Regulation of autophagy-related mechanisms during bacterial infection

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Chapter 1

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Introduction and outline of the thesis

1. Infectious diseases

Infectious diseases pose a significant threat all over the world, with varying prevalence and pathological consequences. These diseases can cause different levels of morbidity and mortality and economic losses (Bloom and Cadarette, 2019). For example, Coronavirus Disease-19 (COVID-19), caused by Severe Acute Respiratory Syndrome Coronavirus-2, emerged recently as an acute respiratory infectious disease that caused more deaths in the past few years than any other infectious disease in the recent history. Apart from several other emerging infectious diseases, we are still combatting longstanding ones, such as tuberculosis (TB) and many types of respiratory and gastrointestinal infections, of which salmonellosis is a prime example. In addition, there are infectious diseases that continue to emerge and reemerge, like influenza.

Among bacterial infectious diseases, TB has the most severe impact on society. From 2000 to 2015, the world economy lost up to $616 billion due to TB. For certain countries in Africa and southeast Asia, the costs of TB have exceeded 1% of their gross domestic product (Burki, 2018). Over 1 billion people died because of TB during the past 200 years and in the last decade around 1-2 million people died annually, often due to co-infection with Human Immunodeficiency Virus (HIV). Despite the disease control programs, TB rates are still relatively high, with around 10 million people worldwide annually exhibiting symptoms of active TB (Chakaya et al., 2022). Additionally, it is estimated that a quarter of the world's population is latently infected, providing a large reservoir for future cases of active TB (Cohen et al., 2019).

Vaccines are considered the most effective measures against many infectious diseases. However, developing effective vaccines for certain diseases can be challenging. Many pathogens, including *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, are skilled at evading immune surveillance (Kennedy et al., 2020). Besides, some of the pathogens, such as influenza virus and HIV, have a strong tendency to mutate, necessitating the development of updated and modified vaccines (Chaudhary et al., 2021). For bacterial infectious diseases, antibiotics are widely used and have proven effective in reducing the number of infections. However, misuse and overuse of antibiotics have led to the emergence of antimicrobial resistance (AMR) in certain pathogens. These pathogens employ various mechanisms to become resistant to antibiotics, such as reducing cell membrane permeability, acquiring resistance genes, or undergoing single-point mutations (Ge et al., 2022). In some cases, emergence of drug resistance can be cured by use of antibiotic combinations. However, pathogens are capable of developing

multi-drug resistance. For example, Mtb strains have emerged that are resistant to both first-line and second-line antibiotics. Multidrug-resistant TB, characterized by resistance to at least rifampicin and isoniazid, accounts for 3.3% of new TB cases. It is reported that around 30,000 children develop multidrug-resistant TB annually, with an estimated death rate of 22% (Jenkins and Yuen, 2018). Antimicrobial resistance (AMR) is also a major problem for hospital-acquired infections. Every year, around 33,000 people die because of AMR and the costs associated with these infections are more than € 1.1 billion in EU countries (OECD, 2019). While the quest for developing new antibiotics continues, the emergence of AMR also calls for novel therapeutic strategies to combat infectious diseases.

One promising strategy to fight infections is host-directed therapy (HDT). The host immune system plays a crucial role in controlling infections and determines if the infections are contained or progress into disease. HDT aims to interfere with host cellular processes that are required by the pathogens to survive and replicate, or to modulate the immune response involved in defense against pathogens (Wallis et al., 2023). For example, HIV enters host cells through CC-chemokine receptor 5 (CCR5) to initiate infection. Inhibiting the CCR5 receptor using a CCR5 inhibitor, such as Maraviroc, is a good example of an HDT approach that can help alleviate associated symptoms (Kaufmann et al., 2018). This approach aims to enhance clinical treatment outcomes and may be applied adjunctively or alternatively to antibiotics. Among HDT approaches, some may involve the modulation of autophagy. As discussed below (section 4), autophagy is an important intracellular defense mechanism against various pathogens. Mtb, for instance, is known to be targeted by the autophagic pathway activated by the cytokine interferon-γ (Gutierrez et al., 2004). Consequently, pro-autophagic molecules that activate autophagy are being considered for treatment of TB (Kaufmann et al., 2018; Zumla et al., 2016). Similarly, autophagy modulation is under investigation for other intracellular pathogens, such as *Salmonella* (Wu et al., 2020). Many other routes for HDT development are currently being explored (Kilinç et al., 2021; Wallis et al., 2023). However, further development and application of these potential new therapies will require a better understanding of the interaction mechanisms between host and pathogen.

2. Mycobacterial pathogens

2.1 Pathological hallmarks of mycobacterial infections

Bacterial pathogens belonging to the genus *Mycobacterium* are the causative agents of a variety of infectious diseases, ranging from TB and leprosy to pneumonia, lymphadenitis, skin and soft tissue infections, and disseminated infections. Among these, TB remains the most serious global health problem. This infectious disease is characterized by symptoms such as persistent coughing, severe weight loss, and fatigue. Pulmonary TB and TB meningitis are the most common forms of the disease, but the infection can also affect other organs, such as the bone (Pott's disease), or spread systemically (miliary TB). The TB pathogen, Mtb, is spread from person to person by coughing or sneezing. After inhalation of the bacilli-containing aerosol droplets, Mtb reaches the lower respiratory tract and is delivered to the alveoli, where it infects epithelial and immune cells, including macrophages, dendritic cells and neutrophils. The infected cells eventually initiate inflammatory responses and recruit more immune cells to form a granuloma, the hallmark of TB (Bussi and Gutierrez, 2019).

2.2 Interaction of *Mycobacterium* and macrophages

The macrophage is the main cell type recognizing and phagocytosing Mtb and is responsible for initiating granuloma formation. Recognition of pathogens by the innate immune system is mediated by the germline-encoded pattern recognition receptors (PRRs). PRRs recognize pathogen-associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs), molecules released through injury or infection by either the pathogen or the host (Tang et al., 2012). Plasma membrane receptors, including Toll-like receptors (TLRs), C-type lectin receptors and scavenger receptors (Stamm et al., 2015), are the first PRRs to recognize Mtb (**Fig.1**). The most extensively studied receptors involved in Mtb recognition are the TLRs. Among the TLR family, TLR2, TLR4, and TLR9 have been shown to play important roles in recognition of Mtb (Kleinnijenhuis et al., 2011). C-type lectin receptors recognizing Mtb include the mannose receptor, Mincle, Dectin-1, and DC-SIGN (DC-specific intercellular adhesion molecule-3 grabbing nonintegrin) (Goyal et al., 2016). The relevant scavenger receptors include scavenger receptor A, CD36, and MARCO (macrophage receptor with collagenous structure) (Stamm et al., 2015). Even though specific roles of each of these receptors in Mtb internalization have been defined by *in vitro* studies, it is

likely that phagocytosis of Mtb *in vivo* and subsequent signaling responses are dependent on multiple receptors (Bussi and Gutierrez, 2019).

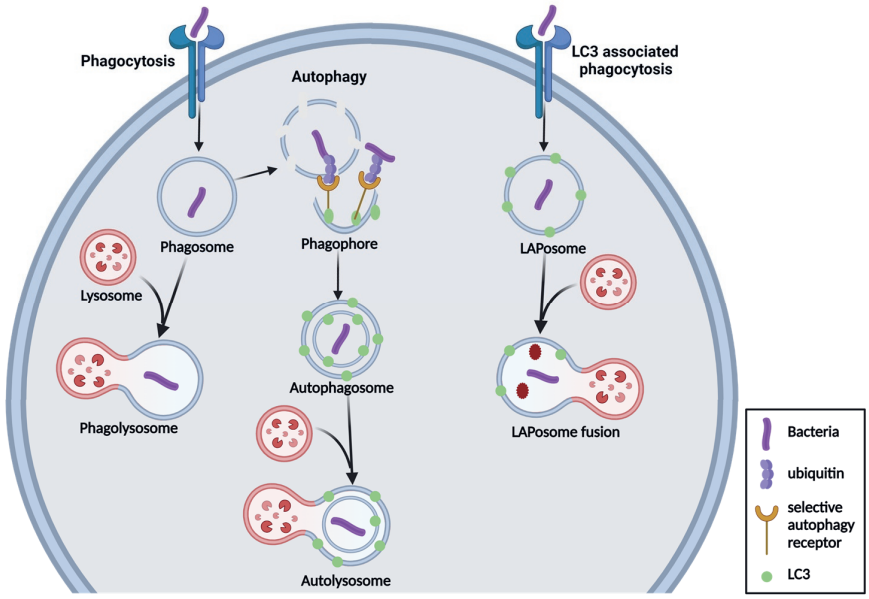

Figure 1. Schematic representation of pathogen interaction with host cellular degradation pathways. Pathogens can be recognized by pattern recogniton and scavenger receptors and phagocytosed into the phagosome. Phagosomes fuse with lysosomes for degradation of the cargo. Intracellular pathogens such as mycobacteria or *Salmonella* can arrest phagsome maturation or cause phagosomal membrane damage to invade the cytosol. This triggers the canonical antibacterial autophagy pathway, also known as selective autophagy or xenophagy. In this pathway, ubiquitinated bacteria are recognized by selective autophagy receptors, which interact with LC3 on phagophore membranes, which then mature into double membrane vesicles named autophagomes that will fuse with lysosomes. LC3-associated phagocytosis (LAP) is another pathway that shares molecular characteristics with both phagocytosis and autophagy to internalize pathogens into single membrane vacuoles named LAPomes, which also fuse with lysosomes for degradation. (figure created with Biorender).

The phagocytic pathway is the primary defense mechanism against bacterial invasion of host cells. The phagosomal environment facilitates several antimicrobial mechanisms, including the production of reactive oxygen and nitrogen species (ROS and RNS) (**Fig.1**). Furthermore, to drive microbial degradation, the phagosomes will fuse with early and late endosomes, and subsequently with lysosomes, and this stepwise process is called phagosome maturation. The small GTPase Rab5A is essential for tethering of early endosomes and their fusion with phagosomes. One of the Rab5 effectors, the type III phosphatidylinositol 3-kinase (PI3K) complex

(PI3KC3), generates phosphatidylinositol 3-phosphate (PI3P) on organellar membranes. PI3P is essential for membrane trafficking and sorting within the endosomal system (Thi and Reiner, 2012). Replacement of Rab5 by Rab7 is called Rab conversion and is necessary for early stages of phagosomes to be transformed into late phagosomes (Rao and Meena, 2011). Eventually, the late phagosomes fuse with lysosomes, turning into phagolysosomes. The main features of phagolysosomes are the abundant presence of proteases and the low pH (luminal pH values as low as 4.5), which favors the activation of hydrolytic enzymes after the maturation steps.

After phagocytosis, Mtb is contained in vesicles of phagosomal origin. However, Mtb is able to subvert this pathway by arresting phagolysosome formation (**Fig.1**) (Mishra and Surolia, 2018). Previous studies showed that Mtb is able to block Rab conversion and thus impairs the maturation of phagosomes into phagolysosomes (Via et al., 1997). In addition, the generation of PI3P on phagosomes is inhibited by Mtb virulence mechanisms (Purdy et al., 2005). The activities of the PI3P effectors, such as early endosome antigen, and further downstream effectors, like the endosomal sorting complex required for transport, are also diminished, which is consistent with the arrest of phagosome maturation (Vieira et al., 2004). The arrested phagosome is characterized by absence of mature lysosomal hydrolases and incomplete luminal acidification (Deretic et al., 2006). Furthermore, Mtb has been shown to be able to withstand at least to a certain extent the acidic environment of lysosomes (Paroha et al., 2018).

Besides arresting phagosome maturation, Mtb can rupture the phagosome membrane and escape into the cytosol. This pathogenic behavior is dependent on the 6 kDa early secretory antigenic target (ESAT6) protein family secretion (ESX) systems (**Fig.1,2**). Among the five ESX systems (ESX-1, ESX-2, ESX-3, ESX-4 and ESX-5), ESX-1 is best studied due to its significant role in virulence, and it is the primary mediator of cytosolic invasion. ESX-1 is encoded by a genomic locus called region of difference 1 (RD1) (Groschel et al., 2016). ESX-1 has been shown to be crucial to evade host immune responses and survive inside the host cells. The ESX-1 system secretes ESAT-6 (also known as EsxA) in conjunction with CFP-10 (also known as EsxB) as a heterodimer. The membranolytic activity of ESAT-6 was first demonstrated in a study by Hsu and coworkers, who constructed a planal lipid membrane, and found that the conductance was disrupted by ESAT-6, with or without CFP-10 (Hsu et al., 2003). Similarly, Jonge and colleagues incubated liposomes with ESAT-6 and observed fragmented membranes from disrupted liposomes (De Jonge et al., 2007). It was further shown that ESAT-6 can undergo a conformational change into a more α-helical and folded structure, and insert

into the phagosomal membrane to form a membrane-spanning pore, which causes phagosomal membrane damage (Ma et al., 2015). The damaged membrane can be recognized by the endosomal sorting complexes required for transport (ESCRT) machinery of the host cell, which facilitates phagosome repair. However, Mtb secretes another effector EsxH to antagonize this process, which accelerates the membrane damage process (Augenstreich and Briken, 2020).

