Examining the Role of Autophagy Proteins in Host Defenses

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Molecular Genetics
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Abstract

Autophagy is an evolutionarily conserved catabolic pathway required for maintaining cellular homeostasis and responding to microbial infections. The autophagy related genes (ATG) play a key role in the autophagy pathway, which culminates in the delivery of cargo to lysosomes for degradation. How autophagy is regulated is not clear.

Salmonella enterica serovar Typhimurium is a Gram-negative pathogen that can be targeted to the autophagy pathway, which involves engulfment of bacteria in membranes decorated with the ATG8 family member LC3. This targeting requires a ubiquitin signal on bacteria and adaptor proteins, such as p62 and NDP52, that can simultaneously bind both ubiquitin- and LC3. In my studies, I showed that these two adaptors are recruited to bacteria with similar kinetics but independently of each other. Depletion of either adaptor leads to impairment of antibacterial autophagy, yet simultaneous depletion of both adaptors does not have a synergistic effect. Importantly, the two adaptors do not colocalize, but rather form non-overlapping microdomains surrounding the bacterium. Hence, p62 and NDP52 act cooperatively to drive efficient antibacterial autophagy by targeting the protein complexes they coordinate to distinct microdomains associated with bacteria.
My studies also examined the role of an IRGQ, a newly identified LC3B-binding protein, IRGQ is a member of the Immunity Related GTPases (IRG) family for which very little is known in humans. I showed that IRGQ localizes to a perinuclear region and was required for autophagy of S. Typhimurium and protein aggregates. These findings identify a new role for IRGQ in the autophagy pathway in humans.

In addition to autophagy, a subset of the ATGs are also involved in a poorly understood process that involves delivery of the LC3 to phagosomes, termed LC3-associated phagocytosis (LAP). Previous studies have suggested that LAP accelerates phagosome fusion with the lysosome, though this has become controversial in the field. Here, I examined the role of ATG proteins in phagosome maturation using mouse embryonic fibroblasts and bone marrow macrophages. In both cell types, the essential autophagy gene Atg5 had no impact on the maturation of phagosomes, as judged by LAMP1 acquisition, acidification, or generation of a degradative phagosome. These findings suggest that the LAP pathway is not universally required for phagosome maturation. Instead, LAP functions are subject to variation due to factors that are not fully understood.
for my parents

Susan and Leo Chemm
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List of Abbreviations

3-MA, 3-methyladenine;

AIM, ATG8-interacting motif;

Alfy, autophagy-linked FYVE protein;

AMPK, 5' AMP-activated protein kinase;

A. phagocytophilum, Anaplasma phagocytophilum;

ATG, autophagy;

B. pseudomallei, Burkholderia pseudomallei;

BAG3, BAG family molecular chaperone regulator 3;

BMDM, bone marrow-derived macrophages;

BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3;

BNIP3L, BCL2/Adenovirus E1B 19kDa Interacting Protein 3-Like;

CMA, chaperone-mediated autophagy;

C. burnetii, Coxiella burnetii;

CSF1, macrophage colony-stimulating factor 1;

CSF2, granulocyte macrophage colony-stimulating factor;

DAG, diacylglycerol;

DQ-BSA, dye quenched-bovine serum albumin;

DUB, deubiquitinating enzyme;

E1, ubiquitin-activating enzyme, the first enzyme in the ubiquitination cascade;
E2, ubiquitin-conjugating enzyme, the second enzyme in the ubiquitination cascade;

E3, ubiquitin protein ligase, the third enzyme in the ubiquitination cascade;

ER, endoplasmic reticulum;

FIP200, FAK family kinase-interacting protein of 200 kDa;

FNBPL1, formin-binding protein 1-like;

*F. tularensis, Francisella tularensis;*

FYCO1, FYVE and coiled-coil domain containing 1;

GABARAP, GABA type A receptor-associated protein;

GAS, Group A *Streptococcus*;

GAS, IFN-γ activated site;

GcAV, GAS-containing LC3+ autophagosome-like vacuole;

GFP, green fluorescent protein;

HOPS, homotypic fusion and protein sorting;

IRGs, Immunity Related GTPases;

InlK, Internalin K;

IFNγ, Interferon γ;

IRSE, interferon-responsive sequence elements;

LAP, LC3-associated phagocytosis;

LC3, microtubule associated protein 1 light chain 3;

LIR, LC3-interacting region;
LLO, listeriolysin O;

*L. monocytogenes, Listeria monocytogenes*;

*L. pneumophila, Legionella pneumophila*;

LysMcre, Cre recombinase from the endogenous lysozyme M locus;

LAMP1, lysosomal-associated membrane protein 1;

LPS, lipopolysaccharide;

MEFs, Mouse Embryonic Fibroblasts;

MHC1, major histocompatibility complex 1;

MREG, melanoregulin;

mTOR, mammalian target of rapamycin;

mTORC1, mTOR complex 1;

*M. tuberculosis; Mycobacteria tuberculosis*;

MVP, major vault protein;

NDP52, nuclear dot protein 52kDa;

NOD1, nucleotide-binding oligomerization domain-containing protein 1;

NOD2, nucleotide-binding oligomerization domain-containing protein 2;

NOX2, NADPH oxidase 2;

OpZ, opsonized zymosan;

PAMP, pathogen-associated molecular patterns;

PE, phosphatidylethanolamine;

PI3K, phosphoinositide 3-kinase;
PI(3)P, phosphatidylinositol-3-phosphate;

POS, photoreceptor outer segments;

PV, parasitophorous vacuole;

RAB7, Ras-related protein Rab-7;

RIG-I, retinoic acid-inducible gene 1;

RIPK3, receptor-interacting serine/threonine-protein kinase 3;

ROS, reactive oxygen species;

RPE, retinal pigment epithelium;

Rubicon, RUN domain protein as Beclin1-interacting and cysteine-rich containing;

*S. aureus, Staphylococcus aureus*;

*S. cerevisiae, Saccharomyces cerevisiae*;

SCV, *Salmonella*-containing vacuole;

*S. flexneri, Shigella flexneri*;

Sifs, *Salmonella*-induced filaments;

SNAP29, Soluble 29 KDa NSF Attachment Protein;

SQSTM1, sequestosome-1;

SRBC, sheep red blood cells;

STX17, syntaxin 17;

*S. Typhimurium, Salmonella enterica* serovar Typhimurium;

TBK1, Tank-binding kinase 1;
TLR, Toll-like receptor;

Ub, ubiquitin;

Ulk1, unc51-like kinase 1;

UVRAG, UV radiation resistance associated gene;

VPS15, vacuolar protein sorting 15;

VPS34, vacuolar protein sorting 34.

WIPI1, WD Repeat Domain, phosphoinositide interacting 1;

WIPI2, WD Repeat Domain, phosphoinositide interacting 2;

Y. pseudotuberculosis, Yersinia pseudotuberculosis.
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Chapter 1 - Introduction

AUTOPHAGY

Autophagy is defined as a process of “self-eating” and can be broadly categorized into three distinct pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). In **macroautophagy**, cargo is sequestered by an isolation membrane (also called phagophore) into a double membrane vesicle (autophagosome). The autophagosome matures by sequential fusion with endosomes and then lysosomes to allow for cargo degradation and recycling of the macromolecules (Choi et al., 2013; Jiang and Mizushima, 2014) (Figure 1A). In **microautophagy**, the lysosomal membrane directly engulfs parts of the cytoplasm (Li et al., 2012). In **CMA**, the autophagic substrate is recognized by chaperone protein Hsc70 and is shuttled to the lysosomal lumen in the absence of autophagosome formation (Cuervo and Wong, 2014). The research in this thesis exclusively focuses on macroautophagy (hereafter referred to as autophagy).

Autophagy is fundamental to both the cellular response to stress and the maintenance of homeostasis. Thus, it has been linked to neurodegeneration, cancer, development, and innate immunity (Choi et al., 2013; Jiang and Mizushima, 2014; Levine et al., 2011). The mechanisms that regulate autophagy are not clear and are the subject of intense investigation.

**Historic perspective on autophagy**

*The beginning of autophagy*

The term “autophagy” was coined by Christian de Duve in 1963 at the CIBA Foundation Symposium on Lysosomes after a number of scientists observed cytosolic material, such as mitochondria, ribosomes, and endoplasmic reticulum (ER), within lysosomes (Clark, 1957; Novikoff et al., 1956). The term autophagy was initially meant to describe a delivery method of cytoplasmic components to the lysosomes. This discovery was enabled by two techniques: (i)
differential centrifugation and (ii) electron microscopy. Centrifugation allowed for purification of organelles of different densities; and electron microscopy allowed for morphological examination of their internal structures. Today, electron microscopy is still considered a gold standard to confirm that a specific cargo is targeted to the autophagy pathway (Klionsky et al., 2016). The canonical double-membrane autophagosome, the intermediate compartment that sequesters cytoplasmic contents for delivery to lysosomes, was first observed five years later, in an elegant electron microscopy study by Arstila and Trump (Figure 1B) (Arstila and Trump, 1968).

**Physiological role of autophagy**

The next major milestone in the autophagy field was the elucidation of how physiological conditions regulate autophagy. Several groups demonstrated that a catabolic hormone, glucagon, induced autophagy in rat hepatocytes (Arstila and Trump, 1968; Ashford and Porter, 1962; Deter et al., 1967), while an anabolic hormone, insulin, inhibited autophagy (Pfeifer and Warmuth-Metz, 1983). Ofeifer and Warmuth-Metz showed that autophagy was induced by fasting and inhibited by feeding (Pfeifer and Warmuth-Metz, 1983). Further studies showed that amino acid levels can regulate protein degradation via autophagy (Mortimore and Ward, 1976). Today, amino acid starvation is a commonly used method to induce autophagy (Klionsky et al., 2016). These findings aligned with the proposed catabolic function of autophagy to degrade cytosolic material. Multiple autophagy inhibitors, such as bafilomycin A, chloroquine, and 3-methyladenine (3-MA), have been identified at that time (Ohsumi, 2014), proving themselves to be useful tools in the early days of autophagy research. These insights were fundamental to our understanding of autophagy as a catabolic pathway.
Figure 1. Autophagosome structure in mammalian cells visualized by electron microscopy.

(A) A schematic presentation of the formation and maturation of autophagosomes in mammalian cells. (B) Two autophagic vacuoles (Av), at high magnification, limited by double membranes isolated from rat liver 1 h after glucagon administration. Glutaraldehyde and OsO₄ fixation; incubation time, 30 min. This inset corresponds to Fig.17 from Arstila and Trump, 1968. (C, D) Fine structure of autophagosomes in plastic embedded murine hepatocytes (C) and mouse embryonic fibroblasts (MEFs) (D). Autophagy was induced by incubation in serum and amino acid free medium for 2 h. (C) The autophagosome on top of the panel contains a mitochondrion, ER, and ribosomes. The limiting membrane (large arrowheads) is visible only partially as a double membrane (arrows). Below the autophagosome, a U-shaped membrane cistern, a putative phagophore (small arrowhead, P), seems to be in the process of wrapping around a peroxisome. (D) Early autophagic vacuoles (AVi) contain morphologically intact cytoplasm, which looks identical to the cytoplasm surrounding the vacuoles. Late autophagic vacuoles (AVd) contain material which can still be recognized as cytoplasmic, in this case ribosomes, and looks partially degraded, i.e., more electron dense than the cytoplasm surrounding the vacuole. Panels A, C, D are adapted from (Eskelinen, 2008) with permission from Springer.
Selective autophagy vs. non-selective autophagy

Bolender and Weibel were the first to observe mitochondria-specific autophagy in 1973 (Bolender and Weibel, 1973). Selective degradation of ER and peroxisomes was later observed (Beaulaton and Lockshin, 1977; Veenhuis et al., 1983). These discoveries shifted the way autophagy was perceived from being a solely non-selective catabolic pathway to a more complex and tightly orchestrated pathway that can degrade a specific cargo. Since then, autophagy is divided into two categories: (i) non-selective autophagy, such as starvation-induced autophagy; and (ii) selective autophagy. Currently, selective autophagy is classified based on the type of cargo. Selective degradation of mitochondria (Kim et al., 2007), endoplasmic reticulum (Bernales et al., 2006), peroxisomes (Dunn et al., 2005), and protein aggregates (Rubinsztein, 2006) are called mitophagy, reticulophagy, pexophagy, and aggrephagy respectively. Microbial invaders, such as viruses, bacteria and parasites, can also be selectively targeted by autophagy in a process called xenophagy (Orvedahl and Levine, 2009).

The way that non-selective autophagy is perceived is also evolving. A proteomic study from 2008 by Kristensen and colleagues revealed that starvation-induced autophagy, previously assumed to be nonspecific bulk degradation, is actually highly regulated and ordered. Early after starvation, autophagy targets primarily cytoplasmic proteins and proteasomes, followed by ribosomes at 12 h post starvation, and organelles at 24 h post starvation (Kristensen et al., 2008). It remains to be seen whether the term non-selective autophagy will continue to be used within the field.

Genetic analysis of autophagy

The early period of autophagy research relied heavily on electron microscopy studies. In the 1990s, however, genetic manipulations allowed for both the identification of autophagy genes and the examination of their role in health and disease. Cornerstone research was performed by Yoshinori Ohsumi, who observed autophagy in yeast via light (Takeshige et al., 1992) and electron microscopy (Baba et al., 1995). In response to starvation, autophagic vacuoles accumulated in the yeast vacuole. Yeast offered a perfect model system to examine autophagy genetics due to the relative ease of genetic manipulation in this organism. Tsukada and Ohsumi
employed a genetic approach to search for mutants that did not accumulate autophagosomes in the vacuole in response to starvation via a light microscopy-based readout (Tsukada and Ohsumi, 1993). Using this laborious method, they identified the first autophagy gene, *apg1* (later renamed *atg1*). Studying an *apg1* mutant, Tsukada observed that it was sensitive to prolonged nitrogen starvation, consistent with the role of autophagy in mediating survival in response to starvation. Using nitrogen starvation as a primary screen, followed by a secondary light microscopy screen, Tsukada identified 14 more autophagy mutants (Tsukada and Ohsumi, 1993). Others quickly followed suit in identifying more autophagy genes, and to date there are at least 41 *atg* genes identified in yeast (Klionsky et al., 2003; Thumm et al., 1994; Yao et al., 2015) and over 35 *Atg* genes identified in humans (Feng et al., 2014).

In the late 90’s, Noboru Mizushima, then a post-doctoral fellow with Yoshinori Ohsumi, characterized two novel ubiquitin-like conjugation systems in yeast: one mediated the conjugation of Atg12 to Atg5 and the other mediated the conjugation of Atg8 to phosphatidylethanolamine (PE) (Mizushima et al., 1998). Both conjugation machineries were subsequently shown to be essential for autophagosomes formation (Suzuki et al., 2001). Assuming that these conjugation systems must be conserved in other eukaryotes, Mizushima used sequence identity to find human homologues of Atg12, Atg5 and confirmed that the ATG12 conjugation system in humans was also essential for autophagy (Mizushima et al., 1998). In 2000, Tamotsu Yoshimori, working alongside Yoshinori Ohsumi, made a seminal discovery that microtubule associated protein 1 light chain 3 (MAP1LC3 or LC3), a mammalian homologue of yeast Atg8, is conjugated to the autophagosomal membrane throughout the autophagic process (Kabeya et al., 2000). This made LC3 a useful autophagy marker; even today, fluorescently labeled LC3 remains the most common method to monitor autophagy (Klionsky et al., 2016). Kabeya et al., 2000 paper became the most-cited research paper in the autophagy field with over 4,000 citations as of today. Ohsumi’s group proceeded to generate GFP-LC3 transgenic mice to study autophagic process in various tissues (Mizushima et al., 2004). Yoshinori Ohsumi was recognized for his pioneering work in molecular elucidation of autophagy with the 2015 Gairdner International Award. A detailed account of autophagic core machinery is described on page 8.
**Understanding the role of autophagy in disease**

The next leap in understanding of the autophagy pathway came from generating *Atg*-specific knockout mice. These studies were crucial in illuminating the role that autophagy plays in health and disease in higher eukaryotes. The *Atg5* knockout (*atg5<sup>-/-</sup>*) was the first mouse to be generated (Kuma et al., 2004). The infant mice died within 24 h from birth attesting to the critical role autophagy plays in mammalian biology. The knockouts of *Atg3, Atg7, Atg9, Atg16L1* exhibited the same phenotype (Levine and Kroemer, 2008), as the mice with defective autophagy are unable to adapt to the neonatal starvation period. To dissect the role autophagy plays in disease, tissue-specific knockout mice were generated by a number of groups. Neuronal cells lacking *Atg5* accumulated proteins aggregates and inclusions, leading to neurodegeneration (Hara et al., 2006). Similarly, mice lacking *Atg7* in their central nervous system accumulated aggregates and inclusions, showed multiple signs of neurodegeneration, including abnormal limb-clasping reflexes and a reduction in coordinated movement, and died at the age of 28 days (Komatsu et al., 2006). A seminal study from Beth Levine’s group implicated BECN1/Beclin1, a mammalian homologue of yeast Atg6, in tumorigenesis in a mouse model (Liang et al., 2006; Liang et al., 1999; Qu et al., 2003). The role of autophagy in human cancer was subsequently confirmed: monoallelic deletion of BECN1 was detected in ovarian, prostate and breast cancers (Liang et al., 1999); altered expression of *Atg5* was also observed in prostate and skin cancer (Kim et al., 2011c; Liu et al., 2013); and altered expression of the UV radiation resistance associated gene (codes for UVRAG, a protein required for autophagosome biosynthesis) was associated with colon cancer (Liang et al., 2006). The autophagy pathway is however a double-edged sword when it comes to cancer progression: in early stages, autophagy appears to be protective from cancer, while in later stages, autophagy can promote survival of tumor cells and resist their killing by chemotherapeutic agents (White, 2012). In addition to cancer and neurodegeneration, autophagy was also implicated in cardiovascular and pulmonary disease, physiological response to exercise, longevity, and infectious diseases (Choi et al., 2013; Jiang and Mizushima, 2014).

The role of autophagy in infectious diseases is further described on page 15.
Autophagy core machinery

Autophagy is characterized by the formation of intracellular double-membrane vesicles, termed autophagosomes, and the presence of ATG8/LC3 on the autophagosomal membrane. In basal conditions, autophagy is maintained at a low level, but it can be upregulated upon various stresses, such as starvation, accumulation of superfluous organelles or protein aggregates, and infection (Huang and Klionsky, 2007). The core autophagy machinery is described in Table 1. Over 35 autophagy proteins (ATG) are required at different stages of this process in humans and can be grouped into several specific stages: initiation, elongation, and maturation.

Initiation

The autophagy initiation complex triggers the formation of an autophagosomal membrane. The initiation complex is comprised of Unc-51 like autophagy activating kinase 1 (ULK1, a homologue of Atg1), ATG13, RB1CC1/FIP200, and ATG101. Under nutrient-rich conditions, mammalian target of rapamycin (mTOR) is also present in the complex where it inhibits autophagy initiation by impairing ULK1 kinase activity through phosphorylation of ULK1 on Serine 757 and hyperphosphorylation of ATG13 (Kim et al., 2011b). Under autophagy-inducing conditions, mTOR is released which leads to the activation of ULK1. When activated, ULK1 can phosphorylate and activate ATG13 and RB1CC1/FIP200 (Figure 2.1) (Hara et al., 2008; Hosokawa et al., 2009a; Hosokawa et al., 2009b). Nutrient levels are also sensed by 5' AMP-activated protein kinase (AMPK), negative regulator of the mTOR signaling cascade. Under low glucose conditions, AMPK phosphorylate the TSC complex, which is a negative regulator of mTOR complex 1 (mTORC1) activation, and directly phosphorylate ULK1 on Serine 317 and Serine 777 that leads to its activation (Russell et al., 2014).

The phosphatidylinositol 3-kinase class III (PI3K) complex is another essential autophagy complex, which generates phosphatidylinositol-3-phosphate (PI(3)P) and is requisite for vesicle nucleation. The phosphoinositide 3-kinase (PI3K) complex consists of vacuolar protein sorting 34 (VPS34, a lipid kinase), VPS15, BECN1, AMBRA1, and either UVRAG or ATG14 (Itakura et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009) (Figure 2.1). When active, ULK1
phosphorylates and activates BECN1, which allows for VPS34 activation and recruitment to the phagophore (Nazarko and Zhong, 2013). PI(3)P enrichment serves as a platform to recruit PI(3)P-binding proteins WD Repeat Domain Phosphoinositide Interacting 1-2 (WIP1I-2), that then recruit downstream effector ATG16L1, thereby recruiting ATG5-ATG12-ATG16L1 complex to the phagophore (Dooley et al., 2014).

**Elongation**

Phagophore elongation requires two ubiquitin-like (Ubl) conjugation systems, ATG12 and ATG8/LC3. In the ATG12 conjugation pathway, ATG12 is activated by the E1-like enzyme ATG7 and E2-like enzyme ATG10, and then conjugated to ATG5 (Mizushima et al., 1998). The ATG12-ATG5 conjugate then interacts non-covalently with ATG16L1 (Mizushima et al., 2003). ATG16L1 oligomerizes, resulting in the formation of a large tetramer complex of four ATG12-ATG5-ATG16L1 units (Kuma et al., 2002).

In the ATG8/LC3 conjugation pathway, ATG8/LC3 is attached to the phosphatidylethanolamine (PE) with the help of ATG7 (an E1-like enzyme), ATG3 (an E2-like enzyme) and the ATG12-ATG5-ATG16L1 complex (an E3-like enzyme) (Hanada et al., 2007; Ichimura et al., 2000). ATG4 is a cysteine protease that processes cytosolic ATG8/LC3 (pro-LC3) to expose a glycine residue (LC3-I), a prerequisite for PE conjugation (Kirisako et al., 2000). Cytosolic ATG8/LC3 with its exposed glycine residue is referred to as ATG8/LC3-I; and ATG8/LC3 conjugated to PE is referred to as ATG8/LC3-II (Kabeya et al., 2000; Kirisako et al., 2000) (Figure 2.2).

In humans, the ATG8 family contains six homologs categorized into two families by sequence identity: (i) LC3s: LC3A, LC3B, LC3C, and (ii) GABA type A receptor-associated proteins (GABARAPs): GABARAP, GABARAPL1, and GABARAPL2/GATE16, with LC3B being the most studied ATG8 in humans. Similarly to yeast, human ATG8s are required for tethering, membrane fusion, and autophagosome biogenesis (Weidberg et al., 2011; Weidberg et al., 2010). In addition, human ATG8s appear to have evolved different functions: the LC3 family is important for elongation of the phagophore membrane, while the GABARAP family contribute
to autophagosome sealing (Weidberg et al., 2010). Why humans have six ATG8s when yeast has only one is not fully understood.

In addition to its role in autophagosome biogenesis, the ATG8 family functions in autophagy cargo recognition and sorting (Birgisdottir et al., 2013). Adaptor proteins, like sequestosome-1 (SQSTM1)/p62 and nuclear dot protein 52 (NDP52), interact with ATG8s and autophagic cargo, such as ubiquitinated proteins, organelles or invading pathogens (Pankiv et al., 2007; Thurston et al., 2009; Zheng et al., 2009).

Adaptor interaction with ATG8/LC3 is mediated through an LC3-Interacting Region (LIR). LIR (also referred to as ATG8-interacting motif or AIM) is an amino acid region of a protein that mediates its interaction with ATG8 protein(s). The canonical LIR, WxxL (x being any amino acid) was originally characterized using deletion mapping and point mutations of autophagy adaptor p62 and crystal structure of p62 binding LC3B (Pankiv et al., 2007). Later, other LIRs were characterized, including a non-canonical LVV LIR present in another adaptor protein, NDP52/CALCOCO2 (von Muhlinen et al., 2012). In addition to autophagy adaptors, LIR-containing proteins include autophagy proteins, proteins associated with vesicles and their transport, Rab GTPase activating proteins (GAPs), and autophagic cargo (Birgisdottir et al., 2013). Once an autophagosome is formed, autophagy proteins are released into the cytosol, with the important exception of ATG8/LC3, and reused for the biogenesis of the new autophagosomes (Webber and Tooze, 2010).

