., 2007
Tamoxifen	Inducer	Xenophagy	Estrogen and G protein coupled receptor GPR30 antagonist shown to inhibit intracellular *Toxoplasma*	Dittmar et al., 2016
Valproic acid	Inducer	Xenophagy	By myo-inositol depletion and AMPK activation	Schiebler et al., 2015
XCT 790	Inducer	Aggrephagy/Xenophagy	ERR alpha inhibitor	Suresh et al., 2018
ZPCK	Inhibitor	General	Inhibition of cargo degradation within lysosomes	Mishra et al., 2017a

that moderate autophagy, identifying small molecule modulators of autophagy is the primary step. Small molecule study will further enhance the understanding of autophagy and related pathways. Thus, having a robust, sensitive assay to monitor autophagic flux that could be performed at a high throughput rate for the purpose of screening modulators of autophagy is of primary importance (**Figure 1**). In this review, we discuss some of the pharmacological strategies undertaken in the recent past to identify novel autophagy modulators (**Table 2**).

CONVENTIONAL AUTOPHAGY ASSAYS

The real time analysis of autophagy in cells tissues principally been performed via qualitative measures. These assays identify autophagosomes or measure the conversion of LC3I to LC3II (Atg8 in yeast) either through western blotting or microscopy (Klionsky et al., 2016). Owing to the conserved nature of autophagy (Mizushima et al., 1998; Kabeya et al., 2000; Meijer et al., 2007), the use of yeast as a model system to study autophagy is still widely recognized, even after the identification of homologous Atg sequences in mammalian cells. This is primarily because of the ease of handling and the vast array of biochemical and genetic tools available to carry out autophagy studies. Several different techniques to monitor autophagy are well established in yeast (Torggler et al., 2017). For example, Pho8Δ60 assay provides readout for bulk autophagy (Noda et al., 1995). Wild type alkaline phosphatase protein moves from ER (inactive) to vacuole where it gets activated. Deletion of first 60 amino acids from the N-terminal makes the mutated protein cytosolic which is taken up by the autophagosome machinery along with other cytosolic contents and delivered to vacuole for bulk degradation. The action of vacuolar proteases activates the Pho8Δ60, which can act on different substrates to dephosphorylate them. Depending on the substrate being used, the readout could be measured using either photometry or fluorimetry.

Other classical assays in yeast include monitoring the degradation of fluorescent tagged Atg8 (GFP-Atg8), either through microscopy or immunoblotting (Kirisako et al., 1999; Suzuki et al., 2001; Meiling-Wesse et al., 2002). Similarly, autophagic degradation of certain different cargoes like PGK1 or radiolabeled long-lived proteins and organelles like peroxisomes (discussed in later sections) and mitochondria can be chased (Tsukada and Ohsumi, 1993; Kissova et al., 2004; Sakai et al., 2006; Welter et al., 2010; Motley et al., 2012).

Although yeast studies provide a reliable and efficient way to study autophagy, considering the complexity in higher eukaryotes, the results cannot be always extrapolated. Keeping the role of autophagy in different physiological and pathological contexts in mind, several different autophagy assays have been developed in cell culture (Tooze et al., 2015; Orhon and Reggiori, 2017). Many of these assays rely on the status of LC3B protein, which is a mammalian homolog of yeast Atg8 protein and is involved in biogenesis and maturation of autophagosome (Kabeya et al., 2000; Mizushima and Yoshimori, 2007; Weidberg et al., 2010; Nguyen et al., 2016). LC3 gets conjugated to phosphatidyl ethanolamine (PE) on the autophagosome membrane and is the sole marker for autophagosomes right from the biogenesis to its degradation. The form of LC3B conjugated to PE is called LC3B II, while the cytosolic, unconjugated form is referred to as LC3B I. This led to development of various LC3 based assays for monitoring the autophagic flux. Other autophagy marker protein widely utilized for the purpose of autophagy assays is p62/SQSTM1, which is an adaptor protein that helps in cargo sequestration (Bjorkoy et al., 2005). Different fluorescent reporters are tagged to these markers (mRFP/GFP-LC3) to visualize them under the microscope (Kabeya et al., 2000). *In vivo* studies have also been conducted in the past using the fluorescently labeled LC3 marker. Mizushima et al., used a transgenic mice model expressing the GFP-LC3 protein to show that autophagy occurs in all the cell types. The basal levels of autophagy vary in different tissues and starvation stimulus induces autophagy over and above the basal levels in all the tissues (Mizushima et al., 2004). Tandem fluorescent tags on these proteins (mRFP/mCherry-GFP-LC3) provide an added benefit of visualizing different stages of autophagic flux (Kimura et al., 2007). This reporter is an indicator of conversion of autophagosomes into autolysosomes upon fusion with lysosomes, wherein the autophagosomes emit both mRFP and GFP signals ($mRFP^+$ GFP^+) whereas the autolysosomes emit only mRFP signal ($mRFP^+$ GFP^-) because GFP is acid-labile and is quenched in the acidic environment of the autolysosomes.