Illustrating the importance of the membranolytic activity of ESAT-6 in Mtb infection, it is demonstrated that virulent Mtb, but not ESAT-6 deficient vaccine strains, translocate from the phagosome to the cytosol of macrophages (van der Wel et al., 2007). Subsequent work showed that this translocation is a general ability of virulent mycobacterial species and that both interleukin 1 signaling and adaptive immune responses are crucial to counteract this invasive behavior (Houben et al., 2012; Simeone et al., 2012; van Der Niet et al., 2021).

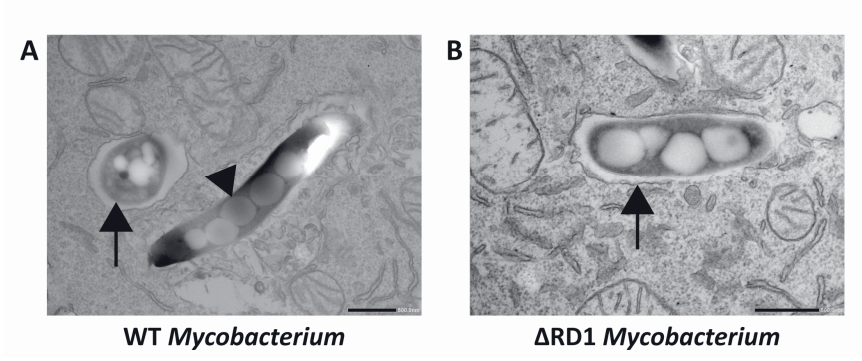

Figure 2. Transmission electron microscopy micrographs of the intracellular localization of *Mycobacterium marinum.* Wildtype (A) and ΔRD1 (B) bacteria are shown inside infected RAW 264.7 macrophages. Arrows indicate bacteria-containing phagosomes and the arrowhead points at a bacterium inside the cytosol. Cytosolic invasion is commonly observed for wild-type bacteria that possess pore-forming activity due to presence of the ESX-1 secretion system and secreted factor ESAT-6, both encoded by the RD1 locus (images courtesy of Gerda Lamers).

Once inside the cytosol, the pathogens can reach the nutrient pool for quick replication. The cytosolic bacteria or bacterial DNA released from the phagosome is then recognized by the host adaptor protein STING, which results in ubiquitination of the bacteria (Watson et al., 2012). The ubiquitination of Mtb, dependent on ubiquitin ligase Parkin (Manzanillo et al., 2013) and Smurf1 (Franco et al., 2017), initiates antibacterial autophagy as a cytosolic mechanism to achieve lysosomal degradation, functioning in parallel

with the phagocytic pathway. TRIM16, another ubiquitin ligase, interacts with galectin-3, a protein recruited to the membrane when it is damaged, which activates autophagy to clear damaged Mtb-containing vesicles (Chauhan et al., 2016).

2.3 *Mycobacterium marinum* infection in zebrafish as a model to study TB

Since Mtb is a human pathogen, using it as a model to study the pathogenesis of tuberculosis causes risks to researchers and the environment. *Mycobacterium marinum* (Mm) is a frequently used model for Mtb that can be used at biosafety level (BSL)2 instead of BSL3. Mm naturally infects cold-blooded hosts, such as fish and frogs. It shows high genetic similarity with Mtb, sharing 3000 orthologous genes and 85% similarity at the amino acid level (Stinear et al., 2008). Mm possesses similar virulence factors as Mtb, including the ESX-1 secretion system, which enables Mm to survive and replicate in host macrophages and produce a chronic granulomatous infection that shares many characteristics with human TB (Chirakos et al., 2020). Mm infection of zebrafish has been widely used as a model system for studying the pathogenesis of TB (Cronan and Tobin, 2014; Ramakrishnan, 2020; Varela and Meijer, 2022). Zebrafish embryos and larvae offer many valuable practical advantages, which are often complementary to the experimental possibilities of mammalian models.

A first practical advantage of working with the zebrafish during the early life stages is that the development of the innate immune system precedes the development of the adaptive immune system. This makes it possible to study the function of innate immunity separately (Harvie and Huttenlocher, 2015; Langenau et al., 2004; Meijer and Spaink, 2011). The first distinguishable macrophages are found at 22 hours post fertilization (hpf) and these are able to phagocytose pathogens at 24 hpf (Herbomel et al., 1999). Neutrophils, distinguished by characteristic cytoplasmic granules, can be recognized from 34 hpf (Willett et al., 1999). The phagocytic behavior differs between macrophages and neutrophils. Macrophages mainly phagocytose microbes that are injected into the blood or a body cavity, while neutrophils are efficient at removing bacteria attached to surfaces, for example injected into the tail fin, subcutaneous tissue or muscle (Colucci-Guyon et al., 2011).

A second advantage of using zebrafish embryos and larvae is that they are suitable for live imaging of phagocytosis and subsequent pathogenesis because of their optical transparency. This property enables *in vivo* real-time imaging using transgenic reporter lines that express fluorescent proteins (Davis et al., 2002; Hosseini et al., 2014; Yang et al., 2012). Exploiting this

property of the zebrafish model, it was found that Mm is phagocytosed by macrophages and induces a systemic infection with formation of granuloma-like structures that can be considered equivalent to the early stages of TB granulomas (Clay et al., 2007; Davis et al., 2002). Neutrophils have been shown to play a protective role by migrating to the nascent granuloma sites and kill Mm-infected macrophages (Yang et al., 2012). In addition to these cellular responses to infection, zebrafish embryos are used to study intracellular host responses to pathogens. For example, by using GFP-Lc3 labelled fish, it was found that Mm colocalizes with GFP-Lc3-positive vesicles, which is a hallmark of the host autophagy response discussed below (section 4) (Hosseini et al., 2014). The advantages of the zebrafish model for imaging have also been exploited in sophisticated high-throughput drug screens (Ali et al., 2011).

A third important advantage of the zebrafish model is that it provides genetic tools that enable the creation of temporary knockdowns of, or permanent mutations in, genes of interest. One widely used method for gene knockdown in zebrafish is the use of morpholinos (MOs). They are synthetic DNA derivatives, which are able to stably pair with mRNA and are resistant to degradation. Injection of MOs into zebrafish embryos can induce transient knockdown that lasts for several days (Bedell et al., 2011). Dependent on the design, MOs can prevent mRNA translation or splicing of targeted transcripts in zebrafish embryos (Timme-Laragy et al., 2012). Although experiments should be carefully controlled for off-target effects, MOs have been widely used because of the ease of delivery and their high efficacy. The CRISPR/Cas9 system is another powerful tool available for manipulating zebrafish genomes. It is derived from endonucleases that exist in archaeal and bacterial genomes, and these endonucleases have been adapted for use in manipulating metazoan genomes (Van der Oost et al., 2009). The Cas9 protein forms a complex with two small RNA molecules: CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA). For genome editing, the crRNA:tracrRNA duplex has been combined into a single guide RNA (sgRNA). The Cas9 protein has also been modified by adding nuclear localization sequences, which enables the endonuclease to target the nucleus in eukaryotes (Li et al., 2016). The CRISPR-Cas9 system is not only used for creating stable loss-of-function mutants, but also for transient knockdown of genes (Crispant technology), which is similar to the MO approach. Other methods, such as Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Targeting Induced Local Lesions in Genomes (TILLING) can also be applied to make mutations in zebrafish (Lawson and Wolfe, 2011), but their use has become less frequent since the CRISPR/Cas9

technology has emerged.

3. *Salmonella* pathogens

3.1 Pathological hallmarks of *Salmonella* infection

Like TB, salmonellosis is a serious condition, resulting from an infection with an intracellular pathogen. It is caused by *Salmonella* species and characterized by fever, abdominal pain, and gastroenteritis. In Europe, *Salmonella* is the second leading cause of foodborne illness, after *Campylobacter* (Ehuwa et al., 2021). Every year, there are approximately 90,000 reported cases of *Salmonella* infection in the EU (Authority et al., 2018). The situation is much worse in less developed countries. In 2017, there were around 90 million reported cases and more than 50,000 deaths worldwide (Stanaway et al., 2019).

The *Salmonella* genus consists of Gram-negative, rod-shaped bacteria, and comprises two species, namely *Salmonella enterica* and *Salmonella bongori*. *S. bongori* has only one subspecies, while *S. enterica* comprises seven subspecies (I, II, IIIa, IIIb, IV, VI and VII.1) with more than 2600 serovars (Garai et al., 2012). Based on the clinical syndrome, *S. enterica* is divided into two categories: typhoidal serotypes (TS) and non-typhoidal serotypes (NTS). TS bacteria, such as *Salmonella enterica* serovar Typhi, are the causative agents of typhoid fever, while infections with NTS bacteria, like *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium, hereafter ST) lead to symptoms like fever and diarrhea (Crump et al., 2015). The conventional treatment for salmonellosis is the use of antibiotics. However, the emergence of antibiotic-resistant strains and increasing costs of treatment due to therapy failures have become major issues (Jajere, 2019). As an alternative, HDT is being explored as an adjunctive treatment. In order to apply HDT for *Salmonella* infection, it is essential to have a comprehensive understanding of the interaction between *Salmonella* and the host. The following sections of the introduction summarize the current knowledge of *Salmonella*-host interplay and are mainly based on ST, which is used in the research of this thesis.

3.2 Introduction to virulence factors of *Salmonella*

Salmonella utilizes an array of virulence factors to manipulate the host response in order to survive intracellularly. The gene cluster encoding *Salmonella* virulence factor type III secretion systems (T3SS) are known as *Salmonella* pathogenicity islands (SPI). Until now, there are 23 characterized

SPIs, of which SPIs 1–5 are common to all types of *Salmonella* (Wang et al., 2020). Among the SPIs, SPI1 and SPI2 encoding T3SS1 and T3SS2, respectively, are important for invasion and dissemination (Carden et al., 2017). T3SSs are needle-like structures located in the bacterial cell wall (Garai et al., 2012). These needles can penetrate the membranes of host cells and form pores to project virulent proteins into the cytoplasm (Wu et al., 2020).

SPI1 is considered the most important *Salmonella* virulence locus and comprises at least 15 reported effectors, translocated by T3SS1. These effectors enable *Salmonella* to invade epithelial cells, modulate the inflammatory response, and induce cytotoxicity in macrophages (Garai et al., 2012; Ibarra and Steele-Mortimer, 2009). On the other hand, SPI2 is responsible for systemic virulence and survival within macrophages. It encompasses over 20 effectors that facilitate the formation of *Salmonella*-containing vacuoles (SCVs) and help to protect the pathogen against ROS and RNS production by the host (Ibarra and Steele-Mortimer, 2009). Although SPI1 and SPI2 are differentially regulated, their effector proteins exhibit overlapping functions in the modification of the SCVs and in promoting bacterial dissemination (Agbor and McCormick, 2011). SPI3 encodes the mgtCB effector, which is a high-affinity Mg^{2+} uptake system for *Salmonella* to adapt to the limited nutrition environment inside phagosomes, thereby promoting bacterial survival (Blanc-Potard and Groisman, 1997). The less characterized SPI-4 and SPI-5 loci are involved in intestinal colonization (Wang et al., 2020).

3.3 Interaction of *Salmonella* with the host

Virulence factors of *Salmonella* have a wide variety of functions in manipulating host processes. After ingestion, *Salmonella* bacteria have to compete with the gut microbiota for colonization. It has been observed that *Salmonella* uses T3SS1 effectors to trigger inflammatory responses, and then exploits the inflammatory response to alter the host commensal microbiota and overcome colonization resistance (LaRock et al., 2015; Stecher et al., 2007). Once breaching the gut epithelia, the bacteria are internalized by phagocytic cells, like macrophages and neutrophils (Garai et al., 2012). While both phagocyte types possess strong abilities to kill *Salmonella*, it is known that especially macrophages can be turned into a replication niche and serve as a vehicle for dissemination into other tissues, leading to systemic disease (Luk et al., 2022).

