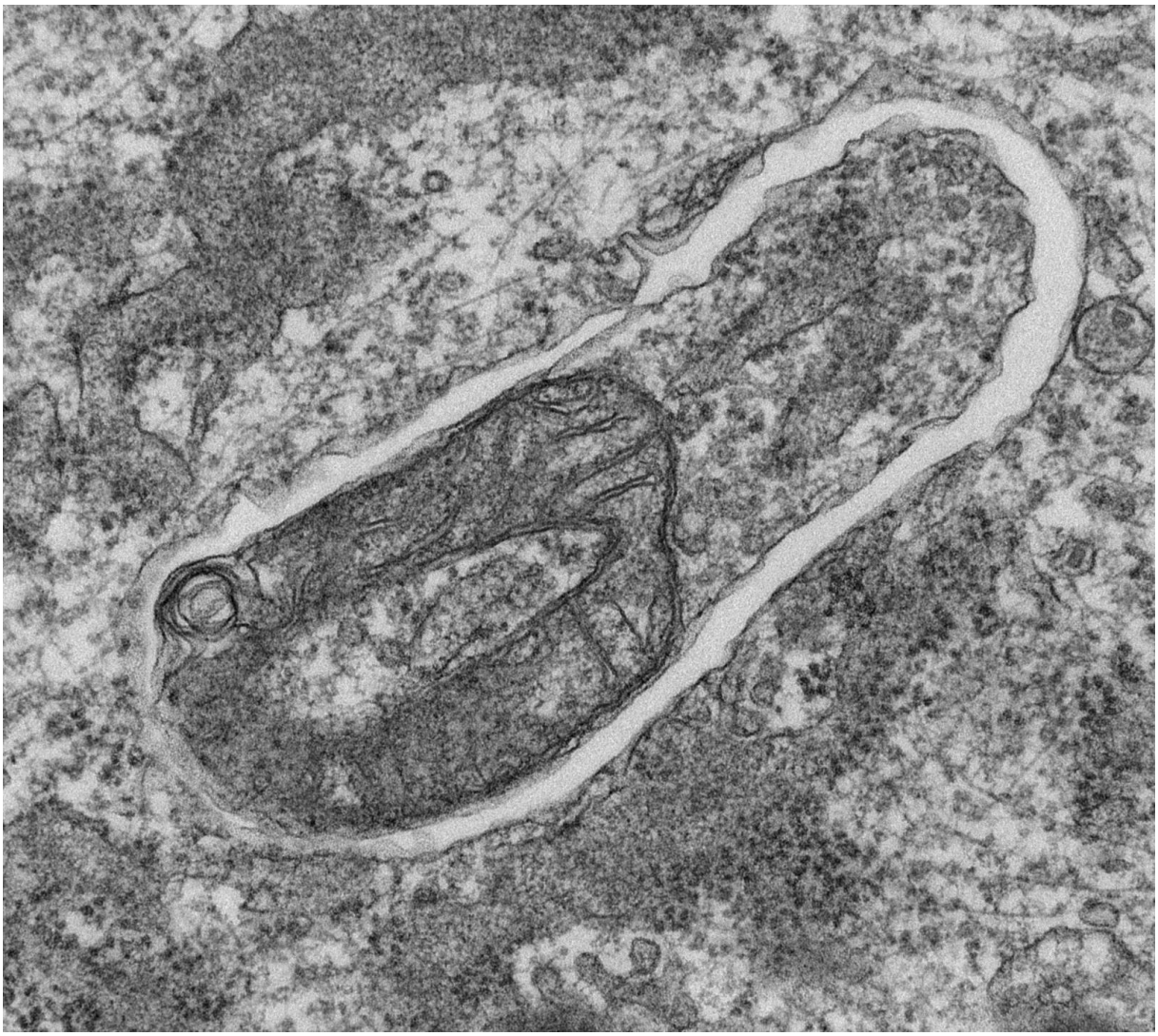
Autophagy as a possible treatment against infections

Looking for bacterial targets that can cause induction of autophagy



Frederick National Laboratory for Cancer Research, USA

Master Thesis

28-03-2013

J.E. Smout 3407748

Under supervision of: Prof. dr. J.B. Helms

Utrecht University

Table of content

Summary for laymen

Autophagy is a process in the body of humans and animals that enables the cell to keep its internal environment stable. Autophagy is the eating of cytosol (cellular liquid) or specific organelles (organs of the cell) for degradation and subsequently the recycling of the building blocks of these compounds. The extended machinery that this process uses is not yet fully understood. Autophagy is important in many processes in the body among which the capturing of invading viruses, bacteria and fungi. These organisms have in turn evolved mechanisms to avoid the autophagy machinery of their hosts. This thesis investigates the possibility of using autophagy as a treatment against infections, specifically bacterial infections, by using targets that are specific for a bacterium.

Overall it is suggested that induction of autophagy can provide an efficient treatment of (bacterial) infections in the human body. However, using specific targets per infection is preferable, since autophagy is involved in many processes throughout the body and general induction of autophagy might lead to unwanted side effects. Treatment of bacterial infection based on autophagy can reduce the use of antibiotics, for which bacteria are becoming more resistant.

The bacteria *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* are food-borne bacteria that are able to cause food poisoning in humans and animals. Salmonella bacteria enter the cell and survive in a *Salmonella* containing vacuole (SCV) where it can grow and replicate and where the bacteria are safe from recognition by the autophagy machinery. When the vacuole is damaged, the human host cell is able to detect the presence of the bacteria and the vacuoles and targets them for degradation by autophagy, repressing the infection. The ability of the human cell to detect damaged vacuoles and free cytosolic bacteria are important for the suggestion to use SifA as a target for repression of *Salmonella* infection. SifA is a protein that is important for maintenance of the SCV. By targeting SifA, the maintenance of the vacuoles will be disrupted, such that the autophagy machinery will be able to detect and eliminate the damaged vacuoles and the bacteria that have escaped the damaged SCV.

Listeria monocytogenes has evolved more strategies to avoid the human autophagy system and is therefore harder to detect by the autophagy system. When the bacterium gets the chance, it is present in the cytosol, where it is covered by different proteins that disguise the surface of the bacterium and therefore prevent recognition by the host cell. Additionally these protecting proteins help the bacterium to move within the cell and to spread to other cells. When the circumstances are less favorable, the bacterium hides in a vacuole that also protects the bacterium from recognition by the host cell and there, the bacterium can grow slowly. Therefore a combination of targets is proposed, together resulting in the deletion of the protection layer. In combination with upregulation of autophagy, this seems to be an efficient way to fight *Listeria* infection. Nevertheless, because of the complicated protection of the bacteria, no simple treatment is possible and additional research is required. Additionally some targets for reduction of cell-to-cell spread and bacterial growth are suggested to reduce infection, which are not relying on the autophagy pathway. Overall induction of autophagy of specific bacterial infections is a promising treatment.

Abstract

Autophagy is a ubiquitous process that is important for homeostasis in eukaryotic cells. It is involved in the clearance of invading microorganisms and viruses from cells. In this thesis macroautophagy, which is the formation of a membrane around the compounds that have to be degraded, is investigated as a target for treatment of infections. First, general induction of autophagy is investigated as a treatment of infections. Additionally induction of autophagy of specific bacteria by using bacterial targets is investigated. Specifically inducing autophagical clearance of bacteria might be an effective alternative for antibiotics.

Not all details of the mechanism of autophagy are known yet, but the key players are determined and the main processes are revealed. The formation starts at the phagosomal assembly site and a membrane is formed around the cargo, creating an autophagosome. In this process many proteins are involved (among which many autophagy related proteins called Atg proteins), which all have their own functions. In case of bulk autophagy the cargo is cytosol, while in case of specific autophagy the cargo can be specific organelles, or in case of xenophagy, invading micro-organisms or viruses. During autophagy, adaptor proteins are used to bind the autophagosome at one side and the invader, which is labeled with ubiquitinated proteins via an unknown mechanism, at the other side.

General upregulation of autophagy as a treatment of infections is considered and recently some promising targets are identified, which are able to specifically induce autophagy without influencing other processes in the cell. Nevertheless, since autophagy is involved in many processes within the body, upregulation might also have negative side effects. Therefor for the bacteria *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* it is investigated if there are possible specific targets for upregulation of autophagy of the invading bacterium in particular.

Salmonella enterica serovar Typhimurium enters the cell in a *Salmonella* containing vacuole (SCV), where it is safe from recognition by the host cell and where it can proliferate. The needle-like structures that the bacterium uses to enter the host cell can also accidentally damage the SCV. This results in increased autophagy. The finding leads to the suggestion to use targets that are involved in the maintenance of the SCV. The SifA protein, which is important for the maintenance of the SCV, or its producer, *Salmonella* Pathogenicity Island II (SPI-2) might be effective targets for the induction of autophagy of specifically *Salmonella enterica* serovar Typhimurium.

Listeria monocytogenes is a cytosolic bacterium, that when it expresses high amounts of Listeriolysin O (LLO) is found in the cytosol, covered by proteins that disguise the bacterium from recognition by the autophagy system. *ActA* is expressed during this stage, leading to protection of the bacterium by ActA proteins and to the formation of an actin tail, enabling motility and cell-to-cell-spread. In case of low LLO expression, the bacterium stays inside a vacuole, where it can proliferate slowly because it is protected from recognition by the host cell. Several infection-restricting targets are proposed, but none of them is based on autophagy. Because of the extensive protection of the *Listeria* bacteria from the autophagy machinery, a combination of targets is needed to induce autophagy. When both virulence factors that recruit protecting proteins (ActA and InlK) are targeted, the bacteria will become targetable. Especially in combination with induced autophagy this might be an efficient treatment of *Listeria* infection. Overall induction of autophagy via specific bacterial targets is a promising treatment of infections.

Introduction

Autophagy (Greek for self-digestion) is a lysosomal pathway of self-digestion in eukaryotes, which is important when extracellular nutrients are limited. This process allows the cell to adapt to environmental changes by degradation of cytoplasmic components, damaged organelles, and invading microorganisms through delivery of these components to lysosomes. Hence, autophagy is a ubiquitous process important for the homeostasis of eukaryotic cells. Autophagy was first described in 1974 (as reviewed in (1)) and is therefore a relatively new research field. Subsequently, it was found to be involved in cancer, neurodegradation, metabolic diseases, aging and immunity. Autophagy is also involved in the clearance of invading microbes from cells. Autophagy is important for the protection of cells, possibly able to prevent cell death. For this reasons it has become an intensive topic for research over the last decades (2, 3).

There are three types of autophagy known, i.e. microautophagy, chaperone-mediated autophagy and macroautophagy (figure 1). Microautophagy is the building of an autophagal body from the lysosomal membrane, which allows the sequestration and elimination of unwanted cytoplasmic components (as reviewed in (4)). In chaperone-mediated autophagy substrate proteins are targeted selectively to the lysosomes and subsequently translocated over the lysosomal membrane (as reviewed in (5)). Macroautophagy (hereafter referred to as autophagy) exists of the formation of a new double membrane containing organelle, the autophagosome, which enables the delivery of different types of cargo molecules into the lysosome (as reviewed in (4)). An autophagosome is an organelle varying in size from 300-900nm (6).

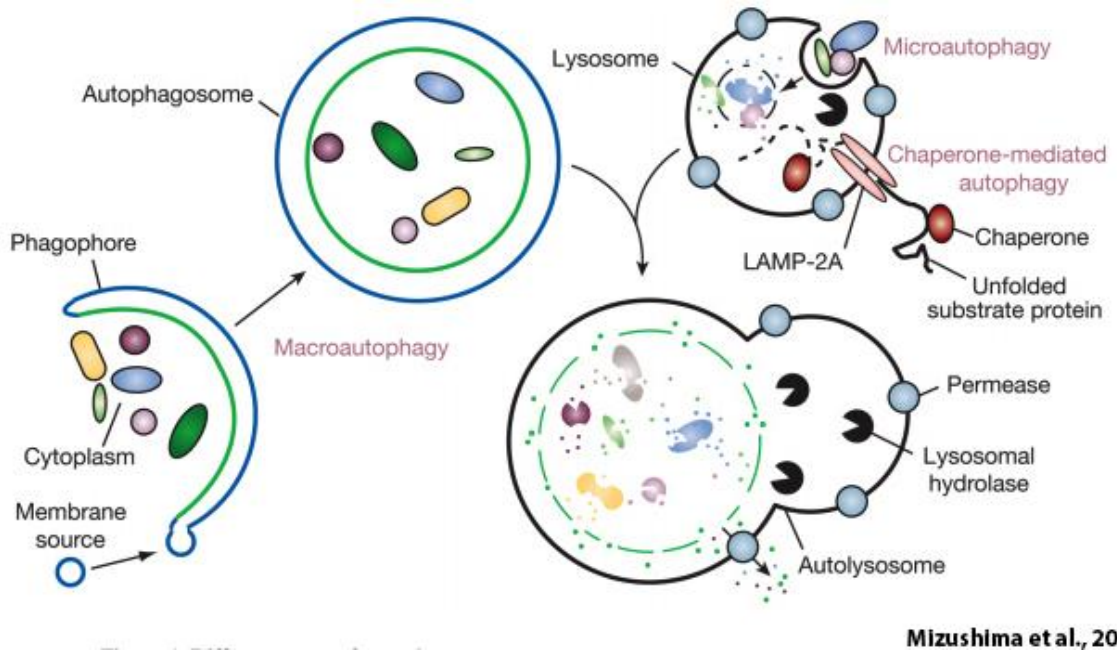


Figure 1: Overview of the three types of autophagy, i.e. macroautophagy, microautophagy and chaperone mediated autophagy. Microautophagy is the invagination of the lysosomal membrane, engulfing unwanted cytoplasmic components. In chaperone mediated autophagy substrate proteins are targeted selectively to the lysosomes and in case of macroautophagy a double membrane vesicle is created, which later fuses with a lysosome for degradation.