**Maturation**

After the phagophore membrane closes around the cargo, autophagosomes mature through a series of fusion events with endosomes and then lysosomes to create autophagolysosomes. Autophagosomes move along the microtubules towards the positive end via binding the protein FYVE and coiled-coil domain containing 1 (FYCO1). FYCO1 binds LC3 (via its LIR), PI(3)P, and Ras-related protein Rab-7 (RAB7), a small GTPase enriched on late endosomes (Pankiv et al., 2010). The actin motor protein myosin VI also appears to be important for autophagosome maturation (Tumbarello et al., 2013; Tumbarello et al., 2015; Tumbarello et al., 2012; Verlhac et
Mature autophagosomes are decorated with LC3, RAB7 and a SNARE protein, syntaxin 17 (STX17) (Itakura et al., 2012). A multiadaptor protein PLEKHM1 brings together proteins on the autophagosome surface (LC3, RAB7, STX17) and homotypic fusion and protein sorting (HOPS) complex, thereby mediating fusion with the lysosome (McEwan et al., 2015). ATG14 also plays a role in the fusion event, as it is able to stabilize the Soluble 29 KDa NSF Attachment Protein (SNAP29)-STX17 binary t-SNARE complex on autophagosomes (Diao et al., 2015). Presumably, the SNAP29-STX17 stabilization on the autophagosome primes it for interaction with a v-SNARE, VAMP8, that can promote an autophagosome-endolysosome fusion event (Diao et al., 2015). Once in the autolysosomes, the cargo and the inner membrane of the original autophagosome are degraded by lysosomal enzymes, and the resulting macromolecules are recycled (Figure 2.3).
**Figure 2. Model of the mammalian autophagy pathway.**

(1) Autophagy induction and vesicle nucleation is achieved by action of ULK1 and BECN1 complexes, respectively. **The induction** of autophagy requires the repression of the mTOR kinase which, when active, inhibits autophagy by hyperphosphorylating and inactivating ULK1 and ATG13. When mTOR is inhibited, a change in the phosphorylation of ULK1, ATG13 and other proteins in the complex, including FIP200, stimulates ULK1 activity and induces autophagy. Vesicle nucleation involves activation of PI3K complex and phosphatidylinositol-3-phosphate generation. ULK1 and PI3K complexes are required for phagophore formation. (2) Two ubiquitin-like conjugation systems, ATG12 and LC3/ATG8, are part of the **vesicle elongation process.** This figure depicts LC3 is conjugation to phosphatidylethanolamine (PE) by the sequential action of the protease ATG4, E1-like enzyme ATG7 and E2-like enzyme ATG3, and E3-like enzyme ATG5-ATG12-ATG16L1 complex. Lipid conjugation converts the soluble processed form of LC3, named LC3-I, to the LC3-II form attached to the phagophore membrane. LC3-II functions in phagophore expansion, and also in cargo recognition (via binding to adaptor proteins). While cargo depicted above is a rod-shaped bacterium, it is meant to represent any autophagic cargo. (3) **Maturation.** Once an autophagosome is completed, ATG proteins that were associated with the expanding phagophore are released in the cytoplasm and reused for the biogenesis of new vesicles. Autophagosomes undergo maturation by fusion with lysosomes to create autophagolysosomes. In the autophagolysosomes, the inner membrane and the lumenal cargo are degraded by lysosomal enzymes.
Table 1. Core molecular machinery of autophagosome formation

<table>
<thead>
<tr>
<th>Atg1/ULK complex</th>
<th>Yeast</th>
<th>Mammals</th>
<th>Characteristics and functions</th>
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<tbody>
<tr>
<td>Atg13</td>
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<td>Regulatory subunit through phosphorylation by M/TORC1 and/or PKA, linker between Atg1 and Atg17</td>
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<td>Atg17</td>
<td>RB1CC1/FIP200 (functional homolog)</td>
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<tr>
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<td>Ternary complex with Atg17 and Atg31</td>
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<tr>
<td>Atg31</td>
<td></td>
<td>Ternary complex with Atg17 and Atg29</td>
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<tr>
<td>Atg11</td>
<td></td>
<td>Scaffold protein in selective autophagy for PAS organization</td>
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<td></td>
<td>C12orf44/Atg101</td>
<td>Component of the complex with ATG13 and RB1CC1</td>
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<th>Mammals</th>
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<td>Atg2</td>
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<td>Atg9</td>
<td>ATG9A/B</td>
<td>Transmembrane protein, directs membrane to the phagophore</td>
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<td>Atg18</td>
<td>WIP11/2</td>
<td>PtdIns3P-binding protein</td>
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<td>PIK3R4/VPS15</td>
<td>Ser/Thr protein kinase</td>
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<td>Atg8</td>
<td>LC3A/B/C, GABARAP, GABARAPL1/2</td>
<td>Ubl, conjugated to PE</td>
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<th>Yeast</th>
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<td>ATG3</td>
<td></td>
<td>E2-like enzyme</td>
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<td>ATG4A/B/C/D</td>
<td>Deconjugating enzyme, cysteine proteinase</td>
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<td>Atg12</td>
<td>ATG12</td>
<td>Ubl</td>
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<td>E2-like enzyme</td>
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<tr>
<td>Atg16</td>
<td>ATG16L1</td>
<td>Interacts with Atg5 and Atg12</td>
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<tr>
<td>Atg5</td>
<td>ATG5</td>
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<td>Conjugated by Atg12</td>
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**Interactions of pathogenic bacteria with the autophagy pathway**

Autophagy is a conserved cellular degradative pathway that functions as a vital part of the host immune response to microbial infection. Yasuko Rikihisa was the first to establish a connection between infection and autophagy in 1984. Using electron microscopy, she observed *Rickettsiae* contained in autophagosomes in polymorphonuclear leukocytes at 4 hours (h) post invasion (p.i.) (Rikihisa, 1984). In 1995, Swanson and Isberg observed *Legionella pneumophila* to be replicating in an autophagosomes-like compartment in macrophages (Swanson and Isberg, 1995). Similarly, Maria Colombo’s group found *Coxiella burnetii* to reside in acidic LC3\(^+\) vacuoles in HeLa cells (Beron et al., 2002). These findings suggested that some pathogens might exploit autophagy for their survival. Conclusive evidence that autophagy can eliminate intracellular pathogens came only in 2004, when Vojo Deretic’s group demonstrated that autophagy suppressed intracellular survival of *Mycoplasma* in macrophages (Gutierrez et al., 2004), and Tamotsu Yoshimori’s group, using *atg5\(^+\)* embryonic stem cells, demonstrated that autophagy can eliminate Group A *Streptococcus* (GAS) (Nakagawa et al., 2004). Since then, numerous studies have examined a number of different interactions of microbial pathogens with the autophagy pathway (Figure 3). The contribution of autophagy to the host response to infection is analogous to a double-edged sword: autophagy can eliminate some pathogens and bacterial toxins, while other pathogens can evade or exploit autophagy for survival and replication within a host.
(a) Vacuolar targeting: 
M. tuberculosis

(b) Cytosolic targeting: 
L. monocytogenes (ΔactA)

S. flexneri

S. pilimurium

S. pyogenes

F. tularensis
Figure 3. Electron microscopy of bacteria targeted by autophagy.

(A) Bacteria targeted by autophagy within an intact or damaged vacuolar compartment.

(A-1) *Mycobacterium tuberculosis* variant *bovis* BCG – Vacuolar *M. tuberculosis* within a double-membrane autophagic structure (MAP – mycobacterial autophagosome). Reproduced with permission from (Gutierrez et al., 2004). (A-2) *S. Typhimurium* within a multi-lamellar structure in wild-type (WT) MEFs (left panel). *S. Typhimurium* within a single-membrane structure in *atg5*<sup>−/−</sup> MEFs (right panel). Reproduced with permission from (Zheng et al., 2009).

(A-3) *Listeria monocytogenes* – *L. monocytogenes* within a single-membrane compartment termed a spacious *Listeria*-containing phagosome. Reproduced with permission from (Birmingham et al., 2008).

(A-4) *Shigella flexneri* – ImmunoTEM of *S. flexneri*, inset is a zoom-in of indicated area. Arrowhead corresponds with Galectin-3 while polyubiquitinated proteins indicated by an arrow. Reproduced with permission from (Dupont et al., 2009).

(B) Bacteria targeted by autophagy within the cytosol.

(B-1) ΔactA *Listeria monocytogenes* – (a) cytosolic ΔactA *L. monocytogenes*. (b) Crescent shaped double membrane structure surrounding bacterium. (c) Double-membrane structure further elongating around bacterium. (d) Cytosolic bacterium fully encompassed within a double-membrane structure. Reproduced with permission from (Rich et al., 2003).

(B-2) ΔicsB *Shigella flexneri* – ΔicsB *S. flexneri* completely surrounded within a multi-lamellar membranous structure. Reproduced with permission from (Ogawa et al., 2005).

(B-3) *Streptococcus pyogenes* – Immuno-TEM of GFP-LC3<sup>+</sup> Group A streptococcus-containing autophagosome-like vacuoles (GcAVs). Reproduced with permission from (Yamaguchi et al., 2009).

(B-4) *Francisella tularensis* – Multiple *F. tularensis* within a double-membrane structure. Reproduced with permission from (Checroun et al., 2006). Reprint from (Shahnazari and Brumell, 2011) with permission from Elsevier.
**Autophagy can restrict intracellular bacterial growth**

Autophagy can restrict intracellular replication of other bacteria. Remarkably, autophagy can target bacteria in different compartments (Figure 4). *Mycobacterium tuberculosis* can be targeted in intact phagosomes by the autophagy pathway in murine macrophages when cells are treated with the autophagy activator, rapamycin, or stimulated by interferon γ (IFNγ) (Gutierrez et al., 2004) (Figure 1). Autophagy also delivers antimicrobial peptides in a p62-dependent manner to the lysosomes to enhance bacterial degradation (Alonso et al., 2007; Ponpuak et al., 2010). Group A *Streptococcus* (GAS) can be targeted by autophagy in the cytosol (Nakagawa et al., 2004) (Figure 4.3). Multiple GAS bacteria are enclosed in a multilamellar compartment called GAS-containing LC3⁺ autophagosome-like vacuole (GcAV) and killed by subsequent fusion with the lysosome (Yamaguchi et al., 2009). *S. Typhimurium* is also targeted to the autophagy pathway and this case is discussed in detail below.

**Salmonella enterica serovar Typhimurium**

*S. Typhimurium* is a Gram-negative, facultative intracellular pathogen that causes disease in a variety of hosts, including humans (Hansen-Wester and Hensel, 2001). Among over 2,000 serovars of *S. enterica*, *S. Typhimurium* is one of the most frequent causes of food-borne gastroenteritis in humans. In fact, there are estimated to be over one million human infections by this pathogen every year worldwide (Ibarra and Steele-Mortimer, 2009).

*S. Typhimurium* uses a type three secretion system (T3SS) for efficient invasion and survival in host cells (Hansen-Wester and Hensel, 2001). T3SS allows for direct transfer of bacterial virulence proteins into host cytoplasm that orchestrates actin cytoskeleton rearrangement resulting in membrane ruffling and bacterial internalization (Hansen-Wester and Hensel, 2001). Once inside the cell, *S. Typhimurium* is contained within the single-membrane Salmonella-Containing Vacuole (SCV). *S. Typhimurium* modifies SCV membrane composition to establish a niche conducive for survival and replication, as it can selectively recruit or exclude various endosome markers (Garcia-del Portillo and Finlay, 1995). Early in infection, SCV is rich in early endosome markers, including early embryonic antigen 1 (EEA1), Rab5, and VPS34.
These later acquire some late endosome markers, including Rab7, lysosomal-associated membrane protein 1 (LAMP1), and vacuolar-type H⁺-ATPase (V-ATPase) (Steele-Mortimer et al., 1999), while excluding other late endosome markers (Brumell et al., 2001; Garcia-del Portillo and Finlay, 1995). At later stages of infection, 6-8 h p.i., a bacterial protein, SifA, induces formation of long filamentous structures emerging from the SCV called *Salmonella*-induced filaments (Sifs). While the function of Sifs is still unknown, it is thought that Sifs function to promote stability of the SCV, to acquire nutrients, or mediate dilution of lysosomal enzymes (Brumell and Grinstein, 2004; Liss and Hensel, 2015; Schroeder et al., 2011). The position of the SCV changes as the maturation process progresses: first, it migrates towards and then resides at the juxtanuclear position by 8 to 14 h p.i. (Abrahams et al., 2006). Afterwards, a subset of SCV move centrifugally towards the host cell periphery at 24 h, presumably to allow for cell-to-cell spread (Szeto et al., 2009).

Interestingly, not all SCV follow this fate (Figure 5). The majority of bacteria follow the path described above, however, at least three other fates have been observed (Figure 5.1). A sub-population of bacteria can damage the SCV and induce Ca²⁺- and synaptotagmin VII-dependent lysosomal membrane repair response that leads to bacteria killing via lysosomal fusion (Roy et al., 2004) (Figure 5.2). Damaged SCV can also get targeted by the autophagy pathway (Birmingham et al., 2006) (Figure 5.3). Finally, a population of bacteria can escape from the SCV into the cytoplasm and associate with ubiquitinated proteins. Depending on the cell type, cytosolic bacteria are either killed (in macrophages) or survive and replicate (in epithelial cells) (Beuzon et al., 2002; Brumell et al., 2002; Perrin et al., 2004) (Figure 5.4).
Figure 4. Autophagy targeting of bacteria in host cells.

1. *M. tuberculosis* is targeted within intact phagosomes following treatment with rapamycin or IFNγ.
2. *S. Typhimurium* is targeted by autophagy in damaged vacuoles.
3. Cytoplasmic bacteria can be targeted by autophagy, including a non-motile mutant (actA) of *L. monocytogenes* (in the presence of chloramphenicol), a mutant of *S. flexneri* lacking the type III secreted effector IcsB, and GAS. Adapted from Cemma and Brumell, 2012.
Figure 5. Model of intracellular S. Typhimurium populations during in vitro infection.

After invasion, at least four different intracellular populations of S. Typhimurium are observed. (1) Majority of S. Typhimurium reside and replicate within an SCV. Sif formation is observed 6 – 8 h p.i. and is associated with these bacteria. (2) SCV injury and Ca^{2+} release into the cytosol triggers recruitment of LAMP1^+ lysosomes via synaptotagmin VII. The lysosomes fuse with the SCV and release their contents into the vacuole. (3) SCV injury can also give rise to a population of bacteria targeted by the autophagy system. Finally, (4) S. Typhimurium can escape into the cytosol and become decorated with Ub^+ proteins. The fate of these bacteria is cell type dependent: S. Typhimurium can grow in the cytosol of epithelial cells but are killed by factors present in the cytosol of macrophages. This figure was originally published in JBC. Birmingham et al. Autophagy Controls Salmonella Infection in Response to Damage to the Salmonella-containing Vacuole. JBC. 2006; 281, 11374-11383. © The American Society for Biochemistry and Molecular Biology.
**Ubiquitin-conjugation system**

Post-translational covalent attachment of ubiquitin is a prevalent cellular regulatory mechanism with important roles in controlling signal transduction, endocytic trafficking and immune response (Pickart, 2001). Ubiquitin is a 76-amino acid protein that can be covalently conjugated to other proteins via sequential interactions among ubiquitin-activating E1, ubiquitin-conjugating E2, and ubiquitin E3 ligase enzymes (Pickart, 2001). The human genome encodes two E1s, around 35 E2s, over 800 E3s, and thousands of substrates for ubiquitin. Ubiquitin is covalently attached to target proteins via an isopeptide bond between its C-terminal glycine (Gly76) and a lysine residue on the acceptor substrate (Ciechanover et al., 1982), though ubiquitin can also be covalently conjugated to cysteine and threonine residues (Kravtsova-Ivantsiv and Ciechanover, 2012). Since ubiquitin itself contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), multiple molecules of ubiquitin can also become linked to each other to form polyubiquitin chains (Shaid et al., 2013). Thus, ubiquitination can take many forms that can differentially control the fate of the target protein. The best understood function of ubiquitination is Lys48 (K48) polyubiquitination that targets substrate for proteasomal degradation (Pickart and Eddins, 2004; Pickart and Fushman, 2004), while Lys63 (K63) chains are important for DNA repair, and monoubiquitination plays a role in endocytosis (Hicke and Dunn, 2003; Jackson and Durocher, 2013; Kolas et al., 2007). Furthermore, there is a strong link between ubiquitination and autophagy. Recent studies have shown that cargo ubiquitination and ubiquitin-binding adaptor proteins are required for efficient autophagy of protein aggregates, peroxisomes and mitochondria (Khaminets et al., 2016; Kirkin et al., 2009b; Shaid et al., 2013).

**Deciphering selective autophagy targeting mechanisms using S. Typhimurium**

In 2006, Cheryl Birmingham and colleagues observed that 20-30% of intracellular S. Typhimurium colocalize with LC3 at 1 h p.i. (Birmingham et al., 2006). Autophagy appeared to target bacteria within damaged vacuoles, as judged by co-localization of LC3 with normal vacuolar markers, such as LAMP1 and major histocompatibility complex 1 (MHC1)
Autophagy restricts bacterial growth, since autophagy-impaired cells (atg5-/- MEFs) are more permissive for S. Typhimurium growth than wild-type cells (Birmingham and Brumell, 2006). S. Typhimurium targeted by autophagy are contained in multilamellar structures (not typical double membrane autophagosomes) at 1 h p.i. (Figure 3A, inset 2) (Kageyama et al., 2011; Zheng et al., 2009). Interestingly, 50% of LC3+ S. Typhimurium colocalize with ubiquitinated proteins, suggesting that ubiquitin might be one of the signals required for autophagy (Birmingham et al., 2006). Protein aggregates were previously shown to depend on adaptor protein, p62/SQSTM1, for bridging ubiquitin-positive (Ub+) cargo and autophagy machinery, as it is able to (i) bind LC3 through an LIR and (ii) bind ubiquitin through an UBA domain. These two features are commonly found among other ubiquitin adaptors (Birgisdottir et al., 2013). An elegant series of experiments by Terrence Zheng and colleagues showed that the same is true in the case of S. Typhimurium; p62 is required to target invading bacteria to the autophagy pathway (Zheng et al., 2009). Using UBA and LIR deletion mutants, Zheng showed that both interactions are needed for effective autophagy. Importantly, in the absence of p62, intracellular replication of S. Typhimurium is enhanced (Zheng et al., 2009) (Figure 6). Yet, even in the absence of p62 (via siRNA knockdown), a small population (8%) of intracellular S. Typhimurium becomes LC3+, suggesting there are other targeting mechanisms. Teresa Thurston and colleagues observed another adaptor protein, NDP52, colocalize with Ub+ S. Typhimurium (Thurston et al., 2009) (Figure 6). NDP52 can interact with LC3 via an LIR and ubiquitin via a Zinc finger domain. That observation led authors to believe that NDP52 functions in a similar way to p62.

Alan Huett and colleagues identified another adaptor protein. Formin-binding protein 1-like (FNBP1L) (Huett et al., 2009) localizes to intracellular bacteria via an unknown mechanism and recruits ATG3 through its HB1 domain. In the absence of FNBP1L, ATG3 is not recruited to intracellular Salmonella and bacteria replication is enhanced (Huett et al., 2009) (Figure 6).

The pertinent questions in the field at the time my research was initiated were: why are two ubiquitin-binding adaptor proteins required for ubiquitin-dependent autophagy of S.
Typhimurium and what is the nature of E3 ubiquitin ligase(s)? The research addressing this question is encompassed in Chapter 3.

In addition to ubiquitin-dependent targeting, *S. Typhimurium* can recruit LC3 via diacylglycerol (DAG) pathway (most likely via LC3 Associated Phagocytosis – discussed on page 38) (Shahnazari et al., 2010). Inhibition of DAG production impairs LC3 recruitment to bacteria. Distinct populations of either Ub⁺ or DAG⁺ *S. Typhimurium* were observed; and inhibition of both pathways leads to a cumulative effect on LC3 recruitment, suggesting that these two pathways act independently of each other (Shahnazari et al., 2010).
Figure 6. Working model of canonical anti-Salmonella autophagy in 2009.

Intracellular S. Typhimurium is targeted by autophagy at 1 h p.i.. Autophagy recruitment is dependent on ubiquitin-binding adaptor proteins, p62 and NDP52, as shown by two independent studies (Zheng et al., 2009) (Thurston et al., 2009). Whether two ubiquitin-binding adaptors were redundant was not clear. The nature of ubiquitin E3 ligase responsible for ubiquitination event(s) was unknown. In addition to p62 and NDP52, FNBP1L localizes to S. Typhimurium and is required for ATG3 recruitment to the SCV (Huett et al., 2009). The current model of anti-Salmonella autophagy is depicted in Figure 28.
**Evasion of autophagy by bacteria**

While some bacteria are killed by autophagy, others can evade or even exploit autophagy to cause disease. For example, *Shigella flexneri* can evade autophagic capture in the cytosol (Figure 7.1). These bacteria enter host cells and escape from the phagosome into the cytosol. The bacterial cell surface virulence protein IcsA is then utilized to promote actin-based motility, but can also bind to the autophagy component, ATG5, thereby targeting *S. flexneri* for autophagy degradation (Ogawa et al., 2005). In order to circumvent degradation via autophagy pathway, another bacterial protein, IcsB competitively binds to IcsA and masks it from recognition by ATG5 and the autophagy pathway (Ogawa et al., 2005). Septins were identified as host factors that, in conjunction with autophagy, might restrict bacterial spreading under conditions that favour antibacterial autophagy (Mostowy et al., 2010). While the majority of *S. flexneri* can escape autophagic capture in the cytosol, its vacuolar membrane remnants are targeted to the autophagy pathway in a p62 and LC3-dependent manner. Autophagic degradation of the membrane remnants suppresses inflammatory responses and necrotic cell death (Dupont et al., 2009).

*Listeria monocytogenes* escapes from phagosomes using a pore-forming toxin, listeriolysin O (LLO) and two phospholipase Cs (Figure 7.2). Once in the cytosol, it uses a cell surface protein called ActA to recruit the host actin-nucleation complex Arp2/3 to promote intracellular motility and cell-to-cell spread (Portnoy et al., 2002). Recruitment of host actin to the bacterial surface by ActA is thought to mask *L. monocytogenes* from autophagy recognition in the cytosol (Yoshikawa et al., 2009). In the absence of ActA, another protein, internalin K (InlK), may also mask intracellular *L. monocytogenes* from autophagic recognition through its interaction with host major vault protein (MVP) complex. Hence, the bacterium becomes surrounded by the host protein and avoids recognition by innate immunity. InlK, however, is only expressed *in vivo* (Dortet et al., 2011).

**Bacteria can block maturation of the autophagosome to create a replicative niche**

Some bacteria can inhibit or delay autophagosome maturation, i.e. fusion with the lysosome, in order to promote bacterial replication. *Yersinia pseudotuberculosis* subverts the autophagy
pathway in macrophages to block autophagy maturation and to establish a replicative niche (Figure 7.3) (Moreau et al., 2010). The bacteria are enclosed in double-membrane or multilamellar LC3⁺, non-acidic vacuoles early after infection (4 h p.i.). In \(\text{atg}5^{+/−}\) MEFs, bacteria are degraded in acidic vacuoles (Moreau et al., 2010). Another \textit{Yersinia} strain, \textit{Yersinia pestis} also localizes to LC3⁺ autophagosome-like vacuoles (in both single and double membrane compartments) and replicates in these non-acidic vacuoles in BMDM (Pujol et al., 2009).

\textit{Anaplasma phagocytophilum} is targeted to autophagy late in infection (3 days p.i.). Bacteria reside in double-membrane, LC3⁺, non-acidic vacuoles in human myelocytic HL-60 cells (Figure 7.3). The role of autophagy in \textit{A. phagocytophilum} infection is highlighted by the use of autophagy modulators: while autophagy induction with rapamycin treatment favours bacterial infection, autophagy inhibition with 3-MA and wortmannin (both are PI3-kinase inhibitors) impairs bacterial replication. Induction of autophagy by \textit{A. phagocytophilum} is dependent on its bacterial Type IV secretion system (Niu et al., 2008).

In HeLa cells, \textit{Staphylococcus aureus} subverts the autophagy pathway (Figure 7.3) (Schnaith et al., 2007). The bacteria replicates in a double-membrane LC3⁺ autophagosome from 3-12 h p.i. and then escapes from the autophagosome to the cytosol to induce apoptosis of host cells to become extracellular. \textit{S. aureus} cannot replicate in \(\text{atg}5^{+/−}\) MEFs (Schnaith et al., 2007). This suggests that autophagy induction inhibits \textit{S. aureus}-containing phagosome maturation and blocks its fusion with the lysosome. Thus, autophagy provides a niche for a protected pathogen to replicate. Interestingly, the \textit{agr}-deficient mutant bacteria that cannot express virulence genes do not induce autophagy and do not survive in the host cell (Schnaith et al., 2007).

While some bacteria block fusion with the lysosome, others delay this event and develop into an acid-resistant form. \textit{Coxiella burnetii} resides in a large and acidic vacuole, the parasitophorous vacuole (PV), within which the bacteria can multiply. These vacuoles have autophagic markers, such as LC3 and BECN1. \textit{C. burnetii} exploits the autophagy pathway for replication, since its inhibition with 3-MA and wortmannin blocks formation of the vacuole. Stimulation of autophagy by starvation increases the number of infected cells as well as bacterial load per cell, and overexpression of autophagic proteins accelerate PV formation. Furthermore, \textit{C. burnetii}
exploits BECN1 to inhibit host autophagy and, thus, establishes a persistent infection (Vazquez and Colombo, 2010).

