Identification of a small molecule inhibitor of autophagic flux

and an automated deep learning workflow for high-throughput autophagic flux analyses

A thesis submitted for partial fulfillment of degree of

Master of Science

as part of Integrated PhD program

(Biological Sciences)

by

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April 2019.

CERTIFICATE

This is to certify that the work described in this thesis entitled **"Identification of a small molecule inhibitor of autophagic flux and an automated deep learning workflow for high throughput autophagic flux analyses"** is the result of investigations carried out by Ms. Irine Maria Abraham in Autophagy lab, 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 **"Identification of a small molecule inhibitor of autophagic flux and an automated deep learning workflow for high-throughput autophagic flux analyses"** is an authentic record of research work carried out by me, under the guidance of Dr. Ravi Manjithaya at Autophagy lab, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore. In keeping with the norm of reporting scientific observations, due acknowledgement has been given wherever work based on the findings of other investigators has been cited. Any omission owing to oversight or misjudgment is regretted.

Date: IRINE MARIA ABRAHAM

Bangalore, India.

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❖ **And most importantly, I thank my Lord who is my refuge, my strength and an ever-present help in trouble.**

TABLE OF CONTENTS

List of Abbreviations

ATG	**Autophagy related**
Cvt	**Cytoplasm to Vacuole Targeting**
PAS	**Phagophore Assembly Site or Pre-Autophagosomal Structure**
mTOR	**Mammalian Target of Rapamycin**
ULK	**Unc-51-Like Kinase**
FIP200	**FAK family kinase-Interacting Protein 200**
UVRAG	**UV irradiation Resistance-Associated Gene protein**
COPII	**Coat Protein complex II**
ESCRT	**Endosomal Sorting Complex Required for Transport**
Bcl-2	**B - Cell Lymphoma/leukemia-2**
ROS	**Reactive Oxygen Species**
NRF2	**Nuclear Factor-like 2**
KEAP1	**Kelch-like ECH Associated Protein 1**
Acetyl-CoA	**Acetyl Coenzyme A**
TCA	**Tricarboxylic Acid cycle**
FDA	**Food and Drug Administration**

Chapter 1
Introduction

Autophagy is an evolutionarily conserved process of cellular homeostasis and survival that takes care of worn out or unnecessary cellular components by delivering them to the lysosome in double-membraned vesicles known as autophagosomes. The autophagic process is important in human health and physiology, as its dysregulation is implicated in neurodegenerative diseases, infectious diseases, ageing, and cancer.[1] Therefore, tackling the many unanswered questions regarding the mechanisms of autophagic flux and developing novel tools to modulate autophagy is of interest.

1.1 Types of autophagy

There are three types of autophagy:

- Macro autophagy
- Micro autophagy and
- Chaperone-mediated autophagy.

Macroautophagy is the most extensively studied of these processes. It is present at low levels in all cells for constitutive turnover of cytosolic components (basal autophagy) and serves as a quality control mechanism. However, it is upregulated or induced by stimuli such as nutrient deprivation and hypoxia (induced autophagy).

Microautophagy involves inward invagination of lysosomal membrane and delivers a small portion of cytoplasm into the lysosomal lumen, whereas chaperone-mediated autophagy involves the direct translocation of cytosolic proteins across the lysosomal membrane, and requires protein unfolding by chaperone proteins.

Furthermore, autophagy is classified into numerous subtypes depending on the specific cargo captured. The process of autophagy is capable of degrading pathogens (xenophagy), misfolded or abnormal proteins (aggrephagy), damaged mitochondria (mitophagy), peroxisomes (pexophagy) or ribosomes (ribophagy). These are all examples of selective autophagy.

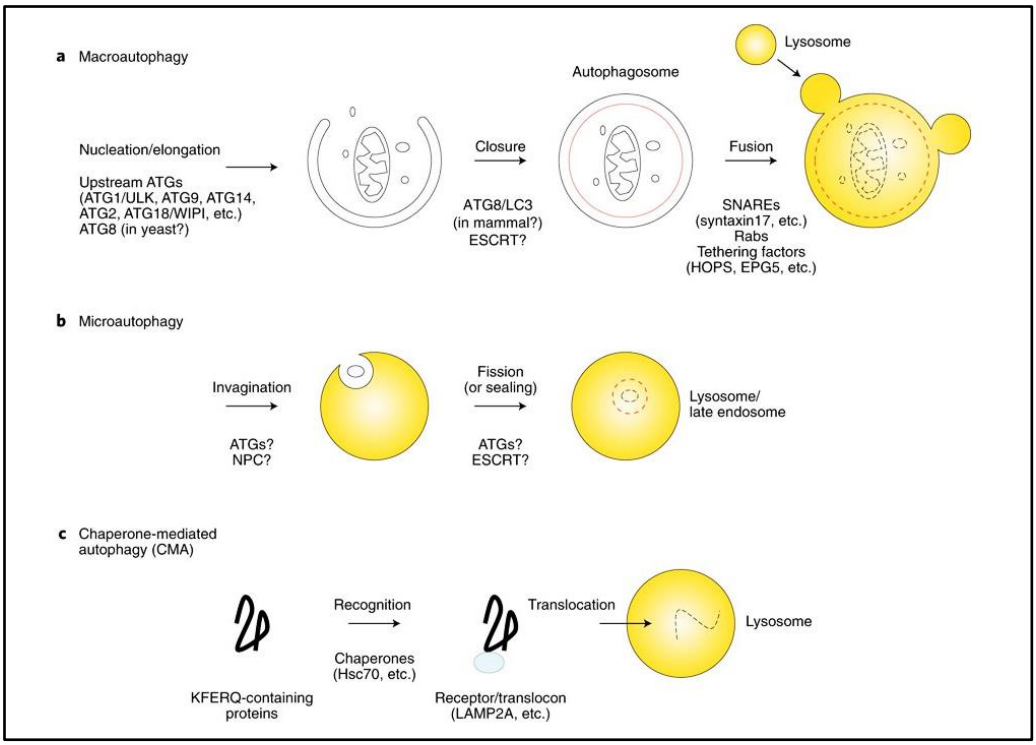

Figure 1: The three types of autophagy.

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1.2 A brief history of autophagy

Christian de Duve discovered the lysosome in 1955 during his investigation into the action of insulin on liver cells. Looking into the functions of the new organelle, he described autophagy as a process where "portions of a cell somehow find their way inside the cell's own lysosomes and are broken down."[2] In 1963, at the first international symposium on lysosomes hosted by the Ciba Foundation in London, Christian de Duve coined the terms 'autophagy' (Greek: auto = self and phagy = eating), 'endocytosis' and 'exocytosis'.[3]

However, the term ‘autophagie’ was in existence a century earlier, in the work of a French physiologist named M. Anselmier.[4] The word was used to describe a mode of nutrition, during food-deprived conditions such as a shipwreck, where “life is maintained for some time at the expense of the substance of the tissues, as is proved by the progressive diminution of the weight of the animal,” according to the ‘Nouveau dictionnaire de médecine et de chirurgie pratiques (New dictionary of medicine and surgery practice).’ Anselmier came upon this observation by performing a strange set of experiments where two dogs of the same age, size, and condition were deprived of nourishment. One died in the usual period from starvation and the other, on which he practiced periodic light bleedings and feeding its own blood, lived several days longer than the first. Nevertheless, it was Christian de Duve who used the word ‘autophagy’ in its modern sense.

Advances in the Transmission Electron Microscopy (TEM) techniques led to the visualization of the structures of the autophagic bodies. In the late 1950s, Alex Novikoff and others observed that mitochondria from mouse kidney cells were encapsulated within membrane-bound compartments termed ‘dense bodies.’ These dense structures were subsequently shown to contain lysosomal enzymes.[5–7] In 1968, Trump and Antti Arstila showed that autophagic structures were formed on administering the hormone glucagon. Initially, acid hydrolase activity was absent in the early autophagic bodies (double-membraned autophagosomes), whereas the enzyme activity was acquired by the later autophagic vacuoles (single-membraned autolysosomes) upon fusion with lysosomes.[8]

Modern science comprehends that the proteins which make up our bodies are replaced almost completely every 1-2 months. This phenomenon is called protein turnover. The central role of autophagy in protein turnover gradually came to light beginning with the experiments of U. Pfeifer and colleagues in 1983. Their study established the link between autophagy and nutrient conditions and how autophagy can be induced by fasting or inhibited by feeding.[9] The same year, G. E. Mortimore and colleagues proved that the status of autophagy is controlled by amino acid levels. The group showed that eight amino acids - Leu, Tyr, Phe, Gln, Pro, His, Trp, and Met (of which Leucine is the most potent[10]) have an inhibitory effect on autophagy.[11,12] Forays into the world of autophagy modulators were set in motion with the identification of 3-methyladenine, an inhibitor of autophagy by P. O. Seglen and P. B. Gordon.[13] This was

followed by two more inhibitors - wortmannin and LY294002 – identified by E. F. Blommaart and colleagues.[14]

Until the 1990s, autophagy research was focused on biochemical or microscopic approaches. However, in 1992-93 Yoshinori Oshumi used yeast to study the morphological changes taking place during autophagy[15] and described a genetic screen in yeast undergoing nitrogen deprivation which led to the isolation of mutants with defective autophagy. The screen identified mutants that fell into 15 complementation groups, which means that at least 15 genes were involved in the yeast autophagic pathway.[16] Around the same time, another group led by M. Thumm also isolated six yeast mutants with defects in autophagy.[17] In 1995, D. Klionsky and colleagues isolated several yeast mutants defective in the transport of the enzyme α-aminopeptidase-I. The transport of the vacuolar enzyme α-aminopeptidase I to the vacuole is known as the Cvt pathway and is an example of selective autophagy.[18]

Although autophagy was discovered in mammals, it was these pioneering studies in yeast that led to the discovery of many overlapping core autophagy genes in the early 1990s and stimulated research in the field. Soon, the autophagic pathway was discovered in many life forms as a conserved process. Today, 41 yeast ATG (AuTophaGy-related) genes have been described and many of these have human orthologues. What eventually ensued was a plethora of investigations into the mechanisms that regulate the autophagic pathway, which led to our current understanding of the process.

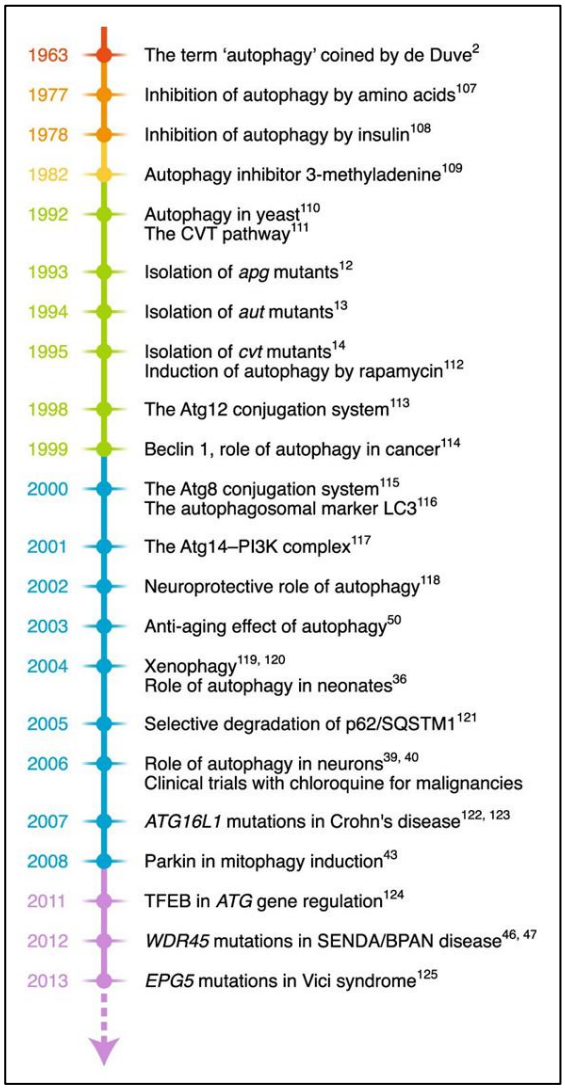

Figure 2: A timeline of important discoveries in the field of autophagy.

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1.3 Mechanisms of autophagy

Autophagosome biogenesis involves multiple membrane remodeling events. The process occurs in several sequential steps:

a) Induction of autophagy and formation of the PAS,
b) Expansion and fusion of the phagophore to form the autophagosome - resulting in sequestration of cargo,
c) Fusion of the mature autophagosome with the lysosome to generate an autolysosome followed by degradation of the autophagic cargo and
d) Utilization of the degradation products.

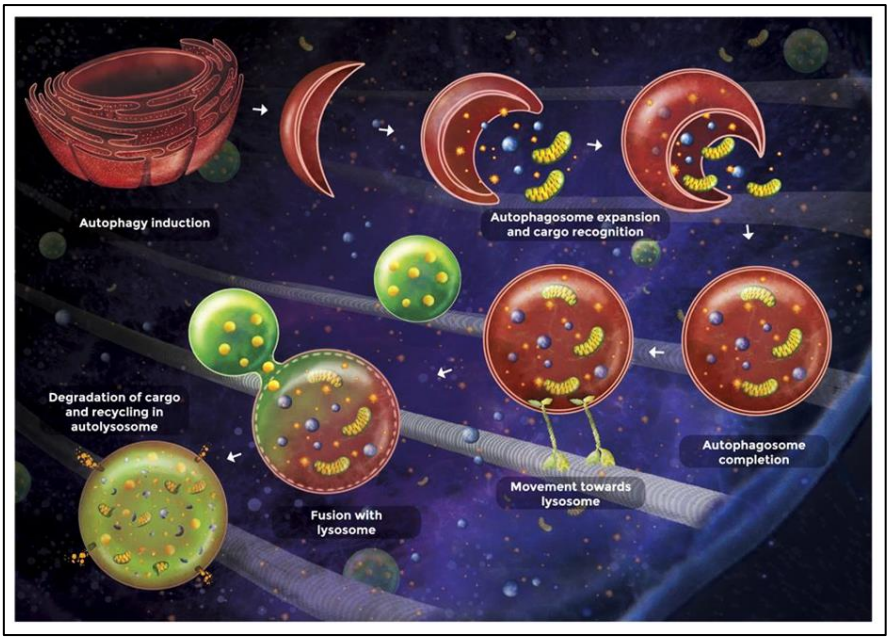

Figure 3: The process of autophagy.

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a) Induction of autophagy

Many signals such as growth factors, amino acids, glucose, and energy status converge on the serine/threonine protein kinase mTOR (mammalian Target Of Rapamycin), which is a master regulator of nutrient signalling and a negative regulator of autophagy.[19] The serine/threonine kinases ULK1 and ULK2 (Unc-51-Like Kinase 1 and 2), which act downstream of the mTOR, exist in two large functional complexes (mTORC1 and mTORC2) that consist of mAtg13 and the scaffold protein FIP200. Atg101, another protein deemed to be part of the ULKs-Atg13-FIP200 complex, binds and stabilizes Atg13. It was identified later than the rest of the core machinery and was observed to be required for mammalian autophagy.