Different stages of autophagy are autophagosome initiation, elongation of the membrane, and closure and maturation of the autophagosome, including the fusion of a lysosome to the autophagosome (figure 2). During the entire process the vesicle receives different names. During initiation and elongation, the vesicle is called a phagophore. A closed vesicle is called an autophagosome and during maturation it is called either an amphisome or an autolysosome, depending on the stage in maturation (figure 3). The maturation step involves potential input from the endocytic pathway, including fusion with early endosomes, multivesicular bodies (MVB) and late endosomes, and input from the lysosomal pathway by fusion of a lysosome with the vesicle. During maturation the pH in the autophagosome decreases due to the fusion with the acidic, hydrolytic endosomes and lysosomes. This promotes the action of the enzymes needed for degradation.

Even though over the last years a lot of research has been performed on autophagy, still the exact mechanism of the process is unknown. Until now, 31 Autophagy-related genes (*Atg*) are identified in yeast, of which 15 genes are found to be required for autophagy (7). In humans, several homologues of these *Atg* genes are found. The process of autophagy is controlled by different pathways, which interpret the status of cellular energy (AMP-dependent protein kinase, AMPK), nutrient/amino acid availability (target of rapamycin, TOR), and growth factors such as insulin (8).

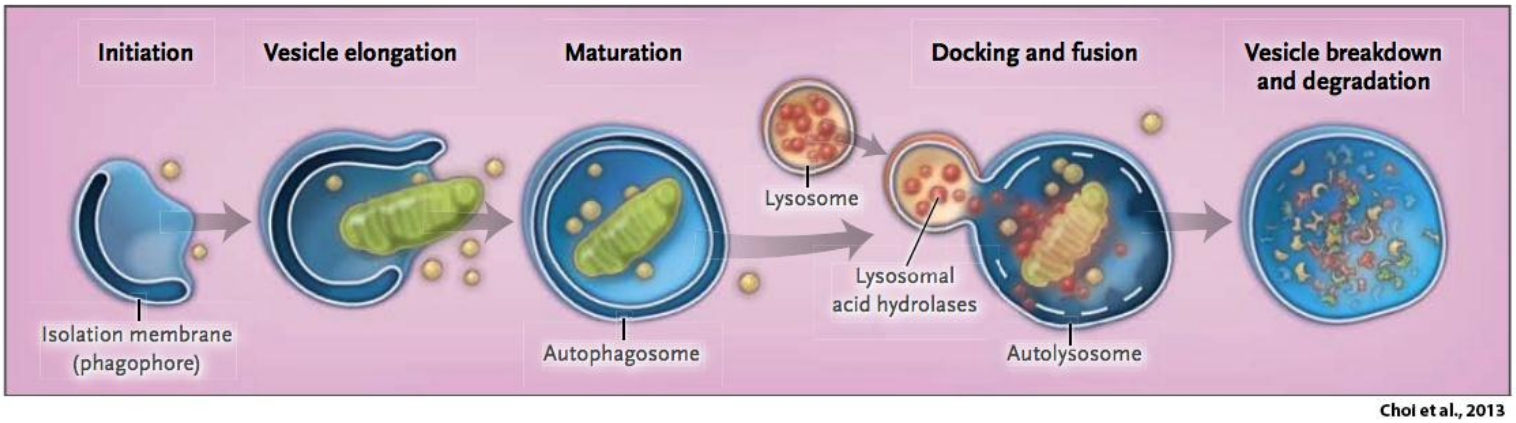


Figure 2: Basic overview of the process of autophagy. Autophagy starts with the formation of a double-layered isolation membrane at the ER-mitochondria contact site (9). The membrane is elongated and cargo recognition occurs. The autophagosome is completed and during maturation a lysosome docks at the autophagosome and subsequently fuses with it, enabling degradation of the cargo.

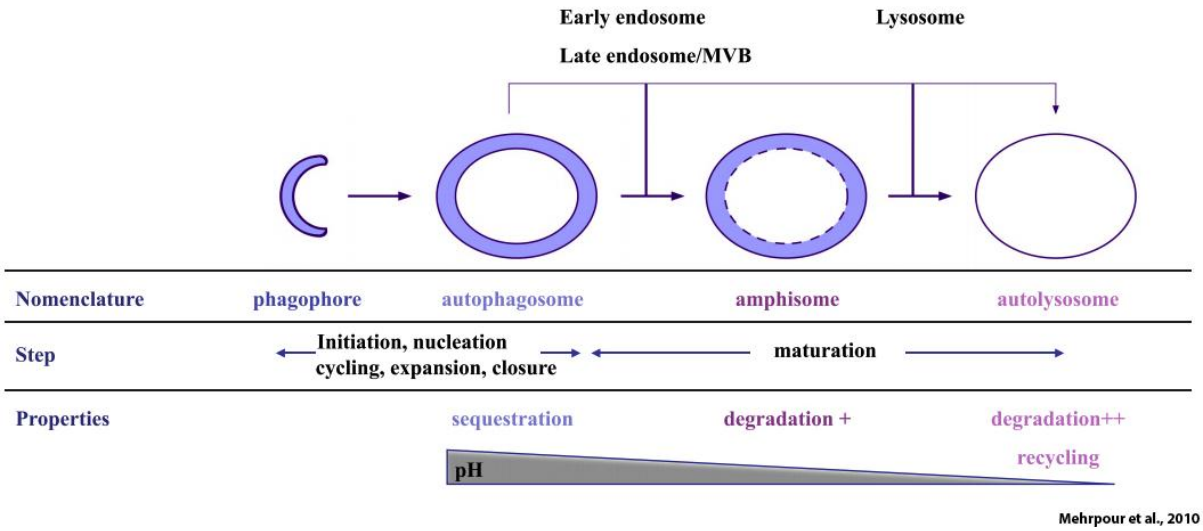


Figure 3: Integrated view of mammalian autophagy. Autophagy is initiated by the formation of an isolation membrane, called a phagophore, which elongates and closes, forming an autophagosome. During maturation the autophagosome receives input from the endocytic and lysosomal pathways, during which the pH of the vesicle decreases.

The question investigated in this thesis is: Can induction of autophagy in human cells be a possible treatment of pathogenic infections? Since this is a broad question that covers many subtopics, the focus will be on specific targets for autophagy of bacteria, covered by the second research question: Can specific targets for autophagy be identified for bacteria?

In order to get insight into the first question, the autophagy machinery is described in the first chapter by the description of the key players and their roles in autophagosome formation and cargo recognition. To be able to answer the second question, the autophagy-based host-pathogen interactions between human cells and *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes* are described in the second chapter. The focus will be on these bacteria, since they are known to be able to infect the human body and induce severe illness and are therefore of clinical significance. In the third chapter, some more background information on the function of autophagy for the human body is described, to provide a more broad view on the possibilities of autophagy induction as a treatment. In the discussion the question if inducing autophagy would be a possible treatment against pathogenic infections, and if it would be possible to specifically induce autophagy of (specific) bacteria will be discussed based on the knowledge acquired and additional relevant literature.

Autophagy

Machinery

A basic overview of the mechanism of autophagy is shown in figure 2. So far, there are four functional groups of genes found to be involved in formation of the autophagosome, i.e. the ULK complex, the class III phosphatidylinositol 3-kinase (PI3K) complex, the Atg9 trafficking system, and the two parallel ubiquitin-like conjugation systems, the Atg16L complex and LC3-PE (4, 10). In the following paragraphs the main mechanism of phagophore assembly site formation, initiation of autophagy, nucleation of the isolation membrane and the expansion and closure of the autophagosome are described in general terms. The details of each process are yet to discovered, so the general mechanisms known so far are described and the depicted by schematic overviews of the key players. At the end of this chapter, the entire process is summarized in a schematic overview (figure 8).

Phagophore assembly site formation

The exact mechanism of membrane delivery to the phagophore assembly site is still unknown. A model of the process of membrane delivery to the phagophore assembly site, which is also known as the pre-autophagosomal structure, (PAS) is depicted schematically in figure 4. The source of the membrane used for autophagosome formation is still unknown. The ULK1 complex is located at the PAS and attracts other Atg proteins that are needed for correct autophagosome formation (11). Recently it was discovered that autophagosomes are formed at ER-mitochondria contact sites, meaning that the PAS is located at this site (9). This does not automatically mean that the membrane used for Autophagosomal formation is of ER and/or mitochondrial origin. Atg5 and mATG14 are located at the PAS during formation of the PAS, suggesting that for all steps in autophagosome formation some proteins are already present during initiation (9). These proteins seem to wait for their turn to perform their functions when needed.

Atg9 is the only Atg protein known that is a transmembrane protein. Atg9 is thought to mediate the delivery of new membrane for the formation of autophagosomes at the PAS. In yeast Atg27 is found to be shuttling between the Golgi complex, mitochondria and the PAS. When Atg27 is lacking, Atg9 is restricted to mitochondria, therefore Atg27 is required for Atg9 cycling (12). In yeast, Atg9 is found on Atg9-vesicles, which are cytoplasmic small vesicles containing Atg9, Atg27, the Rab GTPase Ypt1 and Trs85, which is a specific subunit of the transport protein particle III (TRAPPIII) complex (13). The TRAPPIII complex facilitates the association of Ypt1 to Atg9. Both Trs85 and Ypt1 are localized on the PAS in an Atg9-dependent manner. This presence of Trs85 probably recruits the TRAPPIII complex to the PAS (13). These vesicle-tethering proteins might also be involved in the formation of autophagosomes (13). This function of Atg9 and its vesicles is only described in yeast. It is not known if this process is the same in humans, but it can be expected to be dependent on a similar mechanism.

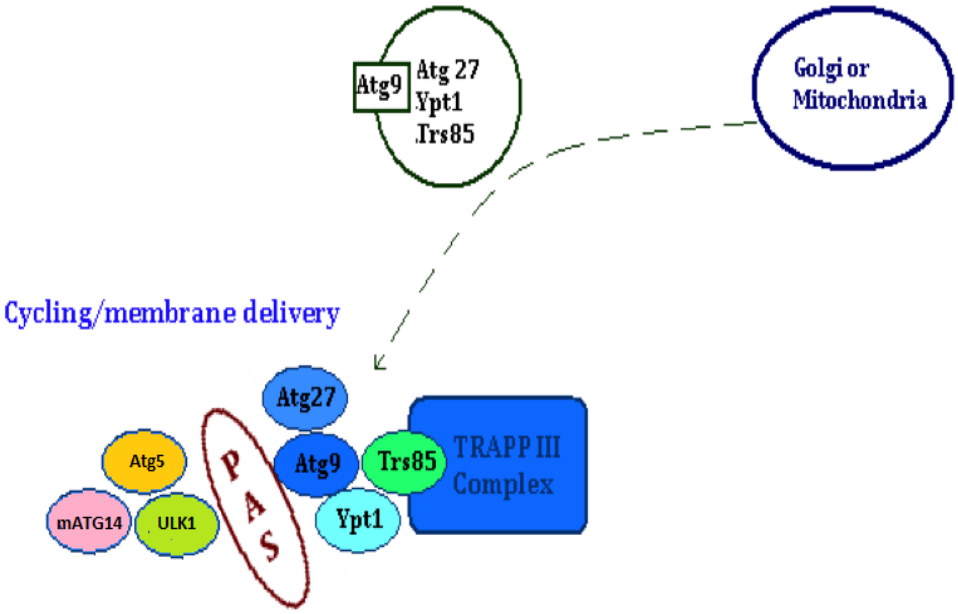


Figure 4: Model of membrane delivery to the phagophore assembly site. Atg9-vesicles carrying Atg9, Atg27, Ypt1 and Trs85 shuttle from the Golgi complex or mitochondria to the phagophore assembly site (PAS). At the PAS, Atg9 is bound to Ypt1 via Trs85, which is part of the TRAPP III complex. All proteins present in the Atg9-vesicle are thought to be of importance for the membrane delivery to the PAS. At the PAS ULK1, mAtg14 and Atg5 are present, waiting to come in action in later stages of autophagy. The exact mechanism is still unknown. Figure is a graphical representation based on information described in the text.

Regulation of Initiation

Initiation of autophagosome formation occurs at the PAS. The process of initiation and the regulation of this process are depicted in figure 5. The ULK1 complex is located at the PAS and is important for the recruitment of other Atg proteins needed for autophagosome formation to this site (11). In humans, the ULK complex exists of ULK1 (Unc-51-like kinase 1), FIP200, Atg13 and Atg101 (14). Atg13 localizes on the phagophore assembly site and is essential for autophagosome formation initiation (15). Atg101 is found to stabilize Atg13 and is therefore also of importance for autophagy. Depletion of ULK1, Atg13 or Atg101 is sufficient to inhibit autophagy (14).

The complex is negatively regulated by nutrient availability via mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is incorporated into the complex through ULK1 and phosphorylates ULK1 and Atg13 in case of sufficient nutrient availability. This phosphorylation is important for the inhibition of autophagy (16). Dephosphorylation of ULK1 occurs during starvation or during treatment with the autophagy inducing component rapamycin (15, 17). This suggests that mTORC1 suppresses autophagy via direct phosphorylation and therefore inactivation of the ULK1 complex (15).