*Legionella pneumophila* is found in ER-derived double-membrane autophagy-like organelles decorated with ribosomes (Amer and Swanson, 2005; Swanson and Isberg, 1995). In human macrophages and mouse A/J macrophages, *L. pneumophila* is able to delay autophagic maturation for 4-6 h in order to differentiate into an acid-tolerant bacterial form (Sturgill-Koszycki and Swanson, 2000)(Figure 7.5). The autophagosome eventually fuses with the lysosome, and *L. pneumophila* is able to replicate in an acidic environment. Other mouse macrophages (except from A/J mice), however, are non-permissive for *Legionella* infection since they have a functional Naip5 gene, which is a cytosolic pattern recognition receptor that senses *L. pneumophila*’s flagella and promotes bacterial killing via autophagy (Amer and Swanson, 2005; Zhao et al., 2011). This does not allow enough time for *L. pneumophila* to induce acid-resistance (Amer and Swanson, 2005).

In murine macrophages, *Francisella tularensis* disrupts the phagosomal membrane early in infection (1-2 h) and escapes into the cytoplasm, where it replicates from 4-20 h p.i.. It then re-enters the endosomal pathway through autophagy (Checroun et al., 2006). At 24 h p.i. bacteria localize to a large fusogenic double-membrane vacuole decorated with LC3 (Checroun et al., 2006) (Figure 7.4). It is speculated that *F. tularensis* uses autophagy to promote its exocytosis from infected cells, thereby promoting cell-to-cell spread (Checroun et al., 2006).
Figure 7. Bacterial evasion or subversion of autophagy pathways.

(1) *S. flexneri* and *B. pseudomallei* escape from the phagosome, gain intracellular motility, and replicate in the cytosol. Bacterial effectors IcsB and BopA prevent targeting of bacteria by autophagy and LAP, respectively. (2) *L. monocytogenes* can escape from the phagosome using a pore-forming toxin, LLO (and other virulence factors). When LLO levels are high, it successfully escapes and multiplies in the cytoplasm. However, when LLO levels are low, *L. monocytogenes* resides in a spacious *Listeria* containing phagosome (SLAP). Fusion of SLAPs with lysosomes is blocked by LLO. (3) *S. aureus*, *A. phagocytophilium*, and *Y. pseudotuberculosis* create a replicative niche in an autophagosome by blocking its fusion with lysosomes. (4) *F. tularensis* initially escapes from the phagosome and replicates in the host’s cytoplasm. It then enters the autophagy pathway and continues to replicate in the mature autophagosome. (5) *L. pneumophila* delays fusion of the autophagosome with lysosomes until it develops into an acid-resistant form and can replicate in the acidic autophagolysosome. Reprint by permission from Elsevier, Cemma and Brumell, 2012.
Role of Immunity Related GTPases in Autophagy

Immunity Related GTPases (IRGs) belong to a group of interferon-inducible GTPases which are induced by all types of interferon (Martens and Howard, 2006). In fact, in IFN-γ-treated murine cells (both macrophages and fibroblasts), transcripts of IRGs are among the most abundant upregulated genes based on suppression subtractive hybridization screen (this method allows for rapid identification of differentially expressed cDNA clones) (Boehm et al., 1998). This family of proteins plays a role in host resistance to bacteria and parasites (Pilla-Moffett et al., 2016). Evolutionary, IRGs occur sporadically in chordates. For example, IRG genes are present in cephalochordata, zebrafish, dog, mouse, and primates, but absent in cat and bird (Li et al., 2009a) (Figure 8A). Of relevance to this thesis, murine and human IRGs have been implicated in regulating autophagy. Interestingly, IRG genes are largely expanded in rodents. Genomic investigation by Howard’s group identified 17 IRG genes and 4 IRG pseudogenes in the genome of the C57BL/6 laboratory mouse (Bekpen et al., 2005; Pilla-Moffett et al., 2016). Murine IRGs have been under investigation since the 1990s, when the first six IRG genes were identified and sequenced (Martens and Howard, 2006). Most murine IRG genes can be induced by interferon as they have interferon-responsive sequence elements (IRSE) and IFN-γ activated site (GAS) sequences in their promoter regions. Murine IRGs promote cell-autonomous defence against intracellular protozoa and bacteria, including *L. monocytogenes*, *M. tuberculosis*, *S. Typhimurium*, *Chlamydia trachomatis*, and *Toxoplasma gondii*. This is clearly demonstrated by the fact that IRG-deficient mice become susceptible to various infections. Surprisingly, deletion of even a single IRG can result in susceptibility to certain infections. In uninfected cells, IRG proteins associate with membranes to different degrees and are found on different compartments, including Golgi, ER, mitochondria, and in the cytosol. Upon infection, IRGs are thought to localize to and disrupt the pathogen-containing vacuole, thus making a pathogen accessible for degradation in the cytosol, likely through the autophagy pathway (Coers, 2013; Deretic, 2011).

To date, three murine IRGs have been linked to the autophagy pathway: *Irgm1* (Feng et al., 2008a; Gutierrez et al., 2004; Singh et al., 2006), *Irga6* (Haldar et al., 2014; Zhao et al., 2008),
and Irgb10 (Haldar et al., 2014). Recruitment of Irga6 and Irgb10 to the pathogen-containing vacuole requires ATG5 and ATG3 (Haldar et al., 2014; Zhao et al., 2008), though the mechanism of this recruitment remains obscure. This suggests that autophagy proteins, ATG3 and ATG5, are upstream of Irga6 and Irgb10 recruitment to the vacuole. How ATGs recruit Irga6 and Irgb10 to the vacuole and allow them to restrict intracellular pathogens is not understood.

The involvement of Irgm1 in autophagy is controversial. A number of labs have demonstrated that Irgm1 can stimulate autophagosome formation and control replication of *Mycobacteria tuberculosis* (Gutierrez et al., 2004; Singh et al., 2006; Traver et al., 2011). In the absence of Irgm1, the autophagic flux is impaired as demonstrated by Western blotting, and the number of autophagosomes is reduced as demonstrated by quantification of LC3$^+$ puncta with immunofluorescence (Singh et al., 2006). This evidence places Irgm1 upstream of autophagy machinery. Mechanistic insights on how Irgm1 stimulates autophagy, however, remain elusive. MacMicking’s group showed that Irgm1 is dispensable for autophagy (at least during early stages of autophagosome formation) in IFN-γ-induced macrophages that overexpress LC3 (Matsuzawa et al., 2012). Instead, Matsuzawa and colleagues posit that IFN-γ promotes autophagy via the p38 MAPK signalling pathway (Matsuzawa et al., 2012). In addition, Irgm1-deficient mice that overexpress LC3 harbor more autophagy puncta rather than less when compared to wild-type counterparts (King et al., 2011).

Endogenous Irgm1 localizes to the ER, mitochondria, endomembrane system, and cytosol, yet, unlike other IRGs, Irgm1 does not localize to the pathogen-containing vacuole in the case of *M. tuberculosis* and *L. monocytogenes* (Springer et al., 2013). This suggests that either (i) it was not captured at the examined time points, (ii) it acts indirectly, or (iii) it might be dispensable in antibacterial autophagy.

In addition to cell-autonomous functions, Irgm1 has a systemic role in protecting the host from infections, as Irgm1-deficient mice experience a dramatic collapse of the lymphomyeolid system. Irgm1 is essential for (i) lymphocyte survival (Feng et al., 2004), (ii) proper macrophage function, including adhesion and motility (Henry et al., 2007), (iii) normal hematopoietic
function (Santiago et al., 2005), and (iv) normal hematopoietic stem cell response to infection with *M. tuberculosis* and *Mycobacterium avium* (Feng et al., 2008a). In addition, (v) Irgm1 regulates survival of mature CD4+ T lymphocytes by preventing IFN-γ-induced autophagic cell death (Feng et al., 2008b). A recent paper suggests that collapse of the lymphomyeolid system in Irgm1-deficient mice can be explained by damage to lysosome function (Maric-Biresev et al., 2016). In summary, though the link between murine IRGs and the autophagy pathway has been established (though controversial in the case of Irgm1), few mechanistic details relating to their function have been uncovered.

Until recently, IRGs were thought to be largely absent from humans (Martens and Howard, 2006). Currently, it is believed that humans have only three IRGs: IRGM, IRGC, and IRGQ. These genes are all syntenic to their murine homologues (*Irgm1, Irgc*, and *Irgq*, respectively), indicating conservation from a common ancestor. However, none of the human IRGs are interferon-inducible, as they lack GAS or ISRE elements in their promoters. IRGM is relatively well studied, while IRGC and IRGQ have yet to be examined.

Given that human *Irgm* gene is truncated and not interferon-inducible (in comparison with the mouse gene), *Irgm* was initially believed to be a pseudogene (Bekpen et al., 2009). In 2006, Singh and colleagues published a groundbreaking study implicating IRGM in response to infection by *Mycobacterium tuberculosis* (Singh et al., 2006). In the absence of IRGM, fewer BCG colocalized with a lysosome marker and bacterial survival was enhanced (Singh et al., 2006). While the mechanism of its action was not clear at the time, it was presumed that it might be through general inhibition of autophagy. Knock down of IRGM expression resulted in less LC3+ puncta and less LC3-II in cells treated with IFN-γ, supporting this hypothesis (Singh et al., 2006).

The role of IRGM in resistance to *Mycobacterium tuberculosis* is corroborated by the fact that SNPs in IRGM are associated with an increased risk of clinical tuberculosis (Intemann et al., 2009). In addition, polymorphisms in IRGM and ATG16L1 have been linked to Crohn’s disease, thereby hinting at the possibility that autophagy and IRGs may be connected (Parkes et al., 2007; Wellcome Trust Case Control, 2007).
Subsequent studies showed that IRGM does not localize to the *M. tuberculosis*-containing phagosome, but rather acts upstream of the actual autophagic degradation. Instead, a fraction of IRGM localizes to the mitochondria via binding to a mitochondrial lipid cardiolipin, where it can affect mitochondrial fission via an unknown mechanism and induce autophagy, presumably through indirectly stabilizing AMPK phosphorylated on Threonine 172 (Chauhan et al., 2015; Singh et al., 2010). AMPK is known to phosphorylate ULK1 (Egan et al., 2011; Kim et al., 2011b) and BECN1 (Kim et al., 2013) resulting in their activation. Also, IRGM assembles with activated forms of ULK1 and BECN1, thereby promoting their co-assembly (Chauhan et al., 2015). IRGM complexes also included BECN1 interactors, AMBRA1, ATG14L, and UVRAG (Chauhan et al., 2015). Hence, IRGM promotes autophagy initiation via multiple pathways. In addition to ULK1 and BECN1, the IRGM complex contained ATG16L1, and NOD2 (Chauhan et al., 2015). This complex is stabilized by autophagy proteins, but not NOD2 (Chauhan et al., 2015). It is noteworthy that three Crohn’s disease associated factors, IRGM, ATG16L1, and NOD2, are in this complex. Thus, IRGM orchestrates core autophagy initiation machinery, including ULK1, ATG16L1 and BECN1 complex, and links it with innate immune receptor, nucleotide-binding oligomerization domain-containing protein 2 (NOD2). Chauhan and colleagues also observed IRGM interact with other innate immune adaptors, including NOD1, retinoic acid-inducible gene 1 (RIG-I), and Toll-like receptor 3 (TLR3), implying that it might be acting as a bridge between pathogen-associated molecular patterns (PAMP) sensing and autophagy (Chauhan et al., 2015) (Figure 9).

IRGC is an ortholog of mouse Irgc with 90% sequence identity; it is not induced by interferons. Expression data indicate that IRGC is strongly expressed only in testes (Martens and Howard, 2006). IRGC is a virtually unstudied protein. Similarly, no studies to date have been published on IRGQ. The reason why IRGQ has remained in the shadow of other IRGs is likely because IRGQ is not predicted to be a functional GTPase, as it lacks the universally conserved G1 motif required for GTP binding (Bekpen et al., 2005; Li et al., 2009a). IRGQ’s aberrant GTP binding site in humans is also identified in IRGQ homologs in zebrafish and mice (Bekpen et al., 2005; Martens and Howard, 2006), IRGQ may have another function independent of GTP-binding. According to the Protein Atlas (http://www.proteinatlas.org/ENSG00000167378-IRGQ/tissue),
IRGQ is expressed in most cell types in human. Examination of the IRGQ amino acid sequence from various organisms indicates that it contains two LC3-Interacting Regions (LIR) that are mostly conserved in organisms where IRGQ is present, except for the zebrafish which has only one LIR (Figure 8B). Given that IRGs are pivotal in immunity and autophagy and IRGQ has two LIRs, I hypothesize that IRGQ has a yet unidentified function in autophagy. The research in Chapter 4 of this thesis will further explore this possibility.
Figure 8. Evolution of Immunity-Related GTPases.

(A). Overview of the evolution of IRGs. Numbers outside the bracket represent the counts of putatively functional genes and those inside the bracket represent counts of pseudogenes. Modified from Li et al., 2009. (B). IRGQ sequences from a number of organisms (from http://www.uniprot.org/) aligned using ClustalW T-Coffee algorithm. LC3-Interacting Regions denoted as LIR1 and LIR2.
Figure 9. Model of human IRGM function in autophagy.

IRGM resides both in the cytosol, as well as in the mitochondrial matrix or inner membrane via its interaction with cardiolipin. IRGM promotes mitochondrial fission, and promotes autophagy via a yet uncharacterized mechanism. AMPK activates autophagy initiation components, ULK1 and BECN1 via phosphorylation. IRGM promotes autophagy induction by orchestrating a complex containing ULK1, BECN1 complex, ATG16L1 and NOD2. This model is based on data from Singh et al., 2010 and Chauhan et al., 2015.
**LC3-ASSOCIATED PHAGOCYTOSIS**

In 2007, Sanjuan and colleagues observed that the autophagy protein LC3-B, is conjugated to a single phagosomal membrane upon stimulation of TLR2 and TLR4 in murine macrophages (Sanjuan et al., 2007). This was remarkable because, unlike canonical autophagy where the cargo is sequestered into a double membrane LC3+ vesicle, LC3 was directly conjugated onto a pre-existing single membrane. Sanjuan later coined this process LC3-Associated Phagocytosis, or LAP (Sanjuan et al., 2009). LAP has been implicated in a number of functions (described below), including resistance to bacterial and fungal infections (Gong et al., 2011; Gong et al., 2015; Li et al., 2013; Ma et al., 2012; Ma et al., 2014; Sanjuan et al., 2009). These recent findings highlight the fact that our cells have multiple systems to dispose of unwanted microbial invaders, and that successful pathogens have evolved mechanisms to evade these systems to promote infection of their host. It also suggests that ATG proteins, including LC3, have non-canonical functions separate from canonical autophagy and warns autophagy researchers against solely relying on LC3 as the maker of autophagy.

**LAP vs. canonical autophagy**

Canonical autophagy and LAP (also referred to as noncanonical autophagy) have key similarities and differences (Figure 10). In canonical autophagy, the cargo is sequestered into a double-membrane compartment that has LC3 conjugated to both the inside and outside membranes, and it requires a host of autophagy proteins for initiation, elongation, and closure of the autophagosomes. In LAP, a pre-existing single membrane phagosome acquires LC3-II, and the process requires some autophagy proteins, but not others (Figure 10). For instance, the autophagy pre-initiation complex, comprised of ULK1/2, ATG13 and FIP200, is dispensable for LAP (Martinez 2011, Henault 2012, Martinez 2015); ATG14, Ambra1 and WIPI-2 are also not required (Martinez 2015). Meanwhile, both canonical autophagy and LAP require the class III PI3K core complex, consisting of VPS34, VPS15, BECN1 and UVRAG (Feng et al., 2014). The core class III PI3K complex contains either ATG14 or UVRAG in a mutually exclusive fashion.
Itakura et al., 2008; Zhong et al., 2009); interestingly only UVRAG is required for LAP, while ATG14 is dispensable (Martinez et al., 2015). In addition to class III PI3K complex, both LAP and autophagy require protein machinery necessary for LC3 conjugation and processing: ATG3, ATG4, ATG5, ATG7, ATG10, ATG12, and ATG16L1 (Feng et al., 2014; Martinez et al., 2015; Sanjuan et al., 2007). In both pathways, the cargo eventually fuses with the lysosome. These similarities and differences between LAP and canonical autophagy are presented in Figure 10.

Two ways to distinguish canonical autophagy and LAP are to (i) visualize the process by electron microscopy analysis, as autophagosomes consist of two membranes (or several membranes in the case of infection by some pathogens such as S. Typhimurium) and LAP phagosomes are single-membrane compartments, and to (ii) knock down a component of the pre-initiation complex, such as ULK1, which should impair canonical autophagy but not LAP.

Canonical autophagy occurs at a basal level in all cells, while LAP is observed under some circumstances when cells uptake a particle via phagocytosis. LAP occurs in hematopoietic cells, that include macrophages, dendritic cells, and neutrophils, and in retinal pigment epithelial (RPE) cells, which are macrophage-like and express some hematopoietic markers (Abnave et al., 2014; Frost et al., 2014; Henault et al., 2012; Huang et al., 2009; Kim et al., 2013; Ma et al., 2012; Ma et al., 2014; Martinez et al., 2011; Martinez et al., 2015; Romao et al., 2013; Sanjuan et al., 2007; Sanjuan et al., 2009). The role of LAP in non-hematopoietic cell types is not yet clear.

In the past 9 years, the field has gained a better understanding of the LAP pathway, including identification of the cell surface receptors and their ligands that trigger LAP, determination of the protein machinery involved, and a better appreciation of the functions of this pathway; yet many questions remain.
### Proteins specifically required for Autophagy

- **Pre-initiation complex:** ULK1, FIP200, ATG13
- **Class III PI3K complex:** ATG14, Ambra1
- **Atg5-12-16L1 recruitment:** WIPI-2

### Proteins required for both Autophagy and LAP

- **LC3/Atg8 family:** LC3A, LC3B, GABARAL2
- **Class III PI3K complex:** BECN1, VPS34, UVRAG
- **LC3 lipidation, processing:** ATG3, ATG4, ATG7, ATG5-12-16L1 complex

### Proteins specifically required for LAP

- **Receptors:** TLRs, FCGR, Dectin-1, TIM4
- **Osmotic imbalance:** V-ATPase
- **NADPH oxidase:** Rubicon
- **Stabilization of NOX2:** Rubicon

<table>
<thead>
<tr>
<th>Proteins specifically required for Autophagy</th>
<th>Proteins required for both Autophagy and LAP</th>
<th>Proteins specifically required for LAP</th>
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<tr>
<td>Pre-initiation complex: ULK1, FIP200, ATG13</td>
<td>LC3/Atg8 family: LC3A, LC3B, GABARAL2</td>
<td>V-ATPase MORN2</td>
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<tr>
<td>Class III PI3K complex: ATG14, Ambra1</td>
<td>Class III PI3K complex: BECN1, VPS34, UVRAG</td>
<td>NADPH oxidase 2: NOX2/CYBB</td>
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<tr>
<td>Atg5-12-16L1 recruitment: WIPI-2</td>
<td>LC3 lipidation, processing: ATG3, ATG4, ATG7, ATG5-12-16L1 complex</td>
<td>Stabilization of NOX2: Rubicon</td>
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</table>
Figure 10. Comparison between LAP and canonical autophagy.

Canonical autophagy and LAP are schematically depicted. In the autophagy pathway, a cargo is sequestered into a double-membrane LC3\(^+\) autophagosome via a signal, such as ubiquitin. It also requires autophagy pre-initiation complex ULK1-FIP200-ATG13, class III PI3K complexes containing ATG14 and UVRAG, and LC3 conjugation machinery. In LAP pathway, LC3 conjugation to the phagosomal membrane requires engaging an extracellular receptor, class III PI3K complex containing UVRAG, NOX2 NADPH oxidase, Rubicon, production of intraphagosomal ROS, functional activity of V-ATPase and potentially osmotic imbalance. Similarly to the canonical autophagy, the resulting LC3\(^+\) compartment matures via fusion with the lysosome and results in eventual degradation of the cargo. Protein machinery required for both pathways is depicted in the middle of the page, while machinery that is unique to each pathway is depicted towards periphery. The table underneath the schematic summarizes the common and distinct protein machinery required for LAP and autophagy.
Table 2. Summary of cargos being targeted by the LAP pathway.

<table>
<thead>
<tr>
<th>CARGO</th>
<th>LIGAND</th>
<th>RECEPTOR</th>
<th>CELL TYPE</th>
<th>REFERENCE</th>
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<td><strong>PARTICLES</strong></td>
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<tr>
<td>Zymosan (uncoated)</td>
<td>β-glycan</td>
<td>TLR2/Dectin1</td>
<td>RAW-GFP-LC3, BMDMs, PBMC</td>
<td>(Sanjuan et al., 2007)</td>
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<td></td>
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<td>(Martinez et al., 2015)</td>
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<td>(Romao et al., 2013)</td>
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<tr>
<td>Zymosan (IgG-coated)</td>
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<td>FCGR</td>
<td>RAW-GFP-LC3, BMDN</td>
<td>(Huang et al., 2009)</td>
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<td>β-glycan particles</td>
<td>β-glycan</td>
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<td>RAW-Dectin-1, BMDM, BMDC</td>
<td>(Ma et al., 2012)</td>
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<td>TLR4</td>
<td>RAW-GFP-LC3</td>
<td>(Sanjuan et al., 2007)</td>
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<td>PAM3CSK4</td>
<td>TLR2</td>
<td>RAW-GFP-LC3, BMDN</td>
<td>(Huang et al., 2007)</td>
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<td>IgG, DNA</td>
<td>TLR9/FCGR</td>
<td>RAW-GFP-LC3, pDC</td>
<td>(Henault et al., 2012)</td>
</tr>
<tr>
<td>IgG-coated beads</td>
<td>IgG</td>
<td>FCGR</td>
<td>RAW-GFP-LC3, BMDN</td>
<td>(Huang et al., 2009)</td>
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<td><strong>FUNGAL PATHOGENS</strong></td>
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<td>Saccharomyces cerevisiae</td>
<td>β-glycan</td>
<td>TLR2/Dectin1</td>
<td>RAW-GFP-LC3, BMDM</td>
<td>(Sanjuan et al., 2007)</td>
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<td>HK Candida albicans</td>
<td>β-glycan, O-mannose</td>
<td>TLR2/Dectin1, TLR4</td>
<td>RAW-dectin-1, PBMCs, human DC</td>
<td>(Ma et al., 2014)</td>
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<td>TLR2/TLR4</td>
<td>BMDM-GFP-LC3</td>
<td>(Romao et al., 2013)</td>
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<td><strong>BACTERIAL PATHOGENS</strong></td>
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<td>LPS</td>
<td>TLR4</td>
<td>RAW-GFP-LC3</td>
<td>(Sanjuan et al., 2007)</td>
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<td>Burkholderia pseudomallei</td>
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<td>TLR4</td>
<td>RAW 264.7</td>
<td>(Gong et al., 2011)</td>
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<td>(Li et al., 2013)</td>
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<td>(Gong et al., 2015)</td>
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<tr>
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<td>TLR4</td>
<td>RAW 264.7</td>
<td>(Lerena and Colombo, 2011)</td>
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<td>TLR4</td>
<td>RAW 264.7</td>
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<td></td>
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<td>RAW 264.7</td>
<td>(Abnave et al., 2014)</td>
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<tr>
<td>Shigella flexneri</td>
<td>TC7</td>
<td></td>
<td></td>
<td>(Campbell-Valois et al., 2015)</td>
</tr>
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<td>Apoptotic-like Leishmania major</td>
<td>PtdSer</td>
<td>TIM4</td>
<td>CD11b⁺ F4/80⁻, BMDM, GFP-LC3</td>
<td>(Crauwels et al., 2015)</td>
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<td><strong>CELLS OR CELLULAR COMPARTMENTS</strong></td>
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<tr>
<td>Apoptotic, necrotic, necroptotic cells</td>
<td>PtdSer</td>
<td>TIM4</td>
<td>CD11b⁺ F4/80⁻, BMDM, GFP-LC3</td>
<td>(Martinez et al., 2011)</td>
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<td>MCF10</td>
<td>J774</td>
<td>Retinal Pigment</td>
<td>(Florey et al., 2011)</td>
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<td>Retinal Pigment, Epithelium</td>
<td>(Florey et al., 2015)</td>
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<td>(Kim et al., 2013)</td>
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<td>(Frost et al., 2014)</td>
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Mechanism of LC3-Associated Phagocytosis

Cell surface receptors that trigger LAP

LAP is triggered in response to particles that stimulate TLRs (TLR2, TLR4, TLR9), FCG2A/FcγR2A, dectin-1, TIM4, and other cargo such as fungi, bacteria, apoptotic and entotic cells (Florey et al., 2011; Henault et al., 2012; Huang et al., 2009; Ma et al., 2012; Ma et al., 2014; Martinez et al., 2011; Martinez et al., 2015; Romao et al., 2013; Sanjuan et al., 2007). Table 2 summarizes all cargos that have been shown to target to the LAP pathway to date. How receptor signaling results in recruitment of LC3 is not entirely clear, though a number of mechanisms have been suggested.