The cytoplasmic autophagic flux of proteins is too small to be chased over a time course using an assay (Welter et al., 2010). The turnover rate of cytosolic proteins through basal autophagy is less and does not provide a broad window or physiological range to carry out a screen using protein degradation as a measure. In turn, having an inducible cargo that is specifically degraded through autophagy provides a higher working range. The inadaptability of the conventional autophagy assays into a high throughput setting presents a major limitation and hence makes the small molecule screening a very cumbersome process (Cheong and Klionsky, 2008; Wang and Subramani, 2017).

HIGH THROUGHPUT ASSAYS TO MONITOR AUTOPHAGY

Multiple aspects and steps of the autophagy pathway have been exploited to establish several different HTS assay systems both in yeast and mammalian cells. These have also led to identification of potent novel autophagy modulators (**Figure 1**). Studies on these modulators have not only revealed their therapeutic potential but led to better understanding of the autophagy process.

Growth Based Autophagy Assays

MTOR is a nutrient sensor and hence is central to cells growth. MTOR also is a regulator of the autophagy pathway (Noda and Ohsumi, 1998; Loewith and Hall, 2011). Rapamycin, an inhibitor of MTOR, activates autophagy pathway (Abraham and Wiederrecht, 1996). This understanding has been widely utilized

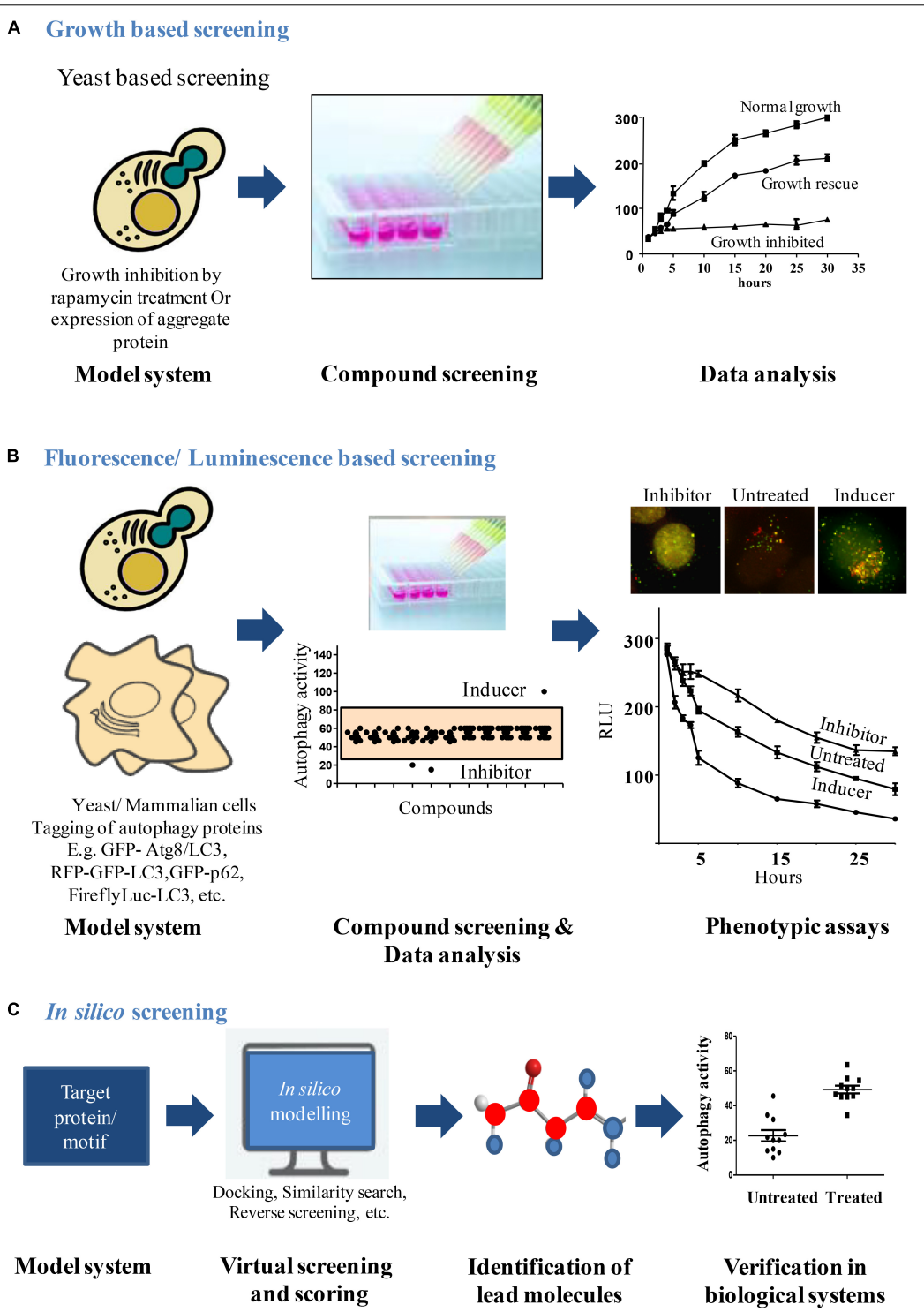

FIGURE 1 | Workflow for screening autophagy modulators. **(A)** Growth based screening: growth inhibition can be induced in yeast due to over expression of aggregate proteins or rapamycin treatment. This cytostatic effect exhibited by yeast can be used as a platform to screen compounds that rescue the growth lag through autophagy induction. After compound treatments, analysis of yeast growth curves identifies the compounds that rescued the growth lag. **(B)** Fluorescence/Luminescence based screening: fluorescent or luminescent reporters are tagged to autophagy proteins for transfection in yeast or mammalian model systems. Modulators of autophagy from chemical libraries are obtained by analyzing the fluorescent/luminescent signal intensities or by visualizing the autophagic vesicle formation by microscopy. **(C)** *In silico* screening: structures of autophagy proteins/motifs of interest can be obtained from data sources like Protein Data Bank and can be used as a model system to identify chemical molecules that bind using *in silico* modeling softwares. The selected lead molecules are then verified in biological system to validate its ability to modulate the process.

TABLE 2 | Summary of HTS assays for compound libraries.

Model system	Assay principle	Read out	Compound(s) identified	Autophagy modulation	Reference
Yeast	Rescue of rapamycin induced growth inhibition	Growth based assay	LY-83583	–	Butcher et al., 2006
	Rescue of rapamycin induced growth inhibition	Growth based assay	SMER 10, 18, and 28	Inducer	Sarkar et al., 2007
	Rescue of rapamycin induced growth inhibition	Growth based assay	SMIRs	Inhibitor	Sarkar et al., 2007
	Rescue of SNCA α-synuclein induced growth lag	Growth based assay	6-Bio	Inducer	Suresh et al., 2017
	Degradation of luciferase tagged peroxisomes	Luminescence	Bay11, ZPCK	Inhibitor	Mishra et al., 2017a