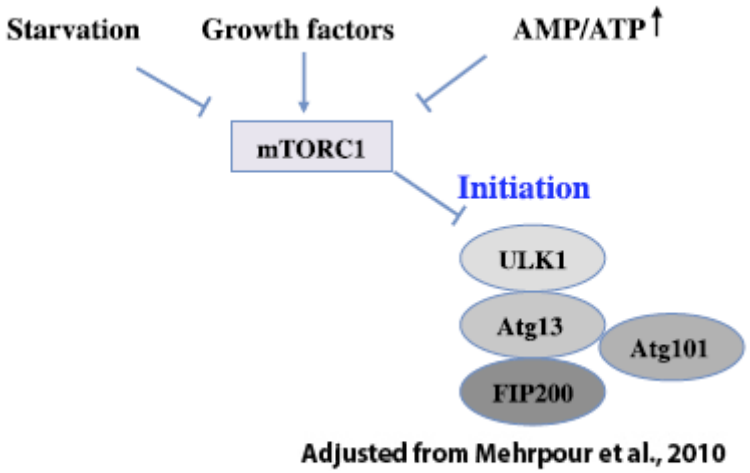
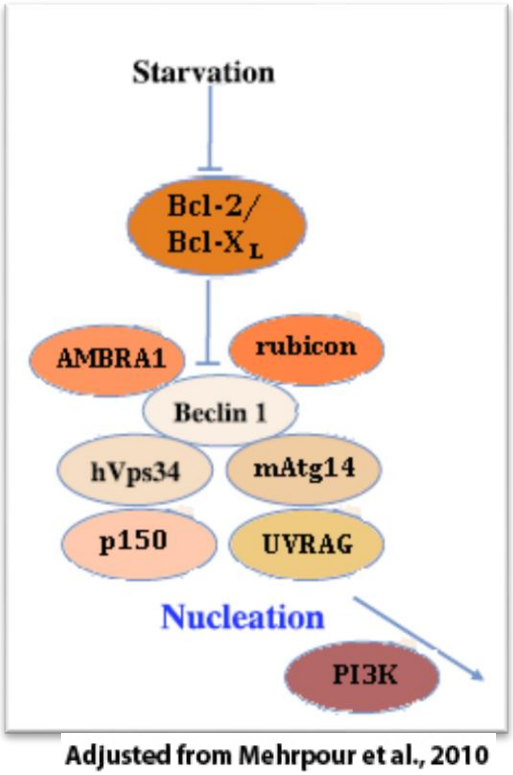


Figure 5: Regulation of Initiation of autophagy. The ULK1 complex, consisting of ULK1, Atg13, FIP200 and Atg13 stabilizing protein Atg101, is needed for the initiation of autophagy. The initiation step is regulated by the mTORC1 pathway, which responds to nutrient availability, growth factors and the AMP/ATP level in the cell.

Nucleation

The link between initiation and nucleation is not clear. It is known that both processes are needed for correct autophagy, but it is not known how they are connected to each other. For nucleation, the class III phosphatidylinositol 3-kinase (PI3K) complex is needed. This complex exists in humans of hVps34 (human vacuolar protein sorting 34), a serine/threonine kinase p150, mAtg14, UVRAG, Beclin 1 and two proteins that interact with Beclin 1, i.e. Rubicon and AMBRA 1 (that also activates Beclin 1) (10) (Figure 6). During formation of the autophagosome, mATG14 is present at the PAS (9)(figure 4), showing that (part of) the PI3K complex is already present at initiation, taking it over as soon as there is a piece of membrane formed. PI3K itself is only needed later in the nucleation for the recruitment of the proteins needed for expansion and closure, while all other proteins of the complex are needed for the nucleation itself.

The activity of Beclin 1 is regulated by Bcl-2 (B-cell lymphoma/leukemia-2), which regulates therefore the nucleation of autophagy. Under normal conditions the BH3-domain of Bcl-2 or of the homolog Bcl-X_L binds to the BH3 domain of Beclin 1 and inhibits its function. Starvation can indirectly stimulate dissociation of Bcl-2 or Bcl-X_L in two ways; either by activation of BH3-only proteins that can competitively disrupt the interaction or by posttranslational modifications of Bcl-2, leading to reduced affinity to BH3 domains of Beclin 1 and BH3-only proteins (18).



Adjusted from Mehrpour et al., 2010

Figure 6: Nucleation of the isolation membrane and regulation of the nucleation step. The PI3K-complex, consisting of hVsp34, mAtg14, p150, UVRAG, Beclin1, and the two Beclin1 associated proteins AMBRA1 and rubicon, are needed for nucleation. PI3K is needed for the transition of nucleation step to the expansion step, while the rest of the complex is needed for nucleation itself. Nucleation is regulated by Bcl-2 or Bcl-X_L that can inhibit the activity of Beclin 1. Nucleation is downregulated in presence of nutrients, while it is upregulated during starvation.

Elongation and closure

The PI3K complex recruits the two ubiquitin-like (Ubl) conjugation systems, i.e. Atg12-Atg5-Atg16 (the Atg16L complex) and LC3 conjugated to phosphatidylethanolamine (LC3-PE or LC3-II) (figure 7). These complexes play an important role in elongation and expansion of the forming autophagosome. The Atg16 complex localization is enabled by Atg7 and Atg10 (17) and specifies the site of LC3 lipidation (addition of the phosphatidylethanolamine, PE) for membrane biogenesis. For elongation, homotypic fusion of Atg16L1 precursors is needed. SNARE protein VAMP7 and some other SNAREs are needed for this fusion step (19).

The interaction of Atg12 with the E2 enzyme Atg3 enables ectopic LC3 lipidation (20). The conjugation of LC3 with PE is enabled by Atg7 and Atg3, while Atg4 helps the conjugation by exposing a glycine residue at the COOH terminus of LC3, a formation known as LC3-I (21). Mutation of Atg3 in mice resulted in absence of conjugation of LC3 with PE. These mutants were unable to produce normal autophagosomes, all were malformed. This indicates that the conjugated LC3 is essential for the correct formation and closure of autophagosomes in mice (22). LC3-PE is tightly associated with membranes and is found on both the external and the internal surface of the newly synthesized membrane (23).

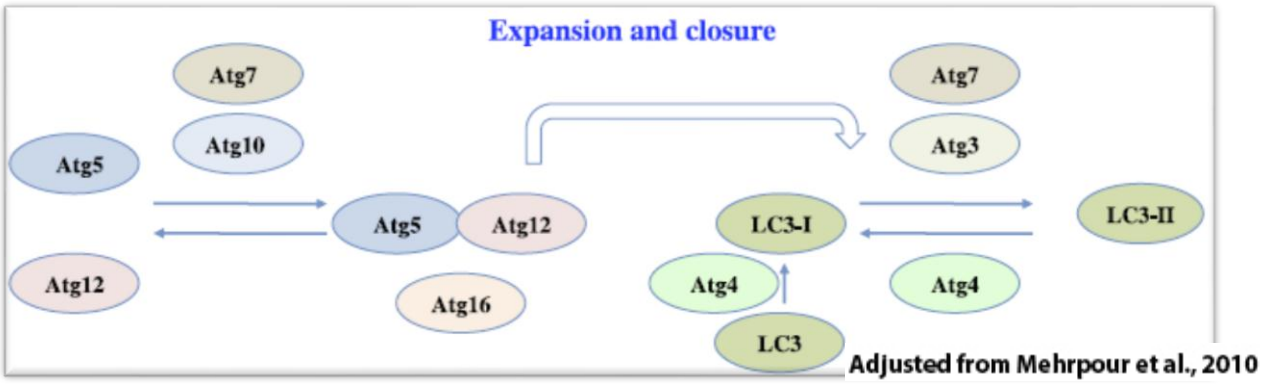


Figure 7: Expansion and closure of the isolation membrane to form an autophagosome. LC3-II (also known as LC3-PE) formation is needed for the correct formation of autophagosomes. The formation of LC3 is changed into LC3-I by Atg4 to expose the lipidation site. For lipidation (the addition of the PE group), Atg3 and Atg7 are needed. Atg3 is recruited by Atg12 of the Atg16L-complex. This Atg16L-complex is recruited by both Atg7 and Atg10 and exists of Atg5, Atg12 and Atg16.

In summary, the exact mechanism of autophagy is not known yet. The main processes of the autophagosome formation are the formation of the PAS, initiation, nucleation, elongation and expansion and closure. The mechanisms of these processes are roughly known. At the PAS there are for all following steps in formation some proteins present. The link between initiation and nucleation is not known. Possibly the ULK1 complex is mainly needed for recruitment of other proteins, since ULK1 is already present at the PAS. An overview of all processes is depicted schematically in figure 8.

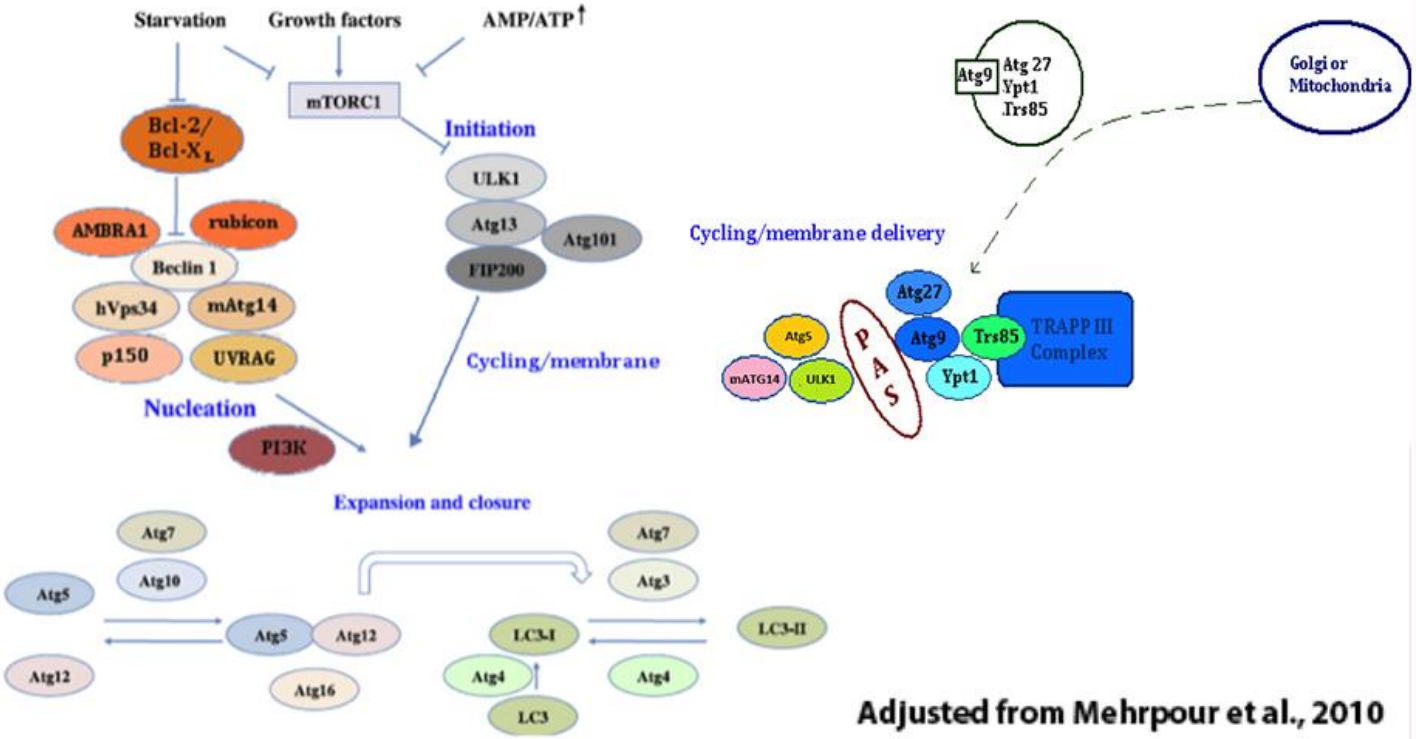


Figure 8: Overview of the molecular mechanism regulating autophagy. Initiation occurs at the phagophore assembly site (PAS) and the membrane is suggested to get delivered to the PAS by the Atg9-vesicles that are cycling between the Golgi apparatus, mitochondria and the PAS. Nucleation occurs via the PI3K-complex after which PI3K recruits the Atg16L-complex and LC3-PE which are together responsible for correct expansion and closure of the autophagosome (figure partly adjusted from (17), and partly self-made).

Cargo recognition

There are two types of macroautophagy known, i.e. selective and nonselective autophagy. Nonselective autophagy, also known as bulk autophagy, is the engulfment of cytoplasm and subsequent degradation (4). Selective autophagy has become a large topic in research since it was found that many organelles are targeted specifically to the autophagy pathway. There are several selective pathways of autophagy that are distinguished by the different organelles that are specifically targeted for degradation by autophagy (table 1)(4). This large amount of selective pathways suggests that autophagy can be a highly selective quality control mechanism of human cells. The mechanisms used for selective and nonselective autophagy are the same (24).

Table 1: Overview of selective autophagy pathways and organelles that are degraded by this pathway (4).