Reactive Oxygen Species

TLRs, FCGR, and dectin-1 receptors initiate reactive oxygen species (ROS) production by the CYBB/NOX2 NADPH oxidase, an event that is required for LAP (Huang et al., 2009; Ma et al., 2014; Martinez et al., 2015; Romao et al., 2013). The original study that drew the connection between CYBB/NOX2 NADPH oxidase observed that IgG-coated beads associated with LC3-II and ATG12 via Western blotting and immunofluorescence, and this association was dependent on CYBB/NOX2-produced ROS (Huang et al., 2009). Cybb/NOX2−/− bone marrow derived neutrophils (BMDNs) did not recruit LC3 to the phagocytosed zymosan, IgG- or LPS-coated beads (Huang et al., 2009). The role for ROS in LC3 recruitment is consistent in both murine and human phagocytes (Huang et al., 2009; Ma et al., 2014; Martinez et al., 2015; Romao et al., 2013) suggesting it is evolutionary conserved. Macrophages from human patients with chronic granulomatous disease (with defective NADPH complex and impaired ROS production) exhibited defects in the LAP pathway (Romao et al., 2013). In fact, intraphagosomal ROS in the absence of other signals is sufficient to recruit LC3 to phagosomes (Martinez et al., 2015).

CYBB/NOX2 NADPH oxidase has a number of characterized positive and negative regulators that can modulate its activity (Gardet et al., 2010; Kim et al., 2011a; Noubade et al., 2014; Sokolovska et al., 2013; Yang et al., 2012). For example, upon TLR2 activation, Run domain Beclin1-interacting and cysteine-rich domain-containing protein, Rubicon, stabilizes
CYBB/NOX2 NADPH oxidase through directly binding to its transmembrane, p22\textsuperscript{phox} subunit, resulting in maximizing the ROS production (Yang et al., 2012). In fact, Martinez and colleagues found that Rubicon is critical for LAP. In Rubicon\textsuperscript{-/-} bone marrow derived macrophages (BMDMs), LC3 failed to localize to zymosan and PAMP3CSK4-beads (Martinez et al., 2015). Rubicon performs two functions on the LC3\textsuperscript{+} phagosomes: (i) it allows sustained Class III PI(3)K activity that leads to enrichment of PtdIns(3)P on the phagosomal membrane and (ii) stabilizes the CYBB/NOX2 complex via biding to its subunit p22\textsuperscript{phox} (Martinez et al., 2015). A cytosolic component of NADPH oxidase, p40\textsuperscript{phox}, contains a PX domain that can bind PtdIns(3)P (Kanai et al., 2001). Rubicon-dependent PtdIns(3)P enrichment leads to p40\textsuperscript{phox} stabilization of the phagosome (Martinez et al., 2015). Hence, Rubicon and PtdIns(3)P stabilize the active CYBB/NOX2 NADPH complex to promote optimal ROS production for LAP.

**Other factors that lead to LAP**

In addition to ROS, LC3 recruitment to single membranes involves functional activity of V-ATPase and osmotic imbalance (Florey et al., 2015). There is evidence to suggest that V-ATPase is recruited early during phagocytosis, consistent with the idea that it can precede LC3 recruitment (Lukacs et al., 1992; Yates et al., 2005). Placing intracellular compartments under hypo-osmotic conditions resulted in their swelling and LC3 recruitment. LC3 recruitment was found to be dependent on functional activity of V-ATPase, since it could be ablated by treatment of bafilomycin A, a V-ATPase inhibitor (Florey et al., 2015). Interestingly a lysosomotrophic agent, chloroquine, and VacA, a pore-forming toxin of Helicobacter pylori, induce LAP-like LC3 lipidation (Florey et al., 2015). These findings also imply that V-ATPase is recruited to the phagosome early in infection, rather than at the later stages after fusion with late endosomes and lysosomes. Whether CYBB/NOX2-dependent ROS production results in membrane damage leading to osmotic imbalance remains to be determined.

**MORN2** is another factor identified to promote LC3 recruitment in LAP, as in the absence of MORN2, phagosomes containing M. tuberculosis or L. pneumophila failed to acquire LC3 and pathogen replication was enhanced (Abnave et al., 2014). MORN2 also physically interacts with
LC3 and p62 via co-immunoprecipitation in macrophages (Abnave et al., 2014). Yet the mechanism of how MORN2 promotes LC3 recruitment to the phagosome is not clear.

In human RPE cells, an intracellular cargo sorting protein, Melanoregulin (MREG), colocalizes with LC3 to phagosomes and contains a canonical LIR motif. It is likely recruiting LC3 to the phagosomes, as it can directly interact with it and in its absence result in a marked reduction of LC3+ phagosomes in RPE (Frost et al., 2014). So far, MREG has been exclusively studied in the retinal cells, and its expression in hematopoietic cells has not yet been experimentally tested.

**Phagosome maturation**

A trafficking protein FYCO1 is an LC3- and PI(3)P-binding protein that can mediate movement of the autophagosomes along microtubules towards the plus-end (Pankiv et al., 2010). FYCO1 contains an LIR and is able to bind directly to LC3A and LC3B (Olsvik et al., 2015). Ma and colleagues found a role for FYCO1 in LAP (Ma et al., 2014). FYCO1 is recruited to dectin-1 phagosomes via binding to LC3 and regulates ROS production and phagosome maturation by destabilizing p40phox component of the NADPH oxidase complex (Ma et al., 2014). In cells lacking FYCO1 phagosomes stay positive for p40phox longer, produce more ROS, and mature slower (Ma et al., 2014).

**Functions of LC3-Associated Phagocytosis**

While LAP is a relatively new field, a number of functions have been already ascribed to this important process. Like autophagy, LAP is an innate immune response to fungal and bacterial infection. Previous studies have described LAP’s role in restricting infections with the following pathogens: *E. coli*, *S. cerevisiae* (Sanjuan et al., 2007), *Burkholderia pseudomallei* (Gong et al., 2011; Gong et al., 2015), *Candida albicans* (Romao et al., 2013), *M. tuberculosis*, *L. pneumophila* (Abnave et al., 2014), *S. flexneri* (Campbell-Valois et al., 2015), and *Aspergillus fumigatus* (Martinez et al., 2015). Yet, some pathogens, like *Mycobacterium marinum* and *L. monocytogenes*, are able to recruit LC3 to phagosomes and co-opt the LAP pathway for their survival via blocking fusion with the lysosome (Lam et al., 2013; Lerena and Colombo, 2011).
In addition to pathogenic cargo, LAP clears apoptotic, necrotic, receptor-interacting serine/threonine-protein kinase 3 (RIPK3)-dependent necrotic and entotic cells, and regulates the inflammatory cytokines and antigen presentation (Florey et al., 2015; Florey et al., 2011; Henault et al., 2012; Ma et al., 2012; Martinez et al., 2011; Romao et al., 2013). Besides, LAP is linked to autoinflammatory, lupus-like disease, at least in response to exposure to dying cells (Martinez et al., 2016). In addition to responding to pathogenic infections and dead cells, LAP is required for bone resorption (DeSelm et al., 2011) and vision (Frost et al., 2014; Kim et al., 2013).

One of the major functions ascribed to LAP is to promote phagosome maturation. This conclusion is based on the observation that autophagy-deficient murine macrophages (RAW264.7 and CSF2/GM-CSF-differentiated BMDMs) fail to degrade live yeast (Sanjuan et al., 2007), apoptotic cells (Martinez et al., 2011), and phagosomes containing beads coated with TLR2 ligands exhibit delayed phagosome fusion with the lysosome (Sanjuan et al., 2007). However, a study in human macrophages observed quite the opposite—LC3 recruitment to zymosan-containing phagosomes was associated with delayed phagosomal fusion with the lysosome (Romao et al., 2013). Therefore, the impact of LC3 recruitment to phagosomes remains unclear. Chapter 5 of this thesis will examine the role of autophagy proteins, ATG5 and ATG7, in phagosome maturation.

Rationale for my studies of ATG proteins
Over the past half-century, research output in the field of autophagy has grown exponentially from few papers a year to a few thousand a year, surpassing the 1,000 papers a year mark in 2008 (Figure 11). As of this moment of writing this thesis, 23,166 papers are published on autophagy. In addition to getting this massive spotlight in the life science research community, autophagy has also captured the attention of the pharmaceutical industry. Currently, multiple clinical trials are testing the role of autophagy modulators in cancer and other diseases (Barnard et al., 2014; Garber, 2011; Klionsky and Thorburn, 2014; Mahalingam et al., 2014; Rangwala et al., 2014a; Rangwala et al., 2014b; Rosenfeld et al., 2014; Vogl et al., 2014).
While significant progress has been made in characterizing the autophagy pathway and its components, many questions still remain. Why are multiple autophagy adaptors required? What other LC3 interacting proteins are involved in autophagy? What is the impact of LAP on the fate of the phagosome?

Thesis Summary

In this thesis, I investigate the role of autophagy machinery in host defence. In Chapter 3, I decipher the relationship between the two LC3- and ubiquitin-binding adaptor proteins, p62 and NDP52, that are both required for anti-Salmonella autophagy. Previous to my work, it was not known why two different adaptors are required to target the same bacterial cargo to autophagy. I showed that both adaptors were recruited to bacteria with similar kinetics independent of each other, and that depletion of either adaptor led to impairment of antibacterial autophagy. In addition, I show that p62 and NDP52 acted in the same pathway, as depletion of both adaptors did not synergistically impair autophagy. Remarkably, these adaptors did not colocalize, but rather formed non-overlapping microdomains surrounding bacteria. This finding shifted the way the field understands how p62, NPD52, and other autophagy adaptor proteins function. I posit that p62 and NDP52 act cooperatively to drive efficient antibacterial autophagy by targeting the protein complexes they coordinate to distinct microdomains associated with bacteria.

In Chapter 4, a dual proximity-dependent biotin identification (BioID)/ affinity purification – mass spectrometry (AP-MS) method was employed to map protein interactions for LC3B. I have followed up on one previously unstudied protein, IRGQ, and characterized it as a novel LC3B interactor. IRGQ interacted with LC3B through its evolutionarily conserved LC3-interacting region and colocalized with LC3B under some conditions that promote accumulation of autophagosomes. The requisite role of IRGQ in autophagy was observed in autophagy of peroxisomes, protein aggregates, and S. Typhimurium. In sum, these studies unraveled IRGQ as an LC3B-interacting protein and an important player of selective, ubiquitin-dependent autophagy.
Finally, in Chapter 5, I examined the role of autophagy components in LC3-Associated Phagocytosis (LAP). During LAP, autophagy machinery is thought to conjugate LC3 onto the phagosomal membrane to promote fusion with the lysosome. However, a recent study has suggested that autophagy proteins may in fact impair phagosome maturation to promote antigen presentation. Hence, I examined the impact of autophagy proteins on phagosome maturation in murine cells using FcGR-dependent phagocytosis as a model. I showed that phagosome maturation is not affected in Atg5-deficient mouse embryonic fibroblasts, or in Atg5- or Atg7-deficient bone marrow-derived macrophages using standard assays of phagosome maturation. Therefore, I propose that autophagy proteins may be required for phagosome maturation under some conditions, but are not universally required for this process.
Figure 11. The growth of the autophagy field and landmarks papers.

Figure was generated using PubMed advanced search option for papers with the word “autophagy” in the title or abstract in a given year. The key studies in the autophagy field discussed in this introduction are highlighted. Inspired by Ohsumi, 2014.
Chapter 2 - Materials and Methods

Cell lines and culture conditions

HeLa human epithelial cells and Mouse embryonic fibroblasts (MEFs) were obtained from the American Type Culture Collection. Wildtype and autophagy-deficient \((\text{atg}^5)\) mouse MEFs have been previously described (Kuma et al., 2004). All cells were cultured in Dulbecco's Modified Eagle Medium (HyClone, SH3024301) supplemented with 10% FBS (Wisent) at 37 °C in 5% CO2 without antibiotics.

Macrophage generation and culture conditions

\(\text{Atg}^5\) flox/flox (WT), \(\text{Atg}^5\) flox/flox -LysMcre\(^{+}\) (\(\text{atg}^5\) ) mice, \(\text{Atg}^7\) flox/flox (WT) and \(\text{Atg}^7\) flox/flox -LysMcre\(^{+}\) (\(\text{atg}^7\) ) mice were previously described (DeSelm et al., 2011; Zhao et al., 2008). All experimental protocols involving mice were approved by the Animal Care Committee of The Hospital for Sick Children. Mice were euthanized by cervical dislocation. The femur and tibia were removed, cleansed of muscle fibers and cut distally. The bone marrow was then removed via a 10 sec pulse of centrifugation at 2000 rpm. The resulting cells were centrifuged at 1500 rpm for 5 min, washed with growth media and plated on 10 cm tissue culture dishes. Media was replaced with fresh RPMI growth media (see below) every 3 days. Typically, \(10^8\) bone marrow-derived macrophages (BMDM) were recovered after 7 days. CSF1/M-CSF differentiated BMDMs were cultured in RPMI-1640 medium (Wisent, SH3002701) supplemented with 10% FBS (Wisent, 090-510), 5% sodium pyruvate (Invitrogen, 11360-070), 5% penicillin/strep (Invitrogen, 15140122), 5% nonessential amino acids (Invitrogen, 11140050), 0.5 \(\mu\)M 2-ME (Invitrogen, 21985023) and 10% CSF1 conditioned media from NIH3T3 cells. CSF2/GM-CSF differentiated BMDM were cultured in RPMI supplemented with 10% FBS, 10 mM HEPES buffer (Life Technologies, 15630-080), 5% penicillin/strep, and non-essential amino acids in the presence of 20 ng/ml murine CSF2 (R&D Systems, 415-ML-010). Phagocytic particles were added in RPMI-1640, cells were washed 3-times with PBS at a 10-min time point after beginning
of phagocytosis, and incubated in media without antibiotics.

**Bacteria strains and Salmonella invasion**

Wild-type S. Typhimurium SL1344 and bacteria expressing RFP were used for these studies. For invasion by S. Typhimurium, late-log bacterial cultures were used for infecting cells and prepared via a method optimized for bacterial invasion (Birmingham et al., 2006). HeLas were seeded in 24-well tissue culture plates on glass coverslips 16–24 h before use at a density of 50,000 cells/coverslip.

**Small interfering RNA (siRNA) treatment, plasmids, and transfection**

p62 (no. M-010230-00) and siGenome nontargeting siRNA pool no. 2 (no. D-001206-14-20) were from Dharmacon. NDP52 (CALCOCO2), 5’-UUCAGUUGAAGCAGCAGCUCUCUGUCUCCC-3’ was custom ordered from Sigma. IRGQ siRNA 5’–CCUCUUGUCUGCGUGCGCA 3’ was ordered from SIGMA (NM_001007561, SASI_Hs01_00173686). ATG12 siRNA, 5’-GUGGGCAGUAGAGCGAACA-3’, was custom ordered from Sigma. siRNA knockdown and transfections were done as described (Zheng et al., 2009).

Constructs used were GFP-LC3 (provided by Tamotsu Yoshimori, Osaka University, Japan) (Kabeya et al., 2000), RFP-LC3 (provided by Walter Beron, Universidad Nacional de Cuyo, Argentina), p62-mCherry (previously described (Zheng et al., 2009)), NDP52-GFP (gift from J. Kendrick-Jones, University of Cambridge), IRGQ-Flag (from Origene), IRGQ^{ALIR2}, IRGQ^{ALIR1ALIR2} (cloned by Nancy Zhou).

**Reagents**

Rapamycin was used at concentration 500 nM for 4 h, puromycin was used at 5µg/ml for 4 h, and bafilomycin A was used at 500 nM this for 4 h.
Immunostaining

Immunostaining was conducted as previously described (Brumell et al., 2001). In brief, after infections, cells were fixed using 2.5% paraformaldehyde for 10 min at 37°C. For some experiments, extracellular S. Typhimurium was detected by immunostaining prior to permeabilization. Cells were then permeabilized and blocked using 0.2% saponin with 10% normal goat serum for 30 min at room temperature and stained for intracellular bacteria and various host proteins.

Mouse monoclonal antibody to p62 was from BD Biosciences (610832); rabbit polyclonal antibodies to NDP52 were from Abcam (ab68588); rabbit polyclonal antibodies to S. Typhimurium (Salmonella O anti- serum group B factors 1, 4, 5, and 12) were from Biodesign (240984); mouse monoclonal antibodies against GFP was from Invitrogen; rat monoclonal antibody against LAMP1 (clone ID4B) was from Developmental Studies Hybridoma Bank (University of Iowa); and mouse monoclonal antibody against poly-ubiquitinated proteins (FK2) was from Enzo (BML-PW0150-0025), rabbit polyclonal antibody to IRGQ was from Novus (NBP1-94049), rabbit monoclonal anti-PMP70 (Epitomics-an Abcam Company). All fluorescent secondary antibodies were AlexaFluor conjugates from Molecular Probes (Invitrogen).

Immunoblotting and Immunoprecipitation

Western blotting was performed as previously described in (Huang et al., 2009) using LC3 (Novus, NB600-1384), GAPDH (Millipore, MAB374), IRGQ (Novus, NBP1-94049), p62 (BD Biosciences, 610832), NDP52 (Abcam, ab68588), PMP70 (Epitomics-an Abcam Company),

The immunoprecipitation protocol was previously described (Pankiv et al., 2007). Mouse anti-GFP (Invitrogen, A11120) was used for IP.

RNA Isolation and Quantitative PCR

RNA was isolated using RNeasy kit (QIAGEN, 74104), and cDNA was synthesized using iScript Reverse Transcription Supermix for RT-qPCR (BioRad, 1708840). Ten nanograms per
reaction were used for quantitative RT-PCR using predesigned Taqman probes for target genes and Hprt1 as housekeeping reference (Life Technologies).

**LAMP1 phagosome maturation assay**

Zymosan particles (Sigma, Z-4250) were opsonized by overnight incubation in 6 mg/mL human IgG (Meridian Life Sciences, A50170H) at 4°C. For unopsonized particles, Zymosan A, *S. cerevisiae* BioParticles, Texas Red conjugate (Molecular Probes, Z2843) were used. On day 1, MEFs were seeded on glass coverslips in 24-well tissue culture plates at 2.5x10⁴ cells/well, and, on day 2, transfected with FcγRIIA-GFP construct using GeneJuice (Novagen, 70967-3) as per the manufacturers' instructions. On day 3, phagocytosis was synchronised by spinning opsonized zymosan (OpZ) at 1,500 rpm for 5 min onto cells. BMDMs were seeded on glass coverslips in 24-well tissue culture plates at 2.0x10⁵ cells/well, and on the following day, phagocytosis was synchronised by spinning OpZ at 1,500 rpm for 5 min. Cells were then fixed with 2.5% paraformaldehyde (EMS, 15710) at 30, 60, 90, or 120 min and stained for OpZ and LAMP1 (DSHB clone 1D4B).

**LysoBrite and DQ-BSA phagosome maturation assays**

SRBC (MP Biomedicals, 55876) were opsonized by 1 h incubation with rabbit anti-sRBC antibody (MP Biologicals, 55806) at room temperature. Cells were seeded in Ibidi microscopy chambers (80827). MEFs were seeded at 1.0x10⁴ cells/chamber and transfected with FcγRIIA-GFP using GeneJuice (Novagen, 70967-3). BMDMs were seeded at 6.0x10⁴ cells/chamber. For the LysoBrite assay, cells were incubated with LysoBrite (AAT Bioquest, 22659) for 30 min, as per the manufacturers' instructions. For the DQ-BSA assay, cells were incubated with 10 µg/ml of DQ-BSA (Life Technologies, D12051) for 1 h, followed by 1 h incubation in complete media. Phagocytosis was synchronised by spinning sRBC at 1,500 rpm for 5 min onto cells. The cells were imaged live at 30, 60, and 90 min time points after beginning of phagocytosis.
Clearance of ubiquitin-positive protein aggregates

HeLa cells were seeded in 24 well plates onto glass coverslips at a density of 25,000 cells/cover-slip on Day 1. The following day (Day 2) an appropriate siRNA treatment was conducted (control, IRGQ or ATG12 siRNA) for 48 h. On Day 4, cells were stressed with puromycin (5µg/ml) in their regular media for 4 h, washed three times with PBS++ and allowed to recover in regular media for another 8 h. Cells were fixed at 0, 4, and 8 h time points and stained antibody against polyubiquitinated proteins (FK2) and DAPI. At least 100 cells were then counted for the presence or absence of at least 4 large Ub+ aggregates at each time point. This protocol was taken from (Zheng et al., 2009).

Microscopy

In Chapter 3, samples were analyzed using a Zeiss Axiovert microscope (63x objective) and LSM 510 software. Confocal images were imported into Adobe Photoshop and assembled in Adobe Illustrator for labeling. Colocalization quantifications were performed using a Leica DMI'RE2 epifluorescence microscopy.

In Chapter 4 and 5, Images were acquired using a Wave-FX-X1 Spinning Disc Confocal, Leica DM16000B inverted research microscope with a Hamamatsu ImagEMx2 (EMCCD) camera using 63x objective.

Statistical Analysis

For quantification studies, at least 100 particles were enumerated for each condition in each experiment for most assays. For the LysoBrite or DQ-BSA assays, 50 particles were enumerated; and for LAMP1 phagosome maturation assay, at least 200 particles were enumerated. At least three independent experiments were performed for each graph, unless otherwise indicated. The mean ± SEM is shown in figures. P values were calculated using two-tailed two-sample equal variance Student’s t-test unless specified otherwise. A p-value of less than 0.05 was considered statistically significant and is denoted by *. p < 0.01 is denoted by ** and p < 0.005 is denoted by ***.
Chapter 3 – Characterizing ubiquitin-dependent antibacterial autophagy of S. Typhimurium

The data from this chapter is published in *Autophagy* (Cemma et al., 2011).

Summary

Autophagy is an innate immune defence against bacterial invasion. Recent studies show that two adaptor proteins, p62 and NDP52, are required for autophagy of the bacterial pathogen *S. Typhimurium*. However, it was not known why two different adaptors are required to target the same bacterial cargo to autophagy. Here I show that both adaptors were recruited to bacteria with similar kinetics, that they were recruited to bacteria independent of each other, and that depletion of either adaptor lead to impairment of antibacterial autophagy. Depletion of both adaptors did not synergistically impair autophagy, indicating they act in the same pathway. Remarkably, I observed that these adaptors did not colocalize, but rather formed non-overlapping microdomains surrounding bacteria. I conclude that p62 and NDP52 act cooperatively to drive efficient antibacterial autophagy by targeting the protein complexes they coordinate to distinct microdomains associated with bacteria.

Introduction

Autophagy is an important degradation pathway that targets long-lived proteins, damaged organelles, and bacteria (Huang and Klionsky, 2007; Klionsky and Emr, 2000). Autophagy plays a key role in innate immunity by restricting bacterial replication in host cells. Previous studies from our lab showed that a population (~30%) of *S. Typhimurium* is targeted by autophagy during infection (Birmingham et al., 2006). It appears that these bacteria are targeted by autophagy within SCVs, since bacteria that colocalize with the autophagy marker LC3 also colocalize with markers of the SCV (Zheng et al., 2009). Past studies also found that bacterial
replication is increased in autophagy-deficient cells (Birmingham et al., 2006). In these cells, more bacteria are observed in the cytosol than control cells, suggesting autophagy acts to prevent bacterial escape from SCVs and/or to promote fusion with lysosomes (Birmingham et al., 2006).

Our research group found that approximately 50% of LC3+ bacteria colocalize with ubiquitinated proteins (Ub+), suggesting that ubiquitin may serve as a signal to promote antibacterial autophagy (Birmingham et al., 2006). In fact, two recent studies support this model (Thurston et al., 2009; Zheng et al., 2009). Randow and colleagues showed that NDP52 is recruited to Ub+ bacteria (Thurston et al., 2009). NDP52 is an adaptor protein that binds both ubiquitin and LC3, and also coordinates a signaling complex including Tank-binding kinase (TBK1), Sintbad and Nap1 (Thurston et al., 2009). Our group showed that p62 (also known as SQSTM1) is recruited to Ub+ bacteria (Zheng et al., 2009). p62 can also bind both ubiquitin and LC3, and also coordinates a signal transduction complex (Moscat and Diaz-Meco, 2009). Both groups showed that depletion of these individual adaptors results in impairment, but not complete suppression, of antibacterial autophagy (Thurston et al., 2009; Zheng et al., 2009). However, it remains unclear why depletion of individual adaptors can cause autophagy impairment. Indeed, these parallel studies prompt many questions. Do these adaptors act in the same pathway? Is their recruitment interdependent? Do they associate with each other in the same complexes? Owing to the general importance of these adaptors to autophagy, I decided to address these questions.