Under nutrient-rich conditions, mTORC1 is incorporated into the large ULK1-Atg13-FIP200 complex, leading to phosphorylation of ULK1 and Atg13 and consequently, inhibition of autophagy. On the other hand, during nutrient deprivation (starvation), mTORC1 dissociates from the ULK1 complex, causing the activation of ULK1. Activated ULK1 auto-phosphorylates, undergoes a conformational change and phosphorylates both Atg13 and FIP200 to initiate autophagy.[20]

b) Expansion of autophagosomes and sequestration of cargo

The biogenesis of autophagosomes begins with the formation of a small cup-shaped membrane called the isolation membrane or PAS ((Pre-Autophagosomal Structure or Phagophore Assembly Site). Upon initiation of autophagy, the mTOR complex moves to the ER (Endoplasmic Reticulum) and recruits the PI(3)P kinase complex. In mammals, the class III PI(3)P kinase complex comprises of the PI(3)P kinase Vps34, the serine/threonine kinase p150, mAtg14 (Barkor), Beclin-1, and Ultraviolet irradiation Resistance Associated Gene (UVRAG).[21,22] Bcl-2 (B-cell Lymphoma/leukemia-2), an anti-apoptotic protein sequesters Beclin-1 under nutrient-rich conditions. Dissociation of Beclin-1 from Bcl-2 is required for autophagy induction. The interaction of Beclin-1 with Vps34 promotes the catalytic activity of Vps34, which uses the substrate phosphatidylinositol (PI) to generate phosphatidyl inositol triphosphate (PI3P). The subdomains of the ER thus enriched with PI(3)P are called omegasomes and provide a platform for recruitment of other Atg proteins involved in autophagosome biogenesis.[23] The membrane sources suggested for the expanding autophagosomes are the COPII vesicles, Atg9 (the only integral membrane protein involved in autophagosome biogenesis) vesicles, ER, plasma membrane, ER-Golgi intermediate compartment and the mitochondria.

Two interrelated ubiquitin-like (Ubl) conjugation systems, Atg12–Atg5-Atg16 and MAP1LC3–PE (phosphatidylethanolamine), are essential for the elongation and expansion of the phagophore membrane. Atg12 is activated by Atg7 (E1 ligase), transferred to Atg10 (E2 ligase) and covalently attached to an internal lysine of the substrate protein Atg5. The Atg12–Atg5 conjugate further interacts with a coiled-coil protein Atg16 and links the Atg12–Atg5-Atg16 complex into a tetramer by self-oligomerization.[24–28] However, unlike the ubiquitination reaction, Atg12–Atg5 conjugation is irreversible, and an E3 ligase is not required.

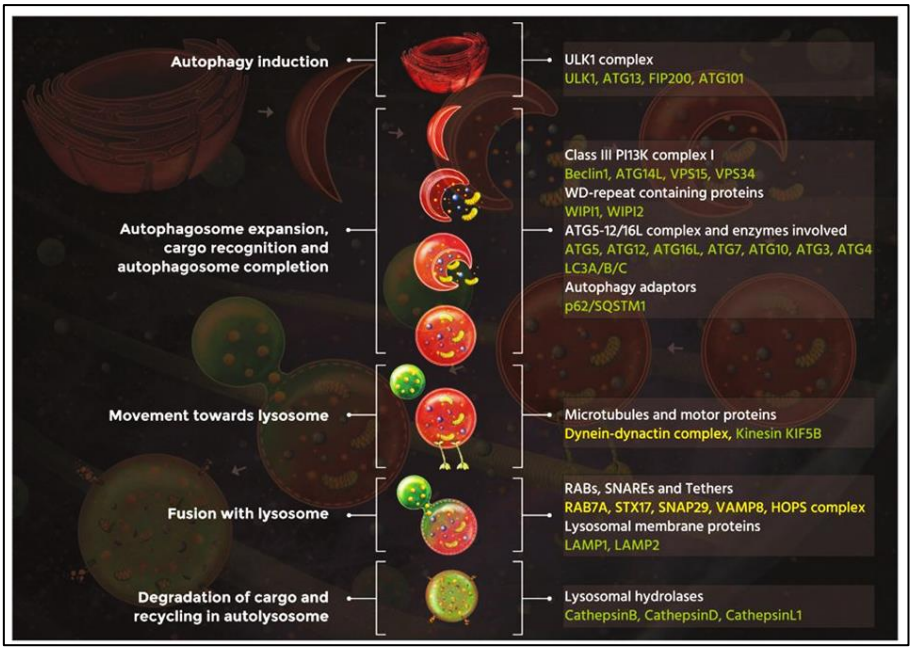

Figure 4: The molecular mechanisms of autophagy.

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MAP1LC3 is processed by a cysteine protease Atg4, exposing the C-terminal glycine residue and forming the cytosolic MAP1LC3-I. Atg7 (E1 ligase) activates MAP1LC3-I, transfers it to Atg3 (E2 ligase) and is finally conjugated to the target lipid PE via an amide bond to derive the MAP1LC3-II form.[28,29] The Atg12–Atg5 conjugate acts as an E3 ligase in this reaction. In nutrient-rich conditions, most of the MAP1LC3 is cytosolic whereas upon autophagy induction, MAP1LC3 largely exists as the lipid-conjugated form and is localized to both sides of the phagophore. MAP1LC3 determines the extent of membrane curvature and therefore, controls the size of the autophagosome. This is why, monitoring the lipidated MAP1LC3 is a good strategy of assessing the autophagic flux.

The closure of the elliptical phagophores into the spherical autophagosomes is completed by membrane abscission by the Endosomal Sorting Complex Required for Transport (ESCRT).[30] At this point, the autophagosomes dissociate from the ER and move towards the lysosome.

(c) Fusion and degradation of cargo

The distribution of lysosomes is altered by nutrient availability. Starvation causes perinuclear clustering of lysosomes, which in turn influences mTORC activity.[31] Autophagosomes, on the other hand, are formed throughout the cytoplasm. The movement of the autophagosomes and the lysosomes are mediated by the motor proteins dynein (minus end transport) and kinesin (plus end transport).[32] The fusion of the mature autophagosomes with the lysosome is driven by the assembly of the SNARE (Soluble N-ethylmaleimide sensitive factor Attachment protein Receptor) proteins. The SNARE proteins are themselves controlled by the Rab GTPase, HOPS (Homotypic fusion and protein sorting complex) and tethering factors. Two cognate SNARE complexes, formed on opposing membranes, mediate autophagosomal fusion:

(i) autophagosomal-localized Stx17 (Qa), SNAP29 (Qbc) and endolysosomal-localized VAMP8[33]
(ii) autophagosomal-localized R-SNARE YKT6, SNAP29 and lysosomal-localized Qa SNARE Stx7.[34]

The structures thus formed upon fusion of the autophagosome and lysosome are called autolysosomes. Upon fusion, the inner membrane of the autophagosome dissolves, the autophagosomal lumen acidifies and the cargo is then degraded by lysosomal acid hydrolases such as cathepsins B, D and L.

(d) Generation of recycled products

Once the cargo has been degraded, the resulting monomeric units (particularly amino acids) are transported back to the cytosol for protein synthesis and maintenance of cellular functions. Therefore, autophagy is a catabolic process which generates intermediates required for the anabolic reactions, by degrading cytosolic materials.

1.4. Role of autophagy in health and disease

The process of autophagy plays many physiologically relevant roles.

1.4.1 Neurodegenerative diseases

Neurons are more vulnerable to accumulation of cellular waste than other cell types because they lack cell division. Neurodegenerative diseases are characterized by accumulation of intra- and extracellular protein aggregates that are not degraded by the ubiquitin-proteasome system. Autophagy clears away these aggregates and plays a neuroprotective role.[35] Inducers of autophagy have been shown to lessen the symptoms of neurodegeneration, e.g., 6-Bio, an inducer of autophagy previously identified in the lab clears away aggregates of synuclein, which is implicated in Parkinson's disease.[36]

1.4.2 Infectious diseases

When pathogens invade and enter a cell during an intracellular infection, autophagy captures the foreign infectious agents and neutralizes them using the lysosome. The use of autophagy for the clearance of pathogens is called xenophagy and studies suggest that xenophagy can limit the extent of infection by various microbes, e.g., *Mycobacterium* spp, *Listeria* spp.[1]

1.4.3 Ageing

Genetic and pharmacological investigations hints at a link between autophagy and rate of ageing. For instance, evaluation of lifespan has been done in models such as flies, worms and mice. Overexpression of a protein (Atg5) essential for autophagy or treatment using an autophagy inducer, rapamycin, in mice shows an extension in the life span of the animals.[37,38]

1.4.4 Cancer

The relationship between autophagy and cancer was first documented by the examination of mice with allelic loss of the essential autophagy gene Beclin-1 (Becn1 or Atg6). These mice were noted to have partially defective autophagy and eventually develop hepatocellular carcinomas.[39–41]

Cells with defective autophagy accumulate ubiquitylated protein aggregates, abnormal organelles, particularly mitochondria, as well as excess organelles such as peroxisomes, endoplasmic reticulum (ER) and ribosomes. These effects lead to the production of reactive oxygen species (ROS), metabolic insufficiency, and increased proteotoxicity, thereby promoting cellular damage, reduced stress tolerance and compromised survival.

Autophagy acts as the guardian of the genome by preventing DNA mutations, gene copy number variations and gene amplification, and suppressing the initiation of tumours. Two important ways by which this occurs is as follows:

(a) Mitophagy reduces ROS and oxidative stress

Dysfunctional mitochondria lose membrane potential, which triggers the activation of PTEN-induced putative kinase 1 (PINK1). PINK1 activates the E3 ligase parkin (PARK2) to ubiquitylate mitochondrial outer membrane proteins, which is recognised by the autophagy machinery, leading to the selective elimination of damaged mitochondria and maintenance of mitochondrial quality control.[42]

(b) Transcription of antioxidant-defence genes

NRF2 is a transcription factor which translocates to the nucleus and turns on the expression of numerous ROS-detoxification genes, promoting cell survival. In the absence of oxidative stress, NRF2 is bound to its inhibitor kelch-like ECH-associated protein 1 (KEAP1) and is degraded, and antioxidant-defence genes are not activated.[43]

Oxidative stress causes the modification of KEAP1 that releases NRF2 or causes the upregulation of the autophagic cargo adaptor p62. The binding of p62 to KEAP1 competitively displaces NRF2, which is free to translocate to the nucleus.

However, tumour cells have upregulated autophagy compared to the basal autophagic levels of normal cells and tissues. Autophagy induction promotes survival in the presence of the numerous stressors associated with the tumour microenvironment including starvation, growth factor deprivation, hypoxia, damaging stimuli and proteasome inhibition. Degradation of proteins by autophagy generates amino acids that can feed into the TCA cycle to sustain mitochondrial metabolism lipids harvested from lipid droplets by lipophagy, or from organelle membrane degradation by autophagy, can be used to produce acetyl-CoA to maintain TCA cycle function.

Therefore, autophagy can be detrimental or beneficial to cancer depending on context and hence, is considered a double-edged sword.[44]

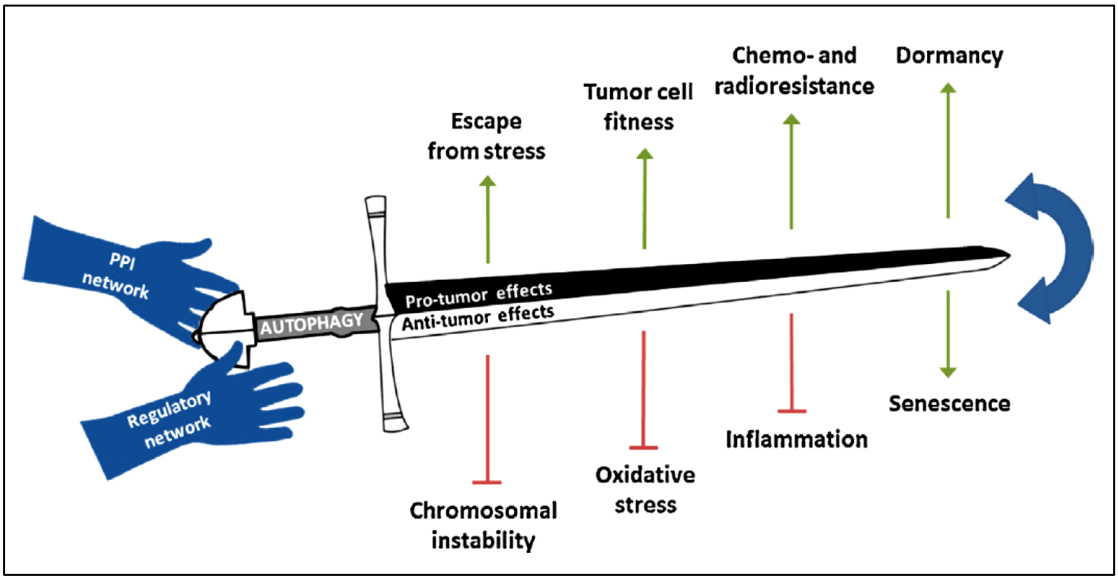

Figure 5: The role of autophagy in cancer is context-dependent. On the one hand, autophagy prevents tumour initiation but on the other, autophagy promotes the growth of tumour cells.

With permission from Kubisch, J. et al., Semin. Cancer Biol. 23, 252–261 (2013).

1.5 Modulation of autophagy

Given its importance in heath and disease, modulation of autophagy is a strategy for treating various conditions. In addition, it provides us insightful observations regarding the mechanisms of autophagic flux. Of the numerous ways to modulate autophagy, the lab had ventured into the chemical biology approach because of its many advantages.

Rebecca Ward at Harvard University was the first to coin the "chemical genetic" approach. According to her, understanding a life process involves perturbing it (using small molecules, for instance) and determining the consequence. Such an approach would strive to have the broad generality and power of genetics.

Small molecules are organic, non-peptide compounds, typically less than 1500 Da in size, that are either synthetic or derived from natural product extracts. Membrane permeability and multi-ring core structure are their common properties.

The advantages of using small molecules in research are mentioned below:

- ✓ Small molecules are cell permeable and affect signalling pathways and processes within the cell.
- ✓ They are cost-effective, stable, easily manufactured, stored and administered.
- ✓ The actions of small molecules are specific, dose-dependent, rapid and reversible.
- ✓ The structural diversity that can be provided by synthetic chemistry allows the functional optimization of small molecules.
- ✓ They can be used in cell culture as well as easily adapted into in vivo models.

The power of biology-oriented chemical design and synthesis, coupled with noteworthy advances in screening technology, has enabled the identification of a growing range of small molecules. These compounds bear the burden of big roles and can pave the way to answering big questions.

Inhibitors of autophagy like chloroquine and hydroxychloroquine have been approved by the FDA for treatment of various cancers. These molecules have been used in conjunction with radiation therapy and chemotherapy in clinical trials.

However, the fallbacks are as follows: (i) the high doses of the drug required for achieving autophagy inhibition causes toxicity-associated symptoms in patients and (ii) these inhibitors inhibit the autophagic flux by blocking autophagosomal fusion with the lysosome and hence, ae not specific to the autophagic pathway. Therefore, the need for novel autophagy-specific inhibitors are of paramount importance.

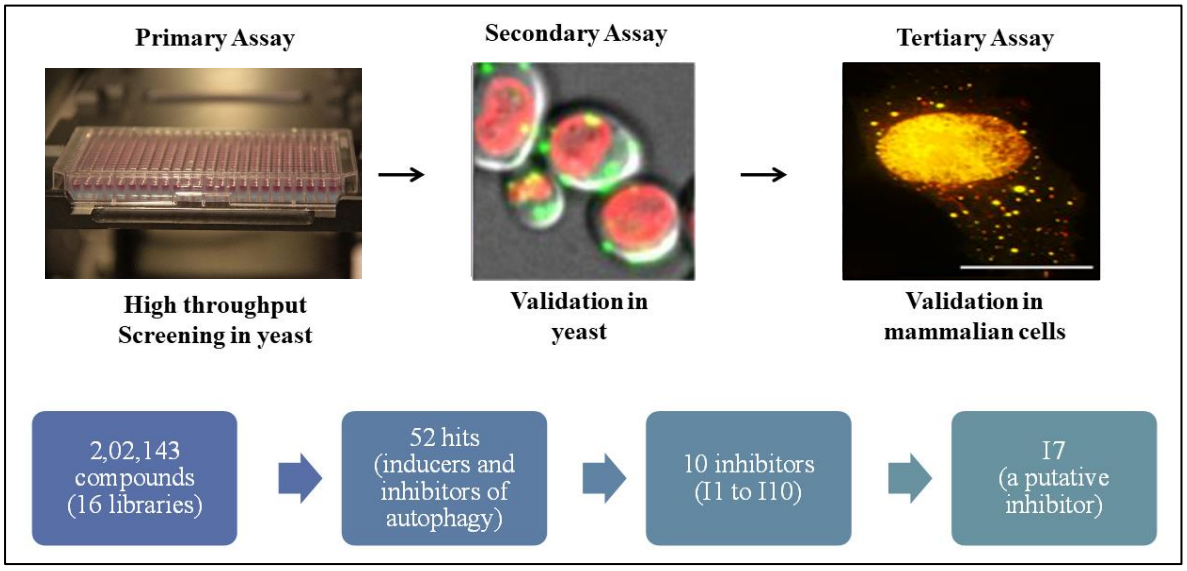

Figure 6: The schematic for the high-throughput screening system.[45] Small molecules from 16 libraries were screened using in the primary assay and the promising candidates from this assay are validated through secondary assays in yeast and tertiary assays in mammalian cells.