Name of the pathway	Organelle targeted for autophagy
Reticulophagy/ERphagy	Endoplasmatic Reticulum
Pexophagy	Peroxisomes
Mitophagy	Mitochondria
Lipophagy	Lipid droplets
Zymophagy	Secretory granules
Nucleophagy	Parts of the nucleus
Ribophagy	Ribosomes
Aggrephagy	Aggregate-prone proteins
Xenophagy	Pathogens

To be able to distinguish between organelles that have to be degraded and functional organelles, recognition of specific marker molecules is needed for organelles that must be degraded. Molecular mechanisms underlying this targeting are still largely unknown. In general, the pathways seem to rely on specific adaptor proteins that have a cargo recognition site on one side, and an LC3-interacting region (LIR) on the other site of the protein. The interaction between the adaptor proteins, the cargo and the LC3 molecule is necessary for the recruitment of the cargo to the phagophore assembly site (PAS) (24).

Xenophagy of bacteria

Via autophagy the human cell is able to maintain homeostasis under varying conditions. During starvation, autophagy is induced to recycle nutrients that are captured in molecules with less important functions. The large amount of selective autophagy pathways suggests that autophagy can also be a highly selective quality control mechanism in human cells (24). Additionally autophagy is used to eliminate invading microbes from the human cells. This pathway is called xenophagy (derived from the Greek words xenos, which means stranger and phago, meaning eating).

The xenophagy pathway is needed to eliminate the invading microbes in human cells. The xenophagy pathway can eliminate bacteria, viruses and fungi. Here we will focus on the recognition of bacteria. We will explore the possibility of induction of autophagy as a treatment against bacterial infections. By fighting infections via the autophagy pathway, the use of antibiotics could be reduced.

Ubiquitin

It is detected in several cell types that polyubiquinated proteins accumulate on bacteria that enter the host cell cytosol (25). Although the mechanism used by the ubiquitin system to target these bacteria is still unknown, the fact that the bacteria become surrounded by ubiquitin is of importance for the induction of autophagy. This ubiquitin tag allows the autophagic machinery to recognize the invading bacterium (26). Four proteins are identified to be able to contribute to xenophagy of bacteria, i.e. p62 (26, 27), NBR1 (28), OPTN (29) and NDP52 (30, 31). All four receptors make use of the mechanism of binding the polyubiquinated bacterium on one side, and a LC3 molecule on the other side via a LC3 interaction region (LIR). LC3 is needed for correct autophagosome formation and therefor enables autophagy of the substrate bound to the other side of the adaptor protein (figure 8). Autophagosomal structures containing the polyubiquinated substrate, the recognition protein and the LC3 molecule are degraded in autolysosomes (27). NBR1 and p62 are able to interact and form hetero-oligomers, but both are also able to function independently of each other (28).

In the following chapter we will explore two examples of clearance of bacteria from human cells by xenophagy.

Host pathogen interactions via the autophagy pathway

Salmonella enterica serovar Typhimurium

Salmonella is a Gram-negative bacterium that is the most common cause of food poisoning. All *Salmonella* bacteria belong to the same species, *Salmonella enterica,* and are divided over different serovars. When taken in orally (by eating infected poultry, meat, dairy or eggs that are raw or not properly cooked), *Salmonella* can cause diseases in humans. *Salmonella enterica* serovar Typhimurium (also referred to as S. Typhimurium) is, together with *Salmonella enterica* serovar Enteriditis, the most common cause of salmonellosis, which is the colonization of the small and large intestine, resulting in gastroenteritis. The symptoms are vomiting, diarrhea, headache and fever (32).

Salmonella-containing vacuole

When S. Typhimurium enters the human body, it first has to invade the cells. A type III secretion system (T3SS) is used to inject effector proteins into the cytosol of the host cell via a needle-like structure (as reviewed(33). Following this injection into the host cell, a specific single membrane vacuolar compartment is formed, called the *Salmonella*-containing vacuole (SCV). Directly after formation, the SCV starts a maturation process in which it interacts with early endosomes (34). The bacterium actively prevents the fusion of this SCV with lysosomes, to prevent degradation of the vacuole (35).

SifA expression is induced upon entry of *Salmonella* into the host cell. SifA is required for the formation of lysosomal glycoprotein-containing structures in epithelial cells (called Sifs) and is an effector protein of the *Salmonella* pathogenicity island II type III secretion system (SPI-2 T3SS)(36). SifA is of importance for the mechanism of replication of *Salmonella* within macrophages. In a *SifA*$^-$ mutant, the SCV is lost several hours after uptake, and the bacteria are found freely in the cytosol. This means that SifA has an important role for maintenance of the membrane of the SCV and for replication of the bacteria in macrophages (37). On the other hand, in epithelial cells *SifA*$^-$ mutants show increased replication compared to wildtype *Salmonella* bacteria, due to the loss of SCV membrane integrity. Interestingly this shows that in epithelial cells the bacteria show increased replication when present in the cytosol rather than in a specialized vacuole (38). Taking these results together, the role of SifA seems to differ between host cell types. In macrophages and fibroblasts bacteria that are present in the cytosol are not able to replicate (39). Additionally in macrophages SifA is needed for colonization and growth in macrophages (36). In epithelial cells SifA$^-$ mutants show increased replication. These data suggest that there is a difference in the cytosol of different cell strains, leading to differences in survival of *Salmonella* bacteria within the cytosol.

When intact, the SCV is mostly not recognized by the autophagy machinery. However, there are ways that the autophagy machinery can detect *Salmonella* and autophagy can be induced.

Intact SCVs can be surrounded by a membrane

Salmonella infection is increased in host cells that are defective in autophagy when compared to wild-type infected host cells (40). This means that *Salmonella* seems to be selectively isolated by the autophagy machinery. An intact vacuole containing *Salmonella* is in approximately 20% of all cases associated with LC3-PE (40, 41) and Atg16L (40). LC3-PE is a membrane associated protein, and is associated to an isolation membrane that is formed around the *Salmonella*-containing vacuole.

LC3-PE and Atg16L are still recruited to the SCV in absence of Atg9L, FIP200 and the PI3K complex, suggesting that the recruitment of LC3-PE is dependent on a different mechanism than the membrane formation usually occurring in autophagy, for which FIP200 and PI3K are needed (figure 8). In absence of Atg7 or Atg3, which are needed to add the PE group to activate the inactive LC3-I form into LC3-PE (Figure 8), there is no recruitment of LC3 to the invading *Salmonella*. When there are no autophagy-related proteins such as LC3 recruited to the *Salmonella*, replication is not reduced. This can lead to cell death of the host cell because of the large amount of new *Salmonella* bacteria formed. This means that LC3-PE recruitment is important for restriction of the replication of *Salmonella* within the host cells (40). The role of Atg16L in this process in unknown. Since Atg16L is localized close to the LC3-PE, it is expected to have a function within the xenophagy. Atg14L, ULK1 and Atg9L1 are found to cycle between the membrane formation site around the SCV and another cellular pool. Not much is known about this enwrapping of the SCV by a membrane, but a model is proposed, which has to be confirmed by further experiments (figure 9)(40). Even though the capture of the bacterium inside a vesicle does not seem to be able to eliminate the bacterium, it is found to restrict the infection.

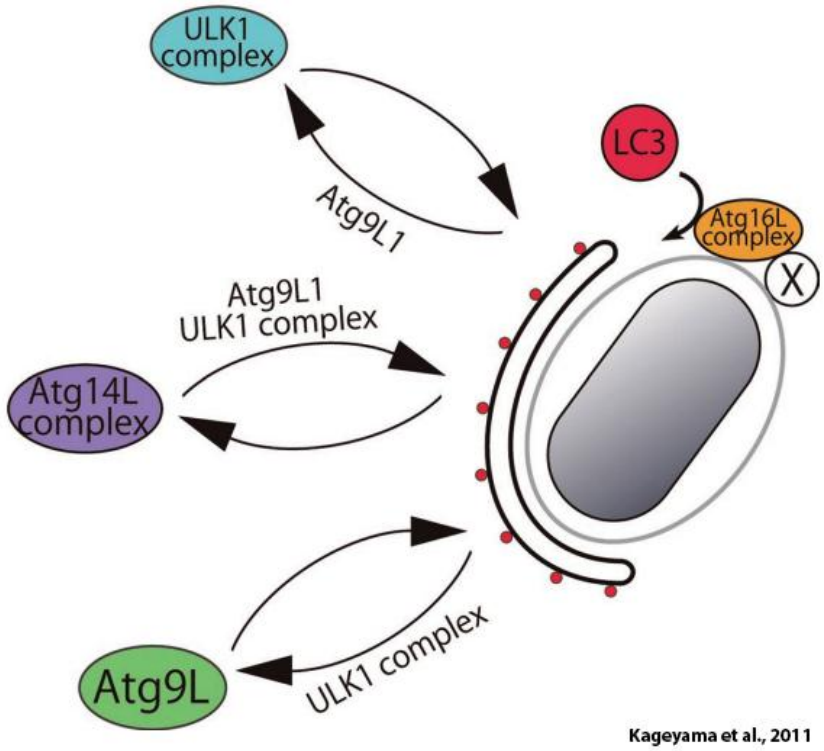


Figure 9: Model of protein dynamics during xenophagy of S. Typhimurium. The ULK1 complex, Atg9L and the Atg14L complex are cycling between the membrane that is formed around a SCV and another cellular pool. This cycling needs the indicated Atg proteins. They all contribute to the formation of a membrane around the SCV and are of importance of the restriction of *Salmonella* growth within the host cell. An unknown recruitment factor (X) is provided by *Salmonella* itself.

Type III Secretion Systems

Salmonella enterica expresses two different Type III Secretion Systems (T3SSs), which are encoded by the *Salmonella* Pathogenicity Islands (SPIs). The T3SS coming from the SPI-1 (SPI-1 T3SS) enables invasion of the host cells via the formation of pores in the host cell membrane by needle-like structures and via targeting different processes in the host cell by its effector proteins. The T3SS coming from the SPI-2 (SPI-2 T3SS) is a multifunctional system that facilitates replication of *Salmonella* within the SCVs (as reviewed in (42)). Recently researchers found that SPI-2 T3SS excretes the deubiquitinase SseL. This deubiquitinase is found to counteract the formation of ubiquitinated structures in infected cells (43). Ubiquinated structures are recognized by the autophagy adaptor proteins during autophagy (as described in the paragraph about ubiquitin). SseL activity deubiquinates the ubiquinated structures and therefore prevents recognition of the structures by the adaptor proteins and subsequently prevents autophagy.

Damaged *Salmonella*-containing vacuoles

One important way in which the host cell recognizes the invading bacteria involves the suggestion that SPI-1 T3SS can accidentally create pores in the membrane of the *Salmonella*-containing vacuole (41), leading to a damaged SCV. The first way in which a host cell can detect a damaged vacuole involves the recruitment of LC3-PE (figure 10), which is essential for the formation of an autophagosome (figure 8).

Until recently it was not known how the recruitment of LC3-PE to the damaged SCV occurred. It was only known that the recruitment of LC3-PE occurred independently of Atg9L1, FIP200 and the PI3K complex since in absence of these molecules LC3-PE and the Atg16L-complex were still recruited (40). In 2012 it was discovered that Galectin 8, a cytosolic lectin, was able to recognize glycans that were exposed on damaged SCVs (44). Under homeostatic conditions these glycans are not present in the cytosol, they are only found extracellular. Therefore the presence of such glycans in the cytosol corresponds to a 'dangerous' situation, which is the presence of extracellular components in the cytosol. Galectin 8 binds the glycans exposed on the membrane of damaged SCV and recruits NDP52 (44). Galectin-1, -3, -8, and -9 are able to bind the glycans associated with damaged endosomes or vacuoles. Recently it is discovered that due to a specific sterical hindrance, only galectin 8 (and not other galectins) is able to bind to NDP52 leading to antibacterial autophagy (45). As described before in the section about cargo recognition, NDP52 is an adaptor protein that is able to bind both the substrate and LC3. Therefore the galectin 8 – NDP52 complex is able to recruit LC3-PE, and is of great importance for antibacterial autophagy and growth restriction of *Salmonella* in human cells. Additionally p62 is found to act cooperatively with NDP52 in the clearance of *Salmonella* from host cells via autophagy. They both target a different microdomain of the bacterium, and are recruited independently of each other to the bacterium at the same time. Both adaptor proteins function via the same autophagy pathway. Both cargo recognition proteins are needed for effective autophagy, suggesting that both proteins bring unique components that are needed for correct ubiquitin-dependent autophagy of the bacteria (46).