**Results**

**NDP52 and p62 are recruited to S. Typhimurium with similar kinetics**

Given that previous studies have found two LC3-binding adaptor proteins, p62 and NDP52, localize to S. Typhimurium, I examined kinetics of their recruitment to S. Typhimurium (Thurston et al., 2009; Zheng et al., 2009). p62 and NDP52 were both recruited to bacteria, with maximal association seen at 60-90 min p.i., the time when bacteria are targeted by autophagy, and declining sharply after 2 h p.i. (Figure 12A). At 60 min p.i. ~37% bacteria were positive for p62 and ~32% bacteria were positive for NDP52 (Figure 12A). When examined at 60 min p.i.,
the vast majority of bacteria that were positive for one adaptor were also positive for the other; when selecting for p62\(^+\) bacteria, 94\% were positive for NDP52 (Figure 12B). When selecting for NDP52\(^+\) bacteria, 91\% were positive for p62 (Figure 12B). Therefore, I showed that p62 and NDP52 are recruited to the same bacteria, and with similar kinetics.

**NDP52 and p62 can associate in a complex, but also localize to distinct compartments in the cell**

Given that both adaptors directly bind ubiquitin and LC3, I expected p62 and NDP52 to associate with each other. Indeed, I was able to co-immunoprecipitate p62-mCherry and NDP52-GFP from transfected HeLa cells with anti-GFP antibody (Figure 13). As a control, I showed that anti-GFP antibody did not pull down p62-mCherry. Rapamycin treatment and *Salmonella*-infection had little effect on the association of p62 and NDP52 when compared to untreated cells. I was also able to co-immunoprecipitate endogenous p62 with NDP52 from non-transfected cells (data not shown). Thus, my data indicate that p62 and NDP52 can associate in protein complexes. However, when I examined the subcellular distribution of these adaptors by immunofluorescence (endogenous levels, detected with antibodies), I observed puncta with one adaptor that did not colocalize with the other. As shown in Figure 12C, I could readily detect NDP52\(^+\) puncta in which p62 was absent (see inset) and p62\(^+\) puncta in which NDP52 was absent. Therefore, p62 and NDP52 can target distinct compartments in the cell.
Figure 12. The ubiquitin-binding adaptors, p62 and NDP52, are recruited to *S. Typhimurium* with the same kinetics.

(A) HeLa cells were infected with *S. Typhimurium* for 1 h. Cells were fixed at the indicated time and stained with antibody against endogenous p62 or NDP52. The percentage of p62⁺ or NDP52⁺ bacteria was enumerated by fluorescence microscopy. At least 100 bacteria were counted for each time point. The experiment was conducted two times and error bars represent the range. (B) HeLa cells were infected for 1 h, fixed, and stained for p62 and NDP52. The percentage of p62 colocalizing with NDP52⁺ bacteria (left panel) and the percentage of NDP52 colocalizing with p62⁺ bacteria (right panel) were enumerated by fluorescence microscopy. At least 100 bacteria were counted for each condition. The average +/- SD for three independent experiments is shown. (C) Untreated HeLa cells were coimmunostained with p62 and NDP52 antibodies. Insets show higher magnification in the area indicated with an arrow.
Figure 13. Transfected p62 and NDP52 interact in untreated, rapamycin-treated and Salmonella-infected HeLa cells.

HeLa cells were transfected with p62-mCherry and NDP52-GFP or p62-mCherry and GFP. Then cells were infected with S. Typhimurium, (1 h), or treated with rapamycin (2 h), as indicated. HeLa lysates were immunoprecipitated (IP) with GFP antibody. Lysates and precipitates were then blotted (WB) for p62 and GFP. The lysate blot also contains the GFP alone band (27 kDa), which is not depicted in the figure.
**NDP52 and p62 are both required for autophagy of *Salmonella***

Previous studies have shown that knockdown of these adaptors results in impairment, but not complete suppression of antibacterial autophagy (Thurston et al., 2009; Zheng et al., 2009). To determine whether p62 and NDP52 function in the same pathway, we performed single and double knockdowns of adaptor proteins and measured the effect on autophagy by quantifying LC3\(^+\) intracellular bacteria. I observed that knockdown of either adaptor caused significant inhibition of LC3 recruitment to bacteria, confirming previous studies (Thurston et al., 2009; Zheng et al., 2009) (Figure 14A). However, double knockdown of both effectors (confirmed by Western blotting in (Figure 14D)) did not have an additive effect on autophagy impairment. Therefore, I concluded that p62 and NDP52 are not redundant and act in the same pathway to promote autophagy of *S. Typhimurium*.

**Adaptor proteins are recruited to the bacteria independently of one another**

To determine if adaptor recruitment is interdependent, I depleted expression of each adaptor singly, and examined its effect on the recruitment of the other adaptor to intracellular bacteria. Upon NDP52 depletion, p62 recruitment was not affected compared to control siRNA-treated cells (Figure 14B). Similarly, p62 depletion did not affect NDP52 recruitment to bacteria (Figure 14C). Since depletion of one adaptor did not affect the recruitment of the other, I conclude that p62 and NDP52 are recruited independently to bacteria.

**NDP52 and p62 localize to non-overlapping microdomains around bacteria**

Upon examination of *S. Typhimurium* positive for both adaptors, I was surprised to discover that they do not colocalize. Instead, I observed that p62 and NDP52 form independent non-overlapping microdomains around the bacteria (Figure 16). The microdomains were easily visible by confocal microscopy, especially using 3D rendering (Figure 16), and often detectable by epifluorescence microscopy using a 100x objective. Adaptor microdomains often, though not always, encompassed the entire bacterium. I conclude that p62 and NDP52 target distinct microdomains associated with *S. Typhimurium*, and therefore do not act in the same complex to promote autophagy of bacteria.
Figure 14. p62 and NDP52 are recruited independently to S. Typhimurium targeted by autophagy.

(A) HeLa cells were treated with control, p62, NDP52, or p62+NDP52 siRNA for 48 h and also transfected with GFP-LC3 for 24 h. Cells were then infected with S. Typhimurium and fixed at 1 h p.i.. Cells were then stained for external and internal bacteria. The percentage of LC3+ intracellular bacteria was enumerated by fluorescence microscopy. At least 100 bacteria were counted for each condition. The average +/- SD is shown for three independent experiments. Asterisk denotes p value < 0.001 calculated by two-tailed Student’s t-test. (B, C) HeLa cells were transfected with the indicated siRNA and then infected with S. Typhimurium for 1 h. The percentage of p62+ bacteria (B) and the percentage of NDP52+ bacteria (C) were enumerated by fluorescence microscopy. At least 100 bacteria were counted for each condition. The average +/- SD for four independent experiments is shown. (D) Protein lysates from siRNA-treated cells were harvested and analyzed by Western blotting using antibodies to p62 and NDP52. GAPDH was used as a loading control.
Figure 15. p62 and NDP52 are recruited to bacteria-associated microdomains on *S.* Typhimurium targeted by autophagy.

HeLa cells were infected with *S.* Typhimurium and fixed at 1 h p.i. Cells were then co-immunostained with p62 and NDP52 antibodies and stained with DAPI to label DNA. Insets show higher magnification in the boxed areas.
Figure 16. p62 and NDP52 are recruited to non-overlapping bacteria-associated microdomains to promote autophagy of *S. Typhimurium*.

HeLa cells were infected with *S. Typhimurium* and fixed at 1 h p.i.. Cells were then co-immunostained with p62 and NDP52 antibodies. All panels depict the same group of bacteria from different angles. Arrows indicate NDP52⁺ microdomains and arrowheads indicate p62⁺ microdomains.
Discussion

These data provide novel insight into the mechanism by which ubiquitin-binding adaptor proteins, p62 and NDP52, target bacteria to the autophagy pathway. I demonstrate that these adaptors are both recruited to the same bacteria at the same time. However, p62 and NDP52 are recruited independently of one another. In addition, I show that p62 and NDP52 are not redundant and function in the same pathway, indicating that each adaptor brings unique components necessary to drive ubiquitin-dependent antibacterial autophagy. To my surprise, I found that the adaptors localize to distinct bacteria-associated microdomains.

How are these microdomains established? Differential ubiquitination events and their specific recognition by the adaptors may play a role. p62 preferentially binds K63-linked poly-ubiquitin chains, though it is also able to bind K48-linked chains and mono-ubiquitin (Babu et al., 2005; Okatsu et al., 2010; Seibenhener et al., 2004; Tan et al., 2007; Wooten et al., 2005). NDP52 binds monoubiquitin in vitro, but there are no studies examining NDP52 binding affinity to different types of ubiquitin (Thurston et al., 2009). It is possible that NDP52 does not bind poly-ubiquitin chains, and is recruited specifically to domains rich in mono-ubiquitin. It is noteworthy that the identity of ubiquitinated proteins associated with S. Typhimurium are not known, nor are the E3 ligases that mediate these post-translational modifications during infection. Alternatively, p62 and NDP52 may be recruited to microdomains via other proteins that they associate with.

How do p62 and NDP52 contribute to autophagy? Through their binding to LC3, p62 and NDP52 may help recruit membrane to generate autophagosomes around bacteria. Our research group found that bacteria targeted by autophagy are present in multilamellar structures, indicating the requirement of extensive membrane recruitment (Zheng et al., 2009). By targeting different microdomains associated with bacteria, p62 and NDP52 may mediate recruitment of membrane from different sources. This might be analogous to micropexophagy in yeast, where a specific membrane called the micropexophagy apparatus (MIPA) contributes to autophagy, in addition to membrane derived from the vacuole (Sakai et al., 2006). Alternatively, p62 and NDP52 may regulate autophagy through recruitment of the signaling complexes they coordinate.
This study highlights an area of growing concern in autophagy that deals with the requirement of multiple adaptors for Ub⁺-selective autophagy. p62 often acts in conjunction with other adaptors, including ALFY (Clausen et al., 2010), BAG3 (Gamerdinger et al., 2009), and NBR1 (Kirkin et al., 2009a), though it has not been clear why both adaptors are required and why autophagy phenotypes are observed with depletion of either adaptor. In conclusion, these new data provided a new perspective on how the field is thinking of p62 and NDP52, LC3-binding adaptor proteins. Despite apparent similarity they are not redundant, and furthermore, localize to different parts of the autophagic cargo. More broadly, this study suggests that adaptor proteins may target microdomains on their cargo (protein aggregates, organelles, etc.) and provide non-redundant signals or membrane recruitment to promote autophagy. This hypothesis, while exciting, requires further study.
Chapter 4 – Elucidating a role of LC3B-binding protein, IRGQ, in the autophagy pathway

The data in this chapter is currently unpublished. Contributions to this chapter are as follows:

Nancy Zhou (in the laboratory of Dr. John Brumell) cloned BirA*-LC3B tagged construct, generated stable HEK293 T-REx BirA*-LC3B cell line, and prepared cell extracts for mass spectrometry. Nancy Zhou also cloned IRGQ_{ΔLIR1}, IRGQ_{ΔLIR2}, IRGQ_{ΔLIR1ΔLIR2} plasmids.

Dr. Étienne Coyaud and Estelle Laurent (in the laboratory of Dr. Brian Raught) processed and analyzed all the BioID and AP-MS experimental samples, including BioID and AP-MS of cell pellets for FLAG-BirA* controls and FLAG-BirA*-LC3B samples and SAINT analysis.

Taoyingnan Li (in the laboratory of Dr. John Brumell) performed IRGQ colocalization experiments with γ-tubulin and GFP-LC3B (Figure 18B, C), and assisted in performance of the aggrephagy clearance experiment (Fig 20C).

Miluska Vissa (in the laboratory of Dr. Peter Kim) performed pexophagy experiments (Figure 20E, F, D).

The contributions of my colleagues were integral to the design of my experiments and are included here to present a complete story of the IRGQ project for the benefit of the readers of this thesis. My contributions (Marija Cemma) included all other data in this chapter, specifically: co-immunoprecipitation studies (Figure 17), immunofluorescence studies (Figure 18A), functional studies of xenophagy (Figure 19A, B), aggrephagy (Figure 19C, D), and autophagy flux studies (Figure 20).
Summary
Autophagy is an essential catabolic pathway important for maintaining cellular homeostasis and for defence against intracellular pathogens. The pathway is carried out by the concerted action of over 35 autophagy proteins, including LC3B. LC3B is covalently conjugated to the autophagosome membranes; it is essential for elongation of the phagophore membrane and cargo selection. In this chapter, using data generated by a dual BioID and AP-MS approache to characterize the LC3B interactome, I identified a novel LC3B interacting protein, IRGQ, for which very little is known. IRGQ interacts with LC3B through its evolutionarily conserved LC3-interacting region (LIR). The colocalization of LC3B and IRGQ was observed when autophagy was induced with rapamycin or when autophagic flux was blocked with bafilomycin A. Furthermore, in the absence of IRGQ, HeLa cells had impaired capacity to target *S. Typhimurium*, protein aggregates, and peroxisomes to the autophagy pathway. These findings establish IRGQ as a functionally important player required for efficient autophagy.

Introduction
The ATG8 family of proteins plays a pivotal role in autophagy, and are responsible for phagophore elongation and sealing, as well as cargo selection (Birgisdottir et al., 2013; Weidberg et al., 2010). Out of six ATG8 family members (LC3A, LC3B, LC3C, GABARAP, GABARAPL1, GABARAPL2/GATE16), LC3B is the most commonly used autophagosome marker (Klionsky et al., 2016). Like other ATG8s, LC3B is an ubiquitin-like molecule that is covalently conjugated to phosphatidylethanolamine on autophagosomal membranes through an enzymatic cascade comparable to ubiquitin conjugation. This LC3 lipidation process requires a subset of ATG proteins, including ATG3, ATG5, ATG7, ATG12, and ATG16L1 (Shpilka et al., 2011). Given the important role that autophagy plays in health and disease, several attempts to examine the LC3B interacting proteins have been already published using yeast 2 hybrid assay and GST-binding (Popovic et al., 2012; Wang et al., 2011). An Affinity Purification followed by Mass Spectrometry (AP-MS) approach was also employed to examine the LC3B interactome (Alemu et al., 2012; Behrends et al., 2010). However, these approaches have limitations. For
instance, AP-MS is not designed to detect unstable interactions or interactions with proteins in poorly soluble compartments. In addition, AP-MS does not necessarily capture proteins in a natural compartment in the cell, which might lead to false positive hits (Morris et al., 2014). This is why, in collaboration with Dr. Brian Raught’s laboratory, I decided to re-examine the LC3B interactome using a combination of AP-MS with another powerful and novel approach - proximity dependent Biotin identification (BioID) (Roux, 2013; Roux et al., 2012). Since the two techniques are complimentary, I surmised that a combination of BioID and AP-MS would yield novel and biologically meaningful LC3B interactors with a functional role in autophagy.

BioID is a proximity biotinylation approach in which bait of interest is fused with E. coli promiscuous biotin ligase, BirA*. Upon addition of exogenous biotin to the media, BirA* biotinylates proteins in the vicinity of the bait (Roux, 2013; Roux et al., 2012). Biotinylated proteins can be selectively isolated by streptavidin pull-down and identified by mass spectrometry. In order for biotinylation to occur, cells are incubated with exogenous biotin, in our case for 16 h. Thus, biotinylated proteins represent a collection of the bait’s vicinal proteins over that period of time, and include stable and transient interactors, as well as proteins that are in the proximity of the bait. Since preservation of protein-protein interactions is not required for their identification, lysis is performed in a high amount of non ionic detergent (Triton X-100), thereby enhancing detection of low solubility proteins or weak interactors, that may remain undetected by traditional AP-MS methods (Lambert et al., 2015; Roux et al., 2012). The caveat of BioID is that biotinylated proteins (the ‘hits’) do not necessary represent physical interactors, but may also identify proximal proteins to the bait that might bear no functional relationship to the bait (e.g. they can be on the same cellular compartment without having a functional relationship). Since BirA* requires accessible lysines to conjugate biotin to, hits might be biased (or missed altogether) depending on the number of accessible lysine residues (Roux, 2013; Roux et al., 2012). While BioID is a relatively new technique and has some limitations, it has been successfully applied to baits located in nuclear lamina and envelope (Kim et al., 2014; Roux et al., 2012), centrosome (Comartin et al., 2013; Firat-Karalar et al., 2014), cilia (Gupta et al., 2015), cytoskeleton (Morriswood et al., 2013), chromatin-associated proteins (Lambert et al., 2015), components of the Hippo signaling pathway
an E3 ligase and a deubiquitinase (Coyaud et al., 2015; Yeh et al., 2015), and focal adhesion complexes (Dong et al., 2016).

As opposed to BioID, AP-MS is great at detecting stable interactions and might miss things that fall apart and are substoichiometric, or interactions with proteins in poorly soluble compartments (Morris et al., 2014). Thus, BioID and AP-MS are two complimentary approaches both useful to detect novel interacting and proximal proteins.

Results

Identification of putative interactors of LC3B by BioID and AP-MS under basal autophagy

In order to look for novel LC3B interacting proteins using two complimentary approaches, Nancy Zhu established a Flp-In T-REx HEK293 cell line that stably expressed FLAG-tagged BirA*-LC3B, as well as the control cell line that expressed FLAG-tagged BirA*. Protein expression of FLAG-tagged LC3B was confirmed by a Western blotting by Nancy Zhu and appropriate localization, under basal and autophagy-inducing conditions, confirmed via immunofluorescence microscopy by me. Pellets from two biological replicas were collected and sent to Dr. Étienne Coyaud and Estelle Laurent for analysis via (i) affinity purification of biotinylated proteins using streptavidin and mass spectrometry for BioID, and (ii) affinity purification using anti-FLAG beads and mass spectrometry for conventional AP-MS. Dr. Étienne Coyaud determined determine high confidence LC3B interactors using ProHits and SAINT (Significant Analysis of INTeractome) analyses.

We found 45 novel putative LC3B interactors identified with both BioID and AP-MS. I have selected one promising hit, IRGQ, for follow-up and confirm it’s putative role as an LC3B binder and characterize its role in the autophagy pathway for a number of reasons. First, IRGQ was a statistically significant hit with a high peptide count in both the BioID and AP-MS approaches. Second, IRGQ is a homolog of IRGM, a protein implicated in autophagy initiation (Figure 9). Third, IRGQ was previously identified to interact with LC3B homolog, GABARAPL2, in an AP-MS screen (Behrends et al., 2010). Fourth, IRGQ contains two LC3-Interacting Regions (LIRs), a canonical WxxL motif and a non-canonical LVV motif, both of which seem relatively conserved
throughout evolution. Fifth, IRGQ is widely expressed in human tissues according to the ProteinAtlas database (http://www.proteinatlas.org/ENSG00000167378-IRGQ/tissue). Sixth, IRGQ has never been studied before. All these reasons make IRGQ an extremely attractive candidate for an LC3B interacting protein that might have a functional role in the autophagy pathway.

**IRGQ binds LC3B via its LIRs**

First, I confirmed the interaction between LC3B and IRGQ with a co-immunoprecipitation experiment. To this end, I overexpressed IRGQ-FLAG and RFP-LC3B (or RFP alone as a negative control) in HEK293 cell line and pulled down IRGQ using anti-FLAG beads. IRGQ-FLAG could co-immunoprecipitate RFP-LC3B but not RFP alone, suggesting that those two proteins can associate either directly or indirectly with one another. To determine if LIRs are required for the interaction, I also examined IRGQ LIR mutants, IRGQ\(^{\Delta LIR2}\) and IRGQ\(^{\Delta LIR1\Delta LIR2}\). Cloning of IRGQ\(^{\Delta LIR2}\) and IRGQ\(^{\Delta LIR1\Delta LIR2}\) plasmids was performed by Nancy Zhou. I observed that in the absence of LIR1, LIR2, or both LIRs, IRGQ could no longer precipitate RFP-LC3B (Figure 17). This finding implied that both LIRs are required for association between IRGQ and LC3B.
Figure 17. Overexpressed RFP-LC3B co-immunoprecipitates with overexpressed FLAG-IRGQ in HEK293T cells.

(A). Schematic of IRGQ constructs used in the co-immunoprecipitation experiment. (B) HEK293T cells were transfected with IRGQ-FLAG (WT, ΔLIR2, ΔLIR1ΔLIR2) and RFP-LC3B or GFP alone. HEK293T lysates were immunoprecipitated (IP) with anti-FLAG M2 beads. Lysates (input) and precipitates (bound) were then blotted for FLAG and RFP. The experiment was repeated three times. A while space represents a spot where the blot was cut.

Nancy Zhou also cloned IRGQ^{ΔLIR2} and IRGQ^{ΔLIR1ΔLIR2} plasmids, while I have performed the co-immunoprecipitation experiment.
IRGQ localization under basal and autophagy-inducing conditions

Since little is currently known about IRGQ, I examined its localization in search for clues about its function. Using affinity-purified antibodies for immunofluorescence staining and with confocal microscopy analysis, I observed that IRGQ localized to a perinuclear compartment in HeLa cells under basal conditions (Figure 18A). This structure resembled a hollow ring or horseshoe and was observed in every cell (Figure 18A). Upon IRGQ siRNA treatment of HeLa cells, the IRGQ structure disappeared, indicating that the immunoreactive signal was indeed specific to IRGQ. This structure did not colocalize with Golgi, ERGIC, mitochondria, late endosomes, or lysosomes. Taoyingnan Li observed that the IRGQ structure was located around γ-tubulin, which marks centrosomes and serves as at the microtubule-organizing center (MTOC) in animal cells (de Forges et al., 2012). Interestingly, the LC3B interactome is comprised of a number of centrosomal proteins that have not been previously identified as LC3B interactors.

We next sought to determine if IRGQ and LC3B co-localize via immunofluorescence. We used Henle-407 that stably expressed GFP-LC3B (Henle-GFP-LC3B) to examine its colocalization with IRGQ. Under untreated conditions, no colocalization was observed between LC3B and IRGQ (Figure 18C). It is possible that a small amount of LC3B is localized to the IRGQ structure that is not detected by immunofluorescence over the background LC3B staining. In fact, Tang and colleagues were able to visualize LC3B at the centrosomal satellite sites upon inhibition of autophagy with bafilomycin A or chloroquine, but not in untreated cells (Tang et al., 2013). Their finding implies that very small amounts of LC3B might be present at the centrosomal satellite sites, which are in the vicinity of IRGQ. Alternatively, small amounts of cytosolic IRGQ might be localized to cytosolic LC3B.

I also examined localization of IRGQ under conditions that induce autophagosome accumulation. First, I examined IRGQ recruitment to LC3⁺ S. Typhimurium in HeLa cells at 1 h p.i..

Surprisingly, little colocalization was observed (~1% of LC3⁺ bacteria was positive for IRGQ). In contrast, Taoyingnan Li examined localization of LC3B and IRGQ in Henle-GFP-LC3B cells under starvation, rapamycin, or bafilomycin A treatment conditions. Starvation induces autophagy via (i) activation of AMPK kinase that phosphorylates/activates components of autophagy
initiation complex and (ii) inactivation of mTOR (mammalian target of rapamycin) complex 1 that functions as an autophagy initiation inhibitor (Russell et al., 2014). As expected, starvation resulted in LC3B⁺ puncta accumulation (Figure 18C). Yet, IRGQ did not localize to the LC3 puncta, but rather remained in the IRGQ structures. In addition, LC3 did not localize to the IRGQ structure (Figure 18C). Rapamycin induces autophagy via inhibition of the mTOR. As expected, rapamycin treatment resulted in GFP-LC3B accumulation. IRGQ seemed to redistribute from the perinuclear structure to the periphery of LC3B⁺ puncta (LC3B and IRGQ partly overlapped in LC3 puncta) (Figure 18C). Bafilomycin A is a V-ATPase inhibitor and impairs lysosome acidification. As a result autophagosomes are unable to fuse with the lysosomes and instead accumulate in the cytosol. As expected, bafilomycin A treatment led to accumulation of GFP-LC3B. As with rapamycin treatment, IRGQ localized to LC3B⁺ puncta and the two markers partially overlapped (Figure 18C). These findings suggest that a subpopulation of IRGQ can colocalize with LC3B under some autophagy inducing conditions.
Figure 18. IRGQ localization in HeLa and Henle cells in untreated and autophagy-inducing conditions.

(A) Control or IRGQ siRNA treated HeLa cells were stained with IRGQ antibody (green) and DAPI (blue). Scale bar = 11µm. (B) Untreated HeLa cells were stained with IRGQ antibody (green) and γ-tubulin (red). Scale bar = 12µm. (C) IRGQ and LC3B localization was examined in embryonic intestinal epithelial cell line, Henle-407, that stably overexpressed GFP-LC3B in untreated or cells treated with starvation medium, EBSS, rapamycin (autophagy inducer) or bafilomycin A, V-ATPase inhibitor, for 4 h.

I have performed the experiment in Figure 18A, while Taoyingnan Li has performed experiments in Figure 18B, C.
**IRGQ is required for autophagy**

Next, I examined whether IRGQ plays a functional role in autophagy of (i) *Salmonella*, (ii) protein aggregates, and (iii) peroxisomes. I used Atg12 siRNA as a positive control, since it is required for LC3B lipidation and for autophagy in general.

First, I investigated the role of IRGQ in autophagy of *S. Typhimurium*. To this end, I infected HeLa cells with bacteria for 1 h, a time point when the peak of autophagy has been previously documented to occur (Birmingham et al., 2006). The population of LC3⁺ bacteria had dropped dramatically from 28% in control to 8% in IRGQ siRNA-treated cells (Figure 19A, B). This result implied that IRGQ is important for targeting *S. Typhimurium* to the autophagy pathway.