Towards this end, the lab has performed high-throughput screening of a vast number of small molecules from 16 libraries using an endogenously developed luciferase-based luminescence assay. The promising candidates from this primary assay have been validated through secondary assays in yeast and in mammalian cells. The inhibitors identified this way were observed to be targeting different steps of the autophagic process. This emphasizes the strength of the high-throughput screening, which is phenotype-based and not target-specific.

From the 52 hits acquired from the screening, 10 compounds (labeled as I1 to I10) were validated in mammalian cells. The thesis follows the identification of I7 as an inhibitor of autophagy.

Chapter 2

Materials and Methods

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a) Plasmids, Antibodies and Chemicals

Plasmids used:
ptfLC3 (mRFP-GFP-LC3 - Addgene plasmid #21074) was a gift from Tamotsu Yoshimori. GFP-LC3 plasmid was generated in the lab by excising out mRFP fragment from mRFP-GFP-LC3 plasmid.

The primary antibodies used:
LC3B (Sigma-Aldrich, L7543), β-Actin (CST, 4970), LAMP1 (CST, 9091), p62/SQSTM1 (Abcam, ab56416), β-Tubulin (DHSB, E7), EGFR (Santa Cruz Biotechnology, sc-03).
Fluorescent secondary antibodies used:
Atto 633 (goat anti-rabbit IgG, Sigma-Aldrich, 41176).

Chemicals used:
I7 (the identity of the compound can't be revealed since it is patent pending), Bafilomycin A1 (Sigma-Aldrich, B1793), EGF (Thermo Fisher Scientific, PHG0311L) and Lysotracker Deep Red (Thermo Fisher Scientific, L12492).

b) Mammalian Cell Culture

HeLa cells were maintained in growth medium comprising of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, D5648) supplemented with 3.7 g/L sodium bicarbonate (Sigma-Aldrich, S5761), 10% Fetal Bovine Serum (FBS) (Gibco, 10270-106) and 100 units/ml of Penicillin and Streptomycin (Gibco, 15140-122) at 5% CO_2 and 37°C.

c) Cell Titer-Glo cell viability assay

Toxicity of I7 was monitored by Cell Titer-Glo cell viability assay (Promega, G7570). Equal numbers (1500 cells/well) of HeLa cells were plated in a 384 well plate with growth medium. The following day, different concentrations of I7 in the range of 1nM to 100μM for 72 hours. After the incubation period, 5μl of Cell Titer-Glo Reagent was added to each well, and luminescence was measured using Varioskan Flash (Thermo Fisher Scientific).

d) Immunoblotting

Equal numbers of sub-confluent HeLa cells were seeded in 6-well dishes and allowed to attach for 24h. The cells were then treated with I7 and/or Bafilomycin A1 at differing concentrations for the respective duration. The negative and positive controls required for the experiment were set up. After the period of incubation, the cells were washed with PBS and lysed with SDS-DTT-protein dye solution to get total protein lysates. Lysates were scraped off the wells, boiled for 15 mins and loaded onto SDS-PAGE gels for protein separation.

The separated proteins were then transferred onto polyvinyl difluoride (PVDF) membranes using a tank transfer system (Bio-Rad, USA). The membranes were blocked for 1h in 5% skimmed milk in PBS, then probed with primary antibodies overnight at 4ºC, and finally incubated with horseradish peroxidase-conjugated, species-specific secondary antibodies at room temperature for 1h. Bands were visualized using an enhanced chemiluminescence kit with a Syngene Gel-Doc system. Quantification relative to β-Actin by densitometric analysis was conducted using Image-J software and the graphs were plotted using GraphPad Prism 5.

e) Immunofluorescence Microscopy

HeLa cells were seeded in 60-mm dishes containing cover slips and were transfected with plasmid encoding the construct eGFP-LC3 and incubated for a period of 24-48 hours. Later, the cells were incubated with I7 at a concentration of 25μM, for 2h at 37°C and 5% CO_2.

For the last 30 mins of incubation, 100nM of Lysotracker Deep Red stain was added to the cells.

Later, the cells were washed with PBS and fixed using 4% paraformaldehyde (PFA). This is followed by three PBS washes of 5 mins each. The cells were then permeabilised by 0.25% Triton X-100 for 15 mins and washed with PBS thrice 5 mins each. The cover slips were then incubated with respective primary antibodies overnight at 4ºC, and finally incubated with fluorophore-conjugated, species-specific secondary antibodies at room temperature for 1 h.

The cover slips were then mounted on slides with a drop (7µL) of Vectashield antifade reagent (H-1000/ H-1200, Vector laboratories) with DAPI and sealed using colourless nail polish. The cells were observed under a widefield fluorescence microscope (Delta Vision Elite) and images were acquired. The intensity of the signal was measured, and the graphs were plotted on GraphPad Prism 5.

f) Traffic Light Assay

Sub confluent HeLa cells were seeded in 60-mm dishes containing 12 cover slips each and were transfected with plasmid (Addgene, 21074) encoding the construct mRFP1-eGFP-LC3 and incubated for a period of 24-48 hours. Later, the cells were treated with the indicated compounds at concentrations of 10µM, 25µM and 50µM, for 2 h at 37°C and 5% CO_2. The cells were washed with PBS and fixed using 4 % paraformaldehyde (PFA). This is followed by three PBS washes of 5 mins each. The cells were then permeabilised by 0.25% Triton X-100 for 15 mins. The cells were again washed with PBS thrice 5 mins each.

The cover slips were then mounted on slides with a drop (7µL) of Vectashield antifade reagent (H-1000/ H-1200, Vector laboratories) without DAPI and sealed using colourless nail polish. The signal was observed under a widefield fluorescence microscope (Delta Vision Elite) and images were acquired. The number of autophagosomes and autolysosomes were counted using Cell Counter plug-in of ImageJ software, and the graphs were plotted on GraphPad Prism 5.

g) EGFR Trafficking

HeLa cells were plated on 6 well plates in equal numbers. The following day, cells were washed with PBS and then starved in DMEM (serum free media) for 3 hours. Pre-treatment with I7 (25μM) was carried out for 1 hour, following which cells were pulsed with 100 ng/ml of EGF and lysates were collected at 0, 1, 2 and 3-hour intervals.

Chapter 3
Characterization of I7 as an autophagy modulator

The small molecule libraries screened using the luciferase based high-throughput assay identified 52 modulators of autophagy. These hits were inducers and inhibitors that act at different steps of the autophagic pathway. This was possible because the assay is not directed at a particular target and therefore, could detect modulators that affect any step of autophagic flux from biogenesis to cargo degradation. This is one of the greatest strengths of this screening system.

To set up this assay in the lab, a dual luciferase positive reporter yeast strain was constructed and grown in the presence of fatty acid containing media. This induces the biogenesis of peroxisomes and drives the expression of firefly luciferase which is targeted to peroxisomes through a PTS-1 signaling sequence. Then the cells are subjected to starvation. The luciferase activity is monitored over time in the absence (black line) or presence of compounds to yield putative inhibitors (red line) or enhancers (green line) of autophagy.[45]

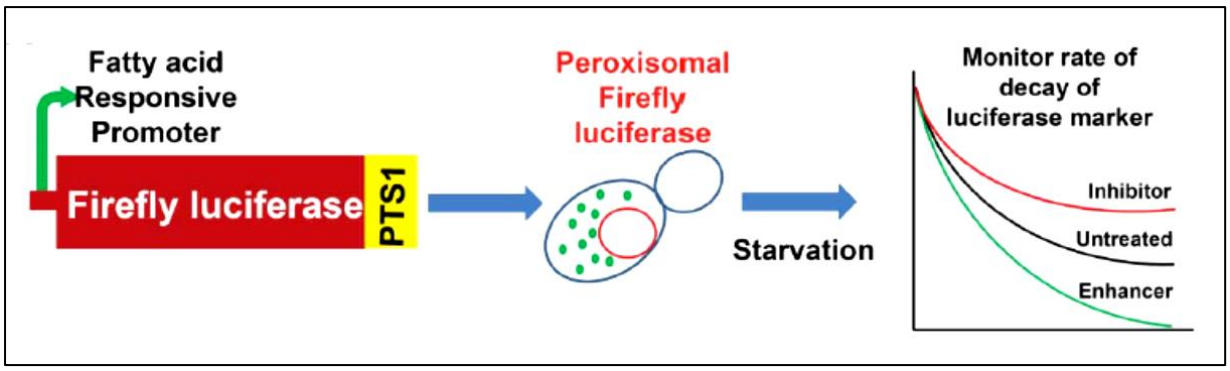

Figure 7: An overview of the dual luciferase high-throughput assay.
A dual luciferase positive reporter yeast strain was constructed and grown in the presence of fatty acid containing media to induce the biogenesis of peroxisomes. Also, peroxisome-targeted firefly luciferase. Under starvation conditions, the cells are monitored over time for luciferase activity. The representative graph shows the rate of decay of luciferase in the absence (black line) of compounds and in the presence of inhibitors (red line) or enhancers (green line) of autophagy.
From Mishra, P. et al., Autophagy 13, 1556–1572 (2017).

From among the 52 hits obtained from the dual luciferase assay, 10 compounds were further tested with the help of secondary assays in mammalian cells. One of the most promising putative inhibitors identified from the screen, a compound referred to as I7 (a halogenated benzene sulfonamide), was taken up for follow-up studies.

3.1 Assessing the cytotoxicity of I7

Before setting up experiments to characterize the compound I7, the toxicity of the compound on HeLa cells was monitored using different concentrations of I7 ranging from 1nM to 100μM, at 72h by Cell Titer-Glo cell viability assay.

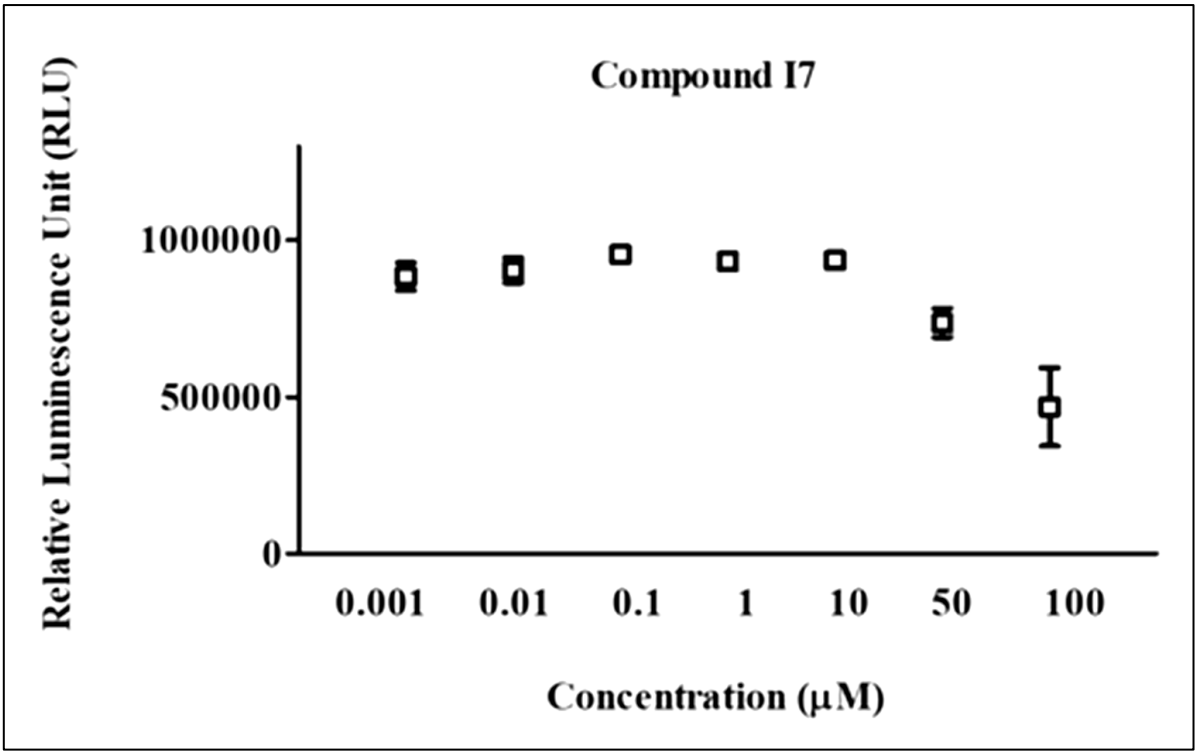

Figure 8: Cytotoxicity Assay for I7. Equal numbers of HeLa cells were seeded in a 384 well plate with growth medium. The following day, the cells were treated with concentrations of I7 in the range of 1nM to 100µM for 72 hours. Post incubation, 5µl Cell Titer-Glo Reagent was added to each well, and luminescence was measured using Varioskan Flash (Thermo Fisher Scientific). The graph was plotted for percent cell viability at 72 hours post I7 treatment. (N = 3)

The HeLa cells were treated for 72 hours with I7 and cell survival was drastically affected only around 100µM. Therefore, the concentrations of 5µM, 10µM, 25µM and 50µM used henceforth for characterizing I7 at 2h treatment duration will not cause cytotoxicity for the mammalian cells.

3.2 Conversion of MAPLC3B-I to MAP1LC3B-II form

Autophagy is a dynamic process and at any given point of time, the rate of autophagic cargo degradation is called the autophagic flux. Monitoring the conversion of MAP1LC3B-I to MAP1LC3B-II is a way to follow the autophagic flux.