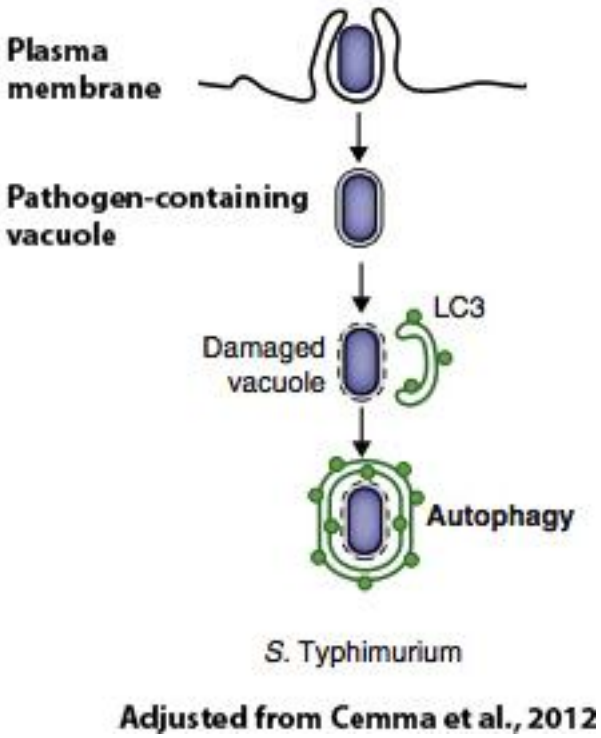
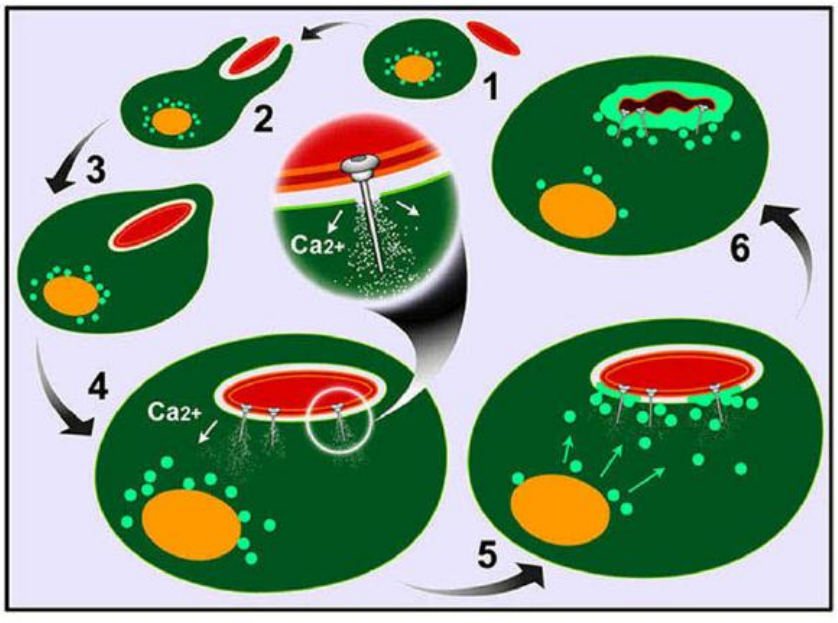


Figure 10: Overview of autophagy of damaged SCV in the host cell. S. Typhimurium enters the host cell in a *Salmonella*-containing vacuole. The vacuole is thought to be damaged by the needle-like structure of the SPI-1 T3SS, which is normally used to enter the host cell. The damaged membrane is recognized by the host cell and via Galectin 8 and NDP52, LC3 is recruited to the vacuole, inducing autophagy.

Secondly there is also a Ca^{2+}-dependent way in which a host cell can recognize and degrade a damaged SCV. Phagolysosome fusion is a Ca^{2+}-dependent process (47). The second way in which a damaged SCV can be detected and targeted for autophagy also depends on the accidental pore forming by the T3SS. The creation of pores enables a Ca^{2+} flux from the Ca^{2+} rich SCV into the cytosol. This elevation in Ca^{2+} level of the cytosol attracts lysosomal synaptotagmin (Syt VII) to the damaged vesicle, which in turn enables fusion of the damaged vacuole with a lysosome for degradation (41, 48)(figure 11) This limits the intracellular growth of the *Salmonella* bacteria that have entered the cell.



Roy et al., 2004, supplementary figures

Figure 11: Model for the Ca^{2+}-dependent degradation of damaged *Salmonella*-containing vesicles. 1. A *Salmonella* bacterium approaches a human cell. 2. The bacterium enters the human cell. 3. Bacterium is present in a *Salmonella*-containing vacuole (SCV). 4. Upon damage of the membrane of a SCV, caused by the needle-like structure of a T3SS, a Ca^{2+} flux streaming from the SCV to the cytosol is induced, elevating the amount of Ca^{2+} in the cytosol. 5. The elevated amount of Ca^{2+} triggers the fusion of lysosomes with the vesicles and the bacteria are killed.

Some bacteria escape the SCV

Both the Ca^{2+} flux and the galectin 8 – NDP52 – LC3-PE complex seem to be important for the clearance of damaged *Salmonella*-containing vacuoles from the cytosol of human cells. Nevertheless, not all *Salmonella* bacteria stay inside the SCV. About 10% of the bacteria escape from the SCV into the cytosol (25). How this escape occurs is unknown, but it might be similar as in case of *Listeria monocytogenes*, in which the bacteria can enter the cytosol via pores in the vacuole membrane (49). This would mean that S. Typhimurium bacteria could escape the damaged vacuoles via the accidentally created pores in the vacuoles.

The escaped bacteria stay in the cytosol and are recognized by the ubiquitin system and become surrounded by ubiquinated proteins (25). As described before, the mechanism of this recognition and labeling is still unknown. Subsequently the ubiquitin around the bacteria can be recognized by NDP52 (31), or p62 (26), inducing autophagy by binding LC3 together with the *Salmonella* bacteria (as described in the paragraph 'Xenophagy of bacteria').

In summary, the human host cell uses autophagy related proteins to create a membrane around *Salmonella*-containing vacuoles and to restrict S. Typhimurium growth within these SCVs. When these vacuoles are damaged (possibly by the needle-like structure of the SPI-1 T3SSs), the damaged vacuole can be either recognized by Galectin 8, which binds to the membrane of the damaged vacuole and recruits NDP52 and subsequently LC3-PE, inducing autophagy, or in a Ca^{2+}-dependent manner, leading to degradation of the vacuole. Additionally about 10% of the bacteria escape from the vacuole and are surrounded by ubiquitinated proteins in the cytosol and can therefore be recognized and targeted for autophagy. To prevent recognition of ubiquitin-labeled structures of *Salmonella*, the SPI-2 also excretes a deubiquitinase that takes off the ubiquitin of structures close to the damaged SCVs. Above findings indicate that autophagy is important for the restriction of S. Typhimurium growth in human host cells and the elimination of the free bacteria and damaged vacuoles.

Listeria monocytogenes

Listeria monocytogenes is a rapidly growing Gram-positive bacterium with a broad host range (50). It causes food poisoning, leading to serious, often fatal, diseases including listeriosis (possibly leading to meningitis and bacteremia), gastroenteritis, and encephalitis. Infection can even lead to spontaneous abortion (32, 51). Most vulnerable are immunocompromised people, pregnant women and newborns (as reviewed in (50)). The bacterium is widely found in water and soil, leading to the fact that no food source is safe from possible contamination. Contamination can occur in every processing stage of the food product. Major food vehicles for *L. monocytogenes* are fresh soft cheeses, unpasteurized dairy products and inadequately pasteurized milk (32).

L. monocytogenes enters the cell in a vacuole, but preferably escapes this vacuole and continues its life within the cytosol. Therefore *L. monocytogenes* is called a cytosolic bacterium. In most cases a macrophage actively engulfs the bacterium via phagocytosis (52), but *Listeria monocytogenes* can also enter other cell types. Subsequently the bacterium escapes this vacuole and enters the cytosol. Entrance into the host cell, escape from the vacuole, replication within the cytosol and manipulation of immune responses occurring in the cytosol are stages of the lifecycle of the bacterium (53).

There are not many cytosolic bacteria known. Many pathogens stay inside a membrane-bound compartment during infection of the host cells. Only a small number of bacteria, including *Listeria monocytogenes* can enter the cytosol and replicate there. This ability of survival is not just dependent on the ability to gain access to the cytosol. Direct microinjection of several different bacteria into the cytosol of host cells results in survival of merely bacteria that are usually able to proliferate in the cytosol. Other bacteria, such as *Salmonella enterica* serovar Typhimurium, are not able to survive in the cytosol. This indicates that pathogenic cytosolic intracellular bacteria have evolved specific mechanisms to grow in the cytosol (54).

Escape from the vacuole

L. monocytogenes escapes the vacuole by making use of listeriolysin O (LLO)(49) among others. Listeriolysin O (LLO) is a cholesterol-dependent haemolysin that can form pores. LLO is able to bind the cholesterol in the membrane of the vacuole, and forms pores in it, leading to a damaged vacuole from which the bacteria can escape. It was shown in animal models that LLO is sufficient for other bacteria to escape from a vacuole into the cytoplasm (55). In case of *Listeria* it is not proven that LLO expression is sufficient. Two more factors involved in escape of *Listeria monocytogenes* bacteria from the vacuole are known, i.e. two types of phospholipases and the host factor GILT.

Two types of phospholipases are found to be involved in the escape of *Listeria* bacteria from the vacuole, i.e. a phosphatidylinositol-specific phospholipase (PI-PLC) and a broad-range phospholipase C (PC-PLC). Mutant bacteria lacking PI-PLC were twofold less virulent in mice than the wildtype. They also had a minor defect in escaping from the vacuole, while there was no effect on the cell-to-cell spread. Mutation in PC-PLC resulted in more severe defects within the bacteria. Bacteria lacking PC-PLC showed a 20-fold decrease in virulence in mice, and there was no cell-to-cell spread anymore. There was no difference in escape from the vacuole. Mutant bacteria lacking both PI-PLC and PC-PLC showed a 500-fold decrease in virulence in mice compared to infection with the wildtype bacteria. Cell-to-cell spread and escape from the vacuole were severely diminished in these double mutant

bacteria (56). These data show that both phospholipids are of importance for the virulence of *L. monocytogenes* and of its escape from the vacuole.

Additionally the bacterium uses the host factor γ-interferon-inducible lysosomal thiol reductase (GILT) for its escape from the vacuole. GILT is constitutively expressed within the lysosomes of antigen presenting cells (as reviewed in (57)). Subsequently GILT accumulates in macrophage phagosomes during maturation into phagolysosomes. To facilitate antigen presentation, the protein reduces disulfide bonds. An enzymatically active precursor of GILT is changed into the mature form in early endosomes. GILT activates the secreted LLO of the bacterium, enabling escape from the vacuole. Lack of this enzyme in host cells induces resistance against *L. monocytogenes* in mice, due to delayed escape of the bacteria from the vacuole(57).

Avoidance of autophagy

Listeria monocytogenes is known to be able to subvert the autophagy system in different ways, depending on if a high or a low amount of Listeriolysis O (LLO) is secreted (figure 12).

When a low amount of LLO is expressed, *Listeria* cannot escape from the phagosome. A spacious *Listeria* containing phagosome (SLAP) is produced (figure 12), in which the bacteria can slowly replicate over a 72h time period (58). Fusion of a SLAP with lysosomes is blocked, preventing degradation of the content of the vacuole. Therefore LLOs seem to play a role in enabling an established persistent infection of *Listeria monocytogenes*. The SLAP formation occurs via the Listeria adhesion protein (LAP) pathway (59).

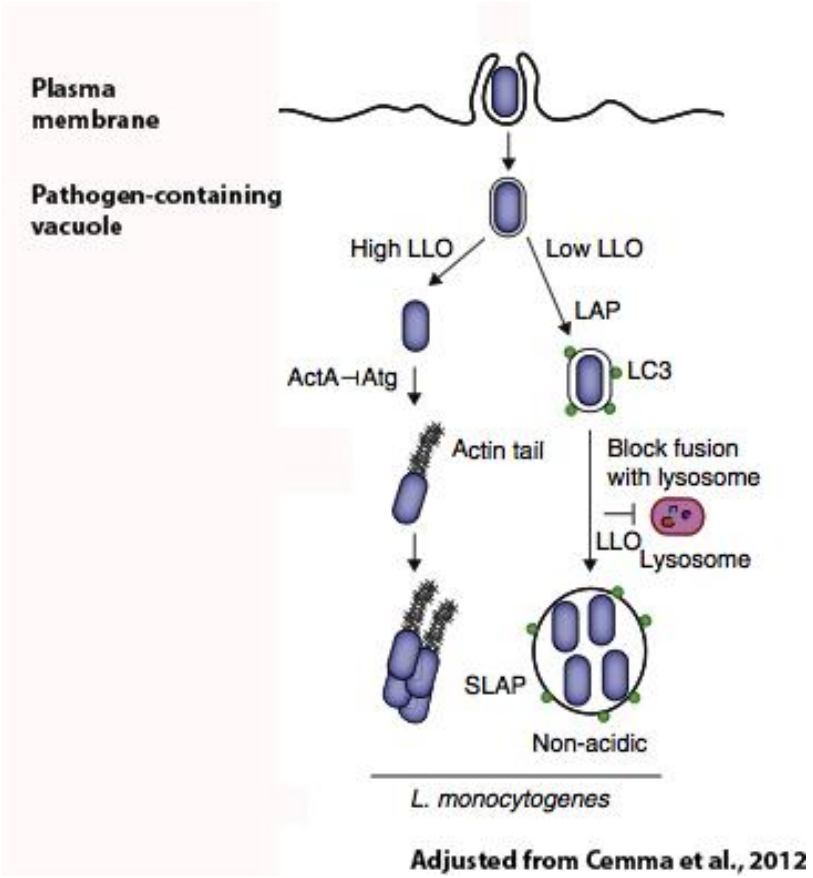


Figure 12: Overview of ways of subverting autophagy by *Listeria monocytogenes.* When *Listeria* bacteria have entered the cell, they start expressing Listeriolysin O (LLO). Depending on the amount of expression, the bacteria either create an actin tail via *ActA* for motility (High LLO) or are caught by a membrane carrying LC3 (low LLO). The excreted LLOs prevent fusion of this created vacuole with lysosomes and the vacuole is transformed into a spacious *Listeria* containing phagosome (SLAP).