Next, I explored if IRGQ plays a role in Ub⁺ protein aggregate clearance in HeLa cells. Puromycin is a structural analog of aminoacyl-tRNA that terminates chain elongation prematurely, resulting in the accumulation of truncated and misfolded polyubiquitinated proteins (Azzam and Algranati, 1973; Lelouard et al., 2004). The number of cells containing more than 4 Ub⁺ protein aggregates was assessed by immunofluorescence after 4 h puromycin treatment and after 8 h recovery in regular media in control, IRGQ, or Atg12 siRNA treated HeLa cells. Interestingly, in control siRNA treated cells, over 60% of cells were able to clear Ub⁺ protein aggregates, while in IRGQ and Atg12 siRNA treated cells this number dropped to 28% and 21%, respectively (Figure 19C, D). This finding indicates that IRGQ is required for efficient clearance of Ub⁺ protein aggregates.

Further, Dr. Peter Kim and Miluska Vissa investigated the role of IRGQ in autophagy of peroxisomes, or pexophagy. Miluska Vissa examined peroxisomal membrane protein PMP70, used as a marker of peroxisomes, in HeLa cells via immunofluorescence. Depletion of ATG12 or IRGQ resulted in statistically significant accumulation of peroxisomes relative to control cells under basal and autophagy inducing conditions, i.e. 24 h amino acid starvation (Figure 19E, F). Miluska Vissa further confirmed her findings by measuring PMP70 protein levels via Western blotting. Depletion of IRGQ resulted in accumulation of PMP70 protein levels (Figure 19D), consistent with immunofluorescence results, reinforcing the idea that IRGQ is required for proper peroxisome turnover.
In summary, IRGQ seems to be required for autophagy of peroxisomes, Ub\(^+\) protein aggregates, and *S. Typhimurium*. Our results from the pexophagy and aggregate clearance assays are consistent with the idea that autophagy is impaired upon depletion of IRGQ. IRGQ depletion also impairs LC3 recruitment to *S. Typhimurium*, suggesting that IRGQ may play a role either upstream or at the cargo sequestration stage.

**IRGQ is not required for LC3 conjugation or autophagic flux**

In order to get insight into how IRGQ mediates its function, I investigated the possibility that IRGQ might play a role in a key step of the autophagy pathway, LC3 lipidation. LC3B can be found in two forms, a cytosolic LC3-I form and the membrane-conjugated LC3-II form. Conjugation of LC3B to PE is an essential step in elongation of autophagosomes. Autophagic flux is measured by assessing both LC3-I and LC3-II forms via Western blotting. The lipidated LC3, LC3-II, is involved in autophagy and correlates with the number of autophagosomes; reduction in the LC3-II levels implies that LC3 lipidation is impaired. I have also examined LC3 lipidation under bafilomycin A treatment, i.e. when autophagy is blocked. This allows one to distinguish between two possible causes of LC3-II level increase, (i) autophagy induction or (ii) a block in autophagy, and is a standard assay in the field (Mizushima and Yoshimori, 2007). The positive control, Atg12 knockdown resulted in depletion of LC3-II band, as expected (Figure 20). The knockdown of IRGQ, however, did not impair formation of LC3-II under control or bafilomycin A treatment conditions in HeLa cells (Figure 20). This finding suggests that IRGQ does not affect the core autophagy machinery involved in formation or turnover of autophagosomes. Instead, IRGQ may regulate the activation of autophagy or enable targeting of specific cargoes to the autophagy pathway.
Figure 19. IRGQ is required for functional autophagy of *S. Typhimurium*, protein aggregates, and peroxisomes.

(A, B) HeLa cells were treated with control or IRGQ siRNA, and invaded with RFP-expressing *S. Typhimurium* for 1 h, then fixed and stained for LC3 (green). The percentage of LC3⁺ bacteria was enumerated by fluorescence microscopy (B). At least 100 bacteria were counted. (C, D) HeLa cells were treated with control, IRGQ or Atg12 siRNA. Cells were then incubated with puromycin (5µg/ml) for 4 h to induce protein aggregates, then puromycin was washed off and cells were incubated for additional 8 h. The cells were fixed at 0, 4, and 12 h time points, and stained with FK2 (anti-ubiquitin) antibody and DAPI. The number of cells with over 4 FK2⁺ puncta were counted at 4 and 12 h, and the percent of cells that cleared protein aggregates was calculated. (E, F, D) HeLa cells were treated with control, IRGQ or Atg12 siRNA, then incubated with regular media or HBSS (amino acid starvation) medium for 24 h. Cells were stained for PMP70, a peroxisomal membrane protein, and DAPI (E). Lysates were then blotted for PMP70, IRGQ and GADPH. A representative Western blot is shown in (D).

I have performed the experiments depicted in Figure 19A, B, C, D. Miluska Vissa from Dr. Peter Kim’s laboratory performed the experiments in Figure 19E, F, D.
Figure 20. IRGQ depletion does not impair autophagy flux under untreated and bafilomycin A treatment conditions.

(A) HeLa cells were treated with control, IRGQ, and Atg12 siRNA, then incubated with regular growth medium or growth medium + bafilomycin A for 4 h. The lysates were then stained for IRGQ, actin or LC3.
Discussion

We have used a dual approach combining BioID and conventional AP-MS to re-examine LC3B interacting proteins under basal autophagy to identify novel LC3B interactor, IRGQ. This dual approach has been successfully used to provide a complimentary set of putative interactors in the Hippo pathway and identify chromatin-associated proteins (Couzens et al., 2013; Lambert et al., 2015).

Validation of BioID/AP-MS approach to find novel autophagy interactors

Both AP-MS and BioID detected previously known LC3B interactors, including a number of autophagy proteins required for LC3B lipidation, microtubule-binding proteins (Mann and Hammarback, 1994), nuclear proteins (Karim et al., 2007; Kraft et al., 2016; Shpilka et al., 2011; Wild et al., 2014), and regulators of ciliogenesis (Kim et al., 2015; Pampliega et al., 2013; Tang et al., 2013). Both BioID and AP-MS identified hits from the above-mentioned categories, thus confirming the validity of the dual approach in our system.

I decided to follow up on a promising hit, IRGQ, to validate this dual BioID/AP-MS approach as a useful tool for identifying novel autophagy-related proteins. It is noteworthy that previous AP-MS studies did not identify IRGQ as an LC3B interactor. This is possibly due to using a different Mass Spectrometer in our analysis. IRGQ appeared to be a strong hit in both BioID and AP-MS LC3B “interactomes” based on statistical analysis, SAINT score, and high peptide number. IRGs have been implicated in host defence and autophagy, making IRGQ a prime suspect in the LC3B interactome. Additionally, IRGQ has two conserved LIRs suggesting that it might bind to LC3B directly. Co-immunoprecipitation experiment confirmed that IRGQ and LC3B can be present in the same protein complexes when overexpressed (Figure 17). I have shown that this association is dependent on the presence of LIR(s) in the IRGQ sequence (Figure 17). While co-immunoprecipitation does not indicate direct interaction, the fact that IRGQ’s LIRs are required for the interaction suggests the possibility that IRGQ and LC3B might interact directly. This remains to be experimentally confirmed with in vitro binding assays. Alternatively, IRGQ’s LIRs
might be required for IRGQ to interact with another protein, like GABARAPL2, that associates with LC3B.

**IRGQ’s interactome**

Although we unraveled that IRGQ, an LC3B interactor, has a function in the autophagy pathway, it does not necessarily mean that IRGQ exerts its function through LC3B. Proteins containing LIR regions are able to interact with various ATG8s (Birgisdottir et al., 2013), and GABARAPL2 was already identified as an IRGQ-interactor in an AP-MS screen (Behrends et al., 2010). Hence, it is likely that IRGQ’s LIR allow for interaction with other ATG8s. Co-immunoprecipitation or *in vitro* binding assays between IRGQ (and IRGQ\(^{ΔLIR1ΔLIR2}\)) and all ATG8s will answer this question. I believe it would be useful to know exactly which ATG8s are interacting with IRGQ to develop a comprehensive model as to how IRGQ might be exerting its function.

IRGM, the only studied homologue of IRGQ in humans, has been implicated in autophagy initiation (Figure 9). The IRGQ protein sequence (623 aa) is roughly three times the length of the IRGM sequence (181 aa) and, unlike IRGM, contains two LIRs. Yet, there are conserved regions among the two. Whether these regions are functionally relevant remains to be determined, as previous studies have not mapped out IRGM regions important for their role in autophagy. Both IRGM and IRGQ appear to be important in antibacterial autophagy against *M. tuberculosis* and *S. Typhimurium*, respectively (Singh et al., 2010)(Figure 19). Chauhan showed that IRGM assembles with activated ULK1 and BECN1 complex (comprised of BECN1, AMBRA1, ATG14L, or UVRAG) and promotes their co-assembly (Chauhan et al., 2015). IRGM can also bind NOD2, a cytosolic PAMP (Chauhan et al., 2015). Hence, it would be particularly important to examine if IRGQ interacts with the proteins important for IRGM’s function in the autophagy pathway. If so, this would imply that IRGQ might function in a similar fashion as IRGM, and that at least some of the conserved amino acid regions are important to carry out that function. Given the lack of data available on IRGQ, learning its interactome via dual BioID/AP-MS approach would yield insights as to its putative mechanism of action. Using an IRGQ\(^{ΔLIR1ΔLIR2}\) in addition to the wild-type IRGQ will allow us to distinguish between LC3B-dependent and LC3B-independent interactors.
**IRGQ and LC3B can localize under certain conditions**

IRGQ localized to a yet uncharacterized perinuclear structure surrounding the centrosome in HeLa cells (Figure 18A, B). That is consistent with the fact that the LC3B interactome contained a subset of centrosomal proteins and suggests that some LC3B localized to the area surrounding the centrosome. Tang et al. observed LC3B at the satellite region of the centrosome under bafilomycin A or chloroquine treatment, conditions that prevented degradation of the autophagosomes, but not under basal conditions (Tang et al., 2013). This observation is consistent with the fact that we were unable to observe LC3B localization with IRGQ in untreated cells (Figure 18C). Hence, it is likely that a fraction of LC3B is present at the IRGQ structure that is functionally important and detected with biotinylation, but remains undetected by confocal microscopy.

Still, we hypothesized that LC3B and IRGQ could colocalize when we induce LC3B accumulation by either inducing autophagy with *S. Typhimurium* infection, starvation, rapamycin, or blocking autophagy with bafilomycin A treatment. *Salmonella* invasion and starvation resulted in LC3B accumulation, yet IRGQ did not localize to the autophagosomes (Figure 18C). On the other hand, under rapamycin and bafilomycin A treatments, IRGQ relocalized to the LC3B+ autophagosomes (Figure 18C). This suggests that LC3 accumulation alone is not enough for IRGQ recruitment. Starvation and rapamycin treatment are known to induce autophagy through deactivation of mTORC1; starvation acts through activation of nutrient-sensing kinase, AMPK, while rapamycin deactivates mTOR directly (Curtis et al., 2005). Yet, the two pathways have not been compared head-to-head in a mammalian system. In *Aspergillus nidulans*, autophagy induced by rapamycin and starvation induced distinct proteomic profiles (Kim et al., 2011d), suggesting that there are differences between the two pathways. Further investigation is warranted into why rapamycin induced IRGQ delocalization, while starvation did not. Under conditions where IRGQ colocalized with LC3, IRGQ was observed on a periphery of LC3B puncta with significant, albeit not complete, overlap. Better resolution microscopy, such as super resolution microscopy is needed to confirm this pattern of colocalization. It is compelling to speculate that IRGQ is in a membrane-bound structure surrounding the centrosome and can relocalize to somehow promote autophagy.
formation. Electron microscopy is needed to further characterize the IRGQ structure and to determine if IRGQ is present on membrane compartments.

IRGQ localization around the γ-tubulin is a noteworthy feature possibly offering clues as to how it functions. LC3B homologue, GABARAP colocalizes with γ-tubulin (Joachim et al., 2015). Under nutrient replete conditions, GABARAP resides on the Golgi via binding to GM130 and hence is unable to participate in autophagy initiation. Under nutrient deplete conditions, another Golgi protein, WAC, suppresses GM130 binding to GABARAP thereby allowing GABARAP to relocate to the MTOC. Once on the MTOC, GABARAP can activate ULK complex and participate in forming autophagosomes (Joachim et al., 2015). Another autophagy protein UVRAG localizes to the centrosome via its association with CEP63 (Zhao et al., 2012). UVRAG induces autophagosome formation through association with BECN1 and PI3K complex activation (Liang et al., 2006; Matsunaga et al., 2009). Independently of BECN1, UVRAG stimulates autophagosome maturation through its interaction with class C vacuolar protein sorting complex (Liang et al., 2008). Future studies should examine if IRGQ is required for autophagy initiation, like GABARAP and WAC, or phagosome maturation, like UVRAG.

**IRGQ’s function in the autophagy pathway**

Finally, we explored the role of IRGQ in three types of selective autophagy: xenophagy, aggrephagy, and pexophagy. Depletion of IRGQ resulted in a dramatic reduction of LC3⁺ S. Typhimurium population (Figure 19A, B), suggesting the IRGQ is perhaps involved in a step upstream of Salmonella targeting to the autophagosome. Interestingly, IRGQ was not detected around LC3⁺ S. Typhimurium implying that either (i) IRGQ does not need to localize to Salmonella for it to exert its function or (ii) only a small fraction of IRGQ is required for LC3B recruitment and it is undetectable by immunofluorescence. We next assessed if IRGQ was required for clearing protein aggregates and turning over peroxisomes. Depletion of IRGQ resulted in a significant defect of Ub⁺ protein aggregate clearance (Figure 19C, D). This impairment was comparable to the one observed in ATG12-depleted cells, consistent with the hypothesis that IRGQ plays a functionally important role in autophagy. Miluska Vissa observed accumulation of peroxisomal protein PMP70 in the case of IRGQ or ATG12 depletion, which
suggested that both autophagy impairment and IRGQ depletion result in accumulation of peroxisomes (Figure 19E, F, G). This observation is consistent with the idea that IRGQ is somehow implicated in effective pexophagy. An alternative explanation is that IRGQ may impair peroxisome biogenesis. Yet, given IRGQ’s role in xenophagy and aggrephagy, the most likely explanation of PMP70 accumulation is that IRGQ is involved in clearing old peroxisomes via the autophagy pathway. We have also examined the possibility that IRGQ might impair LC3 lipidation, and found that not to be the case (Figure 20).

**Examining IRGQ’s function *in vivo***

Finally, it is important to examine the role of IRGQ *in vivo*. Creating an IRGQ knockout mouse would enable investigators to see if IRGQ is required for host defense. All IRGQ experiments in this thesis were performed in human cells. IRGQ is also present in mice, and murine IRGQ has 78% sequence identity to its human homologue. Importantly, the canonical LIR (WxxL) is conserved in mice, while the non-canonical LIR (LVV) has Valine in the third position substituted to Leucine, another hydrophobic branched amino acid that likely also functions to bind LC3B. It is conceivable that murine LIRs are functional, though this has to be experimentally confirmed. Previous studies found that murine IRGs were critical in defence against *Toxoplasma gondii* (Butcher et al., 2005; Collazo et al., 2001; Liesenfeld et al., 2011; Taylor et al., 2000), *Leishmania major* (Liesenfeld et al., 2011), *Listeria monocytogenes* (Collazo et al., 2001; Liesenfeld et al., 2011), and *S. Typhimurium* (Henry et al., 2009). Hence, susceptibility of IRGQ knockout mice to these pathogens should be examined. If IRGQ knockout mice were to be susceptible to some pathogens, some of the critical experiments dissecting IRGQ’s function in human cells will need to be reproduced in mice. These experiments should include co-immunoprecipitation of IRGQ and ATG8 homologues, assessing if this interaction is dependent on the LIRs, and examining the role of murine IRGQ in pexophagy, aggrephagy, and xenophagy.

In addition to examining the role of IRGQ in an animal model, a search for human patients with IRGQ mutations should be undertaken. Polymorphisms in IRGM (another IRG family member)
and ATG16L1 are associated with Crohn’s disease (Deretic and Levine, 2009; Parkes et al., 2007; Wellcome Trust Case Control, 2007). Our data so far implicates IRGQ in protein aggregate clearance. Hence, the patient database search should include patients with Crohn’s disease and neurodegenerative diseases, since autophagy deficiency is linked to their pathogenesis. If coding variants/mutants of IRGQ are identified in patients with these diseases, then the BioID/AP-MS “interactome” of the variant should be compared to wild-type IRGQ to determine if any interactions have been affected in either a positive or negative way by the disease-causing mutation.

While the function of IRGQ remains elusive, we show that IRGQ was able to interact and colocalize with LC3B and, furthermore, was implicated in autophagy of three autophagic cargoes. Our findings clearly demonstrate that a combinatory approach with BioID and FLAG-IP can serve as a powerful tool to unravel functional interactors of autophagy protein, as we exemplified here with LC3B.
Chapter 5 Examining the role of autophagy proteins in phagosome maturation

The data from this chapter is published in *Autophagy* (Cemma et al., 2016).

**Summary**

Phagocytosis plays a central role in immunity and tissue homeostasis. After internalization of cargo into single-membrane phagosomes, these compartments undergo a maturation sequences that terminates in lysosome fusion and cargo degradation. Components of the autophagy pathway have recently been linked to phagosome maturation in a process called LC3-associated phagocytosis (LAP). In this process, autophagy machinery is thought to conjugate LC3 directly onto the phagosomal membrane to promote lysosome fusion. However, a recent study has suggested that ATG proteins may in fact impair phagosome maturation to promote antigen presentation. Here, I examined the impact of ATG proteins on phagosome maturation in murine cells using FCGR2A/FcγR-dependent phagocytosis as a model. I show that phagosome maturation is not affected in *Atg*5-deficient mouse embryonic fibroblasts, or in *Atg*5- or *Atg*7-deficient bone marrow-derived macrophages using standard assays of phagosome maturation. I propose that ATG proteins may be required for phagosome maturation under some conditions, but are not universally required for this process.
Introduction

Macroautophagy (hereafter referred to as autophagy) is a catabolic pathway that targets long-lived proteins, damaged organelles, and pathogens for lysosomal degradation in eukaryotic cells and requires more than 35 autophagy-related (ATG) proteins (Levine et al., 2011). A hallmark of canonical autophagy is that the cargo is sequestered into a double-membrane compartment (the phagophore, which matures into an autophagosome), which is decorated by MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3). LC3 is a ubiquitin-like molecule that is covalently conjugated to phosphatidylethanolamine on phagophore membranes through a series of reactions similar to ubiquitin conjugation and requires a subset of ATG proteins, including ATG5 and ATG7 (Shpilka et al., 2011).

A number of ATG proteins have been implicated in having alternative (noncanonical) functions (Huang and Brumell, 2014). A seminal study by Sanjuan et al. revealed that LC3 can be conjugated to the single-membrane phagosome upon stimulation of TLR2 (toll-like receptor 2) and TLR4 in a process termed LC3-associated phagocytosis (LAP) (Sanjuan et al., 2007). These receptors initiate reactive oxygen species (ROS) production by the CYBB/NOX2 NADPH oxidase, an event that is required for LAP (Huang et al., 2009; Martinez et al., 2015). In fact, intraphagosomal ROS in the absence of other signals is sufficient to recruit LC3 to phagosomes (Martinez et al., 2015). Other receptors implicated in LAP, such as FCGR2A/FcγR2A and CLEC7A/dectin-1, also result in CYBB/NOX2-dependent ROS production (Huang et al., 2009; Ma et al., 2012). LAP is triggered in response to particles that stimulate TLRs, FCGR2A/FcγR2A, CLEC7A/dectin-1, and other cargo such as apoptotic and entotic cells (Huang et al., 2009; Ma et al., 2012; Ma et al., 2014; Martinez et al., 2011; Martinez et al., 2015; Romao et al., 2013; Sanjuan et al., 2007). In addition to ROS, LAP requires a subset of autophagy components, such as ATG5, ATG7, ATG12, ATG16L1, BECN1/Beclin1, and PIK3C3/VPS34, whereas others, such as ULK1 (unc51-like kinase 1), ATG13, and RB1CC1/FIP200 are dispensable (Martinez et al., 2015; Sanjuan et al., 2007). LC3 conjugation to a phagosome is observed in hematopoietic cell types, including macrophages, dendritic cells, and neutrophils (Abnave et al., 2014; Huang et al., 2009; Ma et al., 2012; Ma et al., 2014; Martinez et al., 2011; Martinez et al., 2015; Romao et al., 2013; Sanjuan et al., 2007; Sanjuan et
al., 2009). It has also been observed in RPE cells (Frost et al., 2014; Kim et al., 2013), which are macrophage-like and express some hematopoietic markers (Limb et al., 1997). The role of LAP in nonhematopoietic cell types is not yet clear.

One of the major functions ascribed to LAP is to promote phagosome maturation. This conclusion is based on the observation that autophagy-deficient murine macrophages (RAW264.7 and CSF2/GM-CSF-differentiated BMDMs) fail to degrade live yeast (Sanjuan et al., 2007) and apoptotic cells (Martinez et al., 2011), and phagosomes containing beads coated with the synthetic triacylated lipopeptide PAM3CSK4 or zymosan exhibit delayed phagosome fusion with the lysosome (Sanjuan et al., 2007). However, a study in human macrophages observed quite the opposite—LC3 recruitment to zymosan-containing phagosomes was associated with delayed phagosomal fusion with the lysosome (Romao et al., 2013). Therefore, the impact of LC3 recruitment to phagosomes remains unclear. Hence, I explored how the phagosome maturation is affected in the absence of ATG proteins. We used autophagy-deficient mouse embryonic fibroblasts (MEFs), as well as CSF1/M-CSF- and CSF2-differentiated BMDMs to assess phagosome maturation of IgG-coated zymosan and sheep red blood cells (SRBC) using 3 distinct assays. We did not find a requisite role for Atg5 or Atg7 in phagosome maturation, challenging the notion that autophagy proteins are universally required for phagosome maturation.

Results

Phagosomes in Atg5-deficient MEFs mature normally upon FCGR2A stimulation

To examine if autophagy machinery is required for phagosome maturation in fibroblasts, I performed 2 phagosome maturation assays using wild-type and Atg5-deficient MEFs. It was previously established that heterologous transfection of fibroblastic cells with the FCGR2A receptor confers phagocytic capacity and results in phagosome maturation similar to that observed in professional phagocytes (Downey et al., 1999; Indik et al., 1995). As expected, FCGR2A receptor-transfected wild-type and Atg5-deficient fibroblasts were able to internalize
antibody-opsonized particles and acquire phagosome maturation markers. LAMP1 is localized to late-endosomes and lysosomes and is commonly used as a phagosome maturation marker. FCG2A-transfected MEFs were challenged with IgG-opsonized zymosan particles (OpZ) for 30, 60, 90, and 120 min, then stained for LAMP1 and OpZ. In both wild-type and autophagy-deficient MEFs, OpZ particles acquired LAMP1 with similar kinetics; nearly all became LAMP1-positive at 120 min after the challenge (Figure 21A, B). No significant difference in LAMP1 acquisition was observed between wild-type and Atg5-deficient fibroblasts.

We next explored if phagosome acidification might be impaired in Atg5-deficient fibroblasts. To visualize acidic compartments such as lysosomes and mature phagosomes, I used LysoBrite, a fluorescent indicator that permeates cell membranes and accumulates in acidic compartments. FCG2A-transfected MEFs were incubated with LysoBrite for 30 min and then challenged with antibody-coated SRBC. Anti-SRBC antibody was used to opsonize SRBC in order to facilitate its phagocytosis via FCG2A. Cells were imaged live at 30, 60 and 90 min following phagocytosis. We observed no significant difference in the percentage of LysoBrite-positive phagosomes between wild-type and Atg5-deficient fibroblasts (Figure 21C, D). Hence, I conclude that ATG5 does not play a role in FCG2A-mediated phagosome maturation in murine fibroblasts.

**Phagosomes in autophagy-deficient BMDM mature normally**

Next, I sought to determine the role of autophagy proteins in primary murine bone marrow-derived macrophages (BMDMs). To ensure that my findings are of relevance to most research groups, I have utilized 2 commonly used methods to differentiate macrophages, using either CSF1- or CSF2-containing media. Since conventional knockout of Atg5 (Kuma et al., 2004) or Atg7 (Komatsu et al., 2005) causes neonatal lethality, I used mice where Atg5 or Atg7 was selectively deleted from monocytes/macrophages and granulocytes. This was achieved by crossing Atg5\textsuperscript{flox/flox} or Atg7\textsuperscript{flox/flox} (referred to here as WT) mice with mice expressing the Cre recombinase from the endogenous lysozyme 2/M locus (Lyz2cre\textsuperscript{+}) (DeSelm et al., 2011; Zhao et al., 2008). In all cases I confirmed gene knockout using RT-PCR (Figure 26A-D). As expected, autophagy-deficient macrophages
were unable to process the cytosolic form of LC3 (LC3-I) into the lipidated (and membrane-associated) form of LC3 (LC3-II) as demonstrated by the disappearance of the LC3-II band via Western blotting (Figure 26E, F) (Mizushima and Yoshimori, 2007).