Except for phagocytic cells, *Salmonella* can be taken up by non-phagocytic cells as well, for example epithelial cells, and microfold cells (M cells). M cells

are a preferential site for *Salmonella* entry into the host intestinal epithelium. Typically, the number of M cells is limited. However, after infection, *Salmonella* uses the T3SS1 effector SopB to manipulate the follicle-associated epithelium enterocytes to develop into M cells, facilitating its invasion into the host (Tahoun et al., 2012). Moreover, T3SS1 effectors induce actin cytoskeleton rearrangement and macropinocytosis for bacterial uptake (LaRock et al., 2015).

After phagocytosis, *Salmonella* has the ability to convert the phagosomes into SCVs, which are large dynamic vesicles (vacuoles) that can fuse with endosomes for maturation. In addition, with the help of T3SS2 effectors, *Salmonella* bacteria are able to arrest this fusion process at the late endosome stage, which provides them with a niche to survive and replicate (Garai et al., 2012). In addition, some bacteria are capable of penetrating the SCV membrane using T3SS1 effectors. The resulting damaged membranes can be recognized by galectins for either membrane repair or autophagic degradation. It is also possible that the bacteria cause complete rupture of the SCV membrane, allowing them to be released into the cytosol and reach a higher replication rate. As a countermeasure of the host, the cytosolic *Salmonella* may then be recognized by ubiquitin and bound by autophagy receptors for degradation through the autophagy-lysosomal pathway (Wu et al., 2020). In addition to phagocytosis and autophagy, the LC3-associated phagocytosis (LAP) pathway (**Fig.1** and section 4.3) has also been identified as a defense mechanism against *Salmonella* infection (Huang et al., 2009; Masud et al., 2019).

Adding to studies in cellular and mammalian models, the transparency of zebrafish embryos allows researchers to study and better understand *Salmonella*-phagocyte interactions. Notably, studies have shown that neutrophils are recruited to inflammatory sites, which form in response to *Salmonella* infection. (Tyrkalska et al., 2016). However, macrophages have been identified as the main responders after intravenous infections and have been shown to play a more prominent role than neutrophils in protecting zebrafish against systemic *Salmonella* infection (Masud et al., 2019). Specifically, ablation of macrophages caused 100% mortality of embryos after *Salmonella* infection, while less than 40% mortality was found after neutrophil ablation. As discussed further below (section 4.3), the defense of zebrafish macrophages against *Salmonella* relies heavily on the autophagic defenses, in particular the LAP pathway.

4. Autophagy as a host defense response

4.1 General introduction to autophagy

Macroautophagy (hereafter referred to as autophagy or canonical autophagy) is a catabolic degradative process. It was initially characterized as a bulk degradation process, induced by nutrient deprivation (Ohsumi, 2014). During this process, a double membrane autophagosome is formed to deliver cytoplasmic components to the lysosome (**Fig.1**)(Lamb et al., 2013). The formation of the autophagosome begins with the appearance of a small membrane sac, named isolation membrane or phagophore. Altogether, the process of autophagy includes five steps: initiation, nucleation, elongation, maturation and degradation (**Fig.3**, **Table 1**). In the following sections, each of these steps will be discussed respectively. Throughout the autophagy pathway, membrane structures and vesicles are subject to membrane insertion of a lipidated form of microtubule-associated protein 1A/1B-light chain 3 (LC3), which is a member of the ATG8 family of proteins. LC3 is the most frequently used marker to identify the association of pathogens with the autophagy pathway in experimental settings (Muñoz-Sánchez et al., 2020).

4.1.1 Initiation

Initiation of autophagy involves signal transmission to the membrane source, mostly the endoplasmic reticulum or plasma membrane, from where the formation of phagophores occurs (Lamb et al., 2013). In mammals, phagophore formation is primarily induced by the unc-51 like autophagy activating kinase (ULK) complex, which consists of ULK1/ULK2, autophagy-related genes (ATG)13, ATG101 and focal adhesion kinase family kinase, interacting protein of 200 kDa (FIP200) (Chang and Neufeld, 2009).

There are five ULK1 homologues (ULK1, ULK2, ULK3, ULK4 and serine/threonine kinase 36) identified now, but only ULK1 and ULK2 are involved in the autophagy process. They share 78% sequence identity in their kinase domains and are believed to interact with the same components (Zachari and Ganley, 2017). ULK1 contains a serine-threonine kinase domain at its N-terminal end, a positively charged activation loop, several LC3-interacting region (LIR) motifs and an early autophagy targeting/tethering (EAT) domain at the C-terminal end. The activation loop is involved in regulating the kinase activity and recognizing substrates. The EAT domain contains two microtubule-interacting and transport domains that binds to

ATG13 (Lin and Hurley, 2016). ATG13 forms a heterodimer with ATG101 through its HORMA domain, and bridges the interaction of ULK1 with FIP200. Interaction of ULK1 with ATG13 and FIP200 results in increased ULK1 kinase activity and stability (Zachari and Ganley, 2017).

The activity of the ULK complex is negatively regulated by mammalian target of rapamycin complex 1 (mTORC1), and by other signaling pathways, such as AMP-activated protein kinase (AMPK). In full nutrient conditions, ULK1/2 is phosphorylated by binding to mTORC1, thus inhibiting autophagy initiation. Similarly, ATG13 is phosphorylated by mTORC1 and inhibits the interaction of other components. When under starvation, mTORC1 is inactivated, and thus ULK1 and ATG13 are dephosphorylated, which results in increased ULK1 kinase activity and autophagy initiation. Upon activation, ULK1/2 phosphorylates the substrates FIP200 and ATG13 (Lamb et al., 2013). In addition, ULK1/2 phosphorylates the PI3KC3 complex mediating the subsequent nucleation process (Lin and Hurley, 2016).

4.1.2 Nucleation

The PI3KC3 complex, required for nucleation, consists of three main components: lipid kinase vacuolar protein sorting 34 (VPS34), the serine/threonine protein kinase VPS15, and the regulatory subunit BECN1 (Lamb et al., 2013). VPS34 produces PI3P directly from phosphatidylinositol, which is crucial in several membrane trafficking pathways, including phagosome maturation and autophagosome biogenesis. The activity is regulated by VPS15 (Burman and Ktistakis, 2010; Stjepanovic et al., 2017). In addition, BECN1 can form an interaction with different proteins to modulate the function of PI3KC3, resulting in three distinct PI3KC3 complexes related to autophagy (Wong et al., 2018).

The first PI3KC3 complex contains ATG14L (also called Barkor), which is required for inducing autophagy. The cysteine-rich domain at the N-terminus of ATG14L is found to direct the PI3KC3 complex to the phagophore initiation sites at the endoplasmic reticulum (Matsunaga et al., 2009). The PI3P produced by the PI3KC3 complex can be recognized by early autophagic effector proteins, such as ATG21, WD-repeat domain phosphoinositide-interacting proteins (WIPI) and double FYVE-containing protein 1 (Polson et al., 2010), which recruit LC3, ATG9 and ATG12 for autophagosome formation (Obara et al., 2008). Besides, ATG14L is found to increase VPS34 kinase activity and thereby upregulates autophagy (Zhong et al., 2009).

The second PI3KC3 complex contains UV radiation resistance-associated gene (UVRAG). The role of UVRAG in early autophagy is not entirely clear.

Previous studies suggested that UVRAG upregulates autophagy by interacting with the PI3KC3 complex (Liang et al., 2006). However, it was then found that the interaction of UVRAG with the PI3KC3 complex promotes endosomal trafficking and autophagosome maturation, while not mediating autophagosome formation (Liang et al., 2008).

The third PI3KC3 complex contains both UVRAG and RUN domain and cysteine-rich domain containing BECN1-interacting protein (Rubicon). Unlike the first complex, this complex downregulates autophagy, due to interaction of UVRAG and Rubicon with the PI3KC3 complex (Wirth et al., 2013). In line with this inhibitory function, knockdown of *Rubicon* promotes the maturation steps in autophagy (Matsunaga et al., 2009). The inhibition of autophagosome maturation by Rubicon is mediated by interaction with Rab7, which is known to promote the fusion of autophagic vesicles (Bhargava et al., 2020).

4.1.3 Elongation

After nucleation, there are two essential ubiquitin-like conjugation systems responsible for vesicle elongation: the ATG12 and LC3 pathways. ATG12 is activated by the E1-like enzyme ATG7 and transferred to an E2-like enzyme ATG10, and then is conjugated to ATG5 and binds ATG16L1 to form the dimeric ATG12–ATG5–ATG16L1 complex (Yin et al., 2016). The ATG12–ATG5–ATG16L1 complex is recruited to the membrane, where it functions as an E3-like ligase to mediate the lipidation of LC3 and its subfamily members GABA receptor-associated protein (GABARAP). In the cytosol, LC3 exists in its soluble form as pro-LC3. The C-terminal glycine residue of pro-LC3 is proteolytically cleaved by ATG4 family proteases resulting in the formation of the LC3-I isoform. LC3-I then binds to a cysteine residue in ATG7 and is subsequently transferred to the E2-like enzyme ATG3, which mediates the conjugation of LC3-I to the head of phosphatidyl ethanolamine (PE) with the help of the ATG12–ATG5–ATG16L1 complex. While *in vivo* the main substrate is PE, phosphatidylserine can also act as a substrate for LC3 conjugation *in vitro* (Martens and Fracchiolla, 2020). The lipidated form of LC3-I is named LC3-II. LC3-II is conjugated to both the inner and outer membranes of autophagosomes, where it has distinct functions (Nieto-Torres et al., 2021). The inner membrane LC3-II interacts with different receptors mediating the recognition of specific substrates, while LC3-II in the outer membrane binds to proteins that function in vesicle fusion, such as FYVE and coiled-coil domain containing 1 and PLEKHM1 (Ichimura and Komatsu, 2010; McEwan et al., 2015; Pankiv et al., 2010). The conjugation of LC3-II to the autophagosomal

membrane is reversible by ATG4-mediated cleavage (Martens and Fracchiolla, 2020).

4.1.4 Maturation and degradation

After completing the formation of autophagosomes, they can fuse with endosomes to become amphisomes (Lefebvre et al., 2018). Autophagosomes or amphisomes fuse with lysosomes to form autolysosomes. Maturation requires different regulators such as soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins, tethering proteins, and Rab GTPases. Rab GTPases recruit tethering proteins to form a bridge, where they assist SNARE proteins to physically interact with each other to drive vesicle fusions (Nakamura and Yoshimori, 2017; Zhao et al., 2021).

Rab7 plays important roles in the maturation of phagosomes, late endosomes, and autophagosomes (Zhang et al., 2009). Like other GTPase signaling proteins, Rab7 is activated by conversion from the GDP-bound form into the GTP-bound form. After activation, Rab7 interacts with different proteins involved in vesicle transport and fusion. FYVE and coiled-coil domain containing 1 is reported to interact with Rab7 to regulate transport of autophagosomes along microtubules (Pankiv et al., 2010). PLEKHM1 interacts with Rab7 to facilitate late endosomal and lysosomal vesicles maturation (Van Wesenbeeck et al., 2007). PLEKHM1 has an LC3-interacting region that allows it to bind to autophagosomal membranes and interact with the homotypic fusion and protein sorting (HOPS) complex, whereby it connects the endocytic and autophagy pathways (McEwan et al., 2015). The HOPS complex functions as a tethering factor, mediating autophagosome maturation by interacting with Rab7 (Jiang et al., 2014; Wang et al., 2011).

The SNAREs, which are the actual effectors of vesicle fusions, are categorized into Q-SNAREs and R-SNAREs, based on the Q or R amino acid residue. Q-SNAREs are further subcategorized as Qa-, Qb- and Qc-SNAREs. Q-SNAREs interact with R-SNAREs to form a bridge for the fusion between two vesicles (Nakamura and Yoshimori, 2017). The fusion of autophagosomes with late endosomes and/or lysosomes are facilitated by Qa autophagosomal membrane syntaxin 17, Qbc synaptosome associated protein 29 (SNAP29) and R-SNARE vesicle associated membrane protein 8 (VAMP8) (Itakura et al., 2012). Autophagosome-lysosome fusion is also promoted by R-SNARE YKT6 interacting with SNAP29 and lysosomal-localized syntaxin 7 (Qa) (Matsui et al., 2018), as well as by the interaction of VAMP8 with vesicle transport through interaction with Vesicle Transport through Interaction with t-SNAREs 1B (VTI1B) (Furuta et al., 2010).

Besides Rab GTPases and SNAREs, other proteins on the outer membrane of autophagosomes contribute to promoting vesicle fusions, including LC3 itself and its GABARAP subfamily members. It is suggested that LC3 and GABARAP proteins recruit PLEKHM1 for autophagosome-lysosome fusion (Nguyen et al., 2016). Loss of GABARAPs is found to decrease autophagic flux and lead to the accumulation of ubiquitin aggregates in the cytosol after autophagy induction (Vaites et al., 2018).