When a high amount of LLO is secreted, *ActA* expression induces the formation of an actin tail (figure 12), enabling motility and cell-to-cell spread (60). Additionally the produced ActA protects the bacteria from detection by the autophagy machinery by recruiting the Arp2/3 complex and VASP to the bacterial surface (figure 13)(61). The presence of these proteins disguises the bacterium from recognition by the autophagy machinery. When a form of ActA that is unable to recruit the proteins, is expressed by the bacterium, this protection did not occur. These bacteria become ubiquitinated and were recognized by p62 (adaptor protein) and LC3, followed by autophagy (61).

InlK is a second protein known to contribute to the avoidance of autophagy by *L. monocytogenes* bacteria. InlK is a virulence factor of *L. monocytogenes*, linked to the bacterial surface by sortase A. This InlK attracts the Major Vault Protein (MVP). MVP is a protein that is the main component of vaults; cytoplasmic ribonucleoproteic particules. The presence of MVP at the bacterial surface of the cytosolic bacteria disguises the bacteria of recognition by the autophagy machinery. Bacteria that are overexpressing InlK have an increased survival rate compared to bacteria lacking InlK (62). Based on experiments a model for the escape of autophagic recognition by *Listeria monocytogenes* via ActA and InlK is proposed (figure 13).

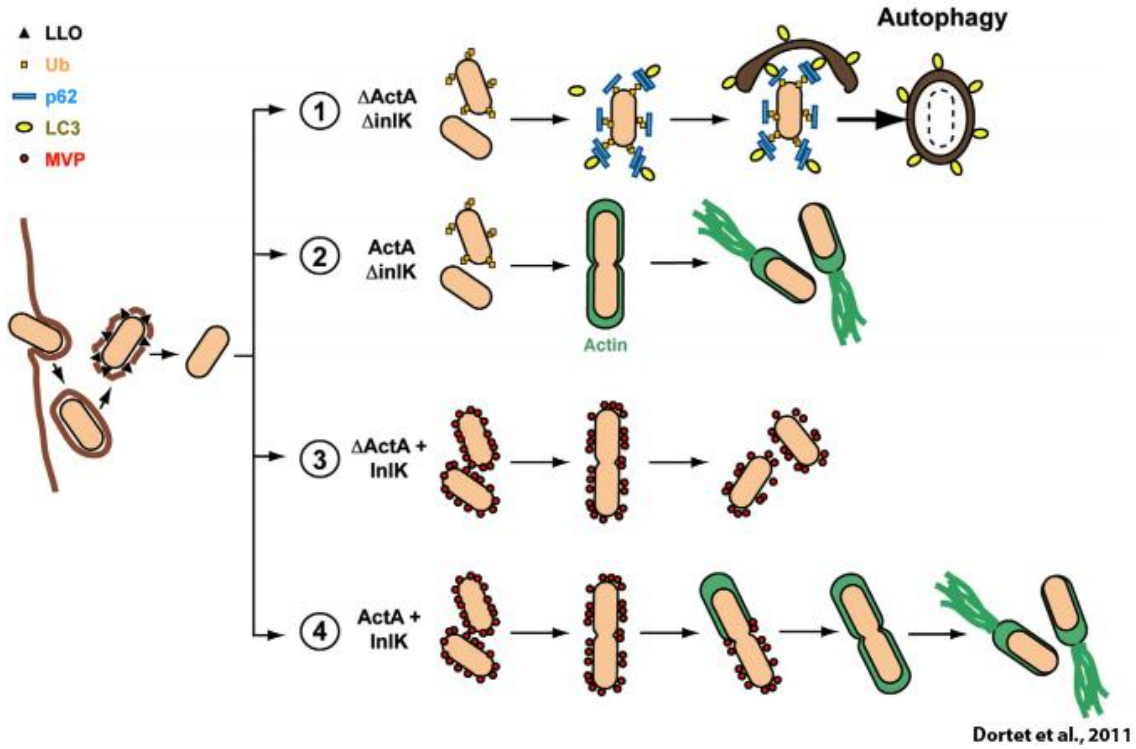


Figure 13: Model for escape of *Listeria monocytogenes* from the autophagy machinery by ActA and InlK. Two independent virulence factors help *L. monocytogenes* bacteria escaping the autophagy machinery during intracellular growth. ActA recruits VASP and Arp2/3, which can mask the bacteria. Therefore they will not be ubiquitinated and recognized by the autophagy machinery (61). InlK recruits MVPs, which also mask the bacteria. Bacteria masked by MVPs are not ubiquitinated and not recognized by the autophagy machinery (62). According to these data a model is proposed for four different scenarios. 1. Both ActA and InlK are absent: the bacterium can be ubiquitinated and subsequently recognized by the autophagy machinery. 2. InlK is absent, ActA is present: *Listeria* bacteria can escape the autophagy. 3. ActA is absent, InlK is present: MVP recruitment efficiently prevents autophagic recognition of the bacteria. 4. Both ActA and InlK are present: the bacterium is protected from recognition by recruitment of MVPs by InlK. Later, the InlK are replaced by ActA, leading to a change of disguise from MVPs to actin.

In summary, after escape of the vacuole by use of GILT, LLO and two phospholipases, the cytosolic *Listeria monocytogenes* bacteria are able to subvert autophagy in two ways. In case of low Listeriolysin O (LLO) secretion, fusion of LC3-bound phagosomes, which are generated via the LAP pathway, with lysosomes is blocked, preventing degradation of the phagosome. When there is high LLO expression, ActA and InlK are expressed. ActA enables formation of an actin tail and therefore it enables bacterial motility. Together with InlK ActA recruit proteins to cover the bacterium, disguising them from recognition by the autophagy machinery (process shown in figure 14).

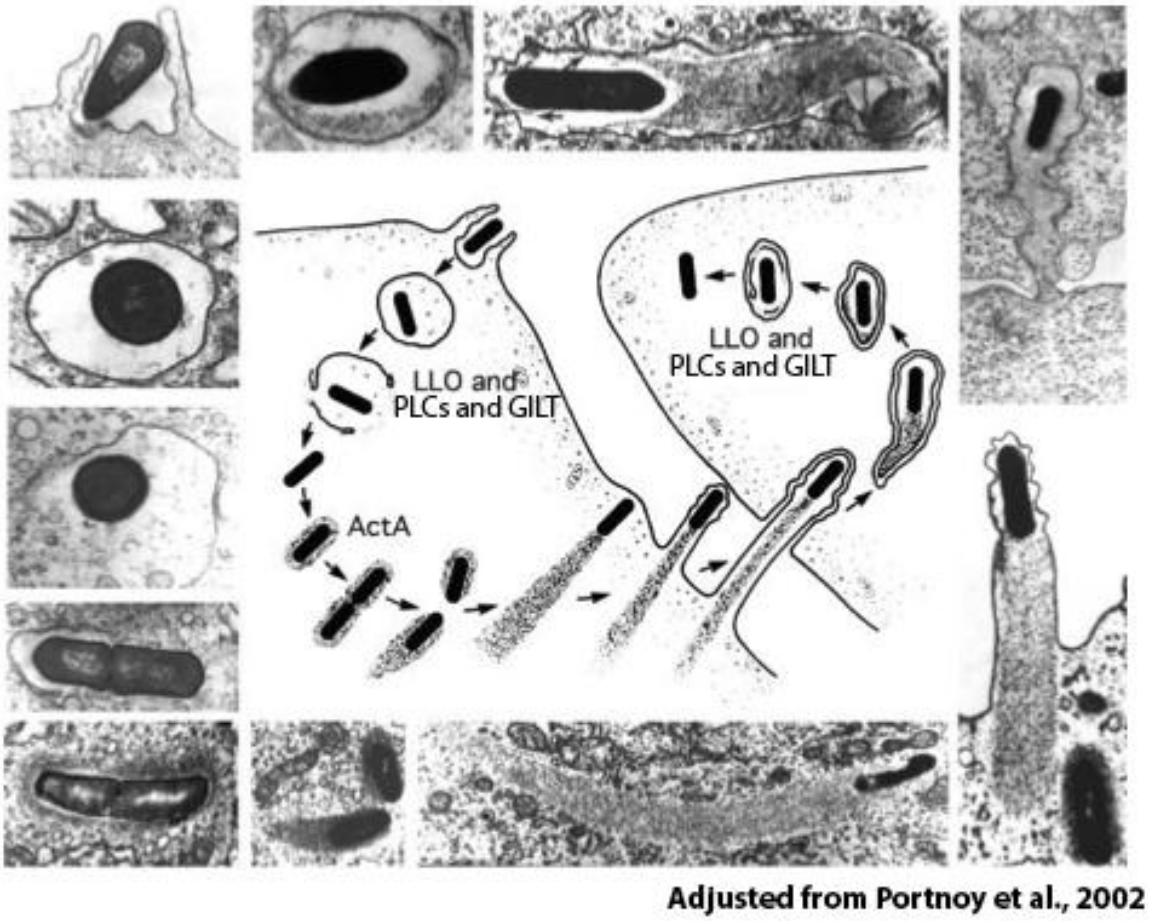


Figure 14: Microscopy pictures and overview of a *Listeria monocytogenes* infection. A *Listeria* bacterium enters a cell in a vacuole. High Listeriolysin O (LLO), phoshpolipases (PLCs) and enable the bacterium to escape from the vacuole the host factor γ-interferon-inducible lysosomal thiol reductase (GILT) enable escape of the bacterium from the vacuole. ActA induces covering of the bacterium by ActA and actin and the formation of an actin tail. The actin tail enables motility and cell-to-cell spread, where the same process of uptake and escape occurs.

Autophagy of Listeria monocytogenes

Although *Listeria* bacteria have mechanisms to avoid autophagic recognition, the host cells have some mechanisms to recognize the bacteria. The exact mechanism of *Listeria* recognition by the host cells is not known, but some proteins involved in the autophagy pathway(s) have been determined.

Infection of bone marrow derived macrophages with wildtype *Listeria* bacteria induces lipidation of LC3-I into LC3-PE, indicating the formation of an autophagic membrane around the bacteria. This autophagic activation was found to be fully dependent on the activity of LLOs by the bacteria, damaging the bacteria containing vacuoles (49). LLOs create pores in the vacuole to give the bacteria access to the cytosol. This means that a damaged vacuole is created, which can be recognized by the autophagic system. The damaged vacuoles are detected by Galectin 8; the same protein that detects damaged *Salmonella*-containing vacuoles (SCVs). Galectin 8 recognizes host glycans exposed on damaged vacuoles and is therefore suggested to serve as a versatile vesicle-damaging pathogen receptor (44). Since Galectin 8 is recognized and bound by NDP52 (45), it can be suggested that via this pathway the damaged *Listeria* vacuoles are recognized and cleared as well.

The adaptor proteins p62 and NDP52 (which are described in the paragraph 'Xenophagy of bacteria') are able to target *Listeria monocytogenes* bacteria to an autophagy pathway that is independent of septin and actin. This finding led to the insight that selective autophagy can occur via different pathways. Both adaptors are found not to act interdependent for clearance of *Listeria* from the host cells. Again it was shown that ActA, which polymerizes actin, prevents the ubiquitination of *Listeria* and therefore prevents autophagy. Furthermore the host cytokine TNF-α, which is produced by the host cell upon bacterial infection, promotes p62-mediated autophagy (63).

Autophagy of *Listeria monocytogenes* occurs via the extracellular signal-regulated kinase (ERK) pathway. When no ERK is expressed, fewer bacteria are targeted for autophagy, and higher bacterial growth is detected. The innate immune receptors TLR2 and NOD/RIP2 are found to activate autophagy via this ERK pathway and are therefore of great importance for degradation of *Listeria monocytogenes* within the host cells (64).

Role of autophagy in other processes

When discovered in 1974 autophagy was thought to be important for the maintenance of homeostasis via self-digestion (as reviewed in (1)). Over the years, autophagy was found to be involved in a lot more processes than just homeostasis. It was found to be involved in cancer, neurodegradation, metabolic diseases, aging, immunity, and protection against - and elimination of - invading bacteria (2, 3).

Caloric restriction, which is reduced food intake without creating malnutrition, is the most physiological inducer of autophagy (65). It induces autophagy via activation of two energy sensors, i.e. AMPK and Sirtuin 1 (SIRT1) or via the inhibition of insulin or the insulin-like growth factor (IGF) leading to mTOR inhibition (as reviewed in (66). Caloric restriction is found to be beneficial for health and to increase the life span in most animals. It is found to have several beneficial effects for the body, summarized in figure 15.