To investigate if autophagy proteins are required for acquisition of LAMP1, I challenged Atg5- or Atg7-deficient CSF1-differentiated macrophages and respective control macrophages with OpZ and quantified the percentage of LAMP1-positive phagosomes at 30, 60, 90, and 120 min post challenge. LAMP1 acquisition by phagosomes followed the same kinetics in wild-type and autophagy-deficient CSF1-differentiated macrophages and significant differences were not observed (Figure 22A-C). This suggests that ATG proteins are not required for and do not play a significant role in LAMP1 acquisition during phagocytosis of OpZ by CSF1-cultured BMDMs. The same results were observed in CSF2-differentiated macrophages (Figure 23A, B). We also assessed phagosome maturation using unopsonized zymosan particles (not coated with IgG as above) in wild-type and Atg5-deficient CSF1- and CSF2-differentiated BMDMs. Phagosomes containing zymosan particles matured at comparable rates in wild-type and autophagy-deficient macrophages (Figure 24A, B).

Next, I assessed phagosome acidification in control and autophagy-deficient macrophages. To this end, I quantified the percentage of LysoBrite+ SRBC-containing phagosomes. As with LAMP1 acquisition, no significant difference was observed between the controls and Atg5- or Atg7-deficient CSF1-cultured macrophages (Figure 22D-F). This indicates that ATG proteins do not play a significant role in acidification of SRBC-containing phagosomes in CSF1-cultured BMDMs. We have further confirmed these findings in CSF2-differentiated macrophages (Figure 23C, D).

Finally, I explored if autophagy proteins might play a role in conferring to the phagosome its degradative properties in CSF1-cultured macrophages by using DQ-BSA dye. DQ-BSA is a self-quenched BODIPY dye conjugate of bovine serum albumin (BSA). The proteolytic digestion of BSA in the lysosome results in dequenching of fluorescence, enabling the visualization of proteolytic compartments. CSF1-differentiated BMDMs were
pulsed with DQ-BSA for 1 h, chased for 1 h in regular medium and then challenged with antibody-coated SRBC for 30, 60, or 90 min. Phagosomes in Atg5- and Atg7-deficient BMDMs acquired the capacity to cleave DQ-BSA with similar kinetics as their respective controls (Figure 22G-I). We have further confirmed theses findings in the CSF2-differentiated macrophages and again saw no impact of ATG proteins on phagosome maturation (Figure 23E, F). In summary, using three distinct phagosome maturation assays, I was unable to detect a significant difference in phagosome maturation of antibody-opsonized particles between control and autophagy-deficient CSF1- and CSF2-differentiated BMDMs.
**Figure 21. Phagosome maturation of IgG-coated particles is unaffected in atg5⁻/⁻ MEFs.**

WT and atg5⁻/⁻ MEFs were transfected FCGR2A-GFP (green) to render them phagocytic. (A, B) MEFs were challenged with OpZ and fixed at 30, 60, 90, or 120 min post challenge, then stained for LAMP1 (red) and OpZ (blue) and imaged (A). A representative image is shown at 60 min post challenge with a LAMP⁺ particle marked by an arrow. (B) The percentage of LAMP1⁺ OpZ was enumerated in WT and atg5⁻/⁻ MEFs. At least 200 OpZ were counted per condition. (C, D) MEFs were incubated with LysoBrite (red) for 30 min and then challenged with SRBC (blue) for 30, 60, or 90 min and imaged live (C). A representative image is shown at 60 min post challenge with a LysoBrite⁺ particle marked by an arrow. (D) The percentage of LysoBrite⁺ SRBC was enumerated in WT and atg5⁻/⁻ MEFs. At least 50 SRBC were counted per condition. Scale bar: 8 μm.
**Figure 22. Phagosome maturation of IgG-coated particles is unaffected in autophagy-deficient CSF1 differentiated BMDMs.**

Phagosome maturation of IgG-coated particles is unaffected in autophagy-deficient CSF1-differentiated BMDMs. (A, B, C) CSF1-derived BMDMs were challenged with OpZ and fixed at 30, 60, 90, or 120 min, then stained for LAMP1 (green) and OpZ (blue) and imaged (A). A representative image is shown at 60 min post challenge with a LAMP\(^+\) particle marked by an arrow. The percentage of LAMP1\(^+\) OpZ was enumerated in WT and *atg5\(^{-/-}\) (B) and WT and *atg7\(^{-/-}\) (C) BMDMs. At least 300 OpZ were counted per condition. (D, E, F) CSF1-derived BMDMs were incubated with LysoBrite (red) for 30 min and then challenged with SRBC (blue) for 30, 60, or 90 min and imaged live (D). A representative image is shown at 60 min post challenge with a LysoBrite\(^+\) particle marked by an arrow. The percentage of LysoBrite\(^+\) SRBC was enumerated in WT and *atg5\(^{-/-}\) (E) and WT and *atg7\(^{-/-}\) (F) BMDMs. At least 50 SRBC were counted per condition. (G, H, I) CSF1-derived BMDMs were pulsed with DQ-BSA (red) for 1 h, chased for 1 h in regular medium, then challenged with SRBC (blue) for 30, 60, or 90 min and imaged live (G). A representative image is shown at 60 min post challenge with a DQ-BSA\(^+\) particle marked by an arrow. The percentage of DQ-BSA\(^+\) SRBC was enumerated in WT and *atg5\(^{-/-}\) (H) and WT and *atg7\(^{-/-}\) (I) BMDMs. At least 50 SRBC were counted per condition. Scale bar: 8 \(\mu\)m.
Figure 23. Phagosome maturation of IgG-coated particles is unaffected in autophagy-deficient CSF2 differentiated BMDMs.

(A, B) CSF2-derived BMDMs were challenged with OpZ and fixed at 30, 60, 90, or 120 min, then stained for LAMP1 and OpZ. The percentage of LAMP1⁺ OpZ was enumerated in WT and \textit{atg}^{5/-} (B) and WT and \textit{atg}^{7/-} (C) BMDMs. At least 300 OpZ were counted per condition. (C, D) CSF2-derived BMDMs were incubated with LysoBrite for 30 min and then challenged with SRBC for 30, 60, or 90 min. The percentage of LysoBrite⁺ SRBC was enumerated in WT and \textit{atg}^{5/-} (C) and WT and \textit{atg}^{7/-} (D) BMDMs. At least 50 SRBC were counted per condition. (E, F) CSF2-derived BMDM were pulsed with DQ-BSA for 1 h, chased for 1 h in regular medium, then challenged with SRBC for 30, 60, or 90 min and imaged live. The percentage of DQ-BSA⁺ SRBC was enumerated in WT and \textit{atg}^{5/-} (E) and WT and \textit{atg}^{7/-} (F) BMDMs. At least 50 SRBC were counted per condition.
Figure 24. Phagosome maturation of uncoated zymosan is unaffected in Atg5-deficient BMDMs.

(A, B) CSF1- (A) and CSF2- (B) derived BMDMs were challenged with uncoated zymosan and fixed at 30, 60, 90, or 120 min, then stained for LAMP1. The percentage of LAMP1⁺ zymosan was enumerated in WT and atg5⁻⁻ BMDMs. At least 300 zymosan particles were counted per condition. The experiment was performed twice (B).
Figure 25. Phagosome maturation of uncoated zymosan particles is enhanced in *Cybb*-deficient CSF1 differentiated BMDMs.

CSF1-derived BMDMs were challenged with IgG-coated zymosan and fixed at 30 or 60 min, then stained for LAMP1 and zymosan. The percentage of LAMP1+ zymosan was enumerated in WT and *cybb*−/− BMDMs. At least 100 OpZ were counted per condition. A p-value of less than 0.05 was considered statistically significant and is denoted by an asterisk (*).
Figure 26. Relative expression of Atg5 and Atg7 in BMDMs.

(A, B) Atg5 mRNA levels were assessed by real time PCR in CSF1- (A) and CSF2- (B) derived BMDMs using Hprt as a housekeeping control gene to allow for comparison. (C, D) Atg7 mRNA levels were assessed by real time PCR in CSF1- (C) and CSF2- (D) derived BMDMs using Hprt as a housekeeping control gene. (E, F) Expression levels of LC3-I and LC3-II in untreated CSF2-derived atg5−/− (E) and atg7−/− (F) BMDMs and their respective controls. Positions of LC3-I and LC3-II are indicated.
Discussion

Our findings challenge the notion that LC3 recruitment is required for phagosome maturation. The difference between my observations and previously reported data (Martinez et al., 2011; Martinez et al., 2015; Sanjuan et al., 2007) could be due to the fact that different phagocytic particles were used in each study. We used antibody-coated particles to engage the FCGR2A and also unopsonized zymosan particles. Previous studies used zymosan, PAM3CSK4- and LPS-coated beads, *Escherichia coli* (Sanjuan et al., 2007), and dead cells (Martinez et al., 2011). Another difference is that macrophages used in this study do not overexpress GFP-LC3, as was the case in some prior studies (Martinez et al., 2011; Sanjuan et al., 2007). Variability in specific reagents during the macrophage differentiation protocol may also underlie the observed differences. We do not regard the LAP model as false, but rather make the point that it is not universally applicable; phagosome maturation, a complex and dynamic process, may have a requirement for ATG proteins under specific conditions.

An important consideration when interpreting these discordant findings may involve the manner whereby the CYBB NADPH oxidase affects phagosome maturation. We previously showed that ROS production by the CYBB NADPH oxidase is required for LC3 recruitment to phagosomes (Huang et al., 2009), an observation confirmed by others (Martinez et al., 2015; Romao et al., 2013). In the present study I examined phagosome maturation in the absence of CYBB-dependent ROS production. Despite the fact that CYBB is required for LAP (Huang et al., 2009)(Martinez et al., 2015; Romao et al., 2013), OpZ phagosomes matured *faster* in the absence of intraphagosomal ROS than in the wild-type counterparts (Figure 25). This suggests two things: first, phagosome maturation does not always require LAP and, second, CYBB NADPH oxidase may actually *impair* phagosome maturation under these conditions. Indeed, CYBB has a controversial role in modulating phagosome maturation; some investigators find that it delays phagosomal maturation (Mantegazza et al., 2008; Savina et al., 2006), whereas others do not (Allan et al., 2014; Rybicka et al., 2012; Rybicka et al., 2010). Recent studies showed that the
maturation status of macrophages dictates the effects that the CYBB NADPH oxidase has on phagosome maturation (Canton et al., 2014). Based on these findings, I hypothesize that both CYBB and ATG proteins, which are linked in phagosomal development, may have effects on phagosome maturation that are condition-dependent and possibly dynamic—altered by status of the macrophages, e.g. in classically activated macrophages (M1) vs. alternatively activated macrophages (M2). An additional layer of complexity is added by the expression of CYBB NADPH oxidase regulators, such as RUBCN/Rubicon (RUN domain and cysteine-rich domain containing, Beclin1-interacting protein). RUBCN is required for LAP (Martinez et al., 2015) and is upregulated in response to TLR2 activation (Yang et al., 2012). Thus, the choice of the phagocytic particle also affects the amount of ROS produced and the destiny of the phagosome. It is possible that other positive and negative regulators of the CYBB NADPH oxidase also play a role in LAP (Gardet et al., 2010; Kim et al., 2011b; Noubade et al., 2014; Sokolovska et al., 2013).

Further studies are required to reveal the reason why ATG proteins are important in phagosome maturation under some conditions but not others. Regardless of the specific reason, I have shown that in our system ATG proteins are not required for conventional phagosome maturation. Hence, I posit that LAP is not universally required for phagosome fusion with endosomes and lysosomes. Instead, the relationship between ATG proteins and phagosome maturation is more complex and likely involves other players.
Chapter 6 – Discussion and Future Directions

**Anti-Salmonella Autophagy**

My findings provide novel insights into the mechanism by which ubiquitin-binding adaptor proteins, p62 and NDP52, target bacteria to the autophagy pathway. I demonstrate that these adaptors are both recruited to the same bacteria at the same time. However, p62 and NDP52 are recruited independently of one another. In addition, I show that p62 and NDP52 are not redundant and function in the same pathway, indicating that each adaptor brings unique components necessary to drive ubiquitin-dependent antibacterial autophagy. To my surprise, I found that the adaptors localize to distinct bacteria-associated microdomains. This study provided a new perspective on how the field is thinking of p62 and NDP52, LC3-binding adaptor proteins. Despite their apparent similarity, they are not redundant, and furthermore, localize to different parts of the autophagic cargo. More broadly, this study suggests that adaptor proteins may target microdomains on their cargo (protein aggregates, organelles, etc.) and provide non-redundant signals or membrane recruitment to promote autophagy.

The data presented in Chapter 3 provided a new perspective on how apparently similar adaptors function and was first to observe heterogeneity in adaptor localization on the surface of a single Salmonella-containing vacuole (SCV). Remarkable progress (Figure 28) has been achieved since Cheryl Birmingham observed S. Typhimurium targeted to the autophagy pathway in 2006 (Birmingham et al., 2006). New adaptors and their mechanism of action have been characterized and an ubiquitin E3 ligase identified. Progress in the field is summarized below.

**Progress in the anti-Salmonella autophagy field to date**

In the years following the publication of Chapter 3 in 2011, the understanding of anti-Salmonella autophagy has advanced significantly; additional adaptor proteins were discovered (optineurin/OPTN, TAX1BP1/T6BP), an ubiquitin E3 ligase characterized (possibly two), and new understanding of NDP52 recruitment and function emerged. This sub-section will summarize progress that has been made.
Adaptor proteins

While it was originally assumed that **NDP52** is recruited to *S. Typhiumium* via its ubiquitin-binding domain (Thurston et al., 2009), Thurston and colleagues later uncovered that early in infection (~1 h p.i.) NDP52 is recruited to *S. Typhimurium* via its interaction with galectin-8, a host lectin and a signal for membrane damage. Only later in infection (~4 h p.i.), ubiquitin-binding zinc-finger can mediate NPD52 recruitment to bacterium (Thurston et al., 2012). In the absence of galectin-8, fewer bacteria acquire LC3 and *S. Typhimurium* replication is enhanced. This finding explains why p62 and NDP52 are found on the different microdomains (Cemma et al., 2011).

NDP52 contains two LIR motifs, DYWE and LVV, which are required in different stages of anti-*Salmonella* autophagy. NDP52 functions early in the autophagy pathway via LVV LIR as it mediates LC3C recruitment (von Muhlinen et al., 2012). In the absence of LC3C, no other ATG8 is recruited to bacterium and replication of *S. Typhimurium* is enhanced (von Muhlinen et al., 2012). It is noteworthy that p62 binds all ATG8 family members with equal affinity (von Muhlinen et al., 2012). NDP52 also functions in autophagosome maturation through DYWE LIR that mediates interaction with LC3B, LC3A, GABARAPL2. NDP52 is required for maturation of the autophagosomes via its interaction with myosin VI via residue Cysteine 425 (Verlhac et al., 2015). Thereby, NDP52 has two functions in *anti-Salmonella* autophagy: (i) LC3 recruitment via LVV motif and (ii) maturation of the autophagosome via DYWE motif.

In addition to p62 and NDP52, another adaptor protein, optineurin (**OPTN**), localizes to intracellular Ub+ positive *S. Typhimurium* (Wild et al., 2011). Like a classical autophagy adaptor, OPTN has an ubiquitin-binding domain (UBAN) and an LIR. In the absence of OPTN, *S. Typhimurium* replication is enhanced suggesting an important biological role in host defence. Interestingly, TANK binding kinase 1, TBK1, can phosphorylate OPTN at Serine 177 that is adjacent to OPTN’s LIR; this modification increases its affinity between OPTN and LC3 and is required for OPTN function in restricting *Salmonella* replication (Wild et al., 2011). It was previously reported that TBK1 is recruited to *S. Typhimurium* via NDP52 (Thurston et al., 2009), so it is possible that OPTN phosphorylation is facilitated by NDP52-dependent TBK1
recruitment. OPTN localizes to the same microdomain as NDP52 (that excludes p62) making this hypothesis plausible. Silencing both OPTN and NDP52 does not have an additive effect on increased bacterial replication relative to single knockdowns, suggesting that they function in the same pathway or are mutually dependent on each other (Wild et al., 2011). In fact, OPTN-deficient mice were more susceptible to infection with S. Typhimurium than wild-type or heterozygous counterparts (Slowicka et al., 2016).

NDP52 paralog, TAX1BP1/T6BP, is also required for efficient antibacterial autophagy (Tumbarello et al., 2012). It localizes to the microdomain around the Salmonella containing NDP52 and OPTN (Tumbarello et al., 2012). Like NDP52, it has a noncanonical LIR and an ubiquitin binding Zinc finger domain, yet unlike its paralog that only binds LC3C and K63 ubiquitin and tetra-ubiquitin, TAX1BP1 also binds LC3B, GABARAPL1 and GABARAPL2 and K48 ubiquitin (Tumbarello et al., 2015). Ubiquitin binding and not LC3 binding is required for TAX1BP1 recruitment to S. Typhimurium (unlike NDP52 that requires LC3 binding but not ubiquitin binding) (Tumbarello et al., 2015). Interestingly, the knockdown of both NDP52 and TAX1BP1 leads to accumulation of Ub⁺ Salmonella in a cumulative manner, i.e. double knockdown has a stronger phenotype that knockdown of either adaptor proteins (Tumbarello et al., 2015). This suggests that NDP52 and TAX1BP1 functions are partly redundant.

Ubiquitin can recruit other autophagic machinery, ATG16L1, ULK1 complex, ATG9L1, to autophagic cargo independently of the LC3-adaptor-ubiquitin interaction. For example, ATG16L1 directly binds ubiquitin around S. Typhimurium (Fujita et al., 2013). ATG16L1 is also recruited to intracellular Salmonella via its interaction with WIPI-2 (Dooley et al., 2014). In the absence of WIPI-2, significantly less p62⁺ S. Typhimurium recruit LC3 and bacterial replication is enhanced (Dooley et al., 2014).

During infection with Shigella flexneri, Tectonin domain-containing protein Tecpr1 acts as an autophagic adaptor binding through direct interaction between ATG5 and WIPI-2 (Ogawa et al., 2011). Tecpr1 is required for restricting bacterial replication, and in addition to anti-Shigella autophagy, is involved in mitophagy and localizes to intracellular S. Typhimurium and GAS (Ogawa et al., 2011). Thus, it is plausible that Tecpr1 functions as an adaptor protein in anti-
Salmonella autophagy.

The ubiquitin-like modifier, FAT10/Ubiquitin D, localizes to the same microdomain around S. Typhimurium as p62 and ubiquitin (that excludes NDP52, galectin-8, OPTN, and TAX1BP1) (Spinnenhirn et al., 2014). It is not surprising since a single moiety of FAT10 can be directly conjugated to multiple Lysines of p62 (Aichem et al., 2012). The caveat, however, is that FAT10 is only expressed upon IFN-γ and TNF-α induction. The challenge is to assess how knockdown of FAT10 impacts bacterial replication, since bacterial clearance is enhanced after cytokine treatment. FAT10-deficient mice are more susceptible to infection with S. Typhimurium (Spinnenhirn et al., 2014).

Role of Myosin VI in autophagosome maturation

Myosin VI is an ATP-dependent motor protein that travels along the actin filaments towards the minus-end of the filament (unlike other myosins that travel toward the plus-end). Myosin is required for transport of endocytic vesicles (Tumbarello et al., 2013). It turns out that NDP52, OPTN, and TAX1BP1 interact with myosin VI through its RRL motif, and myosin VI is recruited to the autophagosome (Tumbarello et al., 2012). In turn, myosin VI brings in endosomes via its interaction with Tom1 (endosomal cargo adaptor), thereby promoting autophagosome maturation and fusion with the lysosome (Tumbarello et al., 2015; Tumbarello et al., 2012; Verlhac et al., 2015). In the absence of myosin VI or Tom1, autophagosome-lysosome fusion of S. Typhimurium cargo is blocked, suggesting a defect in the maturation process (Tumbarello et al., 2015).
Figure 27. Schematic of ATG8-binding adaptor proteins required for anti-Salmonella autophagy.

Adaptor proteins include: p62/SQSTM1, NDP52/CALCOCO2, TAX1BP1/T6BP, and OPTN/optineurin. LIRs, LC3 Interacting Regions, are depicted in green and known LIR interactors of respective adaptors are noted. All ubiquitin-binding domains, UBAN, ubiquitin binding in ABIN and NEMO domain; ZF, zinc-finger domain; UBA, ubiquitin-associated domain, are depicted in purple and preferred ubiquitin chains of respective adaptors are noted. Other domains include: CC, coiled-coil domain (red); NES, nuclear export signal (black); NLS1 and NLS2, nuclear localization signals 1 and 2 (black); SKICH, SKIP carboxyl homology domain (light green); PB1, Phox and Bem1 domain (yellow); ZZ, ZZ-type zinc finger (dark yellow).
Ubiquitin E3 ligases and deubiquitinases (DUBs)

LRSAM1 has been identified as an E3 ligase responsible for recognizing S. Typhimurium via its LRR domain, promoting ubiquitination, and restricting bacterial replication (Huett et al., 2012). LRSAM1 recruitment to S. Typhimurium peaks at 40 min p.i., right before the peak in LC3 recruitment (at 1 h p.i.). Interestingly, LRSAM1 can directly interact with NDP52, yet NDP52 is not required for LRSAM1’s recruitment to bacterium and the two localize to different microdomains around the same bacterium (Huett et al., 2012). In vitro studies indicate that LRSAM1 produces K6 and K27 ubiquitin chains. However, despite LRSAM1 knockdown, 12% of bacteria remain Ub⁺ (Huett et al., 2012). In addition, other studies found that intracellular S. Typhimurium colocalizes with K48, K63, and linear-linked ubiquitin chains (rather than K6 or K27) (Fujita et al., 2013; van Wijk et al., 2012). This suggests that other E3 ligases are likely implicated in the ubiquitination of S. Typhimurium.

Parkin, an E3 ligase implicated in mitophagy (Narendra et al., 2008), is also responsible for autophagy of M. tuberculosis by mediating its K63 ubiquitination and recruitment of adaptors p62, NDP52, NBR1 and a kinase, TBK1, in BMDMs (Manzanillo et al., 2013). Given that PARK2 mutations in humans have been associated with susceptibility to Salmonella enterica serovar Typhi (Ali et al., 2006; Mira et al., 2004) and parkin-deficient Drosophila melanogaster have a 10-fold higher S. Typhimurium burden at 9 h p.i. (Manzanillo et al., 2013), Parkin might play a role in anti-Salmonella autophagy. Given that HeLa cells do not express Parkin, this hypothesis should be examined in a Parkin-expressing cell line, like HEK293.

An siRNA screen in mammalian cells identified an E3 ligase, SMAD Specific E3 Ubiquitin Protein Ligase 1 (SMURF1), to be required for selective viral autophagy and mitophagy (Orvedahl et al., 2011). Interestingly, the E3 ligase activity is not necessary for SMURF1’s function in selective autophagy, as its catalytically inactive mutant (mutation) is able to rescue mitophagy defects in smurf1−/− MEFs (Orvedahl et al., 2011). Instead, SMURF1’s phospholipid-binding domain, C2, is required for autophagy recruitment to damaged mitochondria (Orvedahl et al., 2011). Damaged mitochondria accumulated in the heart, brain, and liver of smurf1−/− mice.
relative to wild-type counterparts (Orvedahl et al., 2011). Whether SMURF is required for anti-
Salmonella autophagy remains to be examined.

There is evidence to suggest that S. Typhimurium can counteract autophagy to some extent. Bacterial deubiquitinase SseL favors bacterial replication and inhibits selective autophagy of cytosolic aggregates that accompany its replication (Mesquita et al., 2012).

**Summary of progress in the anti-Salmonella autophagy field to date**

At least four well-characterized adaptor proteins, p62, NDP52, OPTN, and TAX1BP1, are required for efficient antibacterial autophagy of S. Typhimurium (Cemma et al., 2011; Thurston et al., 2009; Tumbarello et al., 2012; Wild et al., 2011; Zheng et al., 2009). Interestingly, various adaptor proteins have preference for different ATG8s and ubiquitin chains (Figure 27). This might explain why so many adaptors are needed for efficient antibacterial autophagy. All adaptors are able to recruit ATG8 homologues via their LIRs to S. Typhimurium (Birgisdottir et al., 2013). NDP52 is recruited to damaged vacuoles through direct binding to galectin-8, a cytosolic lectin (Thurston et al., 2012), while others are recruited via their interactions with ubiquitin chains (Tumbarello et al., 2012; Wild et al., 2011; Zheng et al., 2009) (Figure 28).