MAP1LC3B exists in two forms inside mammalian cells. MAP1LC3B-I is the cytosolic free form not associated with any membrane. Action of a cysteine protease, ATG4, cleaves the end of MAP1LC3B-I to expose a C-terminal glycine, which is lipidated onto the autophagosomal membrane and becomes the functional form MAP1LC3B-II.[46,47]

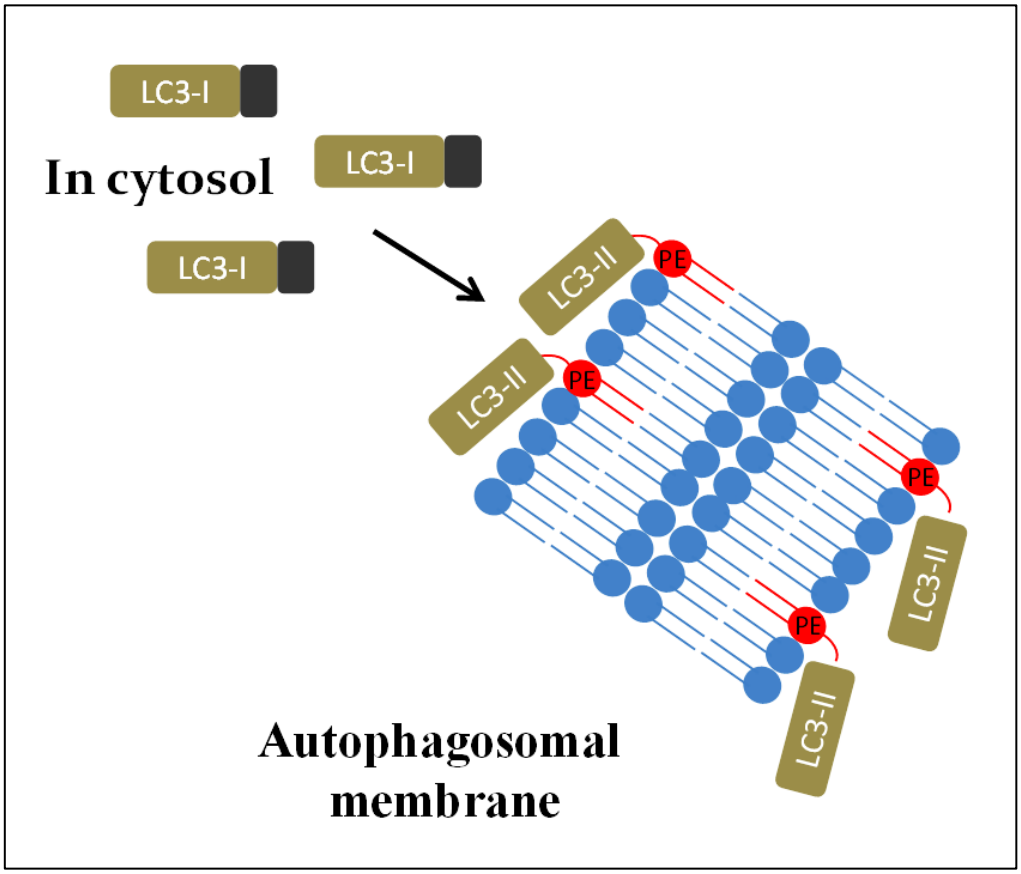

Figure 9: The conversion of cytosolic form MAP1LC3B-I to the lipidated functional form MAP1LC3B-II is one of the standard ways of following the autophagic flux.

For the assay, cell lysates were collected after appropriate treatment with I7 and electrophoresed on 15% percentage of SDS-PAGE. The proteins were transferred onto PVDF membrane and incubated with the appropriate primary and secondary antibodies, and the blots were later developed.

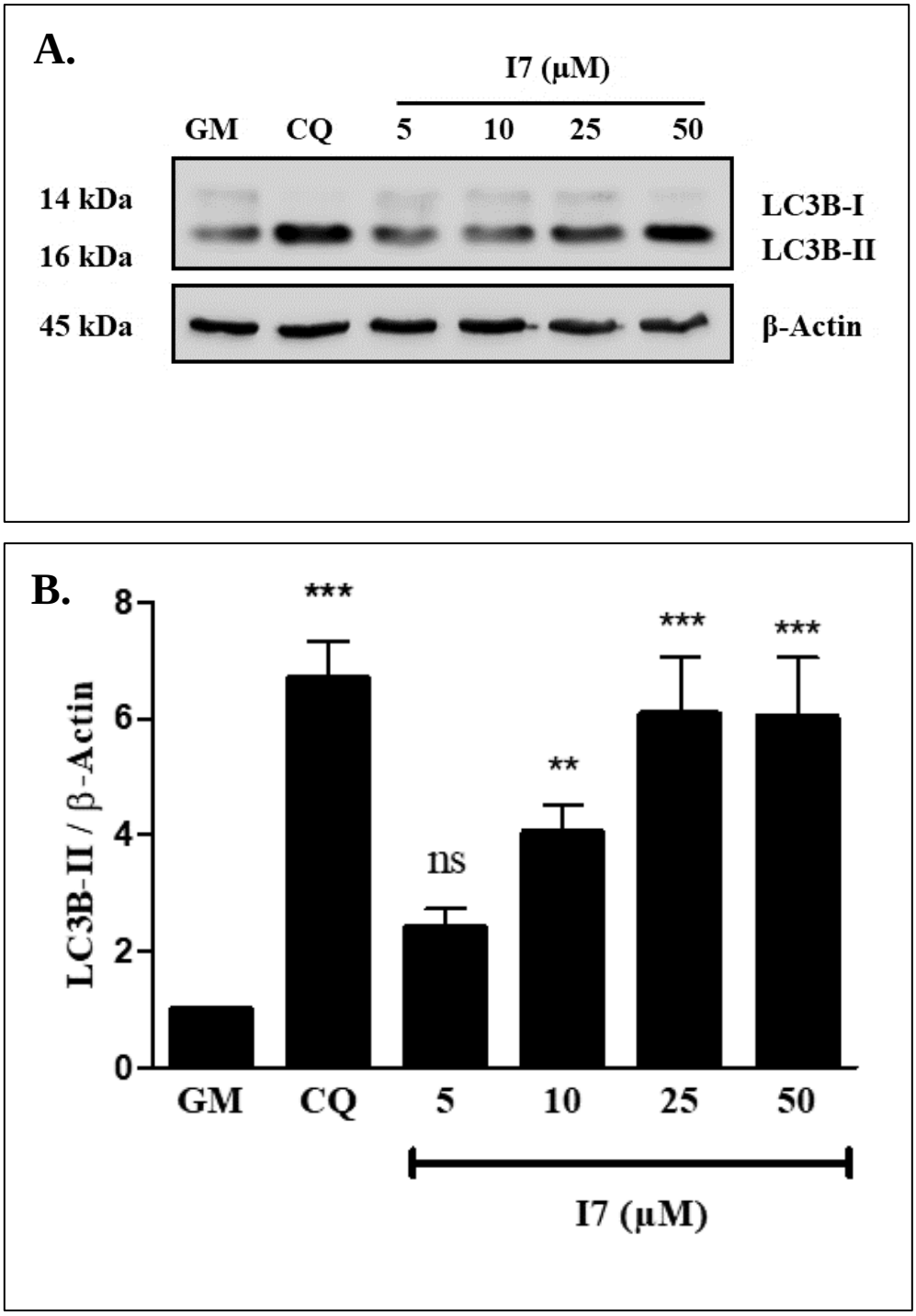

Figure 10: (A) MAP1LC3B conversion assay for untreated, CQ or I7 treated HeLa cells under nutrient rich conditions. (B) MAP1LC3B-II: β-Actin levels were quantified and plotted. ns $p > 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (N = 3, One-way ANOVA, individual means compared with a Dunnett's Multiple Comparison Test.)

It was observed that upon treatment with I7 for 2 hours, there is an increase in the level of MAP1LC3B-II protein in a dose dependent manner. The concentrations of 10µM, 25µM and 50µM were all capable of significantly increasing the MAP1LC3-II form. However, these findings do not tell us whether I7 induces autophagy or blocks the autophagic flux. Either could result in an accumulation of autophagosomes. To resolve this question, a second assay was performed.

3.3 Distinguishing between inducer and inhibitor

To do an autophagic flux assay, cells were treated with I7 for 24h and Bafilomycin A1 was added for the last 4h. The cell lysates were collected along with the appropriate controls. Bafilomycin A1, a well-characterized autophagy inhibitor, blocks autophagosome to lysosome fusion, which causes the accumulation of autophagosomes. If I7 is an inducer, the combination of I7 + Bafilomycin A1 would bring about an increase in MAP1LC3B-II over and above that of treatment with Bafilomycin A1 alone. However, if I7 were an inhibitor, it would lead to an MAP1LC3B-II protein increase like that of Bafilomycin A1. In fact, this was indeed the case.

The immunoblotting results show that I7 + Bafilomycin A1 acts like an inhibitor + inhibitor combination. Thus, I7 is an inhibitor of autophagy.

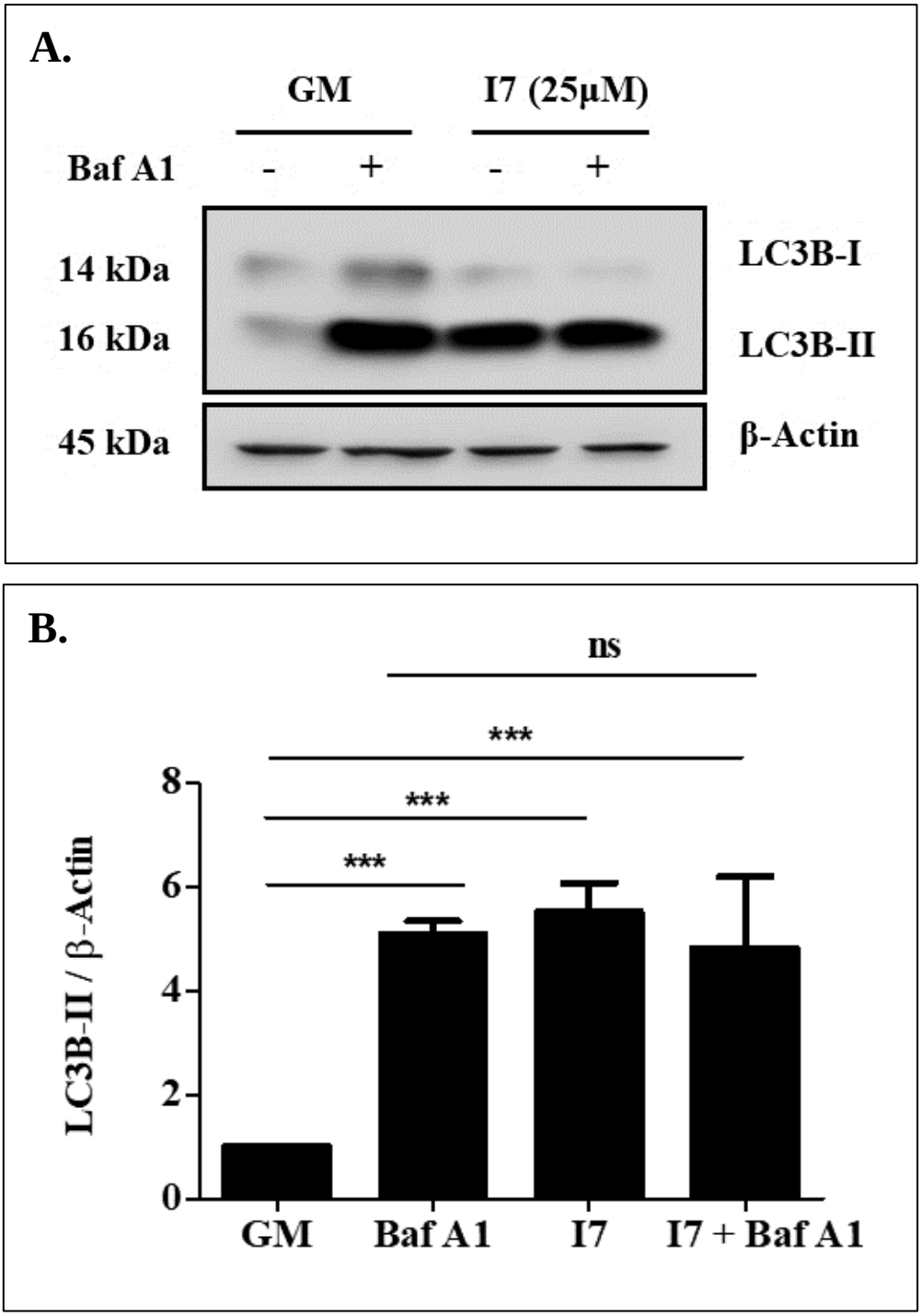

Figure 11: (A) MAP1LC3B conversion assay for untreated, I7 and/or Baf A1 treated on HeLa cells under nutrient rich conditions for 24h. (B) MAP1LC3B-II: β-Actin levels were quantified and plotted. ns $p > 0.05$, *** $p \leq 0.001$.(N = 3, One-way ANOVA, individual means compared with a Dunnett's Multiple Comparison Test.)

3.4 Illustration of autophagic flux by Traffic Light Assay

The tandem fluorescent tagged LC3 reporter protein developed by Kimura and colleagues is one of the most useful approaches to follow autophagic flux and involves overexpression of MAP1LC3B with a double tag construct: GFP-RFP-LC3. The vector uses fluorescent tags with differential pH sensitivity. Due to the acidic pH of the autolysosome, the GFP signal is quenched, whereas the RFP tag is acid-insensitive and is not lost until the proteins are degraded in the autolysosome. Therefore, autophagosomes appear as yellow, while the autolysosomes are labelled in just red. For that reason, the technique is commonly referred to as the Traffic Light Assay (TLA).[48]

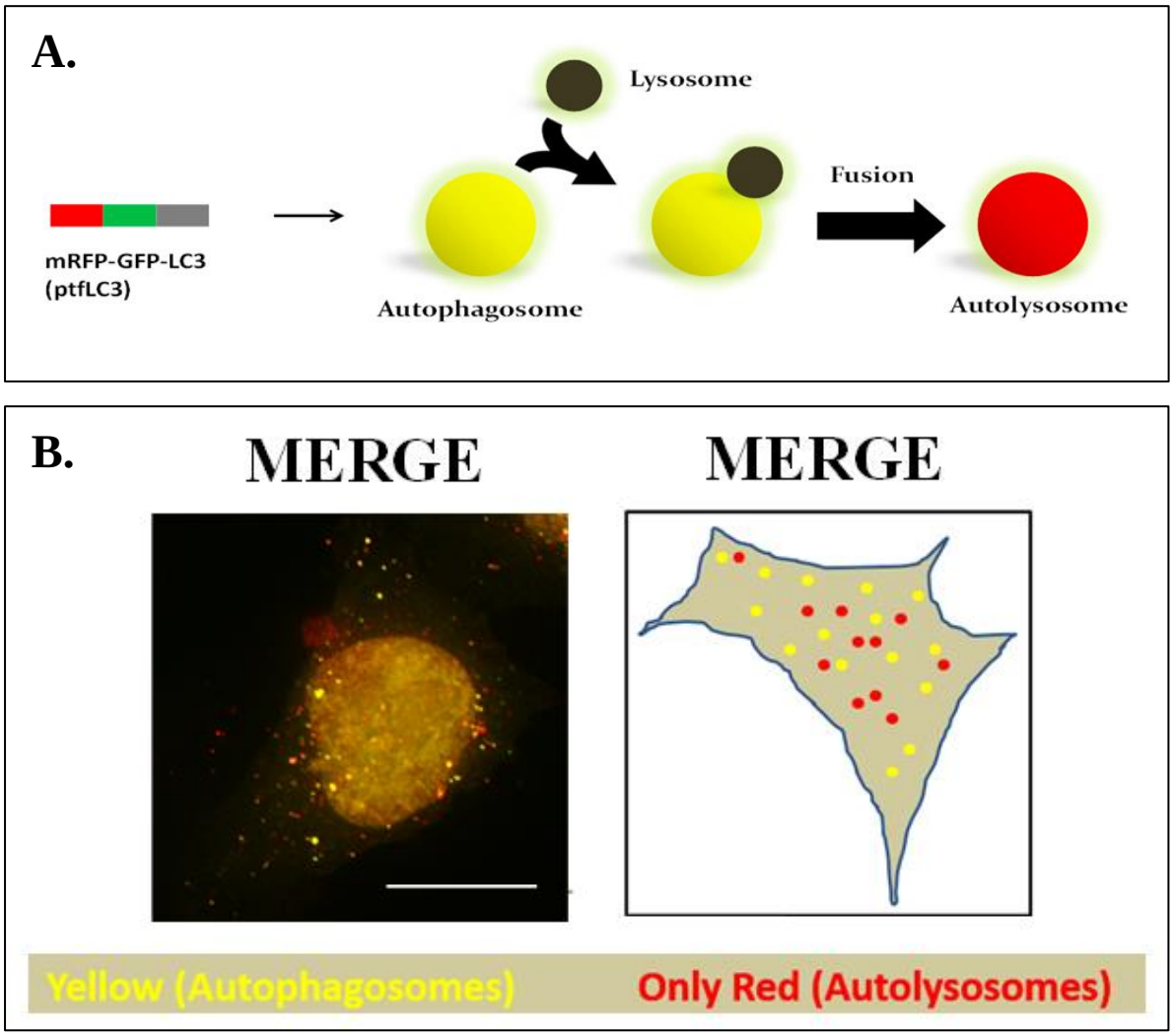

Figure 12: (A) The principle of traffic light assay. (B) Traffic light assay image of a HeLa cell and a diagram – both showing yellow autophagosomes and red autolysosomes. Scale bar:10µm.