Mammalian cells	Increase in number of autophagosomes (GFP-LC3)	Fluorescence microscopy	ARP101	Inducer	Jo et al., 2011
	Increase in number of autophagosomes and autolysosomes (mCherry-GFP-LC3)	Flow cytometry	Cardiac glycosides	Inducer	Hundeshagen et al., 2011
	Degradation of autophagy adaptor proteins (GFP-p62, GFP-NBR1)	Flow cytometry	Lactacystin	Inhibitor	Larsen et al., 2010
	Reduction in intracellular *Mycobacterium tubercluosis*	Fluorescence microscopy	Valproic acid	Inducer	Schiebler et al., 2015
	Increase in autophagosomes and autolysosomes (mCherry-GFP-LC3)	High-content fluorescent microscopy	Flubendazole	Inducer	Chauhan et al., 2015
	Degradation of lipid droplets	Fluorescence microscopy	P23C07	Inhibitor	Lee et al., 2013
	Ratio of GFP-LC3 (autophagosomes) and cytosolic RFP-LC3ΔG (internal control) using the probe GFP-LC3-RFP-LC3ΔG	High-content fluorescent microscopy and flow cytometry	Deslanoside, Cladribine	Inducer	Kaizuka et al., 2016
	Ratio of GFP-LC3 (autophagosomes) and cytosolic RFP-LC3ΔG (internal control) using the probe GFP-LC3-RFP-LC3ΔG	High-content fluorescent microscopy and flow cytometry	Mebendazole Nelarabine	Inhibitor	Kaizuka et al., 2016
	Clearance of A30P α-synuclein	Fluorescence microscopy	Minoxidil and clonidine	Inducer	Williams et al., 2008
	Quantitation of Nuclear LC3	High-content fluorescent microscopy	NSC179818, NSC60785	–	Kolla et al., 2018
	Degradation of luciferase tagged adaptor protein (Luc2p-p62 and Luc2p-p62ΔU)	Luminescence	Temozolomide	Inducer	Min et al., 2018
	Renilla Luc tagged LC3 turnover	Luminescence	KU55933 and Gö6976	Inhibitor	Farkas et al., 2011
	Comparison of data expression pattern	*In silico* data mining	Fasudil	Inducer	Iorio et al., 2010

to develop assays to monitor autophagy via MTOR activity. Butcher *et al.,* developed an assay that monitored the growth of yeast cells each harboring a different plasmid from a pool of 3900 overexpression plasmids in the presence of rapamycin, which is an inhibitor of MTOR (Butcher et al., 2006). Yeast cells when cultured in the presence of rapamycin, undergo growth inhibition, because of block in TOR pathway. From the pool of overexpression plasmids, candidate gene products were identified that suppressed the cytostatic effect of rapamycin and were involved in the TOR pathway. They also characterized the mechanism of LY-83583. LY-83583 is a novel molecule that suppressed the rapamycin-induced growth inhibition and its several candidate targets were also implicated. Sarkar et al. (2007), used yeast to identify small molecule enhancers (SMERs) and inhibitors (SMIRs) of rapamycin using the same strategy. From the screening, 21 SMIRs and 12 SMERs were listed that were structurally non-redundant. They identified SMERs that could enhance autophagy independently of MTOR, and these SMERs (SMER 10, 18, and 28) when tested in mouse and Drosophila models decreased the toxicity associated with mutant Huntington protein, also reflecting on the therapeutic potential of these compounds (Sarkar et al., 2007). The HTS utilized a chemical genetic suppressor platform to rescue or elevate the growth inhibitory properties of rapamycin on wild type yeast cells (Huang et al., 2004). Therefore, because of the involvement of MTOR pathway in regulating autophagy, a simple screen based on the growth of yeast was able to give therapeutically potent small molecule hits.