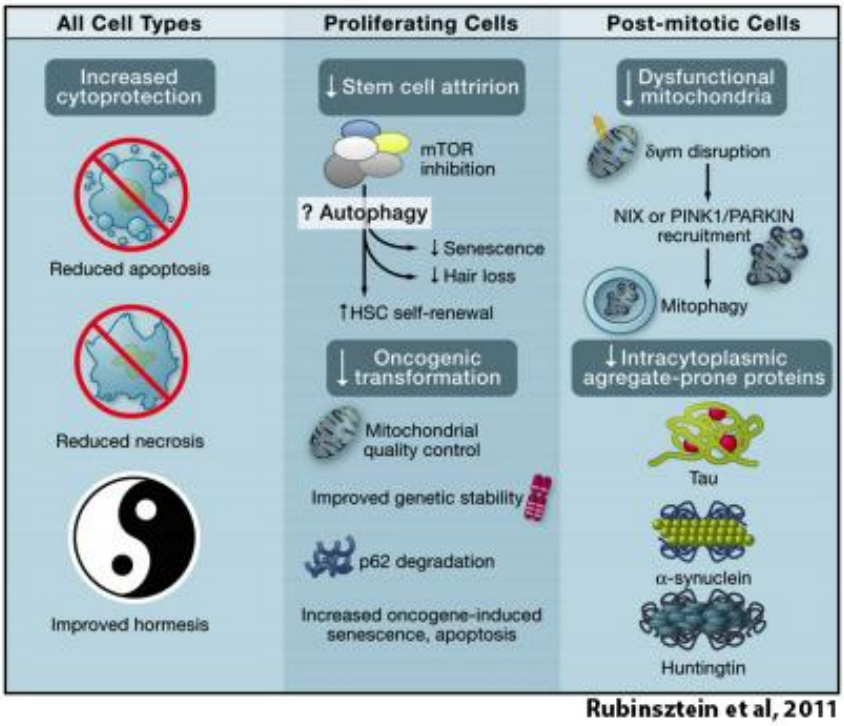


Figure 15: Beneficial effects of caloric restriction for cells. All cell types show reduced apoptosis, reduced necrosis and an improved hormonesis. Proliferating cells show reduced stem cell attrition and reduced oncogenic transformation, leading to among others reduced hair loss and improved genetic stability. In post-mitotic cells the mitochondria function better and less prone-protein aggregates are found.

Even though activation of autophagy seems to be very beneficial for survival and health, it is not without risk. Autophagy may also help to keep cells alive that should die, by preventing apoptosis. Autophagy is usually seen as pro-survival. It enables the cell to deal with starvation and other stresses. This means it might also keep cells alive that are supposed to die, such as chemotherapy-treated tumor cells. Additionally there are pathogens known that make use of the autophagy system to proliferate, including medically important pathogens such as HIV, Hepatitis viruses B and C, and *Coxiella burnetii* (causing Q-fever) (as reviewed in (66)). In case of increased autophagy, patients might become more vulnerable to infections with these pathogens. Therefore it is needed to be cautious when it comes to treating diseases by upregulating autophagy. Unwanted side effects should be monitored during the clinical trials and it should be taken into account that autophagy is involved in many processes throughout the human body and therefore has a great impact.

Discussion

In the following paragraphs the possibility of treating infections by general upregulation of autophagy and the possibility of treating bacterial infections by specifically targeting those bacteria are discussed.

General upregulation of autophagy as a treatment against infection

In 1998 it was shown for the first time that autophagy might be a good target for treatment of infectious diseases. Increased *Beclin* expression led to decreased Sindbis virus replication and virus-induced apoptosis. Consequently this increased *Beclin* expression resulted in protection against lethal encephalitis (67). Back then, Bcl-2 was only known to be involved in apoptosis and it was not known to be involved in autophagy. Over the years it was found out that Beclin 1 is part of the PI3K complex and that it is regulated by starvation via Bcl-2 and Bcl-X_L (figure 6). This was the first experiment that suggested the beneficial effects of increased autophagy for treatment of infections.

Beclin 1

There are many steps of autophagy that are possible targets for induction of autophagy by drugs. Most common mechanisms of autophagy induction targeted are the inhibition of mTORC1, activation of Beclin 1, and lowering of cAMP levels (as reviewed in (6). mTORC1 regulates cell growth and cell differentiation and has a regulatory role in autophagy. cAMP is used in several signal transduction routes and has also a regulatory role in autophagy. Beclin 1 not known to have more functions in the human body besides its involvement in autophagy and is therefore of more interest for upregulation of autophagy.

Ever since this experiments Beclin 1 is an interesting molecule for autophagy research. Very recently a peptide, called Tat-Beclin 1, is found that is able to induce autophagy via a functionally important region of Beclin 1. It interacts with the newly identified negative regulator of autophagy GAPR-1. Activity of Tat-Beclin 1 results in a decreased survival of *L. monocytogenes* within infected cells (68). This peptide seems to be specifically active on Beclin 1, a key protein of the human autophagy machinery. Because of its few functions, having Beclin 1 as a target for autophagy inducing drugs might result in few to no side effects. In diseases that are caused by dysregulation of autophagy this might therefore be a specific and useful peptide for treatment.

Vitamin D

In HIV-1 infected persons, the vitamin D level is found to be low. Low levels of vitamin D are associated with a rapid disease progression and an increased risk for infection with *Mycobacterium tuberculosis.* Recently it was found that the active form of vitamin D significantly inhibits HIV-1 replication in macrophages in a dose-dependent manner. This mechanism is dependent on PI3K, Atg5 and Beclin 1, which are all involved in autophagy and consequently induces autophagy (69). Vitamin D dependent HIV replication and growth of *M. tuberculosis* is found to be dependent on both induction of autophagy and phagosomal maturation (70). This means that sufficient vitamin D is important in HIV- and *M. tuberculosis*-infected patients. This might also be the case for infections with viruses or bacteria that use a similar mechanism as these examples. This is an interesting research-field because vitamin D is a naturally occurring compound, which might therefore provide a very natural treatment of infections.

Autophagy of specific bacteria

It is important to keep in mind that increasing autophagy can fight not all pathogenic infections. Every pathogen is different and is in a different way related to the autophagy machinery. In some cases the pathogen makes uses of the autophagy machinery of the host cell to proliferate. In such cases inducing autophagy would not be a good treatment. Therefore it would be best to specifically target a bacterium for autophagy. This possibility is explored for the bacteria *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* in this paragraph.

Salmonella enterica serovar Typhimurium

In host cells, *Salmonella* infection increases in absence of autophagy (40, 71). This suggests that even though autophagy is not efficient enough to eliminate the bacteria, it is of great importance to restrict growth and prevent excessive infection of host cells. Because autophagy can restrict growth, it might be expected that increased autophagy might be able to eliminate the bacteria from the cells. This means that in case of *Salmonella* general increase of autophagy might be a good treatment for restriction of the infection. To reach total elimination of the bacteria, additional treatment is necessary.

There are proteins that might be specific targets for the clearance of *Salmonella* from cells. A very interesting protein that shows induced expression upon entry of Salmonella into the host cell is SifA. The protein is found to be of importance for the maintenance of *Salmonella*-containing vacuoles (SCV). It was shown that lack of SifA resulted in degradation of the SCV in macrophages (36). Interestingly enough in epithelial cells lack of SifA resulted in increased bacterial replication (38). The increase in expression upon entry of *Salmonella* into the host cell suggests importance of this protein for survival and growth of the bacteria. The finding that disruption of the vacuole leads to increased *Salmonella* growth in epithelial cells suggests that there is a difference in composition between the cytosol of different cell strains. This might be an interesting field for further research. SifA repression might be a useful treatment against *Salmonella* infections in macrophages since it releases the bacteria from their protecting environment. This seems a straightforward approach of treating *Salmonella* infected cells. The main problem in this approach is the finding that in epithelial cells disruption of the vacuole leads to an increase of bacterial replication rather than a decrease. To be able to further develop a treatment based on SifA, further research on the difference between the cytosol of macrophages and epithelial cells leading to this difference in replication is required.

It might also be possible to target the producer of these SifA proteins, which is the *Salmonella* pathogenicity island 2 (SPI-2). Both SPI-1 and SPI-2 are of importance for the ability of *Salmonella* to infect and proliferate within host cells. SPI-1 T3SS enables the entrance of *Salmonella* into the host cell, while SPI-2 T3SS and the other effector proteins of SPI-2 facilitate replication of the bacteria within the SCV (as reviewed in (42)). This means that SPI-1 is most active before and during entrance of the host cell and that SPI-2 is mostly active after invasion of the host cell. Therefore SPI-2 might be an effective target for reducing infection. The effector proteins and translocons of SPI-2 are involved in many processes, among which the deubiquitination of ubiquinated structures, preventing autophagy (43). Therefore SPI-2 and the deubiquinating proteins SseL might be effective targets for the repression of *Salmonella* infection via autophagy.

Intact SCVs are not easily detected in the host cell, while damaged vacuoles are. If there is a possibility to damage the SCVs, this might be an effective treatment of *Salmonella* infection. The damaged vacuoles are detected and targeted for autophagy in two ways as described before. Simply adding membrane-damaging molecules to the cells would damage not only the SCVs, but also the host cell membranes. Therefore it would be optimal if the membrane damaging molecules, such as T3SSs can be targeted to the SCVs specifically. With the current knowledge this seems not possible yet. Optimally, the protein that is recruited by *Salmonella* to the SCV (depicted as factor X in figure 9) can be a target. First it should be investigated what molecule this is and if it has other functions in the host cell as well, to get insight into the possibility of this factor as a target to the SCV.

Listeria monocytogenes

During infection, *Listeria monocytogenes* is able to subvert the autophagic system. Therefore overall upregulation of autophagy is not expected to be a useful treatment. It might be more beneficial to specifically target the mechanisms used for subversion of the autophagic system. Still then it seems hard to upregulate autophagy, but targets for reduction of the infection are suggested.

For *Listeria* bacteria it most favorable to escape from the vacuole and to start to proliferate in the cytosol of the host cell. For this escape the host factor γ-interferon-inducible lysosomal thiol reductase (GILT) is used. Lack of GILT in the host cells induces delayed escape of the bacteria from the vacuole and consequently a higher resistance against *L. monocytogenes* (57). Unfortunately these cells are also deficient in generating specific major histocompatibility complex T-cell responses to specific protein antigens. It is suggested that, due to its ability to reduce disulfide bonds in acidic environments, GILT may have more crucial functions independent of lysosomes and of antigens (72). Therefore although this molecule is of importance for the escape of the bacteria from their limiting vacuole, GILT would not be a good target to restrict *Listeria monocytogenes* infection.

ActA is an important protein for the functioning of *Listeria monocytogenes.* As shown in figure 11, it serves two important functions during *Listeria* infection. Firstly it recruits VASP and Arp2/3 that disguise the bacteria from i.e. recognized by the autophagy machinery. Secondly it enables the formation of an actin tail by the bacterium, enabling transport of the bacterium throughout the cytosol and between cells. Additionally ActA is found to be involved in the disruption of the vacuole from which the bacteria escape (73). Because of the pleiotropic involvement of ActA during *Listeria monocytogenes* infection, this might be an interesting target for treatment of infected cells. Repression of ActA is expected to result in repression of the infection because of the decrease in motility and decreased escape from the vacuole. The proposed model of *Listeria* escape from the autophagy system (figure 13) shows that even in absence of ActA, the bacteria are still sufficiently covered to prevent detection by the autophagy machinery. Therefore targeting ActA will only slow down infection, but will not lead to induction of autophagy. To enable detection of the bacteria by the autophagic machinery and subsequent induction of autophagy, both ActA and InlK should be targeted.

Having LLO as a target will also lead to the reduction of infection, but not to increased autophagy. A low amount of LLO leads to the capture of the bacteria in vacuoles, but has no specific effect on autophagy. Therefore this might be a good target for reduction of the infection and prevention of

cell-to-cell spread of the infection, but for the clearance of the bacteria, additional upregulation of autophagy is needed.

Another suggestion is the upregulation of the expression of ERK in the host cells, or the addition of ERK. It was found that when no ERK is expressed, fewer bacteria are targeted for autophagy (64). Therefore upregulation of ERK might lead to increase of autophagy and subsequently increased clearance of the bacteria. Yet, the bacteria need to be manipulated so they lose their protection. *Listeria monocytogenes* seems to be a bacterium that very efficiently subverts the human autophagy system, based on a protection at different levels. Therefore induction of autophagy of *Listeria monocytogenes* in specific requires a combination of treatments. To reach an efficient combination, more research on the different aspects of its subversion is required.

Conclusion

Autophagy is an important process for cell survival in the human body. Infections can in some cases be fought by induced autophagy. Nevertheless, there are also infections that make use of the autophagic system, and will be enhanced by upregulation of autophagy. Additionally induction of autophagy as a treatment should be considered per patient, since there might also be side effects in combination with other diseases. Autophagy might for example enable cell survival of cells that were targeted by chemotherapy in case of a tumor. It is not possible to draw an overall conclusion for the possibility of induced autophagy for the treatment of infections. The infections should be investigated separately. In some cases induced autophagy might be sufficient to eliminate the pathogenic bacteria from the body. In other cases it might be more efficient to specifically target components of the bacteria that prevent autophagy, to induce clearance via the autophagic pathway. In case of *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes*, there are no proven targets yet. Based on the mechanisms these bacteria use to subvert the autophagic system and the limitations of this system, some suggestions for efficient targets to upregulate autophagy of S. Typhimurium specifically can be made. Further research on these suggested targets is required to investigate if these molecules have additional functions in other processes. Nevertheless, no good targets for induction of autophagy of *L. monocytogenes* could be suggested, since this bacterium makes use of protection at different levels. The most promising suggestion made is the combination of the deletion of the protection by targeting two virulence factors involved in the recruitment of the protection layer, in combination with induction of autophagy. Nevertheless, in case the bacterium can be detected, the autophagy machinery is able to eliminate the bacterium and is therefore still a useful mechanism to fight *Listeria* infections. In conclusion, autophagy is a very promising mechanism for treatment of a broad range of diseases, including infections, although the specific treatment should be adjusted per patient and pathogen.

Literature

1. Klionsky DJ. Autophagy: From phenomenology to molecular understanding in less than a decade. Nature reviews molecular cell biology. 2007;8(11):p931-7p.

2. Levine B, Yoshimori T, Deretic V. Autophagy in infection and immunity. 2009.

3. Choi AM, Ryter SW, Levine B. Autophagy in human healt and disease. New England journal of medicine (NEJM). 2013;14:651-62.