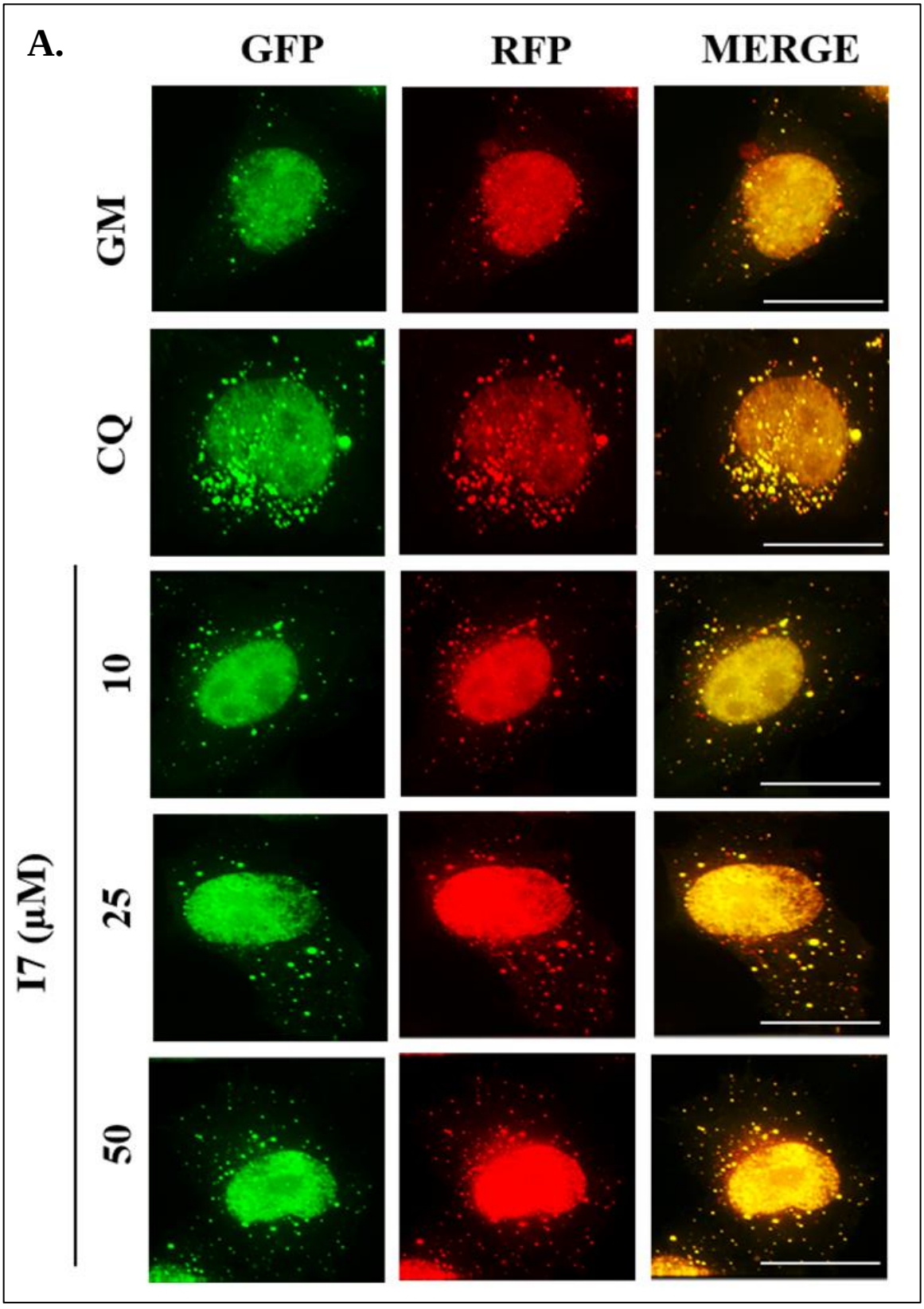

Figure 13: (A) Hela cells transfected with ptf-MAP1LC3B were untreated or treated with either Chloroquine (CQ) or I7 for 2 h under basal conditions and observed under a fluorescence microscope. Autophagosomes appear as yellow dots whereas autolysosomes appear red inside the cells. Scale bar: 10 µm.

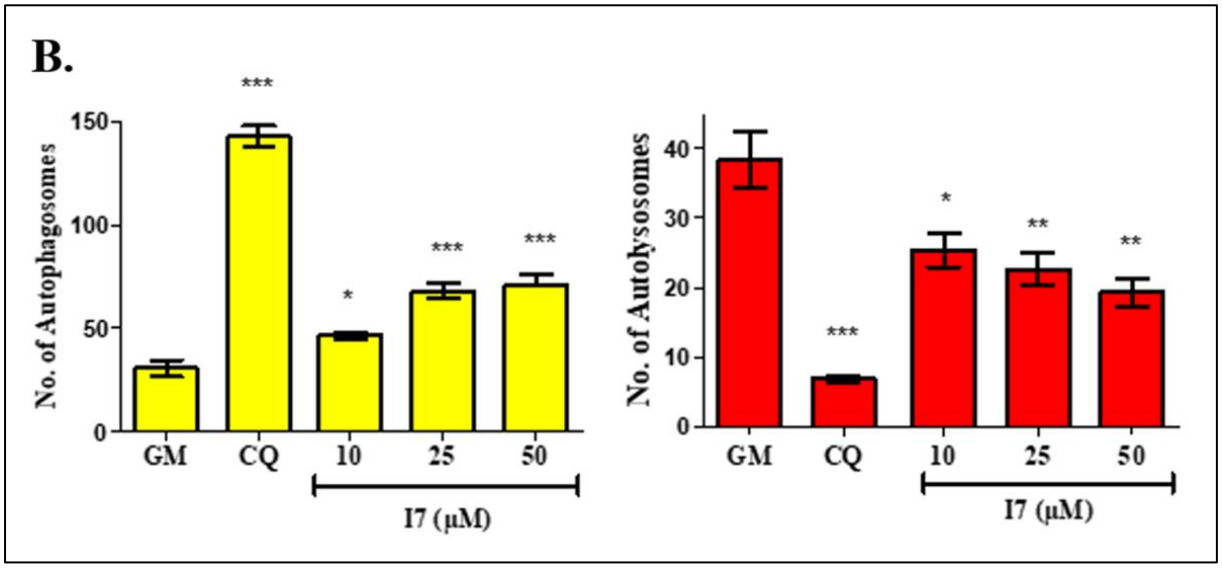

Figure 13: (B) The number of autophagosomes and autolysosomes were quantitated using ImageJ software and plotted. On treatment with I7, autophagosomes increased inside the cells whereas very few autolysosomes were seen. ns $p > 0.05$, ** $p \leq 0.1$, ** $p \leq 0.01$, *** $p \leq 0.001$. (N = 3, One-way ANOVA, individual means compared with a Dunnett's Multiple Comparison Test.)

The drug chloroquine, a well-known autophagy inhibitor, was used as a positive control while 10µM, 25µM and 50µM of the compound I7 were used for the experiment. Chloroquine produced a block in the autophagic flux as expected, resulting in an accumulation of autophagosomes and a decrease in the number of autolysosomes.

A significant dose dependent accumulation of autophagosomes ($mRFP^+/GFP^+$) and a concomitant decrease in the number of autolysosomes ($mRFP^+/GFP^-$) was observed in I7 treated cells. At this point, 25µM was chosen as the optimal dose for I7 treatment on mammalian cells.

3.5 Cargo recognition

The binding of substrates (to be degraded) to the inner surface of the autophagosomal membrane is referred to as cargo loading and this process is mediated by adaptor proteins. One such adaptor protein is p62/SQSTM1. Adaptors associate with both the ubiquitinated cargo (via the Ubiquitin Associated domain) and the lipidated LC3 anchored to the autophagosomal membrane (via the LC3 Interacting Region motif). They have an inverse relationship with the rate of autophagic flux. When autophagy is induced, the adaptors get degraded whereas the inhibition of autophagy leads to an accumulation of the adaptor proteins. To assess the impact of I7 on cargo recognition in HeLa cells, the levels of MAP1LC3B and p62/SQSTM1 were followed over time on treatment with I7.

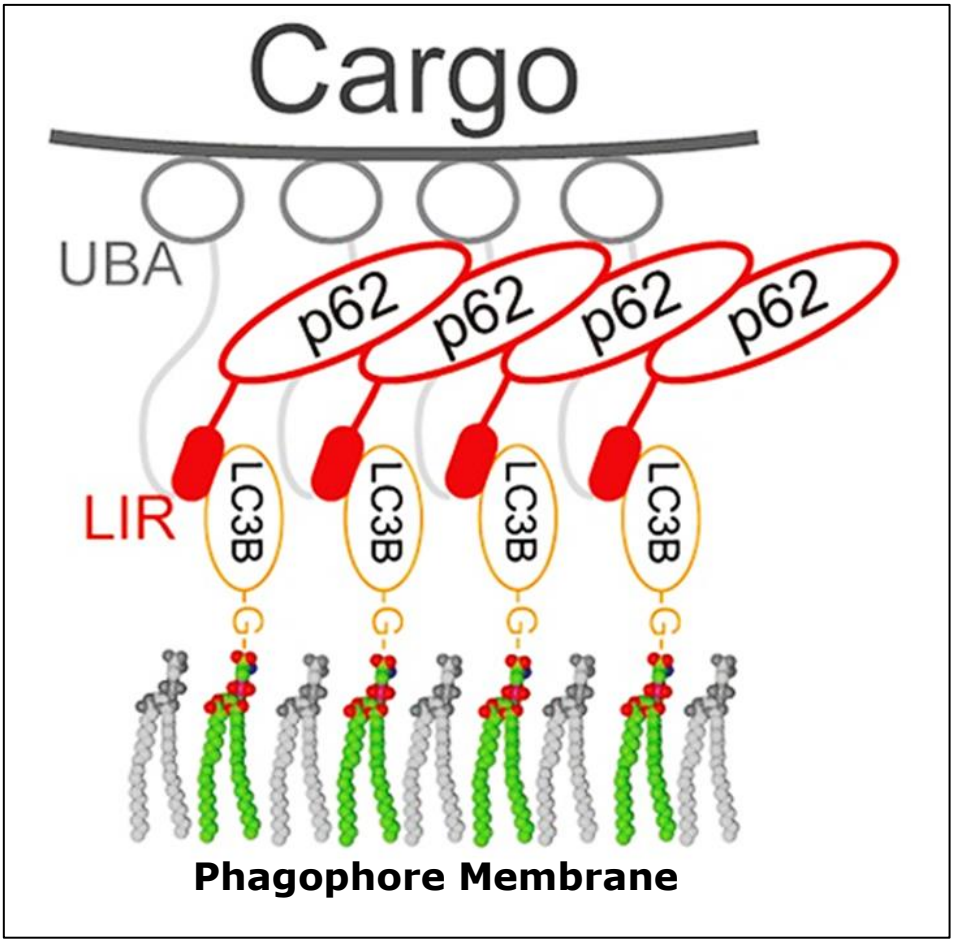

Figure 14: Cargo recognition by p62/SQSTM1. P62/SQSTM1 associate with the ubiquitinated cargo via the Ubiquitin Associated domain) and binds to the lipidated LC3B on the autophagosomal membrane via the LC3 Interacting Region motif.

From Wurzer, B. et al., Elife 4, (2015).

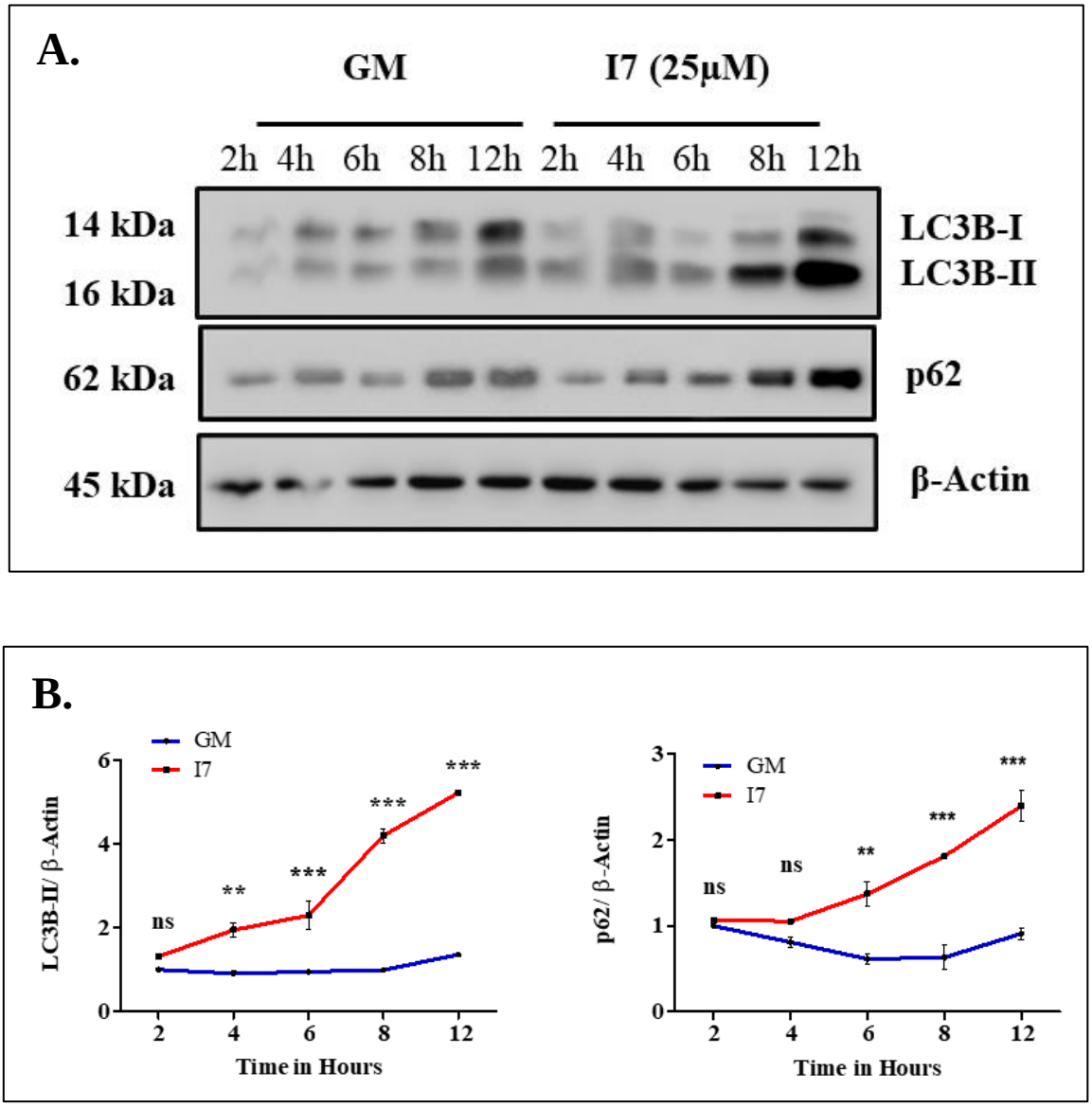

Figure 15: (A) HeLa cells were treated with I7 (25µM) or left untreated for 12h and lysates were collected at 2h intervals. (B) The levels of MAP1LC3B-II: β-Actin and p62: β-Actin over time was plotted. ns $p > 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (N = 3, One-way ANOVA, individual means compared with Bonferroni Post Test.)