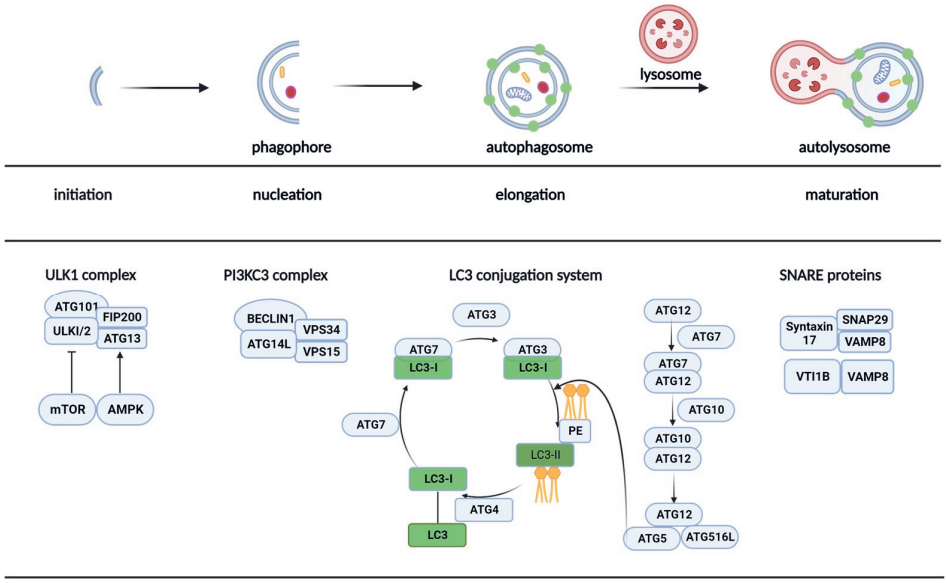

Figure 3. Schematic representation of the autophagy pathway. Basal autophagy is modulated by different signaling pathways, including the mTOR and AMPK pathways. To activate autophagy signal transduction proteins of these pathways interact with the ULK1 complex, which includes ULK1/2, ATG13, FIP2000 and ATG101. The activated ULK1 complex directly interacts with PI3KC3 (VPS34-BECLIN1-ATG14L complex) to form the phagophore. The elongation of the phagophore proceeds with the LC3 conjugation system and the ATG5–ATG12-ATG16L1 conjugation system to form a closed autophagosome. The closed autophagosome then fuses with lysosomes to degrade the caputured cargo. This maturation and vesicle fusion process is mediated by SNARE proteins. (figure created with Biorender).

Table1. The core ATG-related proteins involved in autophagosome formation

	Mammals	Features and function
ULK complex	ULK1 and ULK2	Initiation
	ATG13	
	FIP200	
	ATG101	
PI3KC3 complex (Beclin 1 complex)	VPS34	Nucleation
	VPS15	
	Beclin 1	
	ATG14L	
	AMBRA1	
LC3 conjugation system	LC3A/B/C, GABARAP, GABARAPL1/2/3	Elongation
	ATG7	
	ATG 4A-D	
	ATG3	
ATG12-ATG5-ATG16L1 conjugation	ATG5	
	ATG10	
	ATG12	
	ATG16L1/2	
Others	ATG2A/B	closure of isolation membranes
	ATG9L1/2	autophagosome formation
	WIPI1/2/3/4	binding to PI3P on the autophagosome

4.2 Receptor-mediated selective autophagy

In contrast to bulk autophagy, which manifests itself by random sequestration of substrates (cargo) from the cytosol, selective autophagy captures substrates in a selective, receptor-mediated manner. Many types of selective autophagy are distinguished, based on the substrate specificity, for example aggrephagy (protein aggregates), mitophagy (mitochondria), and xenophagy (microbial invaders). In most cases these substrates are first tagged by ubiquitin, following which they are recognized by the selective autophagy receptors (Sharma et al., 2018). These receptors physically bridge the substrate and the autophagosomal membrane, resulting in the engulfment of the substrate by the autophagic vesicle. To carry out this function, most of the autophagy receptors have ubiquitin-binding domains (e.g., UBA and

UBAN) for substrate recognition and LC3-interacting regions (LIR) (Xu et al., 2015). The LIR motifs interact with LC3 subfamily proteins in autophagy, thus tethering the substrate to the autophagosomal membrane (Kirkin et al., 2009). The sequestome 1-like receptor family consists of many proteins, of which sequestome-1 (p62/SQSTM1), optineurin (OPTN), neighbor of BRCA1 gene 1 (NBR1), nuclear dot protein 52 kDa (NDP52), Tax1 binding protein 1 (TAX1BP1) are well known to be involved in bacterial infection.

The most well-studied mammalian selective autophagy receptor is p62 (Pankiv et al., 2007). p62 has five domains: LIR, UBA, Phox and Bem1p domain (PB1), Keap1-interacting region (KIR), and ZZ-type zinc finger domain (ZZ) (Komatsu et al., 2010). The ZZ domain is related to glucose regulation and NF-κB signaling (Kim and Do Hoon Kwon, 2016). The PB1 domain acts as a scaffold module which forms heterodimers or homo-oligomers through protein-protein interactions (Rogov et al., 2014), which is required for effective degradation of the receptor-substrate complex by autophagy. In fact, the aggregates consisting of ubiquitinated proteins and p62 oligomers may act as a nucleating scaffolds for autophagosome formation (Fujita et al., 2013). p62 selectively targets specific protein aggregates and organelles but also pathogens for autophagic degradation. Besides, p62 has been found to regulate important processes through different signaling pathways, including NF-κB signaling (Xu et al., 2015). The importance of p62 in xenophagy is well demonstrated by its role in controlling the invading bacteria Mtb (Franco et al., 2017), *Shigella flexneri* (Mostowy et al., 2011) and *S.* Typhimurium (Zheng et al., 2009). In agreement, previous work in our laboratory showed that loss-of-function mutation of p62 reduces autophagic targeting of the fish pathogen Mm, and increases susceptibility of the zebrafish host to Mm infection (Zhang et al., 2019).

OPTN, like p62, contains an UBAN domain, a LIR domain, a leucine zipper domain and a C-terminal zinc finger domain (Kim and Do Hoon Kwon, 2016). There is a unique serine residue upstream of the LIR sequence, which may be phosphorylated by Ser/Thr TANK-binding kinase 1 (TBK1), a kinase that also regulates p62 activity (Pilli et al., 2012; Wild et al., 2011). Previous studies have shown that OPTN is required to restrict the growth of *S. enterica* (Wild et al., 2011). Studies in our laboratory have shown that Optn, similar to p62, is required for the host defense against Mm (Zhang et al., 2019).

NBR1, also consists of LIR, UBA, PB1 and ZZ domains, similarly to p62. Through the PB1 domain, NBR1 can interact with itself or with p62, acting individually or cooperatively with p62 in selective autophagy (Lamark et al., 2009). NBR1 mainly works by interacting with LC3 (Rozenknop et al., 2011), and is found to target polyubiquitylated aggregates and organelles for

selective degradation (Odagiri et al., 2012). NBR1 has been shown to link to mycobacterial infection as well but knowledge of its role in antibacterial autophagy remains limited (Franco et al., 2017).

NDP52 is distinguished by binding to myosin VI, and is ubiquitously expressed in various tissues and cells. NDP52 consists of an non-canonical LIR (CLIR), ubiquitin-binding zinc finger (UBZ) domain, galectin-8-binding region, and skeletal muscle and kidney-enriched inositol phosphatase carboxyl homology (SKICH) domain (Kim et al., 2013). NDP52 acts as a part of the TBK1 signaling complex in both *Salmonella enterica* and Mtb infection (Thurston et al., 2009; Watson et al., 2012). Unlike NBR1, NDP52 appears to function independently from p62 and does not function in the same pathway (Cemma et al., 2011). By interacting with ubiquitinated cargoes, NDP52 facilitates the assembly of the autophagic membrane.

TAX1BP1, is considered to be a paralogue of NDP52. During *Salmonella* infection, TAX1BP1 is recruited to ubiquitinate *Salmonella* and thus induce antibacterial autophagy (Tumbarello et al., 2015). Similary, TAX1BP1 is induced by Mtb, which in turn targets ubiquinated Mtb for autophagy. (Budzik et al., 2020).

4.3. LC3-associated phagocytosis (LAP)

While canonical autophagy is characterized by the conjugation of LC3 or other ATG8 family members to the autophagosomal double membrane, the autophagy machinery can also recruit these proteins to vesicles with a single membrane. This process is named conjugation of ATG8 to endolysosomal single membranes (CASM) (Durgan and Florey, 2021). The host defense pathway named LAP is one example of CASM. During LAP, LC3 is recruited to phagosomes, thereby forming LAPosomes (Sanjuan et al., 2007). Studies of LAP in the context of various infections confirm the innate host defense function, although some pathogens exploit the pathway for intracellular survival (Grijmans et al., 2022).

In contrast to canonical autophagy, which is initiated by the ULK1 complex, LAP is independent of this complex. Instead, LAP is induced upon pathogen recognition by different receptors, such as Toll-like receptors, Fc receptors, and scavenger receptors such as TIM4 (**Fig.4**) (Grijmans et al., 2022; Martinez et al., 2011; Sanjuan et al., 2007). After phagocytosis of the pathogen, PI3KC3, consisting of BECN1, VPS15, VPS34, UVRAG and Rubicon, produces PI3P, which is delivered onto the phagosomal membrane (**Fig.4**) (Grijmans et al., 2022; Martinez et al., 2015). PI3P then provides a binding site for LC3 conjugation and stabilizes NADPH oxidase (Martinez et al., 2011; Wang et al.,

2022).

The phagosomal NADPH oxidase is a multiprotein complex consisting of three cytosolic elements (p67 phox, p47 phox, and p40 phox), a low-molecular-weight G protein (Rac 1 or Rac 2), and two membrane-bound components (gp91 phox and p22 phox) (Babior, 2004). In response to the phagocytosis of pathogens, the cytosolic components are recruited to the membrane to assemble the functional NADPH oxidase. Once activated, NADPH oxidase produces ROS, which is considered to be a hallmark of LAP (**Fig.4**). This intraphagosomal ROS production is dependent on the activity of Rubicon (Martinez et al., 2015). Rubicon activates PI3KC3 to produce PI3P, which enables p40 phox to bind to the membrane lipid. Furthermore, Rubicon directly interacts with p22 phox to stabilize the NADPH oxidase (Wong et al., 2018).

The mechanism of LC3 conjugation in LAP is similar to that in canonical autophagy. LC3 is cleaved by ATG4 to form LC3-I, which is then lipidated through the action of two conjugation systems, the ATG12 and the LC3 conjugation system, including ATG12-ATG5-ATG16L1 complex, resulting in the formation of LC3-II on the LAPosome. However, the functional domain of ATG16L1 differs between canonical autophagy and LAP. In canonical autophagy, the coiled coil domain at the N-terminal end of ATG16L1 is responsible for linking to PI3P-enriched sites where LC3 conjugation occurs. In LAP, the WD40 repeat domain at the C-terminal end of ATG16L1 has been found to be involved in the LC3-conjugation process (Wang et al., 2022). This WD40 repeat domain interacts with the mature V-ATPase induced by ROS, which drives subsequent LC3 conjugation (Hooper et al., 2022).

Following the discovery that ROS production is a prerequisite for LC3 conjugation to phagosomes (Martinez et al., 2015), several studies have aimed at understanding the precise connection between LC3 conjugation and ROS. It has been observed that membrane damaging agents can trigger LC3 conjugation to the endosomal membrane (Florey et al., 2015). Similarly, plasma membrane damage induced by *Listeria monocytogenes* has been shown to activate an autophagy-mediated membrane repair mechanism through interaction of ATG16L1 with ATG5 and ATG12 (Tan et al., 2018). Considering that ROS generated during LAP oxidizes lipids and changes the membrane structure, it is hypothesized that such effects of ROS may also induce LC3 conjugation to the phagosomal membrane (Martens and Fracchiolla, 2020). In addition, ROS is proposed to inactivate ATG4B, thereby preventing the cleavage of LC3 and contributing to the stabilization of the LAPosome (Ligeon et al., 2021). Finally, ROS production consumes H+ and thus increases the pH inside the phagosome, which has recently been shown

to promote V-ATPase assembly, which accelerates LC3 conjugation to the phospholipids on the phagosomal membrane (Hooper et al., 2022).