A growth-based neurotoxicity assay in yeast was also utilized by Suresh et al., to identify novel autophagy enhancer 6-Bio that ameliorates α-synuclein toxicity. The compound 6-Bio effectively cleared toxic aggregates in an autophagy dependent manner in both yeast as well as mammalian cells. More importantly, the action of the compound was conserved and showed neuroprotection in a pre-clinical mouse model of Parkinson's disease (Suresh et al., 2017).

Fluorescence Based High Throughput Assays

Fluorescence based microscopy assays are the most commonly used techniques to monitor autophagic flux. Autophagy, being a multistep process involving several molecular players, presents with a number of markers that can be tagged with a fluorescent probe and the autophagy rates can be monitored. Interestingly, this has also been exploited to design several high content-based

imaging strategies to screen for novel autophagy modulators (**Figure 1**).

Clearance of toxic poly glutamine aggregates in cell culture was also demonstrated by using autophagy enhancers obtained from an image based HTS of GFP tagged LC3 puncta representing autophagosomes (Zhang et al., 2007). The number, size and intensity of the autophagosomes were analyzed and quantified using high throughput fluorescence microscopy. GFP-LC3 was used as a probe in an automated microscopy cell-based assay to identify chemical enhancers that rapidly led to an increase in autophagosome content (Balgi et al., 2009). The same reporter was also used by Jo et al. (2011), to identify ARP101, that inhibits matrix metalloproteinase-2 (MMP-2) selectively; as an inducer of autophagy- associated cell death in cancer cells. A high content, flow cytometry based screening approach was used to screen Prestwick Chemical Library containing FDA approved drugs by looking at autolysosome formation and degradation and also endolysosomal activities under basal and stimulated autophagy conditions (Hundeshagen et al., 2011). This study used three different probes to investigate different stages of autophagic flux (GFP-LC3 for autophagosome, mCherry-GFP-LC3 for autophagosome and autolysosome) and endocytic activity (GFP-Rab7). From the screening, cardiac glycosides were validated as potent enhancers of autophagic flux. The same GFP-LC3 probe has also been used by Kuo et al. (2015), to screen 59,541 small molecules prepared by stereoselective diversity-oriented chemical synthesis and identification of enhancers of autophagy.

Larsen et al. (2010), followed the degradation of three fluorescent tagged autophagy markers: GFP-p62, GFP-NBR1, or GFP-LC3 by flow cytometry of live cells after their promoter has been turned off. Relative degradation rates of these three promoters was analyzed under basal autophagy conditions. Through single cell analysis, GFP-LC3B was found to be the most stable protein whereas GFP-NBR1 was the reporter that was most effectively degraded. The degradation of GFP-p62 was observed to show the strongest response to nutrient limitation condition and was reported to be the best reporter out of the three. Chemical screening strategies have also been used to identify novel target processes that activate autophagy (Chauhan et al., 2015). In this study, LC3B puncta in HeLa cells stably expressing mRFP-GFP-LC3B were analyzed using high-content (HC) image analysis of and revealed a novel role of microtubules, which when altered resulted in autophagy induction.

Autophagy dependent degradation of lipid droplets (LDs) was also used for the development of a high content screening platform to discover novel autophagy modulators (Lee et al., 2013). In this study, an indolizine-based fluorescent skeleton called Seoul-Fluor (SF) (Kim et al., 2011) that stains the hydrophobic LDs was used and its subsequent degradation via autophagy was followed.

Two anticonvulsants were discovered as mTOR independent autophagy enhancers, from a functional cell-based screening of FDA-approved drugs that were further shown to clear intracellular population of *Mycobacterium tuberculosis* (Schiebler et al., 2015). In this screen, a library of 214 compounds was screened for its ability to kill intracellular luminescent strain of *M. bovis* BCG (bacille-Calmette-Guerin, live attenuated strain of *M. bovis*) within macrophages. These hits were further validated both in primary macrophages and autophagy null cells and also for their effect on autophagy in an mTOR independent manner. The probe GFP-LC3-RFP-LC3ΔG developed by Kaizuka et al. (2016), serves as a cumulative index for autophagy activity. The probe utilizes the protease activity of the ATG4 family of proteins. Upon cleavage of the fusion protein by ATG4, GFP-LC3 gets associated with the autophagosomes and then degraded upon subsequent fusion to lysosomes. RFP-LC3ΔG on the other hand is cytosolic due to the deletion of glycine at the C-terminal of LC3. This probe can be utilized in different settings like high throughput microscopy, flow cytometry and microplate readers and is also amenable to screening small molecule modulators of autophagy by comparing the ratio of GFP/RFP.