NDP52, OPTN, and TAX1BP1 localize to one microdomain (that excludes p62) and, in addition to recruiting LC3, NDP52 and TAX1BP1 are able to bind myosin VI and promote autophagosome maturation (Tumbarello et al., 2015; Tumbarello et al., 2012; Verlhac et al., 2015) (Figure 28.1). Ubiquitin, p62, and FAT10 localize to the other microdomain around S. Typhimurium (Cemma et al., 2011; Spinnenhirn et al., 2014) (Figure 28.2). In addition, ubiquitin is able to directly recruit ATG16L1; and other autophagy components, ULK1 complex, and ATG9L1, are recruited to S. Typhimurium (Fujita et al., 2013) (Figure 28.4). These findings suggest that there are multiple layers of defence against S. Typhimurium.
The image depicts a complex diagram related to the autophagosome maturation process, focusing on the NDP52/TAX1BP1/OPTN microdomain. The diagram highlights various proteins and their interactions, including ATG3, FNBP1L, and WIPI-2. The S. Typhimurium bacterium is shown in the center, with a focus on E3 ligases such as LRSAM1 and Parkin, which are involved in the ubiquitination of proteins like K6, K27, and mono-Ub.

The Adaptors section includes p62, NDP52, TAX1BP1, OPTN, FNBP1L, and Tecpr1. The Atg8s section lists LC3A, LC3B, and LC3C. The Atg proteins include ATG3, ATG5, ATG16L1, ATG9L1, ULK1, and WIPI-2. The Recruitment signal section highlights Ub, Gal8, and other molecules like myo6, TBK1, LRSAM1, and Parkin.
**Figure 28. The model of Salmonella Containing Vacuole targeted by canonical autophagy.**

This model represents intracellular *S. Typhimurium* in HeLa cells at 1 h p.i. and is based on a number of publications cited in this figure legend. Question marks represent findings in different systems that are relevant (but not yet confirmed) for anti-*Salmonella* autophagy in HeLa cells. **(1)** NDP52, TAX1BP1, and OPTN adaptor proteins localize to one microdomain. NDP52 is recruited via its interaction with galectin-8 (Thurston et al., 2012), while TAX1BP1 and OPTN are recruited via ubiquitin binding (Tumbarello et al., 2012; Wild et al., 2011). All adaptors are able to bind ATG8s, as well as myosin VI. Interaction between OPTN and LC3 is dependent upon OPTN phosphorylation by TKB1 in its LIR region (Wild et al., 2011). Binding to myosin VI is required for autophagosome-lysosome fusion (Tumbarello et al., 2015; Tumbarello et al., 2012; Verlhac et al., 2015). **(2)** Ubiquitin (as stained by FK2 antibody), p62, and FAT10 are localized to the same microdomain. p62 is recruited via binding to ubiquitin (Zheng et al., 2009). Upon treatment with cytokines, FAT10 is covalently conjugated to p62 and might also play a role in efficient antibacterial autophagy (Spinnenhirn et al., 2014). LRSAM1 is one of the E3 ligases responsible for p62 recruitment (Huett et al., 2012). Parkin is a potential candidate as well, as it promotes mitophagy and mediates resistance to *M. tuberculosis* in mice and *S. Typhimurium* in flies (Manzanillo et al., 2013). **(3)** FNBP1L is recruited to intracellular *Salmonella* and mediates recruitment of ATG3. It is not clear how FNBP1L is recruited (Huett et al., 2009). **(4)** ULK1 complex, ATG9L1, and ATG16L1 are recruited to *S. Typhimurium*. ATG16L1 is recruited via direct binding to ubiquitin (Fujita et al., 2013). **(5)** WIPI-2 is recruited to *S. Typhimurium* and brings in ATG16L1-ATG5-ATG12 complex to promote LC3 conjugation via direct interaction with ATG16L1 (Dooley et al., 2014). **(6)** Tecpr1 localizes to *S. Typhimurium*. In the context on *S. flexenri* infection, Tecpr1 directly interacts with WIPI-2 and ATG5 and is required for antibacterial autophagy (Ogawa et al., 2011).
Outstanding questions regarding autophagy adaptors

While it has long been established that Ub⁺ S. Typhimurium gets targeted to the autophagy pathway, the types of ubiquitin chains surrounding the SCV are not thoroughly characterized. To mark Ub⁺ bacteria by immunofluorescence, previous studies have used FK2 antibody, which recognizes K29-, K48-, K63-ubiquitinated chains, and monoubiquitin. What types of ubiquitin linkage are present on the SCV, and in what proportions? It is not clear why NDP52/OPTN/TAX1BP1 microdomain does not colocalize with ubiquitin, as OPTN and TAX1BP1 are supposedly recruited to bacterium via their respective ubiquitin-binding domains. Ubiquitin-binding domains of OPTN and TAX1BP1 were shown to have a preference for K48, K63, and tetra-ubiquitin chains in an in vitro setting (Fujita et al., 2013; van Wijk et al., 2012).

Recently, ubiquitin phosphorylation on Serine 65 was shown to be important for mitophagy (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). Is ubiquitin phosphorylation important for xenophagy, and if so, which kinase is responsible for this post-translational modification? The nature of ubiquitination substrates is completely unknown. Are ubiquitination targets host or bacterial proteins?

One ubiquitin E3 ligase, LRSAM1, generating ubiquitin signal that recruits adaptors to S. Typhimurium has been identified (Huett et al., 2012). LRSAM1 produces K6 and K27 ubiquitin chains (Huett et al., 2012), while adaptor proteins have a preference for K48, K63, and tetra-ubiquitin chains (Fujita et al., 2013; van Wijk et al., 2012) (Figure 27). Given that there are over 600 predicted ubiquitin E3 ligases, it is plausible that other E3 ligases are involved in regulating this process. The perplexing thing is that FK2 is theoretically unable to recognize K6 or K27 (based on manufacturer’s description), yet the population of Ub⁺ Salmonella drops by half in the LRSAM1-depleted cells (Huett et al., 2012). Are there multiple E3 ligases cooperating to promote appropriate ubiquitination of the SCV, where K6 and/or K27 chains result in recruitment of another E3 ligase that promotes K48 and K63 ubiquitination?

Are DUBs involved in regulating ubiquitination? This is an important question since it is now apparent that autophagic turnover of mitochondria (mitophagy) is regulated by the DUBs, USP8, USP15, USP30, and USP35 (Bingol et al., 2014; Cornelissen et al., 2014; Durcan et al., 2014;
Wang et al., 2015). Since autophagy can degrade entire organelles, it makes sense that the host cell would have mechanisms to regulate autophagy even in the face of infection by a microbial pathogen.

Are other adaptors involved? The role of Tecpr1 in *S.* Typhimurium should be investigated. Tecpr1 localizes to LC3\(^+\) *Salmonella* at 1 h p.i., but its functional importance has not been examined. Finally, kinases and phosphatases are likely to regulate anti-*Salmonella* autophagy. TBK1 allows for OPTN to recruit LC3 after it phosphorylates Serine 117 adjacent to OPTN’s LIR (Wild et al., 2011). What are the other kinases and phosphatases involved, and what are the other post-translational modifications involved in regulation of anti-*Salmonella* autophagy?

To answer the outlined questions, I suggest to first identify ubiquitin chains present on the SCV and adaptor proteins they colocalize with. Ubiquitin mutants that are able to undergo only one type of ubiquitination can be used for this purpose. Mass-spectrometry techniques can be used to identify the proteome of the SCV at 1 h p.i., followed by a secondary microscopy screen. This approach has the potential to identify other relevant regulators, including DUBs, E3 ligases, kinases, phosphatases, and other adaptor proteins present on the SCV.

**Examining anti-*Salmonella* autophagy dynamically**

While there are numerous publications examining adaptor and LC3 recruitment to *S.* Typhimurium at 1 h p.i., the data is scant about what precedes or follows the peak of LC3 recruitment. Shun Kageyama delineated in a series of live-cell imaging experiments that ATG9L1 is recruited to the SCV prior to the LC3 peak and is required for canonical autophagy (Kageyama et al., 2011), however, not much else is known. Our current understanding is that membrane damage in *Salmonella*-containing vacuoles (detected via galectin-8) is the first sequence in a series of events necessary for targeting *S.* Typhimurium to the autophagy pathway (Thurston et al., 2012). Yet, surprisingly, the kinetics of when SCV become positive for galectin-8 has not been examined. Following live-cell imaging studies of *S.* Typhimurium with relevant markers (adaptors, E3 ligases, kinases, and other markers identified in the sub-section above) can provide a more precise dynamic analysis and delineate the sequence of events. Is there
heterogeneity among Salmonella-containing autophagosomes? How long does fusion with the lysosome take? While there is evidence to support the idea that Salmonella is targeted for degradation by the autophagy pathway (Birmingham et al., 2006), it has not been thoroughly characterized. More importantly, what are the key events in the pathway? Membrane damage (detected by galectin-8) and ubiquitination are two events required for xenophagy. But are there others?

Only a third of intracellular bacteria are targeted to the autophagy pathway (Birmingham et al., 2006; Cemma et al., 2011). The pertinent question hence becomes how can we manipulate the balance towards targeting more intracellular bacterial for degradation? For instance, does overexpression of key players, such as ubiquitin E3 ligase, enhance bacterial targeting to the autophagy pathway? Or alternatively, can small molecule agents be used to modulate the antimicrobial pathway as a novel treatment of infection?

Finally, can these findings be applied to other ubiquitin-dependent types of autophagy? Selective autophagy of pexophagy, ubiquitin aggregates, ribosomes, and mitochondria also depend on ubiquitin as a signal (Shaid et al., 2013). p62 is the first characterized adaptor and has been implicated in all the above-mentioned types of autophagy (Shaid et al., 2013). It is, however, unclear if other adaptors are as universal as p62. NDP52 and OPTN, for example, were originally characterized in anti-Salmonella autophagy (Thurston et al., 2009; Wild et al., 2011), but recently were shown to be required for mitophagy as well (Lazarou et al., 2015; Richter et al., 2016). Is it the case also for other ubiquitin-binding adaptors? Another question is are the same E3 ligase, kinase, and adaptor proteins used in targeting other pathogens to the autophagy pathway?

Using BioID to further characterize the autophagy pathway

The characterization of IRGQ as a novel LC3B-interacting protein with a function in the autophagy pathway is the first precedent of using the dual BioID/AP-MS approach to uncover new autophagy players. BioID and AP-MS are powerful and complementary approaches that detect both proximal and physical interacting proteins, and have been successfully applied to
identifying putative interactors in the Hippo pathway and chromatin-associated proteins (Couzens et al., 2013; Lambert et al., 2015; Varnaite and MacNeill, 2016).

In the next few years, I imagine the dual approach will be applied to all core autophagy proteins to answer some outstanding questions and identify new autophagy regulators. The fundamental question is what different autophagy types have in common and what sets them apart. Analysis of the autophagy interactome under various autophagy-inducing conditions can be compared for similarities and differences. When we stimulated autophagy with starvation or rapamycin, LC3B accumulated in both cases, yet IRGQ only localized to LC3B\(^+\) puncta in the case of rapamycin treatment (Figure 18C). The reasons for this observation are not clear, as both treatments activate autophagy through mTORC1 deactivation (Curtis et al., 2005). Insights into what sets them apart is likely to explain this surprising observation, though follow-up work will be required. Another question is why do humans have six ATG8s, while yeast have one, and what makes them different from one another? I would also extend this analysis to adaptor proteins, such as p62, NDP52, and OPTN. As a secondary assay, the functional role of hits should be examined in a microscopy-based high-content screening measuring LC3\(^+\) puncta. In addition to eliminating functionally irrelevant proteins, this approach will also enable classification of hits into positive and negative regulators of autophagy.

**The role of IRGQ in the autophagy pathway**

Using the above-mentioned techniques, I have identified IRGQ as an LC3B-interacting protein with a role in the autophagy pathway. Prior to the study described in this thesis, IRGQ had no ascribed function. The meager information on IRGQ included a protein sequence and a few putative interactors, including GABARAPL2, identified in an AP-MS screen, but never confirmed with other techniques (Behrends et al., 2010). These studies identified IRGQ as an LC3B interactor via AP-MS and as an LC3B proximal protein via BioID. I determined that IRGQ and LC3B can associate to the same protein complex via co-immunoprecipitation and this interaction (whether direct or indirect) can be impaired by removal of IRGQ’s LIRs (Figure 17). IRGQ forms a perinuclear structure that surrounds but does not overlap with the centrosome in HeLa cells (Figure 18A, B). Upon rapamycin or bafilomycin A treatment IRGQ re-localized to
LC3B\(^{+}\) puncta (Figure 18C). Finally, IRGQ seems to be functionally important for at least three types of selective autophagy: anti-Salmonella autophagy, pexophagy, and aggrephagy (Figure 19). Despite the advancements in knowledge presented in this thesis, the molecular mechanism of IRGQ-dependent autophagy remains to be discovered. Given that IRGQ did not colocalize with LC3 during the peak of anti-Salmonella autophagy, it is unlikely that it acts as an LC3-binding adaptor protein. Measuring levels of Ub\(^{+}\) and adaptor\(^{+}\) S. Typhimurium in control or IRGQ knockdown cells will answer this question. However, IRGQ may still be involved in cargo recognition. I have ruled out the possibility that IRGQ may impair LC3 lipidation (Figure 20), yet many others remain. IRGQ may act in any stage of the autophagy pathway, including autophagy initiation, phagophore elongation, cargo recognition, and autophagosome maturation (Figure 29). Given that another IRG family member, IRGM, is required for autophagy initiation, it makes sense to explore the possibility that IRGQ might do the same. To test this, the ULK1 complex activation state should be examined in the presence and absence of IRGQ. If IRGQ is required for efficient autophagy initiation, it will be important to delineate if this action is through mTOR, as active mTOR hyperphosphorylates and represses autophagy initiation kinase ULK1. IRGQ might also function through other means such as inhibiting autophagosome maturation. Insights into the IRGQ mechanism of action are likely to be obtained from the AP-MS/BioID approach to delineate IRGQ’s “interactome.” In vivo studies using Irgq-deficient mouse will further establish the biological importance of IRGQ.
Figure 29. Model of IRGQ’s function in the autophagy pathway.

IRGQ localizes to a perinuclear structure composed of five pillars that surrounds the centrosome and is required for efficient autophagy. While anti-Salmonella autophagy is depicted, the autophagic cargo is meant to represent S. Typhimurium, protein aggregates, or peroxisomes. The mechanism of how IRGQ impacts autophagy remains to be discovered, some possibilities are depicted above: (i) it may promote adaptor protein recruitment, (ii) stabilize/activate autophagy initiation complex, and (iii) inhibit mTOR to allow for autophagy initiation. I have ruled out the possibility that IRGQ might impair LC3 lipidation.
Non-hierarchical canonical autophagy

Another big shift in the autophagy field is that it is stepping away from a hierarchical organization of autophagy genes. I have described in the introduction (Figure 2) that autophagy machinery can be subdivided into three categories, such as (i) initiation, (ii) elongation, and (iii) maturation. Yet, borders between these categories are quickly fading. A recent report from Sharon Tooze’s group unraveled the role of GABARAP in autophagy initiation via activating ULK1 kinase (Joachim et al., 2015). This constitutes a non-hierarchical activation since GABARAP is considered to be downstream of ULK1. Another study showed a component of the PI3K complex, ATG14, also functions in autophagosome maturation through stabilizing SNAP29-STX17 binary t-SNARE complex on autophagosomes (Diao et al., 2015). This suggests that ATG14 functions both at early and late stages of the autophagy pathway. Also, in anti-Salmonella autophagy, it was believed that ubiquitin recruits ubiquitin-binding adaptor proteins that in turn bring in the LC3\(^+\) autophagic membrane to sequester the cargo. However, looking at the updated anti-Salmonella autophagy model (Figure 28), in addition to Ub/p62 and NDP52/OPTN/TAX1BP1 microdomains, SCV also contains autophagy conjugation machinery on site, including ATG3, ATG5, and ATG16L1, and an autophagy initiation kinase ULK1. Perhaps, it is just easier for the cell to activate autophagy at the site of where autophagy is happening. What recruits ULK1 to the SCV? One can speculate that possibly adaptor proteins are involved. Finally, a study by Richard Youle’s group demonstrated that adaptor proteins, NDP52 and OPTN, recruit upstream autophagy factors including ULK1 and WIPI-1 to focal spots around mitochondria during mitophagy (Lazarou et al., 2015). These recent observations are likely to be just the tip of the iceberg. Interactome of core autophagy proteins will reveal the interconnected nature of autophagy proteins from various stages, which will likely change the way we characterize autophagy stages.
3. LC3-Associated Phagocytosis

**LAP and phagosome maturation**

The field of LAP research emerged after a seminal study by Sanjuan *et al.* revealed that LC3 can be conjugated to the single-membrane phagosome (Sanjuan *et al.*, 2007). This process of LC3 recruitment to phagosome is observed upon stimulation of various phagocytic receptors, including TLR2, FCGR2A/FcγR2A, and dectin-1 (Florey *et al.*, 2011; Henault *et al.*, 2012; Huang *et al.*, 2009; Ma *et al.*, 2012; Ma *et al.*, 2014; Martinez *et al.*, 2011; Martinez *et al.*, 2015; Romao *et al.*, 2013; Sanjuan *et al.*, 2007). In addition to receptor stimulation, LAP requires NADPH oxidase-derived ROS (Huang *et al.*, 2009; Ma *et al.*, 2014; Martinez *et al.*, 2015; Romao *et al.*, 2013) and a subset of autophagy proteins, including ATG5, ATG7, and LC3B (Sanjuan *et al.*, 2007). The major function ascribed to LAP is to promote phagosome maturation. Publications from Doug Green’s group noted delayed phagosome maturation in LAP-deficient cells (Martinez *et al.*, 2011; Sanjuan *et al.*, 2007). One study in human macrophages observed the opposite - LC3 recruitment actually delayed phagosomal maturation (Romao *et al.*, 2013).

In Chapter 5, I systematically examined if autophagy machinery is required for phagosome maturation. I undertook this analysis in various cell types (*Atg5*-deficient MEFs and *Atg5*- or *Atg7*-deficient CSF1- or CSF2-derived BMDMs), using different particles (unopsonized zymosan, IgG-opsonized zymosan, and IgG-opsonized SRBC), and assessing phagosome maturation with three standard assays (LAMP1 recruitment, LysoBright, and DQ-BSA).

Surprisingly, I was unable to observe a significant difference in phagosome maturation in wild-type and LAP-deficient *atg5*/* and *atg7*/* cells. Hence, I conclude that autophagy proteins are not universally required for phagosome maturation. While this chapter contains largely negative data, it is informative as it rebuts the common assumption in the field. LAP should not be assumed to promote phagosome maturation; instead this process should be examined under each set of circumstances.

Why was I unable to replicate previously published results (Sanjuan *et al.*, 2007) when using the same particle and cells? One possibility is that there is a technical difference either in the way
cells are handled or the environment (potentially resulting in different macrophage priming). A more likely explanation is overexpression of GFP-LC3B in the cells used by Doug Green’s group. This hypothesis is based on previous findings that LC3 overexpression induces autophagy in murine neurons (Hung et al., 2015) and can also affect LAP. Using the same model of LAP of photoreceptor outer segments (POS) in RPE cells, Kim and colleagues observed almost twice the amount of LC3+ phagosomes in cells overexpressing GFP-LC3 (Kim et al., 2013) than Frost and colleagues did in cells with endogenous levels of LC3 (Frost et al., 2014), albeit the studies were performed in different laboratories and direct comparison cannot be made. It is plausible that the level of LAP depends on the pool of available LC3B (and/or other ATG8 homologs). It will be important to examine how overexpression of GFP-LC3B (using an inducible promoter) impacts LAP. The LC3B interactome includes factors that impact trafficking. Perhaps overexpression of LC3B creates these complexes, and in the absence of the ATGs these LC3B-signaling complexes are sequestered away and impair normal signaling pathways on the phagosome, thereby impairing their maturation.

**What separates LAP from regular phagocytosis?**

LAP is a recently characterized pathway and many fundamental questions remain to be addressed by the field. In my view, a major question is what makes LAP different from regular phagocytosis? The current rudimentary understanding is that the LAP phagosome contains LC3B, while a regular phagosome does not. The protein machinery involved in LAP (described in Figure 10) is required for LC3B conjugation. Even the simplest question: *Do other ATG8s get recruited to LAP?* has not yet been addressed.

An easy way to compare phagocytosis and LAP is to understand what proteins are present on the phagosome throughout the maturation process. While phagosome proteome has been assessed in a number of studies (Garin et al., 2001; Jutras et al., 2008), the proteome of LAP phagosomes *vs.* non-LAP phagosomes is completely unknown. Hence, it will be important to assess phagosome proteome in LAP-sufficient and LAP-deficient cells (*atg7°* and *cybb°* knockout macrophages) via latex bead phagosome purification followed by mass spectrometry. Both ATG7 and CYBB/NOX2 NADPH oxidase are required for LC3 recruitment to phagosomes (Huang et al.,
This is a relatively easy technique that would provide the whole proteome of the phagosome, yet does not distinguish between LC3+ and LC3− beads in the LAP-sufficient cells. Hence, this system will be effective only if an overwhelming majority of phagosomes are LC3+ in the LAP-sufficient cells. The findings should be then confirmed by immunofluorescence and live-cell microscopy. Once other differences are established, one can form hypotheses as to upstream signaling events present in one pathway and not another.

**Does LAP differ depending on receptor stimulation**

Another obvious question is whether LAP resulting from engagement with different phagocytic receptors are the same or different? LC3 recruitment to phagosome is observed upon stimulation of TLR2, TLR4, TLR9, FCGR2A/FcγR2A, dectin-1, and TIM4 (Florey et al., 2011; Henault et al., 2012; Huang et al., 2009; Ma et al., 2012; Ma et al., 2014; Martinez et al., 2011; Martinez et al., 2015; Romao et al., 2013; Sanjuan et al., 2007), yet it has not been examined if receptor type can somehow influence the LAP pathway. In other words, are there differences in the LAP pathway triggered by TLRs, dectin-1, TIM4, or FcγR2A?

One way to address this question is to repeat the above-mentioned approach comparing glass beads opsonized with ligands of the previously mentioned receptors. An easier alternative is to follow the recruitment of key LAP markers (identified in a previous experiment) via live cell imaging. This will allow for quantification of the proportion of phagosomes that acquire/loose these markers, as well as identify the timing of these events. I am particularly intrigued to learn the extent of the variability both within and between groups. If receptor determines the fate of phagosome, future studies should dissect the underlying mechanisms. For instance, it would be informative to examine whether the observed differences are correlated with the amount of intraphagosomal ROS or V-ATPase recruitment. ROS levels can be measured by various assays including luminol or nitroblue tetrazolium assays.

Finally, there are a dozens phagocytic receptors whose role has not been yet examined in LAP. Can all phagocytic receptors induce LAP or are there some that cannot? If so, what sets them
apart? A study from Mary Dinauer’s group observed only ~50% of phagosomes to have a fully assembled and functioning NADPH oxidase (Li et al., 2009b), and a report from Sergio Grinstein’s group suggests that this heterogeneity might be due to variation in local lipid composition (Schlam et al., 2013). In addition, NADPH oxidase activity and stability is tightly controlled by a variety of negative and positive regulators (Di et al., 2012; Gardet et al., 2010; Honda et al., 2012; Kim et al., 2011b; Ma et al., 2012; Noubade et al., 2014; Sokolovska et al., 2013; Wang et al., 2012; Yang et al., 2012). For example, Rubicon protein is known to bind the transmembrane component, p22<sub>phox</sub>, and stabilize it on the phagosome resulting in an increased and prolonged ROS production in response to TLR2 and not FCGR signaling (Yang et al., 2012). Since ROS can induce LC3 recruitment to the phagosome (Huang et al., 2009; Martinez et al., 2016; Romao et al., 2013), is LAP just a subset of regular phagocytosis that had a robust and prolonged CYBB/NOX2 NADPH response? Or is LAP truly different from phagocytosis?

**Conclusion**

Findings presented in this thesis extend the understanding of autophagy proteins in host defence. Autophagy adaptor proteins p62 and NDP52 were localized to separate microdomains, both required for efficient anti-*Salmonella* autophagy. Additionally, for the first time, the BioID technique was used on an autophagy protein and resulted in characterization of a novel, previously unstudied, LC3B-interacting protein, IRGQ. Finally, the role of autophagy proteins, ATG5 and ATG7, were shown to be dispensable for LAP, at least under some circumstances. These advances open a new perspective and pose new questions. The LAP pathway is still far from being characterized – proteomics on the various stages of LC3<sup>+</sup> and LC3<sup>−</sup> phagosome will inform the field of the fundamental differences between LAP and phagocytosis. Future studies will need to dissect the mechanism of IRGQ’s function in the autophagy pathway. And proximity-dependent labeling mass spectrometry techniques will most certainly uncover more autophagy-regulating proteins and allow for a fundamental understanding of what defines different types of autophagy. It will inform the medical research field of how autophagy can be upregulated to protect the host from infectious diseases, especially relevant given the increasing threat of antibiotic resistance.
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