Previous studies in our laboratory have demonstrated the role of LAP in zebrafish embryos in response to intravenous *Salmonella* infection (Masud et al., 2019). Knockdown of *atg5*, which participates in both autophagy and LAP, reduced Lc3 colocalization with *Salmonella,* whereas knockdown of *atg13*, which is involved in initiation of canonical autophagy, has no effect on infection. Additionally, knockdown of the gene encoding Rubicon and of Cyba, encoding the p22 phox component of NADPH oxidase, significantly impaired the defense system. Therefore, it was concluded that LAP, rather than canonical autophagy, is the predominant defense mechanism during systemic *Salmonella* infection in this model. In case of Mm infection, LAPosome formation has been observed in RAW 264.7 macrophages and this response was dependent on the ESX-1 virulence system (Lerena and Colombo, 2011). However, LAP has less impact on Mtb due to the secretion of CpsA by this pathogen, which inhibits NADPH oxidase (Köster et al., 2017).

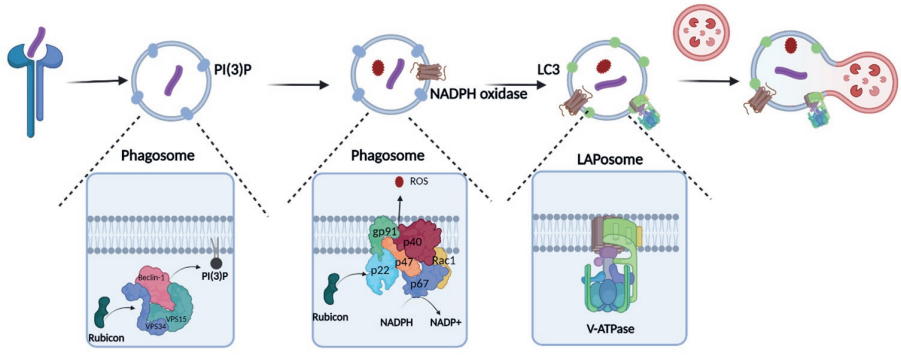

Figure 4. Schematic representation of the LC3-associated phagocytosis (LAP) pathway. LAP is a modified form of phagocytosis that relies on part of the autophagy machinery. During LAP, after phagocytosis, PI3P is generated by the PI3KC3 complex interacting with Rubicon and delivered onto the phagosomal membrane. Rubicon is binding to NADPH oxidase subunit p22 phox to stabilize NADPH oxidase (NOX2) for production of reactive oxygen species (ROS), which is an hallmark step in LAP. ROS production consumes H+ and thus increases the pH inside the phagosome, which promotes V-ATPase assembly on the phagosomal membrane. V-ATPase then binds to ATG16L1, forming the ATG5-ATG12-ATG16L1 complex for LC3 conjugation to the phagosomal membrane.

5. Introduction to the DRAM family

5.1 DRAM1

While autophagy is orchestrated by the core autophagy machinery discussed above, other proteins have been shown to exert regulatory functions. Damage Regulated Autophagy Modulator 1 (DRAM1) is one of such autophagy modulators. Human DRAM1 was first identified as a protein induced by UV-stress and consists of 238 amino acids with six hydrophobic transmembrane regions (Crighton et al., 2006). DRAM1 is highly conserved among different species. Its isoforms are found to localize on the lysosomal membrane, but also on peroxisomes, endoplasmic reticulum, the plasma membrane, and autophagosomes (Mah et al., 2012).

In the UV-stress response and during various malignancies, DRAM1 mediates autophagy and induces apoptosis after induction by transcription factors p53 and p73 (Crighton et al., 2007; Crighton et al., 2006). *DRAM1* knockdown decreased the association of p62 to autophagosomes as well as autophagy-mediated degradation (Galavotti et al., 2013; Nagata et al., 2018). In our laboratory, utilizing the Mm zebrafish infection model and human M2 macrophages infected with Mtb, it was found that Dram1/DRAM1 is induced after mycobacterial infection by the TLR-adaptor MYD88 and transcription factor NF-κB, whereas it was independent of p53 (van der Vaart et al., 2014). Furthermore, Dram1 induction after Mm infection in zebrafish was found to mediate the autophagic defense against Mm proliferation (van der Vaart et al., 2014). Overexpression of *dram1* increased the colocalization of Lc3 and Mm, while knockdown of *dram1* decreased Lc3 colocalization with Mm. Furthermore, we observed that Dram1 deficiency would eventually lead to increased levels of pyroptotic cell death in Mm-infected macrophages, thereby disseminating the mycobacterial infection (Zhang et al., 2020).

To understand the mechanism of DRAM1-mediated autophagy regulation, *ULK1*, *ATG13* and *DRAM1* were overexpressed in HEK293 cells, and it was found that DRAM1 increased the ULK1-ATG13 interaction in a dose-dependent manner (Lu et al., 2019). These results indicate that DRAM1 helps to initiate autophagy and increases autophagosome formation (Lu et al., 2019). Correspondingly, several studies showed that overexpression of DRAM1/Dram1 increases the presence of punctae formed by endogenous LC3 or GFP-LC3 (Crighton et al., 2006; Lu et al., 2019; Mah et al., 2012; van der Vaart et al., 2014). Besides promoting autophagosome formation, DRAM1 is thought to facilitate fusion events in the autophagosomal-lysosomal pathway as well. Using mRFP-GFP tandem fluorescently tagged LC3 to

monitor the process of autophagy maturation, it was shown that *DRAM1* knockdown inhibits the degradation of this marker, suggesting that DRAM1 is required to mediate autophagosome-lysosome fusion (Zhang et al., 2013). Similarly, studies from our laboratory using the zebrafish model showed that *dram1* overexpression increases lysosomal acidification of Mm, while this was impaired by *dram1* knockdown or mutation (van der Vaart et al., 2014; Zhang et al., 2020). It is known that conversion of PI3P to PI(3,5)P2 by PIKFYVE is required for maturation of early endosomes to late endosomes. By expressing an mCherry-Dram1 fusion protein, Dram1-positive vesicles were found to interact and fuse with early endosomes that subsequently mature to acidic vesicles in a PIKFYVE-dependent manner (van der Vaart et al., 2020). However, further details of the molecular mechanisms underlying the role of DRAM1/Dram1 in autophagic defense remain to be elucidated.

5.2 DRAM2

DRAM2 is the closest homologue of DRAM1. It also has a six transmembrane domain structure and shows 37% amino acid similarity with DRAM1. It is mainly localized in the lysosome, like DRAM1. Functionally, DRAM2 is found to downregulate tumor growth, reminiscent of the association of DRAM1 with several types of cancer (Park et al., 2009). DRAM2 also resembles DRAM1 functionally, since it has been shown to play a role in inducing autophagy. Specifically, overexpression of *DRAM2* induced the formation of GFP-LC3 punctae and increased the level of LC3-II, while silencing of *DRAM2* reduced the number of GFP-LC3 punctae, indicating that DRAM2 is required for efficient autophagosome formation (Yoon et al., 2012). Corroborating these results, DRAM2 was found to interact with BECN1 and UVRAG to replace Rubicon from the BECN1 complex, thereby promoting the activity of PI3KC3 and activating autophagy (Kim et al., 2017). In addition, DRAM2 was found to increase the acidification of Mtb-containing phagosomes and antimicrobial activities in human macrophages. Knockdown of *DRAM2* decreased the colocalization of LC3 and LAMP2 with Mtb, indicating that DRAM2 is also involved in autophagosme maturation (Liu et al., 2020). Mtb is found to induce microRNAs MIR144* and miR-125b-5p to inhibit antimicrobial and inflammatory responses by targeting DRAM2 (Kim et al., 2017; Liu et al., 2020). DRAM2 is also targeted by microRNA MIR125B1, which blocks the autophagy-lysosomal pathway and induces acute promyelocytic leukemia (Zeng et al., 2014).

5.3 Other DRAM family members

DRAM3 shares 30% amino acid identity with DRAM1 and is predicted to have six transmembrane domains, similarly to DRAM1. DRAM3 is found to localize to the plasma membrane, endosomes and lysosomes, but not to the endoplasmic reticulum, Golgi apparatus, phagophores or autophagosomes (Mrschtik et al., 2015). DRAM3 regulates autophagic flux and promote cell survival in a similar way as DRAM1, but independent of p53. Different from DRAM1, DRAM3 modulates autophagy in unstarved conditions and is degraded in starved cells (Mrschtik and Ryan, 2016). DRAM4 and DRAM5 have recently been identified. They are encoded by *TMEM150C* and *TMEM150A*. DRAM4 and DRAM5 are 48% similar to each other in amino acid sequence, and show 38% and 35% similarity with DRAM1. DRAM4 is found to localize to endosomes and DRAM5 to the plasma membrane. DRAM4 and DRAM5 are not regulated by p53, like DRAM3, but induced by nutrient deprivation. DRAM4 blocks the autophagic pathway at a phase after autophagosome formation, whereas DRAM5 enhances both autophagy and cell survival (Barthet et al., 2022).

6. Outline of the thesis

While the autophagy modulator DRAM1 has been implicated in the autophagic host defense, the mechanisms underlying its mode of action remain to be elucidated. The aim of the work described in this thesis was to gain a better understanding of the autophagy-related pathways and cellular processes that are dependent on the function of DRAM1. We focused on infections with two intracellular bacterial pathogens, *Mycobacterium marinum* (Mm) and *Salmonella* Typhimurium (ST). Furthermore, we exploited a combination of *in vitro* and *in vivo* models using loss- and gain-of-function approaches in RAW 264.7 mouse macrophages and zebrafish embryos.

The introductory Chapter 1 describes the characteristics of the studied pathogens and the molecular and cellular details of autophagic host defense pathways, including xenophagy and LC3-associated phagocytosis (LAP). It also reviews the current knowledge about xenophagy and LAP in infections with mycobacteria and *Salmonella* and the function of DRAM1/Dram1 in the control of bacterial infection.

In Chapter 2 we demonstrate that DRAM1/Dram1 is required for the host resistance to ST infection and that it promotes both LC3/Lc3 and reactive oxygen responses to this pathogen in RAW 264.7 macrophages and zebrafish. These data support a role for DRAM1/Dram1 in the LAP-mediated host

defense.

In Chapter 3, we set out to study the interaction between Dram1 and two xenophagy receptors, p62 and Optn. Using zebrafish mutant lines and overexpression experiments we show that Dram1, p62 and Optn can protect against Mm independently of each other.

In Chapter 4 we generated *Dram1* knockdown RAW 264.7 macrophage cell lines and found that DRAM1 is required for the recruitment of LC3, acidification of mycobacteria-containing vesicles, and fusion of lysosomes with mycobacteria-containing vesicles. Furthermore, we show that *Dram1* knockdown impairs the ability of macrophages to control Mm infection.

In Chapter 5 we continue the research using RAW 264.7 macrophages and show that DRAM1 is necessary for the antimicrobial peptide Fau to be delivered into Mm-containing vesicles. In addition, we studied Rab GTPases and lysosomal markers to investigate how DRAM1 deficiency impacts on different vesicle maturation stages. Finally, we were able to connect a SNARE family protein, VTI1B, to the DRAM1-mediated antibacterial mechanism.

Finally, Chapter 6 summarizes the results of the thesis, discusses the findings, and provides ideas for further research into the mechanism of DRAM1 action in the defense against bacterial infections.

References

Agbor, T.A., and McCormick, B.A. (2011). Salmonella effectors: important players modulating host cell function during infection. Cellular microbiology *13*, 1858-1869.

Ali, S., Champagne, D.L., Spaink, H.P., and Richardson, M.K. (2011). Zebrafish embryos and larvae: a new generation of disease models and drug screens. Birth Defects Research Part C: Embryo Today: Reviews *93*, 115-133.

Augenstreich, J., and Briken, V. (2020). Host cell targets of released lipid and secreted protein effectors of Mycobacterium tuberculosis. Frontiers in Cellular and Infection Microbiology *10*, 595029.

Authority, E.F.S., Prevention, E.C.f.D., and Control (2018). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSa Journal *16*, e05500.

Babior, B.M. (2004). NADPH oxidase. Current opinion in immunology *16*, 42-47.

Barthet, V.J., Mrschtik, M., Kania, E., McEwan, D.G., Croft, D., O'Prey, J., Long, J.S., and Ryan, K.M. (2022). DRAM-4 and DRAM-5 are compensatory regulators of autophagy and cell survival in nutrient-deprived conditions. The FEBS Journal *289*, 3752-3769.

Bedell, V.M., Westcot, S.E., and Ekker, S.C. (2011). Lessons from morpholino-based screening in zebrafish. Briefings in functional genomics *10*, 181-188.