A high content screening in HeLa cells using EGFP-LC3 reporter identified several autophagy inhibitors. These compounds were then further analyzed using an array of phenotypic cell-based assays. The screening strategy identified several hitherto unknown target proteins amongst the well defined targets like Vps34 and ULK1 (Peppard et al., 2014). In a first of a kind, a high content screening using the fluorescent LC3 reporter, a library of 1539 chemical compounds was aimed to identify modulators that affected the nuclear localization of LC3. Potent modulators were identified that may help in the understanding of LC3 nuclear-cytoplasmic localization (Kolla et al., 2018).

Parkinson's disease associated protein A30P α-synuclein is a substrate for autophagy and has been used to study aggregate clearance by autophagy in the past. One such study used A30P α-synuclein clearance by autophagy as a primary screen to identify novel autophagy enhancers. Using this screen L-type Ca^{2+} channel antagonists, the K^{+}_{ATP} channel opener minoxidil, and the G_i signaling activator clonidine were identified as autophagy inducers that work independent of MTOR. This important discovery revealed that MTOR is dispensable for autophagy induction. The authors showed that cAMP can modulate autophagy by controlling IP3 activity (Williams et al., 2008). As MTOR is central to several other pathways as well, identification of an alternative pathway opened the scope of controlling autophagy independent of MTOR.

Luminescence Based High Throughput Assays

Luciferase being a sensitive reporter protein comes in handy when an assay has to be scaled to a high throughput format (**Figure 1**). Availability of different luciferase variants further helps in the design of an assay according to the needs. These luciferase variants have different degrees of sensitivity (Nanoluc is more sensitive to Firefly luciferase), different sizes and spectra (*Renilla* luciferase is smaller in size to Firefly luciferase) or different properties (Gaussian luciferase is secretory in nature while Renilla luciferase is cytosolic and Firefly luciferase naturally has a peroxisomal targeting signal). Depending on the need of the assay and the process to be studied, an appropriate luciferase

variant may be used in the study. A *Gaussian* luciferase reporter based assay that quantitatively measures the autophagy rate by monitoring proteolytic activity of ATG4B, can be done at a large scale and is quantifiable (Ketteler and Seed, 2008). This luciferase release assay is well suited for upstream signaling events that either increase or decrease the rates of autophagy. A luciferase-based assay that exploited the property of long lived proteins to be solely degraded via autophagy pathway provided a direct relevance of the autophagy modulation in aggregate prone cells. This assay demonstrated autophagic clearance of an expanded polyglutamine *in vitro* and *in vivo* conditions (Ju et al., 2009). This assay takes into account the selective degradation of autophagy cargo using a sensitive luciferase-based reporter. Dynamic and sensitive assay could be achieved by following the cargo that is selective for degradation through autophagy. Peroxisomes provide highly inducible cargo with high turnover rates which are specifically degraded through autophagy machinery under starvation conditions (Sakai et al., 2006). This high turnover of peroxisomes when combined to the sensitivity of luciferase reporter, provides a very sensitive assay to monitor autophagic flux which is also amenable to high throughput setting. Based on this principle, Mishra et al., designed a screening strategy that allows measurement of autophagic cargo (facultative organelle, peroxisomes) clearance rather than ATG8 based changes in autophagosome number. The principle of the assay is based on detection of the levels of firefly and *Renilla* luciferase activities to monitor the flux of selective and general autophagy, respectively, in *S. cerevisiae* (Mishra et al., 2017b). Reporter strains were constructed that expressed Renilla luciferase and firefly luciferase with a peroxisome targeting signal (PTS1) under a fatty acid responsive promoter. These cells when grown in the presence of fatty acid or glycerol containing media, leads to the expression of peroxisomal firefly luciferase and *Renilla* luciferase which is cytosolic. These cells are then subjected to starvation to induce autophagy. Induction of autophagy leads to selective autophagic degradation of peroxisomes (pexophagy) and also non-selective bulk degradation of cytoplasm. The rate of decay in firefly luciferase activity depicts pexophagy whereas *Renilla* levels depict general autophagy. The dual luciferase assay provides the added advantage of monitoring autophagy in real time, is more sensitive and gives kinetic assessment of two different types of autophagy processes simultaneously. Interestingly, the action of the autophagy modulators identified from the screen was conserved across higher eukaryotes (Mishra et al., 2017a). The autophagy inhibitor Bay11 identified from the screen acted at the autophagosome biogenesis step and ZPCK inhibited the degradation of cargo inside the vacuole/lysosome. These inhibitors had a conserved mode of action across yeast, animals and plants.

Luciferase based HTS autophagy assay has been reported for mammalian cells as well. In a study by Min et al. (2018), a luciferase variant Luc2p was fused with the wild type p62/SQSTM1 or a deletion version of p62 (p62 lacking the ubiquitin binding domain) and transfected into glioma cells. The lysates from the two populations (wild type and mutant p62) were compared to monitor the autophagic flux. The performance of this probe was reported to be comparable to GFP-LC3-RFP-LC3ΔG probe described earlier in the review (Min et al., 2018).

In vitro and *in silico* Assays

In recent years, many groups have also carried out a target driven autophagy screen using purified proteins and substrates. To identify substrates for ULK1 that might be involved in the process of autophagy, Egan et al. (2015), screened degenerate peptide libraries to identify a consensus motif for ULK1 mediated phosphorylation. After identifying novel phosphorylation sites, multiple targets for ULK1 were discovered. These substrates were then used to screen for potent inhibitors of ULK1 phosphorylation.