HeLa cells were untreated or treated with I7 (25µM) and lysates were collected at 2h intervals. The levels of MAP1LC3B-II as well as p62/SQSTM1 were followed over a period of 12h. HeLa Cells treated with I7 (25µM) showed an increase in MAP1LC3B-II over time (2h - 12h) as well as an accumulation of the adaptor protein p62/SQSTM1.

For further validation, the fluorescence intensity of endogenous p62/SQSTM1 and its colocalization with GFP-LC3 was analyzed using immunofluorescence microscopy experiments. Treatment with I7 (25µM) caused an increase in LC3 puncta and an accumulation of the adaptor p62 in 2h. This is due to the block in the autophagic flux which leads to accumulation of autophagosomes containing the adaptor p62. Also, there was decreased colocalization of p62 with LC3 which suggests that I7 possibly prevents affects the recognition of cargo by p62 or loading of p62 onto autophagosomes. However, further validation of this observation needs to be carried out by follow-up experiments.

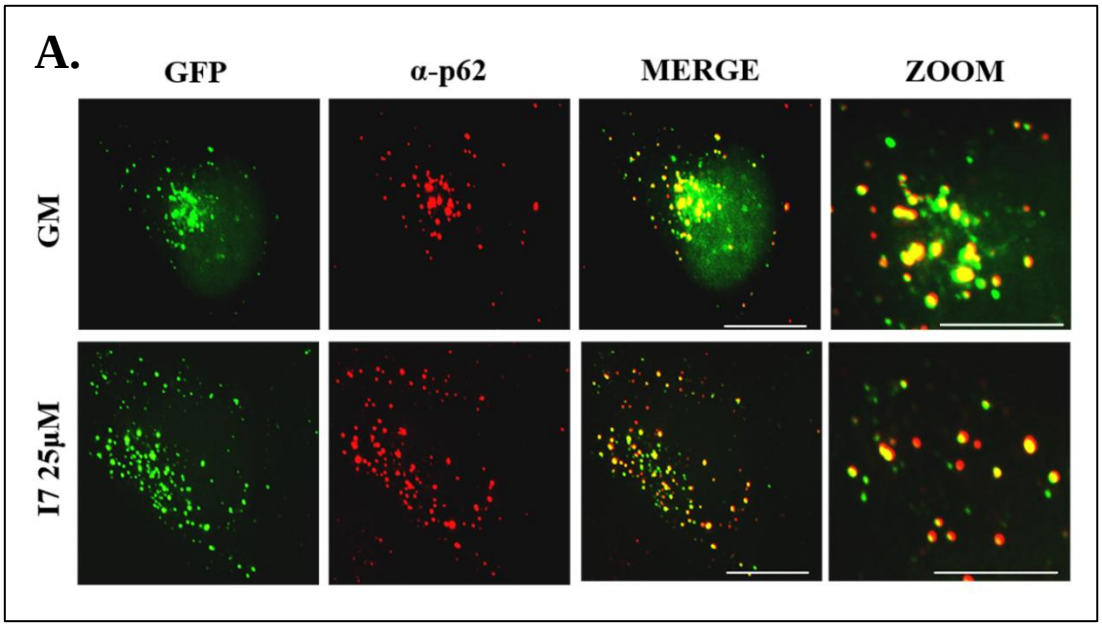

Figure 16: (A) Immunostaining with p62/SQSTM1 antibody in GFP-MAP1LC3B transfected HeLa cells to assess colocalization. Scale bar: 15µm.

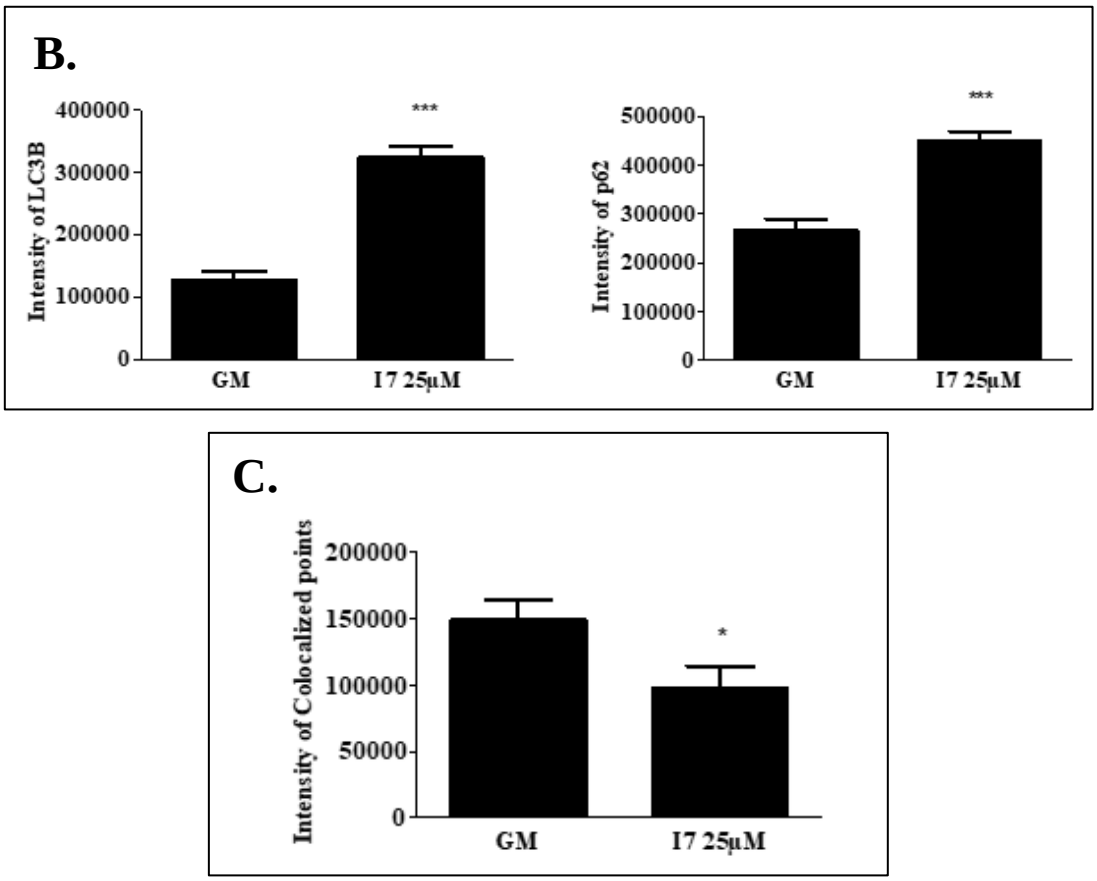

Figure 16: Graphs showing (B) the amount of p62, MAP1LC3B and (C) the colocalization between p62 and MAP1LC3B were plotted. The mean intensity was calculated using the ImageJ analysis software and the colocalization analysis was performed using the colocalization plug-in of ImageJ software. * $p \leq 0.05$, *** $p \leq 0.001$. (N = 3, n = 20 cells. Student's t test.)

Chapter 4
Effect of I7 on lysosomes and vesicular trafficking

A plethora of autophagy inhibitors of varying strengths and differing targets of action have been identified of which chloroquine and its derivatives are the most extensively studied. Hydroxychloroquine is an FDA approved drug used in treatment of various cancers such as glioblastoma, melanoma and solid tumors, in conjunction with radiation therapy and chemotherapy. However, the improvement in overall survival is limited since the drug must be used in high doses to achieve significant autophagy inhibition and this often leads to side effects due to toxicity in patients. Moreover, chloroquine and derivatives block the autophagic flux by preventing the fusion of autophagosomes with the lysosome. These drugs act on the lysosome and are promiscuous inhibitors of autophagy. Hence, there is a need for identification of autophagy specific inhibitors.

The effect of I7 treatment on lysosomes is studied in three ways:

4.1 Lysosomal number using LAMP1 marker

Lysosomal-associated membrane protein 1 (LAMP1) is a glycoprotein localized to the lysosomal membrane. The number of lysosomes per cell can be observed by immunostaining with LAMP1. Immunofluorescence imaging experiments showed that cells treated with I7 (25µM) had a comparable number of lysosomes as that of untreated cells.

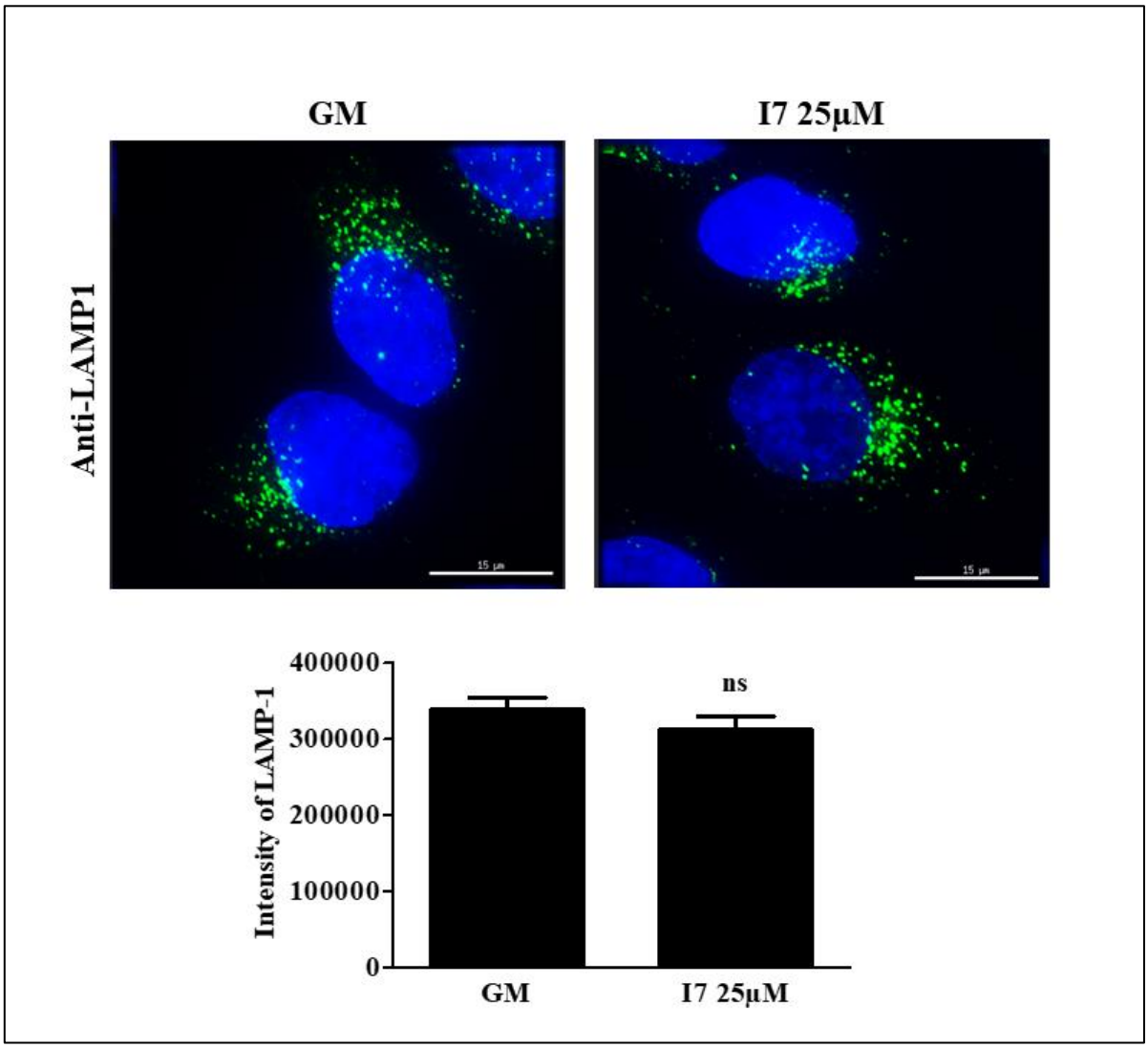

Figure 17: Immunostaining with LAMP1 antibody in DAPI stained HeLa cells to assess the number of lysosomes with or without I7 treatment (25µM). Scale bar: 15µm. Graph showing the intensity of LAMP1 was plotted. The mean intensity was calculated using ImageJ software. ns $p > 0.05$. (N = 3, n = 20 cells. Student's t test.)

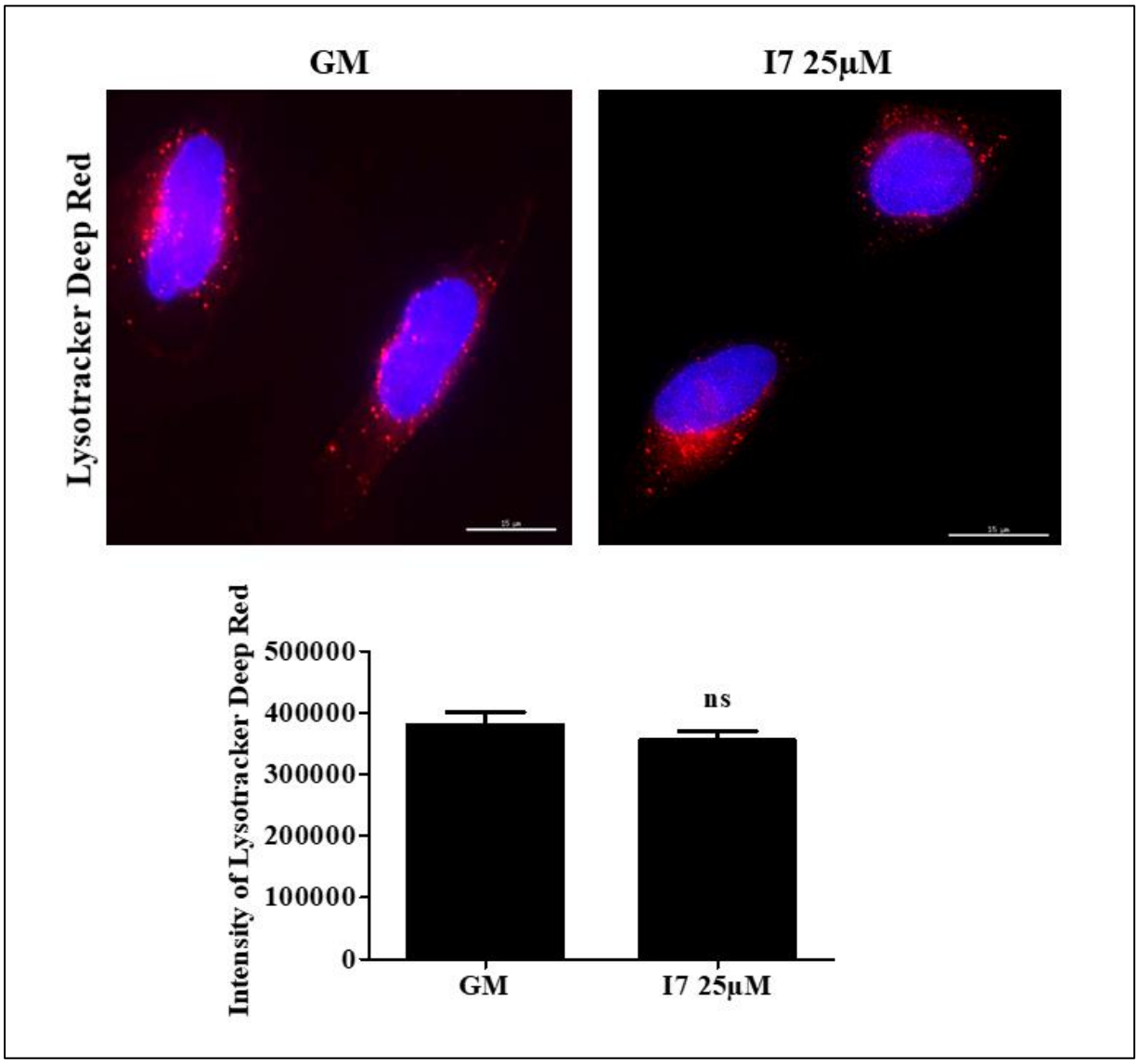

Figure 18: HeLa cells with or without I7 treatment (25µM) were used to assess the lysosomal pH. Lysotracker Deep Red stain was added to the cell culture medium in the last 30min of incubation and the cells were fixed and stained with DAPI. Scale bar: 15µm. Graph showing the intensity of Lysotracker Deep Red was plotted. The mean intensity was calculated using ImageJ software. ns $p > 0.05$. (N = 3, n = 20 cells. Student's t test.)