4. Reggiori F, Komatsu M, Finley K, Simonsen A. Selective types of autophagy. International journal of cell biology. 2012.

5. Arias E, Cuervo AM. Chaperone-mediated autophagy in protein quality control. Curr Opin Cell Biol. 2011;23(2):184-9.

6. Rubinsztein DC, Codogno P, Levine B. Autophagy modulation as a potential therapeutic target for diverse diseases. Nature reviews drug discovery. 2012;11(9):p709-22p.

7. Nakatogawa H, Suzuki K, Kamada Y. Dynamics and diversity in autophagy mechanisms: Lessons from yeast. Nature reviews molecular cell biology. 2009;10(7):p458-10p.

8. Behrends C, Sowa ME, Gygi SP. Network organization of the human autophagy system. Nature. 2010;466(7302):p68-9p.

9. Hamasaki M, Furuta N, Matsuda A, Nezu A, Yamamoto A, Fujita N, et al. Autophagosomes form at ER-mitochondria contact sites. Nature. 2013;495:389-93.

10. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature. 2011;469:323-35.

11. Mizushima N. The role of the Atg1/ULK1 complex in autophagy regulation. Curr Opin Cell Biol. 2010;22(2):132-9.

12. Klionsky D, Yen W, Legakis J, Nair U. Atg27 is required for autophagy-dependent cycling of Atg9. Mol Biol Cell. 2007;18(2):581-93.

13. Ohsumi Y, Kakuta S, Yamamoto H, Negishi L, Kondo-Kakuta C, Hayashi N. Atg9 vesicles recruit vesicle-tethering proteins Trs85 and Ypt1 to the autophagosome formation site. Journal of biological chemistry (JBC online). 2012;287(53):44261-9.

14. Mercer CA, Kaliappan A, Dennis PB. A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. Autophagy. 2009;5(5):649-62.

15. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, et al. Nutrient-dependent mTORC1 association with the ULK1–Atg13–FIP200 complex required for autophagy. Mol Biol Cell. 2009;20(7):1981-91.

16. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009;43(1):p67-27p.

17. Codogno P, Mehrpour M, Esclatine A, Beau I. Autophagy in health and disease. 1. regulation and significance of autophagy: An overview. American journal of physiology.Cell physiology. 2010;298(4):C776-85.

18. Levine B, Sinha S, Kroemer G. Bcl-2 family members; duals regulators of apoptosis and autophagy. Autophagy. 2008;4(5):600-6.

19. Moreau K, Ravikumar B, Renna M, Puri C, Rubinsztein DC. Autophagosome precursor maturation requires homotypic fusion. Cell. 2011;146(2):303-17.

20. Fujita N, Itoh T, Omori H, Fukuda M, Noda T, Yoshimori T, et al. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. Mol Biol Cell. 2008;19(5):2092-100.

21. Kirisako T, Ichimura Y, Okada H, Kabeya Y, Mizushima N, Yoshimori T, et al. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol. 2000;151:263-76.

22. Komatsu M, Sou Y, Waguri S, Iwata J, Ueno T, Fujimura T, et al. The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. Mol Biol Cell. 2008;19(11):4762-75.

23. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 2000;19(21):5720-8.

24. Jin M, Liu X, Klionsky DJ. SnapShot: Selective autophagy. Cell. 2013;152(1-2):368,368.e2.

25. Perrin AJ, Jiang X, Birmingham CL, So NS, Brumell JH. Recognition of bacteria in the cytosol of mammalian cells by the ubiquitin system. Current biology. 2004;14(9):806-11.

26. Brumell J, Zheng Y, Shahnazari S, Brech A, Lamark T, Johansen T. The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. Journal of immunology. 2009;183(9):5909-16.

27. Johansen T, Pankiv S, Clausen T, Lamark T, Brech A, Bruun J, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. Journal of biological chemistry (JBC online). 2007;282(33):24131-45.

28. Kirkin V, Lamark T, Sou YS, Bjorkoy G, Nunn JL, Bruun JA, et al. A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol Cell. 2009;33(4):505-16.

29. Dikic I, Wild P, Farhan H, McEwan D, Wagner S, Rogov V, et al. Phosphorylation of the autophagy receptor optineurin restricts salmonella growth. Science. 2011;333(6039):228-33.

30. von Muhlinen N, Akutsu M, Ravenhill BJ, Foeglein A, Bloor S, Rutherford TJ, et al. LC3C, bound selectively by a non-canonical LIR motif in NDP52, is required for anti-bacterial autophagy. Mol Cell. 2012;48(3):329-42.

31. Ivanov S, Roy CR. NDP52: The missing link between ubiquitinated bacteria and autophagy. Nat Immunol. 2009;10(11):p1137-3p.

32. Brock TD, Madigan MT, Marchalonis JJ. Biology of microorganisms. 12th ed. Pearson International Edition; 2009.

33. Takeshi Noda, Shun Kageyama, Naonobu Fujita, Tamotsu Yoshimori. Three-axis model for atg recruitment in autophagy against salmonella. International journal of cell biology. 2012;2012.

34. Brumell JH, Grinstein S. Salmonella redirects phagosomal maturation. Curr Opin Microbiol. 2004;7(1):78-84.

35. Buchmeier NA, Heffron F. Inhibition of macrophage phagosome-lysosome fusion by salmonella typhimurium. Infect Immun. 1991;59(7):2232-8.

36. Brumell JH, Rosenberger CM, Gotto GT, Marcus SL, Finlay BB. SifA permits survival and replication of *Salmonella typhimurium* in murine macrophages. Cell. Microbiol. 2001;3:75-84.

37. Beuzón CR, Méresse S, Unsworth KE, Ruíz-Albert J, Garvis S, Waterman SR, et al. Salmonella maintains the integrity of its intracellular vacuole through the action of SifA. EMBO J. 2000;19(13):3235-49.

38. Brumell JH, Tang P, Zaharik ML, Finlay BB. Disruption of the salmonella-containing vacuole leads to increased replication of salmonella enterica serovar typhimurium in the cytosol of epithelial cells. Infect Immun. 2002;70(6):3264-70.

39. Holden D, Beuzón C, Salcedo S. Growth and killing of a *Salmonella enterica* serovar Typhimurium sifA mutant strain in the cytosol of different host cell lines. Microbiology (Reading). 2002;148(Pt 9):2705-15.

40. Kageyama S, Omori H, Saitoh T, Sone T, Guan J, Akira S, et al. The LC3 recruitment mechanism is separate from Atg9L1-dependent membrane formation in the autophagic response against salmonella. Mol Biol Cell. 2011;22(13):2290-300.

41. Brumell J, Birmingham C, Smith A, Bakowski M, Yoshimori T. Autophagy controls salmonella infection in response to damage to the salmonella-containing vacuole. Journal of biological chemistry (JBC online). 2006;281(16):11374-83.

42. Waterman SR, Holden DW. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. Cellular Microbiology. 2003;5(8):501-11.

43. Mesquita FS, Thomas M, Sachse M, Santos AJM, Figueira R, Holden DW. The *Salmonella* deubiquitinase SseL inhibits selective autophagy of cytosolic aggregates. Plos Pathogens. 2012;8(6).

44. Thurston TLM, Wandel MP, von Muhlinen N. Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. Nature. 2012;482(7385):p414-5p.

45. Randow F, Li S, Wandel M, Li F, Liu Z, He C, et al. Sterical hindrance promotes selectivity of the autophagy cargo receptor NDP52 for the danger receptor galectin-8 in antibacterial autophagy. Science signaling. 2013;6(261):ra9.

46. Cemma M, Kim PK, Brumell JH. The ubiquitin-binding adaptor proteins p62/SQTM1 and NDP52 are recruited independently to bacteria-associated microdomains to target salmonella to the autophagy pathway. Autophagy. 2011;7:341-5.

47. Jaconi MEE, Lew DP, Carpentier JL, Magnusson,K.E.,Sjögren,M., Stendahl O. Cytosolic free calcium elevation mediates the phagosome-lysosome fusion during phagocytosis in human neutrophils. The Journal of Cell Biology. 1990;110:1555-64.

48. Roy D, Liston DR, Idone VJ. A process for controlling intracellular bacterial infections induced by membrane injury. Science. 2004;304(5676):p1515-4p.

49. Meyer-Morse N, Robbins JR, Rae CS, Mochegova SN, Swanson MS, Zhao Z, et al. Listeriolysin O is necessary and sufficient to induce autophagy during *Listeria monocytogenes* infection. PLoS ONE. 2010(6).

50. Portnoy DA, Auerbuch V, Glomski IJ. The cell biology of *Listeria monocytogenes*infection: The intersection of bacterial pathogenesis and cell-mediated immunity . JCB. 2002;158(3):409-14.

51. Birmingham CL, Canadien V, Gouin E, Troy EB, Yoshimori T, Cossart P, et al. *Listeria monocytogenes*Evades killing by autophagy during colonization of host cells. Autophagy. 2007;3(5):442-51.

52. Pizarro-Cerda J, Sousa S, Cossart P. Exploitation of host cell cytoskeleton and signalling during listeria monocytogenes entry into mammalian cells. Comptes rendus biologies. 2004;327(6):523-31.

53. Ray K, Marteyn B, Sansonetti PJ. Life on the inside: The intracellular lifestyle of cytosolic bacteria. Nature reviews microbiology. 2009;7(5):p333-8p.

54. Monika Goetz, Andreas Bubert, Gefu Wang, Isabel Chico-Calero, Jose-Antonio Vazquez-Boland, Markus Beck, et al. Microinjection and growth of bacteria in the cytosol of mammalian host cells. Proceedings of the National Academy of Sciences of the United States of America (PNAS). 2001;98(21):12221-6.

55. Bielecki J, Youngman P. Bacillus subtilis expressing a haemolysin gene from listeria monocytogenes can grow in mammalian cells. Nature. 1990;345(6271):p175-2p.

56. Smith GA, Marquis H, Jones S, Johnston NC, Portnoy DA, Goldfine H. The two distinct phospholipases C of listeria monocytogenes have overlapping roles in escape from a vacuole and cell-to-cell spread. Infect Immun. 1995;63(11):4231-7.

57. Singh R, Jamieson A, Cresswell P. GILT is a critical host factor for *Listeria monocytogenes* infection. Nature. 2008;455(7217):p1244-4p.

58. Birmingham CL, Canadien V, Kaniuk NA. Listeriolysin O allows *Listeria monocytogenes* replication in macrophage vacuoles. Nature. 2008;451(7176):p350-5p.

59. Cemma M, Brumell JH. Interactions of pathogenic bacteria with autophagy systems. Current biology. 2012;22(13):R540-5.

60. Monack DM, Theriot JA. Actin-based motility is sufficient for bacterial membrane protrusion formation and host cell uptake. Cell Microbiol. 2001;3:352-7.

61. Yoshikawa Y, Ogawa M, Hain T. *Listeria monocytogenes* ActA-mediated escape from autophagic recognition. Nat Cell Biol. 2009;11(10):p1233-8p.

62. Dortet L, Mostowy S, Samba Louaka A, Gouin E, Nahori MA. Recruitment of the major vault protein by InlK: A *Listeria monocytogenes* Strategy to avoid autophagy. Plos Pathogens. 2011;7(8).

63. Cossart P, Mostowy S, Sancho-Shimizu V, Hamon M, Simeone R, Brosch R, et al. p62 and NDP52 proteins target intracytosolic shigella and listeria to different autophagy pathways. Journal of biological chemistry (JBC online). 2011;286(30):26987-95.

64. Kanneganti T, Anand P, Tait S, Lamkanfi M, Amer A, Nunez G, et al. TLR2 and RIP2 pathways mediate autophagy of *Listeria monocytogenes* via extracellular signal-regulated kinase (ERK) activation. Journal of biological chemistry (JBC online). 2011;286(50):42981-91.

65. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008;132:27-42.

66. Rubinsztein DC, Marino G, Kroemer G. Autophagy and aging. Cell. 2011;146(5):682-95.

67. Liang XH, Kleeman LK, Jiang HH, Gordon G, Goldman JE, Berry G, et al. Protection against fatal sindbis virus encephalitis by beclin, a novel bcl-2-interacting protein. J Virol. 1998;72(11):8586-96.

68. Shoji-Kawata S, Sumpter R, Leveno M. Identification of a candidate therapeutic autophagy-inducing peptide. Nature. 2013;494(7436):p201-6p.

69. Spector S, Campbell G. Hormonally active vitamin D3 (1α,25-dihydroxycholecalciferol) triggers autophagy in human macrophages that inhibits HIV-1 infection. Journal of biological chemistry (JBC online). 2011;286(21):18890-902.

70. Campbell GR, Spector SA. Vitamin D inhibits human immunodeficiency virus type 1 and *Mycobacterium tuberculosis* Infection in macrophages through the induction of autophagy. PLoS Pathog. 2012;8.

71. Levine B, Jia K, Thomas C, Akbar M, Sun Q, Adams-Huet B, et al. Autophagy genes protect against *Salmonella* typhimurium infection and mediate insulin signaling-regulated pathogen resistance. Proceedings of the National Academy of Sciences of the United States of America (PNAS). 2009;106(34):14564-9.

72. West LC, Cresswell P. Expanding roles for GILT in immunity. Curr Opin Immunol. 2013;25(1):103-8.

73. Poussin MA, Goldfine H. Evidence for involvement of ActA in maturation of the *Listeria monocytogenes* phagosome. Cell Res. 2010;20(1):109-12.