Renilla luciferase based turnover of LC3 was used to screen two kinase inhibitor libraries for identifying inhibitors of autophagic flux (Farkas et al., 2011). This study identified specific and more potent inhibitors of the upstream signaling component; class III phosphatidylinositol 3-kinase. Inhibitors specific to Ulk1 kinase activity, an upstream protein involved in autophagy initiation were obtained from a screen that utilized purified stress-activated Ulk1 and then looked at the phosphorylation of its substrate, Atg13 at Serine 318 position (Rosenberg et al., 2015).

Iorio et al. (2010) used the large dataset of drug expression pattern integrated into "drug network" and identified the previously hitherto unknown functions of several well characterized drugs. This is a dataset of expression profiles constructed while comparing the transcriptional responses induced by different small molecules in human cell lines. Through data mining, they identified fasudil as a novel autophagy enhancer taking the help of the same drug network (Iorio et al., 2010).

DISCUSSION

Although the core autophagy machinery and the proteins involved in disease conditions might be known, but the exact mechanism of action and how the autophagic flux is regulated is not completely understood which leads to many unanswered questions. Understanding and controlling the autophagic flux either through a genetic or pharmacological approach is a highly promising approach and of great scientific interest. Studies with genetic modulations of autophagic flux have been carried out in the past with immense success. Yoshinori Ohsumi, a pioneer in autophagy field was awarded the Nobel Prize in 2016 for his contribution to the study of autophagic flux. However, chemical modulation has an advantage over genetic manipulations that the phenotype could be observed just on the addition of the compound and the action could 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. However, there are limitations associated with the chemical approach because of the bioavailability issues, toxicity and the secondary or off-target effects associated with the chemical compound. Also, tissue specific effects are difficult to monitor.

To identify novel small molecule modulators of autophagy having a robust and sensitive screening system is the primary step. Therefore, HTS assays for autophagy are of utmost importance as they enable us to screen several small molecules in a small space of time with the inclusion of all possible biological and technical replicates. The data obtained from these assays should be amenable for direct comparison between the control and test groups and statistical analysis. Several high throughput assays have been developed in the recent past to identify small molecule modulators of autophagy. But some limitations associated with these assays must be overcome for a highly potent and effective HTS assay system. Many of these assays have issues with sensitivity and range. They do not directly look at the cargo or possess a higher physiological working range to detect smaller changes in autophagic flux. Although these assays are quantitative but may lack in one of the many parameters required to attain an ideal autophagy assay. An ideal assay would incorporate all these properties such as cargo build up, high sensitivity, ease of experimentation, broader physiological range, and live cell readout in a single high throughput format. Dynamic, sensitive and highly effective assay could be achieved by following the cargo that is inducible and selective for degradation through autophagy.

AUTHOR CONTRIBUTIONS

PM and RM conceived the idea and wrote the manuscript. VA conceptualized and contributed to the figure.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article · February 2017

DOI: 10.1007/s41745-016-0015-z

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Journal of the Indian Institute of Science
A Multidisciplinary Reviews Journal

ISSN 0970-4140

J Indian Inst Sci
DOI 10.1007/s41745-016-0015-z

Springer

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ISSN: 0970-4140 Coden-JIISAD

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.

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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