4.2 Lysosomal pH using Lysotracker Deep Red stain

Lysotracker Deep Red is a deep red-fluorescent dye that labels the acidic organelles of cells and tracks only the acidic functional lysosomes. Incubating HeLa cells with the dye can help understand whether the lysosomal pH is affected by treatment with I7. Fluorescence imaging experiments showed that the lysosomal pH of cells treated with I7 (25µM) was comparable to that of untreated cells.

4.3 Lysosomal function by EGFR degradation assay

The Epidermal Growth Factor Receptor (EGFR) is expressed on the surface of the plasma membrane, which gets internalized on binding to its ligand EGF. The endocytosis of the ligand-bound receptor finally leads to the degradation of EGFR on fusion with the lysosome. If I7 affects the lysosomal function, the endocytic pathway would be blocked, and an accumulation of EGFR would be observed.

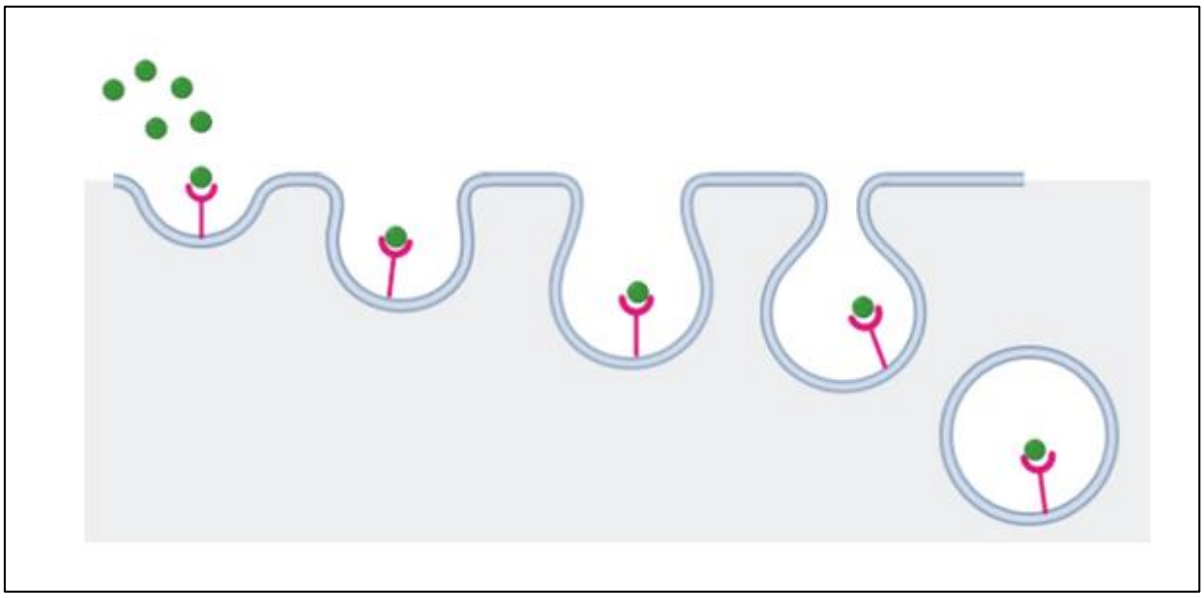

Figure 19: The endocytosis of the ligand-bound EGFR. The negative feedback loop associated with Epidermal Growth Factor Receptor (EGFR) signaling involves endocytosis of the ligand-bound receptor. This internalization of the receptor finally leads to the degradation of EGFR in the lysosome.

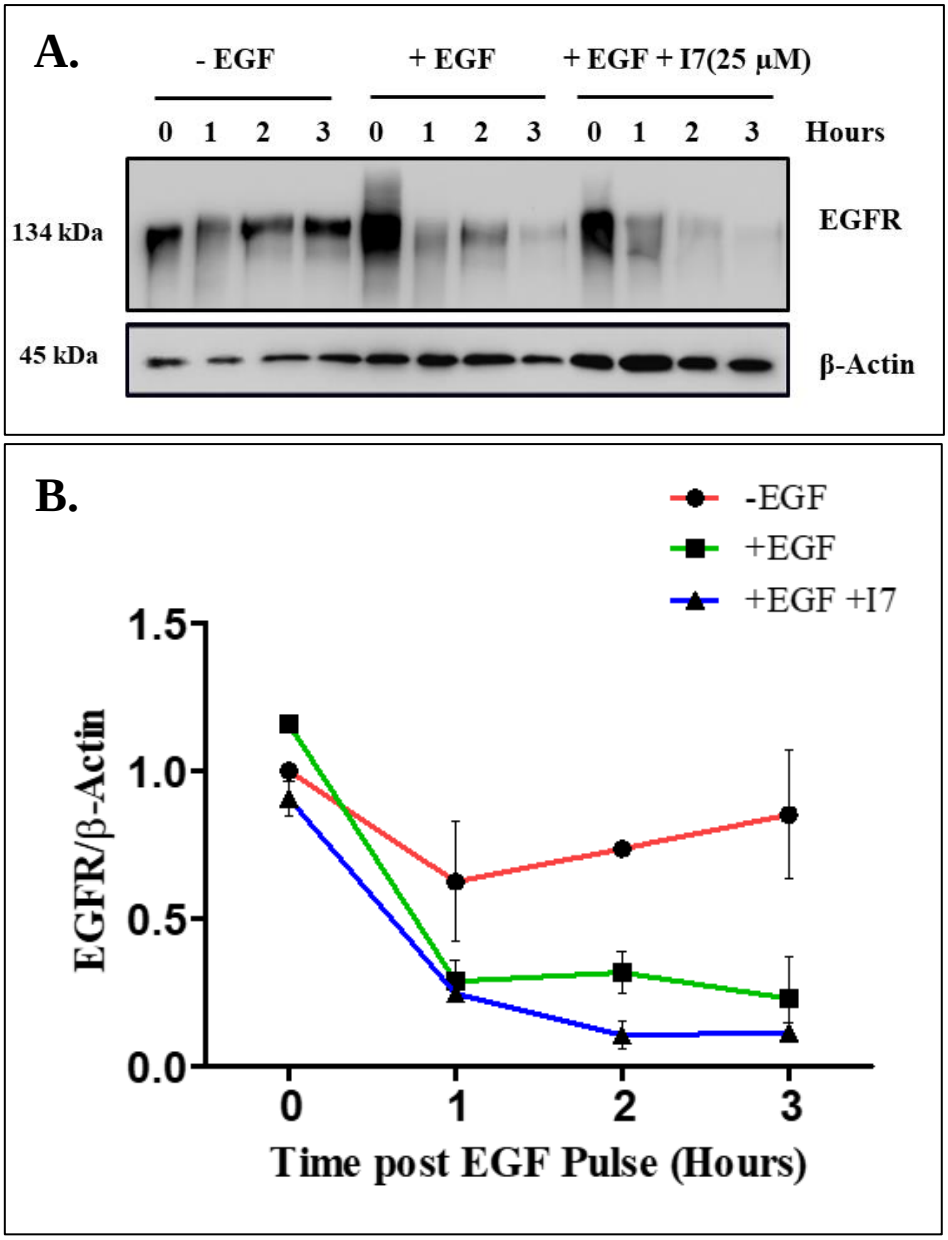

Figure 20: (A) Pretreatment of HeLa cells with I7 (25µM) was carried out for 1 hour, following which they were pulsed with 100 ng/ml of EGF. Samples were collected post EGF pulse at intervals of 1h and an immunoblot was performed against EGFR.
(B) The levels of EGFR-II: β-Actin over time was plotted. (N = 2.)

Upon EGF treatment, there was no difference in the degradation of EGFR over time between I7 treated and untreated groups, suggesting that the compound does not affect the endocytic trafficking pathway. Hence, the lysosomal proteolytic activity is not perturbed.

Chapter 5
Automation of traffic light assay quantitation

The characterization of a putative modulator of autophagy involves acquisition and analysis of traffic light assay images, which provides us with important information regarding the status of the autophagic flux. For instance, the assay showed that I7 causes an accumulation of autophagosomes and a decrease in the number of autolysosomes.

However, a set of traffic light assay experiments typically consists of three biological replicates, each evaluating around 20-25 cells. Considering the green and red channels of each image, the total number of images to be analyzed = 3 x 20 x 2 = 120 images per compound to be tested. This seriously undermines any effort for a high-throughput screening of compounds using the traffic light assay and turns out to be a technical bottleneck.

Currently, the analysis is aided by the ImageJ software which has a cell counter plug-in. The user opens each image on ImageJ and clicks on the visible puncta to enable counting. The counts are then copied to an Excel sheet and plotted. This process is time consuming and may introduce human error.

5.1 The Automated workflows

In collaboration with Mr. Marc Busse from APEER (Zeiss Microscopy), two independent workflows were developed to automate the quantitation of images. Both the workflows require the batch of images to be uploaded in zip format. The images are unzipped, the red and green channels are split and converted to a common .png format.

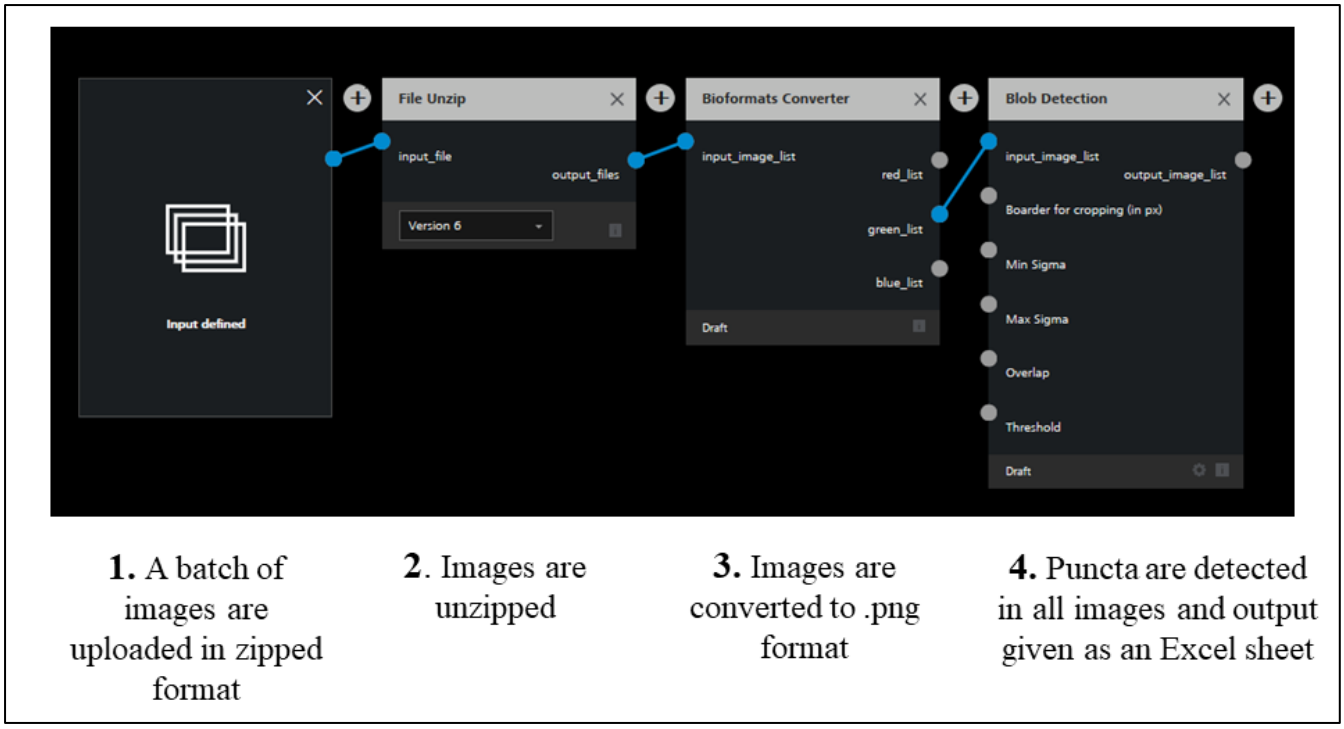

Figure 21: The outline of the blob detection workflow.

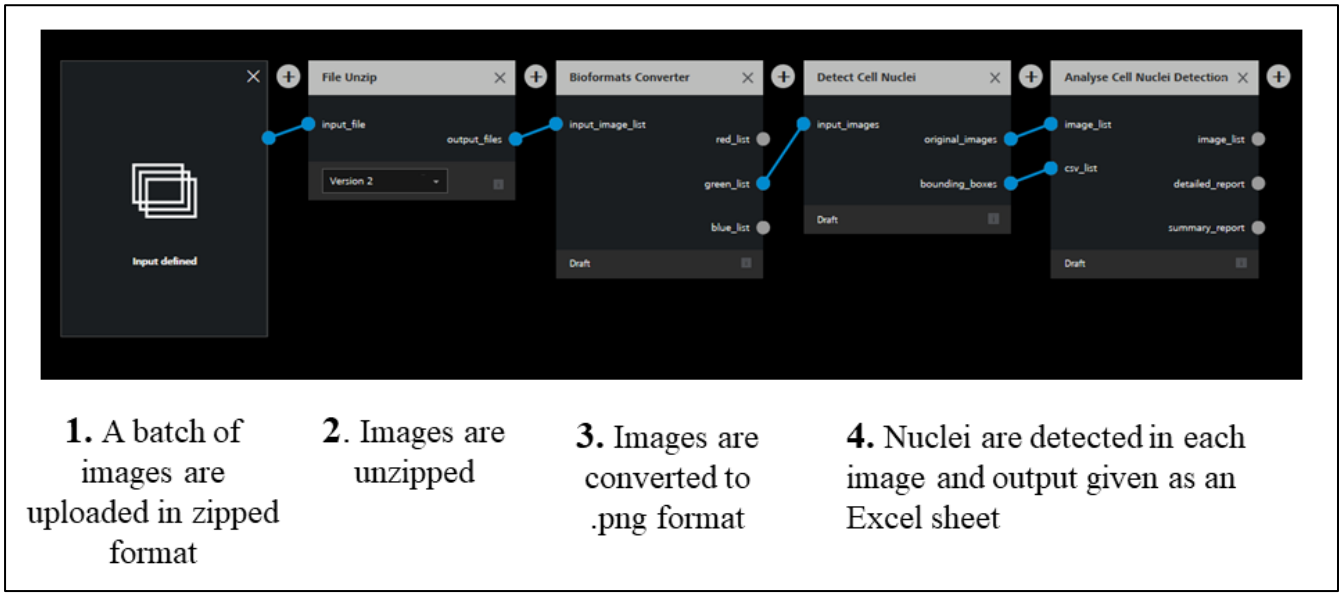

Figure 22: The outline of the cell nuclei detection workflow.

The blob detection workflow counts the puncta in each image and provides an output of the value in a downloadable Excel sheet whereas the cell nuclei detection workflow makes use of the green channel of each image to count the number of cells in the image and output the same in an Excel sheet. The green channel is chosen because it is brighter and clearer than its red counterpart. The nuclei can be detected directly from the GFP-LC3 channel due to the nuclear localization of LC3B-I form. The total number of puncta divided by the number of cells gives us the average number of puncta per cell.