Bhargava, H.K., Tabata, K., Byck, J.M., Hamasaki, M., Farrell, D.P., Anishchenko, I., DiMaio, F., Im, Y.J., Yoshimori, T., and Hurley, J.H. (2020). Structural basis for autophagy inhibition by the human Rubicon–Rab7 complex. Proceedings of the National Academy of Sciences *117*, 17003-17010.

Blanc-Potard, A.-B., and Groisman, E.A. (1997). The Salmonella selC locus contains a pathogenicity island mediating intramacrophage survival. The EMBO journal *16*, 5376-5385.

Bloom, D.E., and Cadarette, D. (2019). Infectious disease threats in the twenty-first century: strengthening the global response. Frontiers in immunology *10*, 549.

Budzik, J.M., Swaney, D.L., Jimenez-Morales, D., Johnson, J.R., Garelis, N.E., Repasy, T., Roberts, A.W., Popov, L.M., Parry, T.J., and Pratt, D. (2020). Dynamic post-translational modification profiling of Mycobacterium tuberculosis-infected primary macrophages. Elife *9*, e51461.

Burki, T.K. (2018). The global cost of tuberculosis. The Lancet Respiratory Medicine *6*, 13.

Burman, C., and Ktistakis, N.T. (2010). Autophagosome formation in mammalian cells. Paper presented at: Seminars in immunopathology (Springer).

Bussi, C., and Gutierrez, M.G. (2019). Mycobacterium tuberculosis infection of host cells in space and time. FEMS microbiology reviews *43*, 341-361.

Carden, S.E., Walker, G.T., Honeycutt, J., Lugo, K., Pham, T., Jacobson, A., Bouley, D., Idoyaga, J., Tsolis, R.M., and Monack, D. (2017). Pseudogenization of the secreted effector gene sseI confers rapid systemic dissemination of S. Typhimurium ST313 within migratory dendritic cells. Cell host & microbe *21*, 182-194.

Cemma, M., Kim, P.K., and Brumell, J.H. (2011). The ubiquitin-binding adaptor proteins p62/SQSTM1 and NDP52 are recruited independently to bacteria-associated microdomains to target Salmonella to the autophagy pathway. Autophagy *7*, 341-345.
Chakaya, J., Petersen, E., Nantanda, R., Mungai, B.N., Migliori, G.B., Amanullah, F., Lungu, P., Ntoumi, F., Kumarasamy, N., and Maeurer, M. (2022). The WHO Global Tuberculosis 2021 Report–not so good news and turning the tide back to End TB. International Journal of Infectious Diseases *124*, S26-S29.
Chang, Y.-Y., and Neufeld, T.P. (2009). An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. Molecular biology of the cell *20*, 2004-2014.
Chaudhary, N., Weissman, D., and Whitehead, K.A. (2021). mRNA vaccines for infectious diseases: principles, delivery and clinical translation. Nature reviews Drug discovery *20*, 817-838.
Chauhan, S., Kumar, S., Jain, A., Ponpuak, M., Mudd, M.H., Kimura, T., Choi, S.W., Peters, R., Mandell, M., and Bruun, J.-A. (2016). TRIMs and galectins globally cooperate and TRIM16 and galectin-3 co-direct autophagy in endomembrane damage homeostasis. Developmental cell *39*, 13-27.
Chirakos, A.E., Balaram, A., Conrad, W., and Champion, P.A. (2020). Modeling tubercular ESX-1 secretion using Mycobacterium marinum. Microbiology and Molecular Biology Reviews *84*, 10.1128/mmbr. 00082-00019.
Clay, H., Davis, J.M., Beery, D., Huttenlocher, A., Lyons, S.E., and Ramakrishnan, L. (2007). Dichotomous role of the macrophage in early Mycobacterium marinum infection of the zebrafish. Cell host & microbe *2*, 29-39.
Cohen, A., Mathiasen, V.D., Schön, T., and Wejse, C. (2019). The global prevalence of latent tuberculosis: a systematic review and meta-analysis. European Respiratory Journal *54*.
Colucci-Guyon, E., Tinevez, J.-Y., Renshaw, S.A., and Herbomel, P. (2011). Strategies of professional phagocytes in vivo: unlike macrophages, neutrophils engulf only surface-associated microbes. Journal of cell science *124*, 3053-3059.
Crighton, D., O'Prey, J., Bell, H., and Ryan, K. (2007). p73 regulates DRAM-independent autophagy that does not contribute to programmed cell death. Cell Death & Differentiation *14*, 1071-1079.
Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P.R., Gasco, M., Garrone, O., Crook, T., and Ryan, K.M. (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell *126*, 121-134.
Cronan, M.R., and Tobin, D.M. (2014). Fit for consumption: zebrafish as a model for tuberculosis. Disease models & mechanisms *7*, 777-784.
Crump, J.A., Sjölund-Karlsson, M., Gordon, M.A., and Parry, C.M. (2015). Epidemiology, clinical presentation, laboratory diagnosis, antimicrobial resistance, and antimicrobial management of invasive Salmonella infections. Clinical microbiology reviews *28*, 901-937.

Davis, J.M., Clay, H., Lewis, J.L., Ghori, N., Herbomel, P., and Ramakrishnan, L. (2002). Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity *17*, 693-702.
De Jonge, M.I., Pehau-Arnaudet, G., Fretz, M.M., Romain, F., Bottai, D., Brodin, P., Honoré, N., Marchal, G., Jiskoot, W., and England, P. (2007). ESAT-6 from Mycobacterium tuberculosis dissociates from its putative chaperone CFP-10 under acidic conditions and exhibits membrane-lysing activity. Journal of bacteriology *189*, 6028-6034.
Deretic, V., Singh, S., Master, S., Harris, J., Roberts, E., Kyei, G., Davis, A., De Haro, S., Naylor, J., and Lee, H.H. (2006). Mycobacterium tuberculosis inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. Cellular microbiology *8*, 719-727.
Durgan, J., and Florey, O. (2021). A new flavor of cellular Atg8-family protein lipidation–alternative conjugation to phosphatidylserine during CASM. Autophagy *17*, 2642-2644.
Ehuwa, O., Jaiswal, A.K., and Jaiswal, S. (2021). Salmonella, food safety and food handling practices. Foods *10*, 907.
Florey, O., Gammoh, N., Kim, S.E., Jiang, X., and Overholtzer, M. (2015). V-ATPase and osmotic imbalances activate endolysosomal LC3 lipidation. Autophagy *11*, 88-99.
Franco, L.H., Nair, V.R., Scharn, C.R., Xavier, R.J., Torrealba, J.R., Shiloh, M.U., and Levine, B. (2017). The ubiquitin ligase Smurf1 functions in selective autophagy of Mycobacterium tuberculosis and anti-tuberculous host defense. Cell host & microbe *21*, 59-72.
Fujita, N., Morita, E., Itoh, T., Tanaka, A., Nakaoka, M., Osada, Y., Umemoto, T., Saitoh, T., Nakatogawa, H., and Kobayashi, S. (2013). Recruitment of the autophagic machinery to endosomes during infection is mediated by ubiquitin. Journal of Cell Biology *203*, 115-128.
Furuta, N., Fujita, N., Noda, T., Yoshimori, T., and Amano, A. (2010). Combinational soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins VAMP8 and Vti1b mediate fusion of antimicrobial and canonical autophagosomes with lysosomes. Molecular biology of the cell *21*, 1001-1010.
Galavotti, S., Bartesaghi, S., Faccenda, D., Shaked-Rabi, M., Sanzone, S., McEvoy, A., Dinsdale, D., Condorelli, F., Brandner, S., and Campanella, M. (2013). The autophagy-associated factors DRAM1 and p62 regulate cell migration and invasion in glioblastoma stem cells. Oncogene *32*, 699-712.
Garai, P., Gnanadhas, D.P., and Chakravortty, D. (2012). Salmonella enterica serovars Typhimurium and Typhi as model organisms: revealing paradigm of host-pathogen interactions. Virulence *3*, 377-388.
Ge, H., Wang, Y., and Zhao, X. (2022). Research on the drug resistance mechanism of foodborne pathogens. Microbial Pathogenesis *162*, 105306.
Goyal, S., Klassert, T.E., and Slevogt, H. (2016). C-type lectin receptors in tuberculosis: what we know. Medical microbiology and immunology *205*, 513-535.

Grijmans, B.J., van der Kooij, S.B., Varela, M., and Meijer, A.H. (2022). LC3-associated phagocytosis and the arms race against bacterial pathogens. Frontiers in Cellular and Infection Microbiology, 1343.
Groschel, M.I., Sayes, F., Simeone, R., Majlessi, L., and Brosch, R. (2016). ESX secretion systems: mycobacterial evolution to counter host immunity. Nat Rev Microbiol *14*, 677-691.
Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I., and Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell *119*, 753-766.
Harvie, E.A., and Huttenlocher, A. (2015). Neutrophils in host defense: new insights from zebrafish. Journal of Leucocyte Biology *98*, 523-537.
Herbomel, P., Thisse, B., and Thisse, C. (1999). Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development *126*, 3735-3745.
Hooper, K.M., Jacquin, E., Li, T., Goodwin, J.M., Brumell, J.H., Durgan, J., and Florey, O. (2022). V-ATPase is a universal regulator of LC3-associated phagocytosis and non-canonical autophagy. Journal of Cell Biology *221*.
Hosseini, R., Lamers, G.E., Hodzic, Z., Meijer, A.H., Schaaf, M.J., and Spaink, H.P. (2014). Correlative light and electron microscopy imaging of autophagy in a zebrafish infection model. Autophagy *10*, 1844-1857.
Houben, D., Demangel, C., Van Ingen, J., Perez, J., Baldeón, L., Abdallah, A.M., Caleechurn, L., Bottai, D., Van Zon, M., and De Punder, K. (2012). ESX-1-mediated translocation to the cytosol controls virulence of mycobacteria. Cellular microbiology *14*, 1287-1298.
Hsu, T., Hingley-Wilson, S.M., Chen, B., Chen, M., Dai, A.Z., Morin, P.M., Marks, C.B., Padiyar, J., Goulding, C., and Gingery, M. (2003). The primary mechanism of attenuation of bacillus Calmette–Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proceedings of the National Academy of Sciences *100*, 12420-12425.
Huang, J., Canadien, V., Lam, G.Y., Steinberg, B.E., Dinauer, M.C., Magalhaes, M.A., Glogauer, M., Grinstein, S., and Brumell, J.H. (2009). Activation of antibacterial autophagy by NADPH oxidases. Proceedings of the national academy of sciences *106*, 6226-6231.
Ibarra, J.A., and Steele-Mortimer, O. (2009). Salmonella–the ultimate insider. Salmonella virulence factors that modulate intracellular survival. Cellular microbiology *11*, 1579-1586.
Ichimura, Y., and Komatsu, M. (2010). Selective degradation of p62 by autophagy. Paper presented at: Seminars in immunopathology (Springer).
Itakura, E., Kishi-Itakura, C., and Mizushima, N. (2012). The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. Cell *151*, 1256-1269.
Jajere, S. (2019). A review of Salmonella enterica with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. Vet World 12: 504–521. Salmonella enterica.