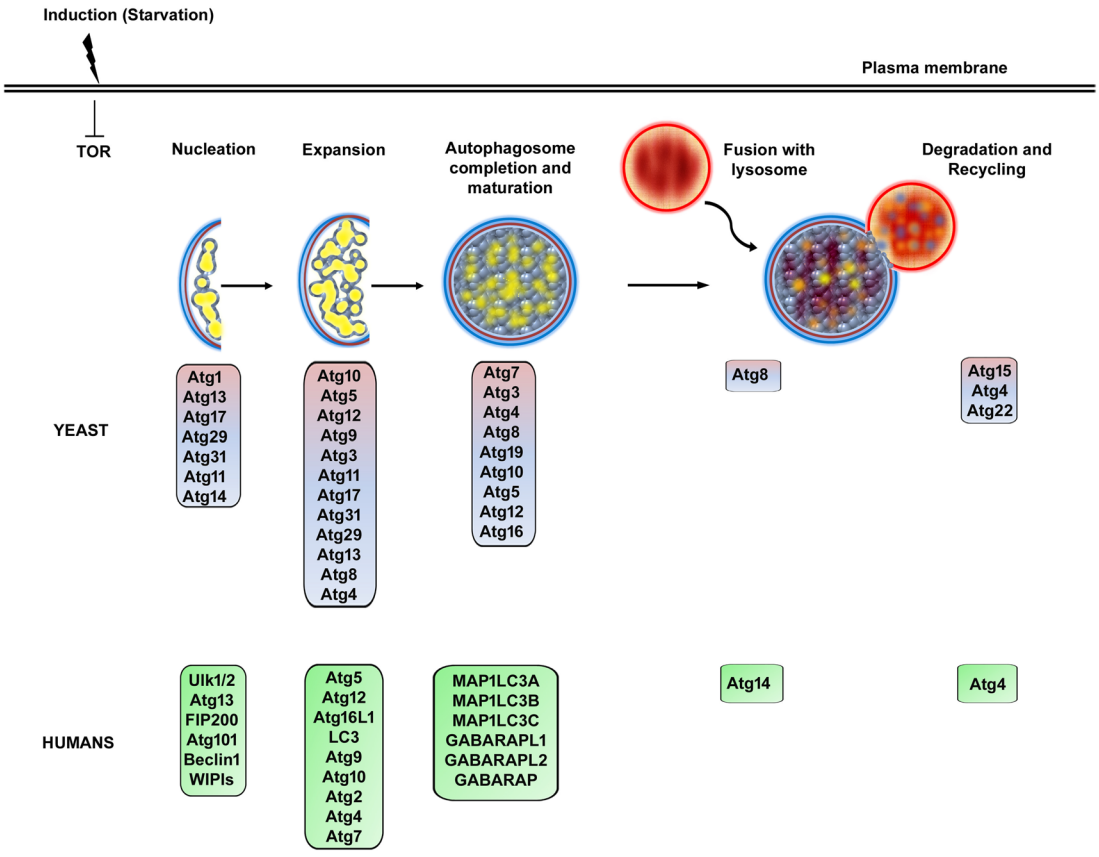

Figure 1: Schematic demonstrating the various steps in the autophagy process. The yeast and human autophagy proteins involved in nucleation, expansion, autophagosome maturation and completion, fusion, and degradation processes are mentioned.

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

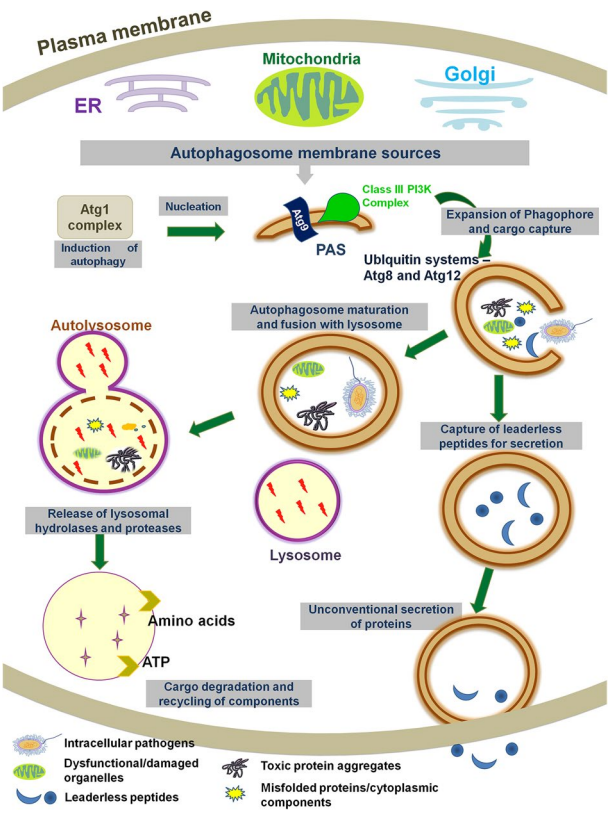

Figure 2: Canonical and non-canonical autophagy flux: under basal levels, autophagy helps in maintaining the cellular homeostasis by getting rid of cellular waste and superfluous components. Stimulation through several factors, such as starvation, stress, or chemicals, leads to induction of autophagy. 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.

Received: 16 October 2016. Accepted: 15 November 2016
Published online: 28 February 2017

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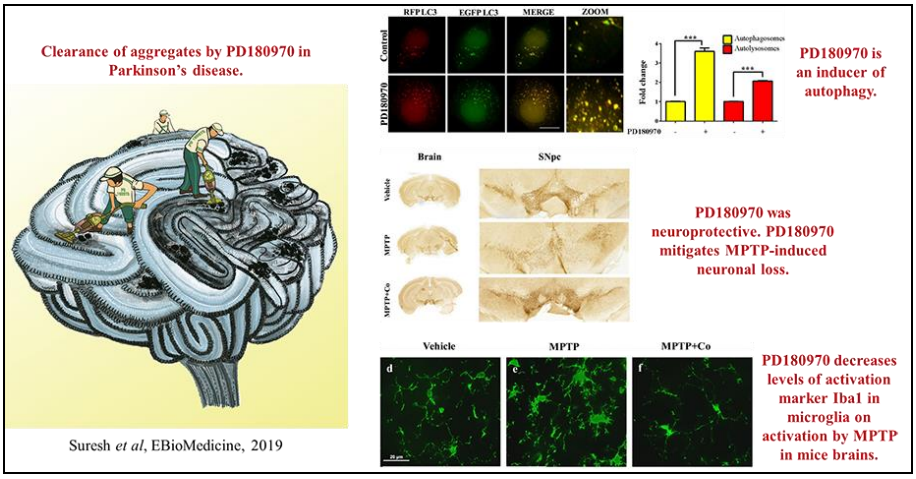
JNCASR scientists find a way to boost innate immune system against intracellular infection

A promising way to deal with intracellular pathogens including multi-drug- resistant bacteria is to induce a pathway of the human immune system known as xenophagy say researchers at the Autophagy laboratory, Molecular Biology and Genetics Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, an autonomous unit of the Department of Science and Technology.