The two underlying principles that is used to build the framework of the workflows are the Laplacian of Gaussian edge detector and the Deep Learning strategy.

5.2 Laplacian of Gaussian edge detector

Laplacian of Gaussian is an edge detection method. The detector processes the images beforehand by passing them through a Gaussian filter, which blurs the images to remove details and noise. This is performed by averaging the intensities of groups of pixels. Following this, the Laplacian detects edges by means of zero crossings.

If an area has uniform intensity, it is considered zero. As the intensity gets darker, the Laplacian assigns a negative value and when the intensity is lighter, the detector assigns a positive value. Hence, at the boundary of an object, a field of zeros is crossed by a positive or negative value.[49]

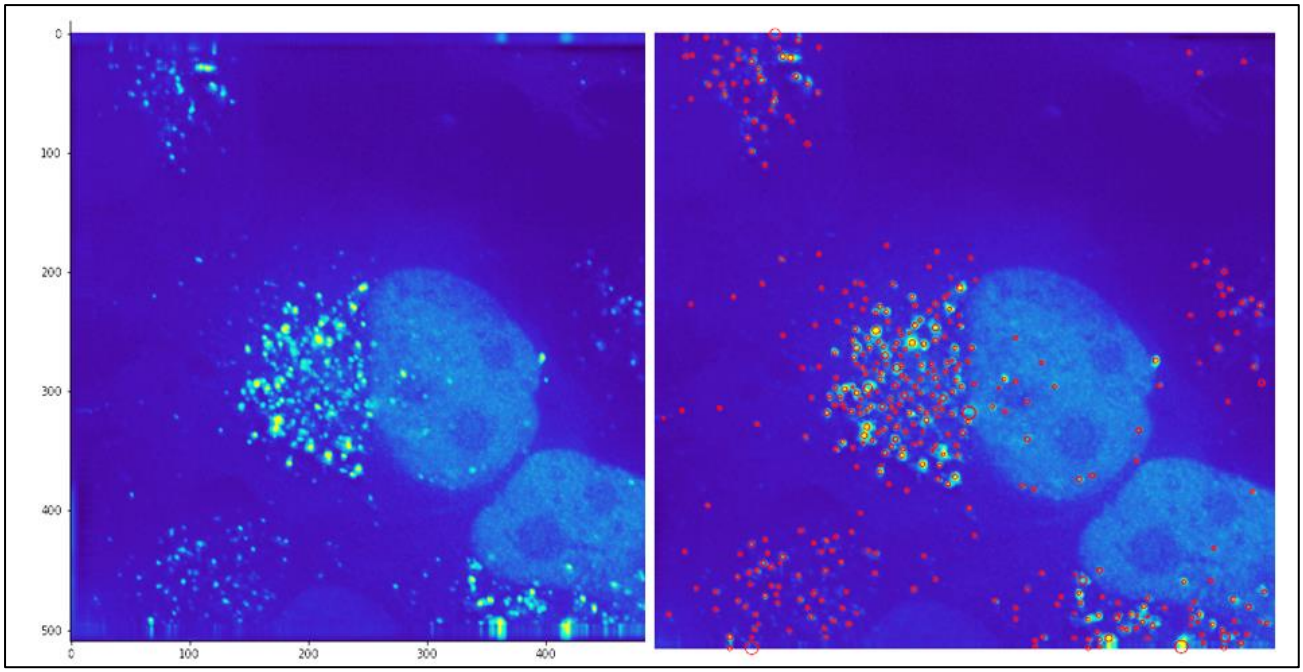

Figure 23: The specificity of the blob detection workflow is illustrated by the blobs/puncta circled in red. These were detected by the automated workflow.

5.3 Deep Learning

Artificial neural networks are computing systems inspired by the biological neural networks that constitute animal brains. A convolutional neural network is a class of deep neural networks, most commonly used for image processing applications. A standard convolutional neural network cannot be used of object detection because the objects of interest might have different spatial locations aspect ratios within the image. Therefore, to detect the object, a huge number of regions must be selected for analysis and this would computationally blow up.

A solution was proposed by a data scientist named Ross Girshick and his colleagues.[50] They came up with a method where a selective search algorithm was used to extract just 2000 regions from the image (called as region proposals) and work with that to classify the presence of an object. This was called R-CNN (Region based convolutional neural network). The same group cleared some of the drawbacks of R-CNN by feeding the input image to the CNN to generate a convolutional feature map and eliminating the need for 2000 region proposals. This reduced the test time for object detection from 49s to 2.3s, which is quite significant in real-time object

detection like vehicle detection and surveillance. This new network was called the Fast R-CNN.[51]

Fast R-CNN was used to train the cell nuclei detection workflow by marking the nuclei in images with bounding boxes and training the workflow to detect nuclei. For this purpose, 500 traffic light images of untreated and treated cells (both inhibitor and inducer treated) were used.

5.4 A comparison of manual and automated quantitation

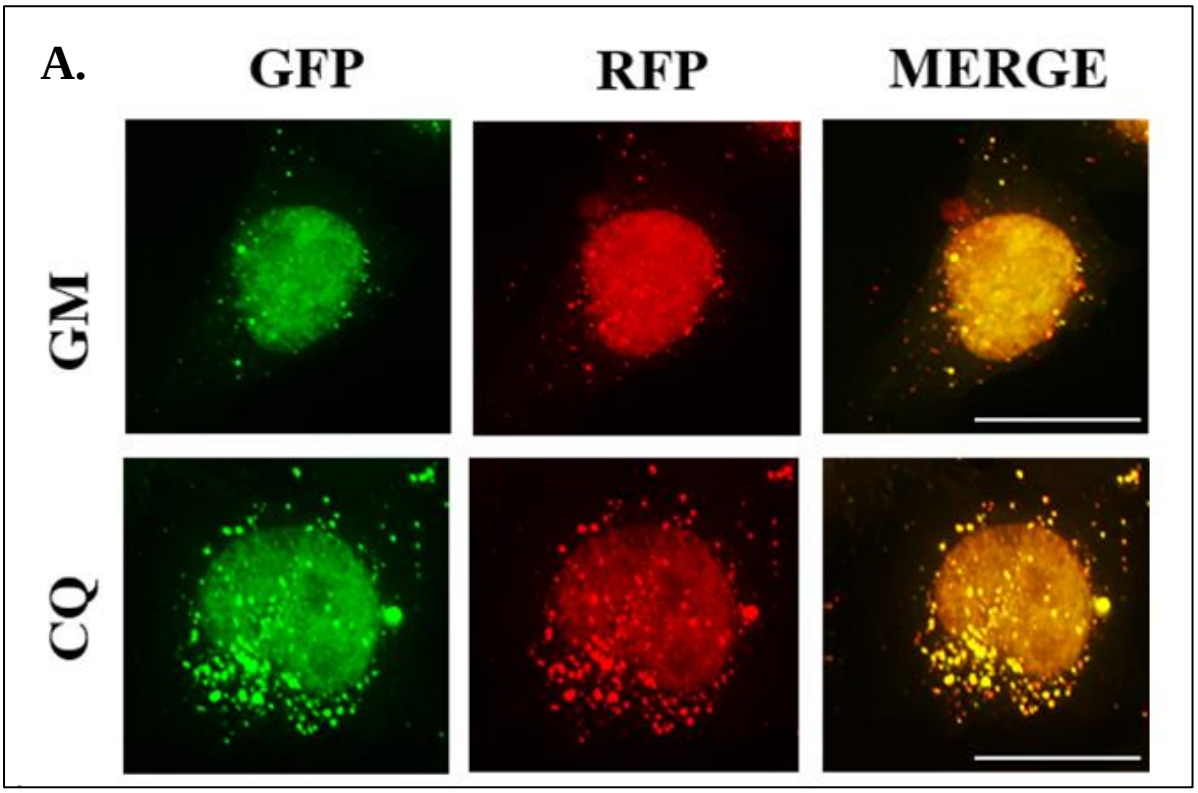

Figure 24: (A) Hela cells transfected with ptf-MAP1LC3B were untreated or treated with either Chloroquine (CQ) or I7 for 2 h under basal conditions and observed under a fluorescence microscope. Autophagosomes appear as yellow dots whereas autolysosomes appear red inside the cells. Scale bar: 10 μm.

B.

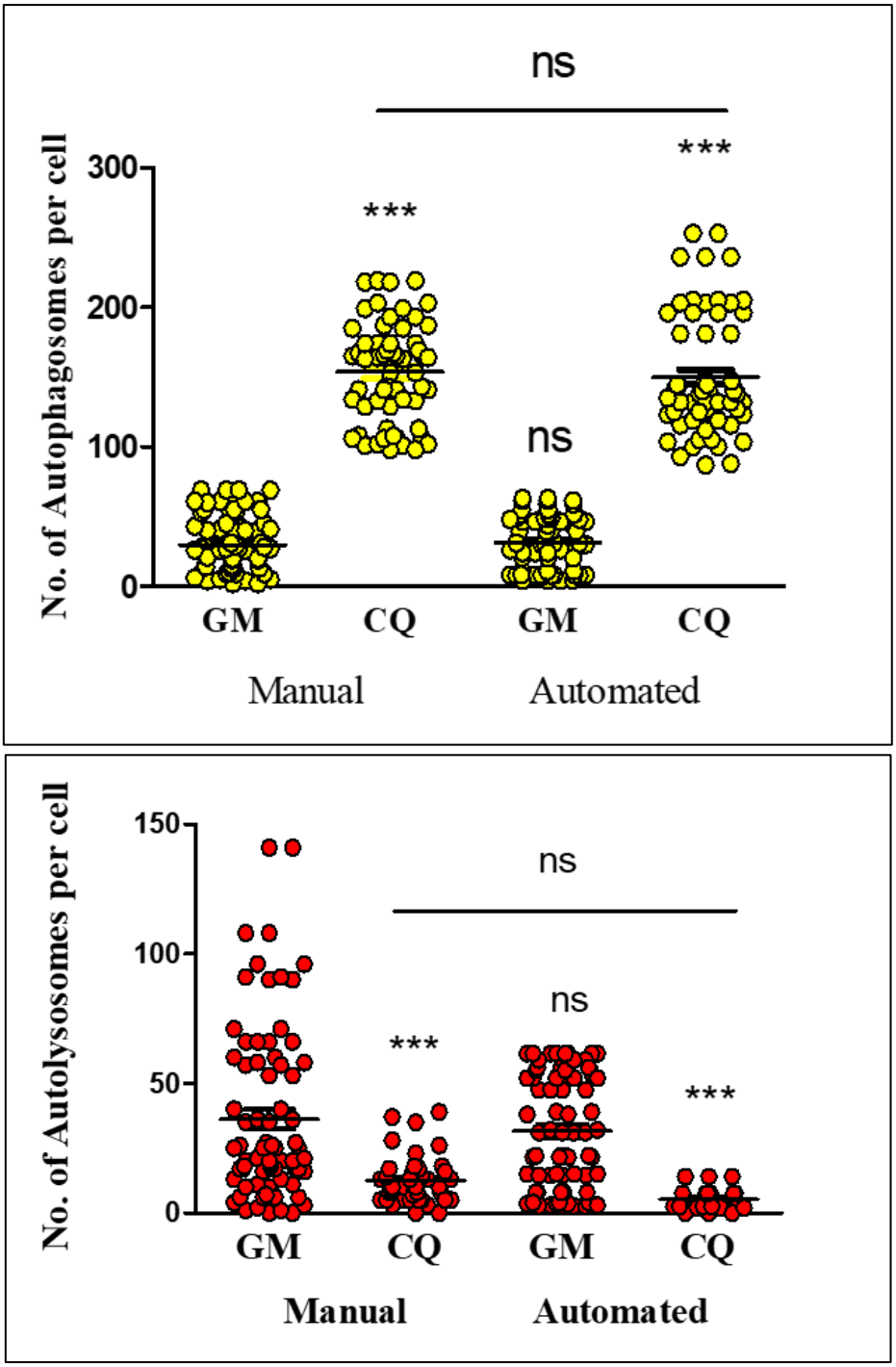

Figure 24: (B) The number of autophagosomes and autolysosomes were manually quantitated as well as analyzed through the automated workflows. Both the methods show that upon treatment with I7, autophagosomes accumulated inside the cells whereas very few autolysosomes were seen. ns $p > 0.05$, *** $p \leq 0.001$. (N = 3, One-way ANOVA, individual means compared with a Dunnett's Multiple Comparison Test.)

Manual and automated quantitation agree with each other while comparing untreated cells against Chloroquine treated cells. The workflow accurately reproduces the result, that is - I7 treatment causes an increase in autophagosomes and a decrease in autolysosomes.

The workflow runs on the cloud and does not require high processing power. Analyzing the data using the workflow saves time and could streamline the analysis of a high-throughput screening system. Therefore, the advantages of the automated quantitation of traffic light assay images are clearly apparent and this system can be used as a common route for autophagic flux assay analysis.

Chapter 7
Discussion and conclusions

The process of autophagy plays a crucial role in human health and physiology, and its modulation has been employed to alleviate symptoms in various disease models.[1] Investigations of novel modulators of autophagy comprises of (a) characterization of the compound as an inducer or inhibitor of autophagy, (b) establishment of its promiscuous or autophagy-specific nature and (c) elucidation of its target and mechanism of action.

The experiments set up to study the modulation of autophagy by I7 have characterized the compound as an autophagy inhibitor. I7 blocks the autophagic flux, which leads to the accumulation of autophagosomes and a concomitant decrease in the number of autolysosomes. This accumulation of autophagosomes explains the increase in MAP1LC3-II levels observed in I7 treated cells. Upon I7 treatment, MAP1LC3-II protein increases proportionately with concentrations of 5µM, 10µM, 25µM and 50µM of I7 and when followed over time, MAP1LC3-II is shown to be increasing over a time period from 2h to 12h. Therefore, the effect of I7 is dose dependent as well as treatment-time dependent. Moreover, the autophagic cargo adaptor SQSTM1 accumulates in cells treated with I7. This observation is corroborated by immunoblotting results and immunofluorescence imaging.

Interestingly, biochemical and immunofluorescence imaging experiments have shown that I7 does not affect lysosomal biology and endocytosis. Therefore, this inhibitor is apparently autophagy-specific and does not alter the number, pH or the degradative function of the lysosomes, unlike the known autophagy inhibitors such as Chloroquine and Bafilomycin A1.

Moving forward, the exact step of the autophagic flux which is affected by I7 needs to be identified. The colocalization study of SQSTM1 and LC3 hints at decreased cargo recognition. However, the status of the autophagosome biogenesis itself is unknown. A protease protection assay must be performed with I7 treated cells to understand whether the autophagosomes that accumulate are complete or not. If the autophagosomes are indeed complete, I7 might be acting upon the components of the fusion machinery. On the other hand, if the autophagosomes are

found to be incomplete, the action of I7 maybe targeting the early steps of autophagosomes biogenesis. Further experiments must be done to arrive at this understanding.

I7 and other previously characterized inducers/inhibitors from the lab have been identified from several small molecule libraries by means of an endogenous high-throughput assay in yeast, followed by secondary assays in yeast and mammalian cells for the hits. Tandem tagged fluorescence-based microscopy analysis is routinely used as secondary assays to characterize the promising candidates, for which the limiting factor has always been the manual quantitation involved. The workflows, developed in collaboration with APEER, identify changes in autophagy flux from a vast array of images in an unbiased manner and enable routine high-content screening for modulators of autophagy. Using these workflows to illustrate the trends of induction or inhibition in libraries of fluorescence-based images will help us improve the accuracy of the automated method and rectify the weaknesses. In the future, it would be very valuable to create a similar workflow for colocalization studies.

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