Jenkins, H.E., and Yuen, C.M. (2018). The burden of multidrug-resistant tuberculosis in children. The International Journal of Tuberculosis and Lung Disease *22*, S3-S6.
Jiang, P., Nishimura, T., Sakamaki, Y., Itakura, E., Hatta, T., Natsume, T., and Mizushima, N. (2014). The HOPS complex mediates autophagosome–lysosome fusion through interaction with syntaxin 17. Molecular biology of the cell *25*, 1327-1337.
Kaufmann, S.H., Dorhoi, A., Hotchkiss, R.S., and Bartenschlager, R. (2018). Host-directed therapies for bacterial and viral infections. Nature reviews Drug discovery *17*, 35-56.
Kennedy, R.B., Ovsyannikova, I.G., Palese, P., and Poland, G.A. (2020). Current challenges in vaccinology. Frontiers in immunology *11*, 1181.
Kilinç, G., Saris, A., Ottenhoff, T.H., and Haks, M.C. (2021). Host-directed therapy to combat mycobacterial infections. Immunological reviews *301*, 62-83.
Kim, B.-W., and Do Hoon Kwon, H.K.S. (2016). Structure biology of selective autophagy receptors. BMB reports *49*, 73.
Kim, B.-W., Hong, S.B., Kim, J.H., Kwon, D.H., and Song, H.K. (2013). Structural basis for recognition of autophagic receptor NDP52 by the sugar receptor galectin-8. Nature communications *4*, 1-8.
Kim, J.K., Lee, H.-M., Park, K.-S., Shin, D.-M., Kim, T.S., Kim, Y.S., Suh, H.-W., Kim, S.Y., Kim, I.S., and Kim, J.-M. (2017). MIR144* inhibits antimicrobial responses against Mycobacterium tuberculosis in human monocytes and macrophages by targeting the autophagy protein DRAM2. Autophagy *13*, 423-441.
Kirkin, V., McEwan, D.G., Novak, I., and Dikic, I. (2009). A role for ubiquitin in selective autophagy. Molecular cell *34*, 259-269.
Kleinnijenhuis, J., Oosting, M., Joosten, L.A., Netea, M.G., and Van Crevel, R. (2011). Innate immune recognition of Mycobacterium tuberculosis. Clinical and Developmental Immunology *2011*.
Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., Sou, Y.-S., Ueno, I., Sakamoto, A., and Tong, K.I. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nature cell biology *12*, 213-223.
Köster, S., Upadhyay, S., Chandra, P., Papavinasasundaram, K., Yang, G., Hassan, A., Grigsby, S.J., Mittal, E., Park, H.S., and Jones, V. (2017). Mycobacterium tuberculosis is protected from NADPH oxidase and LC3-associated phagocytosis by the LCP protein CpsA. Proceedings of the National Academy of Sciences *114*, E8711-E8720.
Lamark, T., Kirkin, V., Dikic, I., and Johansen, T. (2009). NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. Cell cycle *8*, 1986-1990.
Lamb, C.A., Yoshimori, T., and Tooze, S.A. (2013). The autophagosome: origins unknown, biogenesis complex. Nature reviews Molecular cell biology *14*, 759-774.
Langenau, D.M., Ferrando, A.A., Traver, D., Kutok, J.L., Hezel, J.-P.D., Kanki, J.P., Zon, L.I., Look, A.T., and Trede, N.S. (2004). In vivo tracking of T cell development, ablation, and engraftment in transgenic zebrafish. Proceedings of the National Academy of Sciences *101*, 7369-7374.

LaRock, D.L., Chaudhary, A., and Miller, S.I. (2015). Salmonellae interactions with host processes. Nature Reviews Microbiology *13*, 191-205.
Lawson, N.D., and Wolfe, S.A. (2011). Forward and reverse genetic approaches for the analysis of vertebrate development in the zebrafish. Developmental cell *21*, 48-64.
Lefebvre, C., Legouis, R., and Culetto, E. (2018). ESCRT and autophagies: Endosomal functions and beyond. Paper presented at: Seminars in cell & developmental biology (Elsevier).
Lerena, M.C., and Colombo, M.I. (2011). Mycobacterium marinum induces a marked LC3 recruitment to its containing phagosome that depends on a functional ESX-1 secretion system. Cellular microbiology *13*, 814-835.
Li, M., Zhao, L., Page-McCaw, P.S., and Chen, W. (2016). Zebrafish genome engineering using the CRISPR–Cas9 system. Trends in Genetics *32*, 815-827.
Liang, C., Feng, P., Ku, B., Dotan, I., Canaani, D., Oh, B.-H., and Jung, J.U. (2006). Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. Nature cell biology *8*, 688-698.
Liang, C., Lee, J.-s., Inn, K.-S., Gack, M.U., Li, Q., Roberts, E.A., Vergne, I., Deretic, V., Feng, P., and Akazawa, C. (2008). Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. Nature cell biology *10*, 776-787.
Ligeon, L.-A., Pena-Francesch, M., Vanoaica, L.D., Núñez, N.G., Talwar, D., Dick, T.P., and Münz, C. (2021). Oxidation inhibits autophagy protein deconjugation from phagosomes to sustain MHC class II restricted antigen presentation. Nature Communications *12*, 1508.
Lin, M.G., and Hurley, J.H. (2016). Structure and function of the ULK1 complex in autophagy. Current opinion in cell biology *39*, 61-68.
Liu, G., Wan, Q., Li, J., Hu, X., Gu, X., and Xu, S. (2020). Silencing miR-125b-5p attenuates inflammatory response and apoptosis inhibition in mycobacterium tuberculosis-infected human macrophages by targeting DNA damage-regulated autophagy modulator 2 (DRAM2). Cell Cycle *19*, 3182-3194.
Lu, T., Zhu, Z., Wu, J., She, H., Han, R., Xu, H., and Qin, Z.-H. (2019). DRAM1 regulates autophagy and cell proliferation via inhibition of the phosphoinositide 3-kinase-Akt-mTOR-ribosomal protein S6 pathway. Cell Communication and Signaling *17*, 1-15.
Luk, C.H., Enninga, J., and Valenzuela, C. (2022). Fit to dwell in many places–The growing diversity of intracellular Salmonella niches. Frontiers in Cellular and Infection Microbiology *12*, 989451.
Ma, Y., Keil, V., and Sun, J. (2015). Characterization of Mycobacterium tuberculosis EsxA membrane insertion: roles of N-and C-terminal flexible arms and central helix-turn-helix motif. Journal of Biological Chemistry *290*, 7314-7322.
Mah, L.Y., O'Prey, J., Baudot, A.D., Hoekstra, A., and Ryan, K.M. (2012). DRAM-1 encodes multiple isoforms that regulate autophagy. Autophagy *8*, 18-28.

Manzanillo, P.S., Ayres, J.S., Watson, R.O., Collins, A.C., Souza, G., Rae, C.S., Schneider, D.S., Nakamura, K., Shiloh, M.U., and Cox, J.S. (2013). The ubiquitin ligase parkin mediates resistance to intracellular pathogens. Nature *501*, 512-516.
Martens, S., and Fracchiolla, D. (2020). Activation and targeting of ATG8 protein lipidation. Cell discovery *6*, 23.
Martinez, J., Almendinger, J., Oberst, A., Ness, R., Dillon, C.P., Fitzgerald, P., Hengartner, M.O., and Green, D.R. (2011). Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proceedings of the National Academy of Sciences *108*, 17396-17401.
Martinez, J., Malireddi, R.S., Lu, Q., Cunha, L.D., Pelletier, S., Gingras, S., Orchard, R., Guan, J.-L., Tan, H., and Peng, J. (2015). Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. Nature cell biology *17*, 893-906.
Masud, S., Prajsnar, T.K., Torraca, V., Lamers, G.E., Benning, M., Van Der Vaart, M., and Meijer, A.H. (2019). Macrophages target Salmonella by Lc3-associated phagocytosis in a systemic infection model. Autophagy *15*, 796-812.
Matsui, T., Jiang, P., Nakano, S., Sakamaki, Y., Yamamoto, H., and Mizushima, N. (2018). Autophagosomal YKT6 is required for fusion with lysosomes independently of syntaxin 17. Journal of Cell Biology *217*, 2633-2645.
Matsunaga, K., Saitoh, T., Tabata, K., Omori, H., Satoh, T., Kurotori, N., Maejima, I., Shirahama-Noda, K., Ichimura, T., and Isobe, T. (2009). Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. Nature cell biology *11*, 385-396.
McEwan, D.G., Popovic, D., Gubas, A., Terawaki, S., Suzuki, H., Stadel, D., Coxon, F.P., De Stegmann, D.M., Bhogaraju, S., and Maddi, K. (2015). PLEKHM1 regulates autophagosome-lysosome fusion through HOPS complex and LC3/GABARAP proteins. Molecular cell *57*, 39-54.
Meijer, A.H., and Spaink, H.P. (2011). Host-Pathogen Interactions Made Transparent with the Zebrafish Model. Current Drug Targets *12*, 1000-1017.
Mishra, A., and Surolia, A. (2018). Mycobacterium tuberculosis: surviving and indulging in an unwelcoming host. IUBMB life *70*, 917-925.
Mostowy, S., Sancho-Shimizu, V., Hamon, M.A., Simeone, R., Brosch, R., Johansen, T., and Cossart, P. (2011). p62 and NDP52 proteins target intracytosolic Shigella and Listeria to different autophagy pathways. Journal of Biological Chemistry *286*, 26987-26995.
Mrschtik, M., O'prey, J., Lao, L., Long, J., Beaumatin, F., Strachan, D., O'prey, M., Skommer, J., and Ryan, K. (2015). DRAM-3 modulates autophagy and promotes cell survival in the absence of glucose. Cell Death & Differentiation *22*, 1714-1726.
Mrschtik, M., and Ryan, K.M. (2016). Another DRAM involved in autophagy and cell death. Autophagy *12*, 603-605.
Muñoz-Sánchez, S., van der Vaart, M., and Meijer, A.H. (2020). Autophagy and Lc3-associated phagocytosis in zebrafish models of bacterial infections. Cells *9*, 2372.

Nagata, M., Arakawa, S., Yamaguchi, H., Torii, S., Endo, H., Tsujioka, M., Honda, S., Nishida, Y., Konishi, A., and Shimizu, S. (2018). Dram1 regulates DNA damage-induced alternative autophagy. Cell Stress *2*, 55.
Nakamura, S., and Yoshimori, T. (2017). New insights into autophagosome–lysosome fusion. Journal of cell science *130*, 1209-1216.
Nguyen, T.N., Padman, B.S., Usher, J., Oorschot, V., Ramm, G., and Lazarou, M. (2016). Atg8 family LC3/GABARAP proteins are crucial for autophagosome–lysosome fusion but not autophagosome formation during PINK1/Parkin mitophagy and starvation. Journal of Cell Biology *215*, 857-874.
Nieto-Torres, J.L., Leidal, A.M., Debnath, J., and Hansen, M. (2021). Beyond autophagy: the expanding roles of ATG8 proteins. Trends in biochemical sciences *46*, 673-686.
Obara, K., Sekito, T., Niimi, K., and Ohsumi, Y. (2008). The Atg18-Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. Journal of Biological Chemistry *283*, 23972-23980.
Odagiri, S., Tanji, K., Mori, F., Kakita, A., Takahashi, H., and Wakabayashi, K. (2012). Autophagic adapter protein NBR1 is localized in Lewy bodies and glial cytoplasmic inclusions and is involved in aggregate formation in α-synucleinopathy. Acta neuropathologica *124*, 173-186.
OECD (2019). Antimicrobial Resistance: Tackling the Burden in the European Union.
Ohsumi, Y. (2014). Historical landmarks of autophagy research. Cell research *24*, 9-23.
Pankiv, S., Alemu, E.A., Brech, A., Bruun, J.-A., Lamark, T., Øvervatn, A., Bjørkøy, G., and Johansen, T. (2010). FYCO1 is a Rab7 effector that binds to LC3 and PI3P to mediate microtubule plus end–directed vesicle transport. Journal of Cell Biology *188*, 253-269.
Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.-A., Outzen, H., Øvervatn, A., Bjørkøy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. Journal of biological chemistry *282*, 24131-24145.
Park, S.-M., Kim, K., Lee, E.-J., Kim, B.-K., Lee, T.J., Seo, T., Jang, I.-S., Lee, S.-H., Kim, S., and Lee, J.-H. (2009). Reduced expression of DRAM2/TMEM77 in tumor cells interferes with cell death. Biochemical and biophysical research communications *390*, 1340-1344.
Paroha, R., Chourasia, R., Mondal, R., and Chaurasiya, S.K. (2018). PknG supports mycobacterial adaptation in acidic environment. Molecular and cellular biochemistry *443*, 69-80.
Pilli, M., Arko-Mensah, J., Ponpuak, M., Roberts, E., Master, S., Mandell, M.A., Dupont, N., Ornatowski, W., Jiang, S., and Bradfute, S.B. (2012). TBK-1 promotes autophagy-mediated antimicrobial defense by controlling autophagosome maturation. Immunity *37*, 223-234.