Xenophagy is the selective degradation of intracellular pathogens such as bacteria which are captured and degraded by autophagy — an intracellular process responsible for lysosomal mediated degradation of cellular cargoes. Autophagy related discoveries earned Prof. Yoshinori Ohsumi the 2016 Nobel prize for physiology or medicine. JNCASR scientists have found a pharmacological tool to modulate autophagy, which can help induce xenophagy. They have filed a patent for this process, which is pending at present.

Inducing xenophagy is effective against a wide range of bacterial species and also in viruses and parasites — unlike antibiotics that act only on select pathogens. Furthermore, combining the effects of xenophagy with antibiotics has shown positive effects in treating infections.



While xenophagy has been studied to an extent, the molecular principles behind this process are poorly understood. This is further complicated by the fact that each pathogen has its unique way of subverting xenophagy, necessitating the need to identify tools to induce the process during infection.

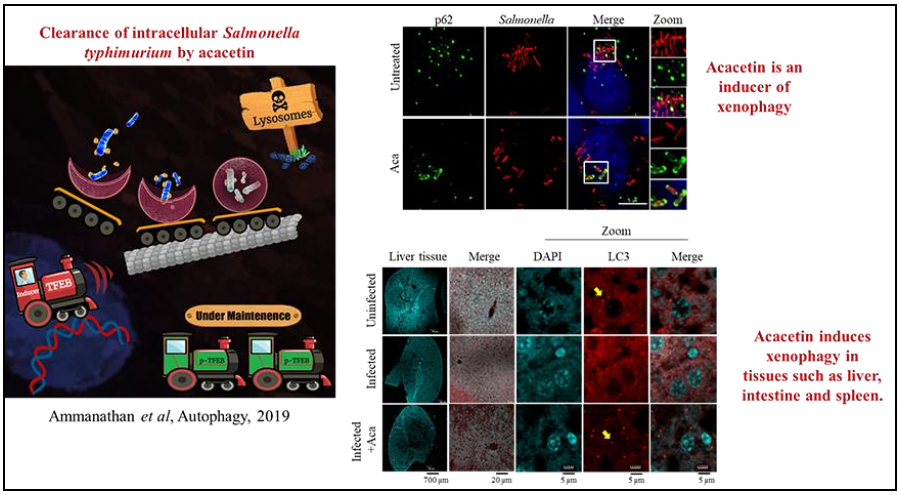
In the present work, acacetin, a plant-derived flavonoid, was used to induce xenophagy against *Salmonella typhimurium* infection. The efficacy of this small-molecule compound has been demonstrated both *in cellulo* and *in vivo*.

While xenophagy occurs in the cytoplasm, the present work published in *Autophagy* on 19 November 2019, highlights nuclear control of the process through a master transcriptional regulator called TFEB.

TFEB, considered as a master regulator of autophagy and lysosomal genes, was kept in a transcriptionally inactive form during infection by typhoid causing bacteria. Rescuing TFEB function during infection-induced xenophagic capture of bacteria as well as lysosomal biogenesis, and both these steps are critical in eliminating the intracellular bacteria.

The identified drug-like compound was further tested for their potential in disease models such as intracellular *S. typhimurium* infection in epithelial and macrophage cell lines. The compound does not directly affect the growth of bacteria but induced host xenophagy machinery to capture the bacteria and increased its fusion with lysosomes. Additional experiments revealed activation of TFEB to induce active lysosomal population inside cells.

Although the process is shown to restrict infection, there are no well-studied compounds that could induce the process during *in vivo* infection. Interestingly, the basal level of xenophagy in tissues such as intestine, liver, and spleen are low, which are also regions that are commonly affected during bacterial infection. It is, therefore, essential to induce the process transiently during infection to be effective in curbing intracellular infection.



This research group has also identified novel genetic as well as pharmacological modulators of autophagy. Pilot-scale screening of essential genes identified two protein complexes, namely septin (Barve *et al.*, J Cell Sci., 2018) and exocyst (Singh *et al.*, J Mol Biol., 2019) that are involved in autophagosome biogenesis. Furthermore, a high throughput screening of more than 2,00,000 compounds comprising of 16 libraries have identified multiple autophagy modulators that could temporally and transiently regulate the process (Mishra *et al.*, Autophagy, 2017; Suresh *et al.*, Autophagy, 2017; Vats *et al.*, Mol Biol Cell, 2019).

Publication:

Ammanathan V, Mishra P, Chavalmane AK, Muthusamy S, Jadhav V, Siddamadappa C, Manjithaya R. Restriction of intracellular Salmonella replication by restoring TFEB-mediated xenophagy. Autophagy. 2019. Nov 19:1-14. doi: 10.1080/15548627.2019.1689770.

Patent details:

“Method for modulating autophagy and applications thereof” Patent number: US20180369186A1.

Funding:

The work is funded by Wellcome Trust/DBT India Alliance Intermediate Fellowship (509159-Z-09-Z), LSRB-DRDO grant (LSRB-310/BTB/2017) and JNCASR intramural funds from DST.