Polson, H.E., de Lartigue, J., Rigden, D.J., Reedijk, M., Urbé, S., Clague, M.J., and Tooze, S.A. (2010). Mammalian Atg18 (WIPI2) localizes to omegasome-anchored phagophores and positively regulates LC3 lipidation. Autophagy *6*, 506-522.
Purdy, G.E., Owens, R.M., Bennett, L., Russell, D.G., and Butcher, B.A. (2005). Kinetics of phosphatidylinositol-3-phosphate acquisition differ between IgG bead-containing phagosomes and Mycobacterium tuberculosis-containing phagosomes. Cellular microbiology *7*, 1627-1634.
Ramakrishnan, L. (2020). Mycobacterium tuberculosis pathogenicity viewed through the lens of molecular Koch's postulates. Current opinion in microbiology *54*, 103-110.
Rao, N., and Meena, L.S. (2011). Biosynthesis and virulent behavior of lipids produced by Mycobacterium tuberculosis: LAM and cord factor: an overview. Biotechnology research international *2011*.
Rogov, V., Dötsch, V., Johansen, T., and Kirkin, V. (2014). Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. Molecular cell *53*, 167-178.
Rozenknop, A., Rogov, V.V., Rogova, N.Y., Löhr, F., Güntert, P., Dikic, I., and Dötsch, V. (2011). Characterization of the interaction of GABARAPL-1 with the LIR motif of NBR1. Journal of molecular biology *410*, 477-487.
Sanjuan, M.A., Dillon, C.P., Tait, S.W., Moshiach, S., Dorsey, F., Connell, S., Komatsu, M., Tanaka, K., Cleveland, J.L., and Withoff, S. (2007). Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature *450*, 1253-1257.
Sharma, V., Verma, S., Seranova, E., Sarkar, S., and Kumar, D. (2018). Selective autophagy and xenophagy in infection and disease. Frontiers in cell and developmental biology *6*, 147.
Simeone, R., Bobard, A., Lippmann, J., Bitter, W., Majlessi, L., Brosch, R., and Enninga, J. (2012). Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death. PLoS pathogens *8*, e1002507.
Stamm, C.E., Collins, A.C., and Shiloh, M.U. (2015). Sensing of Mycobacterium tuberculosis and consequences to both host and bacillus. Immunological reviews *264*, 204-219.
Stanaway, J.D., Parisi, A., Sarkar, K., Blacker, B.F., Reiner, R.C., Hay, S.I., Nixon, M.R., Dolecek, C., James, S.L., and Mokdad, A.H. (2019). The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet Infectious Diseases *19*, 1312-1324.
Stecher, B., Robbiani, R., Walker, A.W., Westendorf, A.M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A.J., Buer, J., and Parkhill, J. (2007). Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS biology *5*, e244.
Stinear, T.P., Seemann, T., Harrison, P.F., Jenkin, G.A., Davies, J.K., Johnson, P.D., Abdellah, Z., Arrowsmith, C., Chillingworth, T., and Churcher, C. (2008). Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis. Genome research *18*, 729-741.

Stjepanovic, G., Baskaran, S., Lin, M.G., and Hurley, J.H. (2017). Unveiling the role of VPS34 kinase domain dynamics in regulation of the autophagic PI3K complex. Molecular & cellular oncology *4*, e1367873.
Tahoun, A., Mahajan, S., Paxton, E., Malterer, G., Donaldson, D.S., Wang, D., Tan, A., Gillespie, T.L., O'Shea, M., and Roe, A.J. (2012). Salmonella transforms follicle-associated epithelial cells into M cells to promote intestinal invasion. Cell host & microbe *12*, 645-656.
Tan, J.M., Mellouk, N., Osborne, S.E., Ammendolia, D.A., Dyer, D.N., Li, R., Brunen, D., van Rijn, J.M., Huang, J., and Czuczman, M.A. (2018). An ATG16L1-dependent pathway promotes plasma membrane repair and limits Listeria monocytogenes cell-to-cell spread. Nature microbiology *3*, 1472-1485.
Tang, D., Kang, R., Coyne, C.B., Zeh, H.J., and Lotze, M.T. (2012). PAMP s and DAMP s: Signal 0s that spur autophagy and immunity. Immunological reviews *249*, 158-175.
Thi, E.P., and Reiner, N.E. (2012). Phosphatidylinositol 3-kinases and their roles in phagosome maturation. Journal of leukocyte biology *92*, 553-566.
Thurston, T.L., Ryzhakov, G., Bloor, S., Von Muhlinen, N., and Randow, F. (2009). The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. Nature immunology *10*, 1215-1221.
Timme-Laragy, A.R., Karchner, S.I., and Hahn, M.E. (2012). Gene knockdown by morpholino-modified oligonucleotides in the zebrafish (Danio rerio) model: applications for developmental toxicology. Developmental Toxicology: Methods and Protocols, 51-71.
Tumbarello, D.A., Manna, P.T., Allen, M., Bycroft, M., Arden, S.D., Kendrick-Jones, J., and Buss, F. (2015). The autophagy receptor TAX1BP1 and the molecular motor myosin VI are required for clearance of salmonella typhimurium by autophagy. PLoS pathogens *11*, e1005174.
Tyrkalska, S.D., Candel, S., Angosto, D., Gómez-Abellán, V., Martín-Sánchez, F., García-Moreno, D., Zapata-Pérez, R., Sánchez-Ferrer, Á., Sepulcre, M.P., and Pelegrín, P. (2016). Neutrophils mediate Salmonella Typhimurium clearance through the GBP4 inflammasome-dependent production of prostaglandins. Nature communications *7*, 12077.
Vaites, L.P., Paulo, J.A., Huttlin, E.L., and Harper, J.W. (2018). Systematic analysis of human cells lacking ATG8 proteins uncovers roles for GABARAPs and the CCZ1/MON1 regulator C18orf8/RMC1 in macroautophagic and selective autophagic flux. Molecular and cellular biology *38*, e00392-00317.
van Der Niet, S., van Zon, M., de Punder, K., Grootemaat, A., Rutten, S., Moorlag, S.J., Houben, D., van der Sar, A.M., Bitter, W., and Brosch, R. (2021). IL-1R1-dependent signals improve control of cytosolic virulent mycobacteria in vivo. Msphere *6*, 10.1128/msphere. 00153-00121.
Van der Oost, J., Jore, M.M., Westra, E.R., Lundgren, M., and Brouns, S.J. (2009). CRISPR-based adaptive and heritable immunity in prokaryotes. Trends in biochemical sciences *34*, 401-407.

van der Vaart, M., Banducci-Karp, A., Forn-Cuní, G., Witt, P.M., Willemse, J.J., Sánchez, S.M., Hosseini, R., and Meijer, A.H. (2020). DRAM1 requires PI (3, 5) P2 generation by PIKfyve to deliver vesicles and their cargo to endolysosomes. bioRxiv.
van der Vaart, M., Korbee, C.J., Lamers, G.E., Tengeler, A.C., Hosseini, R., Haks, M.C., Ottenhoff, T.H., Spaink, H.P., and Meijer, A.H. (2014). The DNA damage-regulated autophagy modulator DRAM1 links mycobacterial recognition via TLR-MYD88 to autophagic defense. Cell host & microbe *15*, 753-767.
van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Brenner, M., and Peters, P.J. (2007). M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. Cell *129*, 1287-1298.
Van Wesenbeeck, L., Odgren, P.R., Coxon, F.P., Frattini, A., Moens, P., Perdu, B., MacKay, C.A., Van Hul, E., Timmermans, J.-P., and Vanhoenacker, F. (2007). Involvement of PLEKHM1 in osteoclastic vesicular transport and osteopetrosis in incisors absent rats and humans. The Journal of clinical investigation *117*, 919-930.
Varela, M., and Meijer, A.H. (2022). A fresh look at mycobacterial pathogenicity with the zebrafish host model. Molecular Microbiology *117*, 661-669.
Via, L.E., Deretic, D., Ulmer, R.J., Hibler, N.S., Huber, L.A., and Deretic, V. (1997). Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. Journal of Biological Chemistry *272*, 13326-13331.
Vieira, O.V., Harrison, R.E., Scott, C.C., Stenmark, H., Alexander, D., Liu, J., Gruenberg, J., Schreiber, A.D., and Grinstein, S. (2004). Acquisition of Hrs, an essential component of phagosomal maturation, is impaired by mycobacteria. Molecular and Cellular Biology *24*, 4593-4604.
Wallis, R.S., O'Garra, A., Sher, A., and Wack, A. (2023). Host-directed immunotherapy of viral and bacterial infections: past, present and future. Nature Reviews Immunology *23*, 121-133.
Wang, M., Qazi, I.H., Wang, L., Zhou, G., and Han, H. (2020). Salmonella virulence and immune escape. Microorganisms *8*, 407.
Wang, T., Ming, Z., Xiaochun, W., and Hong, W. (2011). Rab7: role of its protein interaction cascades in endo-lysosomal traffic. Cellular signalling *23*, 516-521.
Wang, Y., Ramos, M., Jefferson, M., Zhang, W., Beraza, N., Carding, S., Powell, P.P., Stewart, J.P., Mayer, U., and Wileman, T. (2022). Control of infection by LC3-associated phagocytosis, CASM, and detection of raised vacuolar pH by the V-ATPase-ATG16L1 axis. Science Advances *8*, eabn3298.
Watson, R.O., Manzanillo, P.S., and Cox, J.S. (2012). Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. Cell *150*, 803-815.
Wild, P., Farhan, H., McEwan, D.G., Wagner, S., Rogov, V.V., Brady, N.R., Richter, B., Korac, J., Waidmann, O., and Choudhary, C. (2011). Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. Science *333*, 228-233.

Willett, C.E., Cortes, A., Zuasti, A., and Zapata, A.G. (1999). Early hematopoiesis and developing lymphoid organs in the zebrafish. Developmental dynamics: an official publication of the American Association of Anatomists *214*, 323-336.
Wirth, M., Joachim, J., and Tooze, S.A. (2013). Autophagosome formation—the role of ULK1 and Beclin1–PI3KC3 complexes in setting the stage. Paper presented at: Seminars in cancer biology (Elsevier).
Wong, S.W., Sil, P., and Martinez, J. (2018). Rubicon: LC 3-associated phagocytosis and beyond. The FEBS journal *285*, 1379-1388.
Wu, S., Shen, Y., Zhang, S., Xiao, Y., and Shi, S. (2020). Salmonella interacts with autophagy to offense or defense. Frontiers in Microbiology *11*, 721.
Xu, Z., Yang, L., Xu, S., Zhang, Z., and Cao, Y. (2015). The receptor proteins: pivotal roles in selective autophagy. Acta biochimica et biophysica Sinica *47*, 571-580.
Yang, C.-T., Cambier, C., Davis, J.M., Hall, C.J., Crosier, P.S., and Ramakrishnan, L. (2012). Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. Cell host & microbe *12*, 301-312.
Yin, Z., Pascual, C., and Klionsky, D.J. (2016). Autophagy: machinery and regulation. Microbial cell *3*, 588.
Yoon, J.-H., Her, S., Kim, M., Jang, I.-S., and Park, J. (2012). The expression of damage-regulated autophagy modulator 2 (DRAM2) contributes to autophagy induction. Molecular biology reports *39*, 1087-1093.
Zachari, M., and Ganley, I.G. (2017). The mammalian ULK1 complex and autophagy initiation. Essays in biochemistry *61*, 585-596.
Zeng, C.-W., Chen, Z.-H., Zhang, X.-J., Han, B.-W., Lin, K.-Y., Li, X.-J., Wei, P.-P., Zhang, H., Li, Y., and Chen, Y.-Q. (2014). MIR125B1 represses the degradation of the PML-RARA oncoprotein by an autophagy-lysosomal pathway in acute promyelocytic leukemia. Autophagy *10*, 1726-1737.
Zhang, M., Chen, L., Wang, S., and Wang, T. (2009). Rab7: roles in membrane trafficking and disease. Bioscience reports *29*, 193-209.
Zhang, R., Varela, M., Forn-Cuní, G., Torraca, V., van der Vaart, M., and Meijer, A.H. (2020). Deficiency in the autophagy modulator Dram1 exacerbates pyroptotic cell death of Mycobacteria-infected macrophages. Cell death & disease *11*, 1-16.
Zhang, R., Varela, M., Vallentgoed, W., Forn-Cuni, G., van der Vaart, M., and Meijer, A.H. (2019). The selective autophagy receptors Optineurin and p62 are both required for zebrafish host resistance to mycobacterial infection. PLoS pathogens *15*, e1007329.
Zhang, X.-D., Qi, L., Wu, J.-C., and Qin, Z.-H. (2013). DRAM1 regulates autophagy flux through lysosomes. PLoS one *8*, e63245.
Zhao, Y.G., Codogno, P., and Zhang, H. (2021). Machinery, regulation and pathophysiological implications of autophagosome maturation. Nature reviews Molecular cell biology *22*, 733-750.

Zheng, Y.T., Shahnazari, S., Brech, A., Lamark, T., Johansen, T., and Brumell, J.H. (2009). The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. The Journal of Immunology *183*, 5909-5916.
Zhong, Y., Wang, Q.J., Li, X., Yan, Y., Backer, J.M., Chait, B.T., Heintz, N., and Yue, Z. (2009). Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1–phosphatidylinositol-3-kinase complex. Nature cell biology *11*, 468-476.
Zumla, A., Rao, M., Wallis, R.S., Kaufmann, S.H., Rustomjee, R., Mwaba, P., Vilaplana, C., Yeboah-Manu, D., Chakaya, J., and Ippolito, G. (2016). Host-directed therapies for infectious diseases: current status, recent progress, and future prospects. The Lancet Infectious Diseases *16*, e47-e63.