Autophagy and Lc3-associated phagocytosis in host defense against Salmonella

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Chapter 6

Summary and discussion

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The control of infectious diseases continues to pose major challenges to the human health sector, even with the availability of modern drug treatments. Ineffective control of antibiotic-resistant bacterial strains emphasizes the need for alternative therapies, which might include targeting of host factors involved in the immune defense mechanisms that are subverted by pathogens. Developing such host-directed therapies will require a better understanding of host-pathogen interactions and the underlying mechanisms involved. Autophagy is a fundamental process that promotes cell survival by degradation and recycling of waste products, and recently, this process has emerged also as an important host defense mechanism against intracellular infections (reviewed in **Chapter 1**). Pharmacological stimulation of autophagy is therefore considered as a possible therapeutic approach for controlling drug-resistant infectious agents. However, it remains to be established if stimulating autophagy will be effective against pathogens that have evolved virulence mechanisms to restrict the host autophagy response, evade it, or even manipulate it to facilitate intracellular survival and replication. The dual role of autophagy as a host-protective or pathogen-promoting mechanism demands further investigation into host pathogen interactions in order to determine the therapeutic potential of autophagy modulating strategies. The zebrafish provides a useful model to study the host autophagy response to human pathogens. The transparent embryos of this model organism are widely used for studies of the innate immune system (as reviewed in **Chapter 2**) and are especially useful for non-invasive imaging of autophagy activation with the aid of a transgenic reporter for autophagosome formation (GFP-Lc3). Furthermore, the activity of host factors that mediate the autophagic defenses can be efficiently studied in zebrafish using gene knockdown or knockout technologies. In this thesis the zebrafish model has been applied to study the role of the autophagy machinery in host defense against *Salmonella* infection.

The zebrafish-*Salmonella* model

Salmonella enterica serovarTyphimurium (*S*. Typhimurium) infection is a common cause of food-borne disease affecting millions of people per year and there is an alarming rise in drug-resistant strains both in devel-

oping and developed parts of the world (Threlfall, 2002). *S*. Typhimurium is a facultative intracellular pathogen that can invade and persist in both non-phagocytic and phagocytic cell types (Finlay & Brumell, 2000; Ibarra & Steele-Mortimer, 2009; LaRock et al., 2015). The virulence mechanisms of *Salmonella* have been widely studied using a number of *in vitro* and *in vivo* models, to which the zebrafish is a recent addition. The zebrafish-*Salmonella* model was first described by van der Sar and coworkers, who demonstrated that *S*. Typhimurium can replicate inside macrophages following intravenous delivery into embryos at 1 day post fertilization (dpf) (van der Sar et al., 2003). With doses of just 50 bacterial cells, this infection also spreads extracellularly and becomes lethal within one day. Earlier work in our laboratory has shown that *S*. Typhimurium infection elicits a strong pro-inflammatory innate immune response under these conditions that is majorly TLR-Myd88 dependent (Stockhammer et al., 2010; Stockhammer et al., 2009; van der Vaart et al., 2013).

In this study we modulated the zebrafish-*Salmonella* infection model to investigate the host autophagic defense response over a longer period of infection **(Chapter 3)**. To this end, we introduced systemic infection at 2 dpf and followed the host response until 5 dpf. We preferred infections at 2 dpf over 1 dpf as this infection model allowed better discrimination between knockdown effects of host factors **(Figure 1A)**, which was not possible with the rapidly lethal infections at 1 dpf. When embryos are infected at 2 dpf with 200 bacteria, this leads to up to a 10^3 exponential rise of *Salmonella* cells, where 60-70 % of hosts succumb to this infection burden and 30-40 % survivors are left at 3 days post infection (dpi) **(Chapter 3)**. The increased immune competence of embryos at 2 dpf in comparison with 1 dpf might be explained by the presence of higher numbers of phagocytes at this stage or by further development of the anti-microbial properties of these cells.

We used the 2 dpf infection model to study a set of *S.* Typhimurium strains with mutations in virulence factors **(Chapter 4)** and the results are summarized in **Figure 1B**. Consistent with results in mouse models (Miller et al., 1989; Miller & Mekalanos, 1990; O'Collaghan et al., 1998; Thompson et al., 2011), we found that *S*. Typhimurium requires the metabolic factor PurA and the two-component sensor PhoP for effective infection establishment in zebrafish embryos. Mutation in the flagellin regulator

FlhD causes hypervirulence in the zebrafish host, which is also in agreement with studies in mouse models (Lockman & Curtiss, 1990; Schmitt et al., 2001). This observation suggests that flagellin recognition by the TLR pathway is important for mounting an effective immune response by the zebrafish host. This assumption is in line with previous results of our laboratory showing that injection of *Salmonella* flagellin induces expression of pro-inflammatory genes in zebrafish embryos, which is reduced under knockdown conditions of the zebrafish homologs of the flagellin receptor TLR5 (Stockhammer et al., 2009). Finally, the virulence of *S.* Typhimurium was not significantly affected by mutation of SPI1 and SPI2 type III secretion system (T3SS) factors **(Chapter 4)**, for which the likely explanation is that *Salmonella* parasitism of macrophages during systemic infection does not require active T3SS-mediated invasion.

The role of macrophages and neutrophils in host defense against *Salmonella*

In zebrafish embryos it is generally observed that macrophages are more efficient in phagocytosing intravenously injected bacteria, whereas subcutaneously injected bacteria are better taken up by neutrophils as their phagocytic behavior requires microbes to be surface-associated (Colucci-Guyon et al., 2011). However, in our comparison of intravenous and subcutaneous injections we observed macrophages to be the main phagocytes containing *Salmonella* bacteria independent of the route of infection **(Chapter 3)**. By knockdown of the transcription factor *irf8* the developmental fate of macrophages can be shifted to the production of neutrophils (Li et al., 2011). Using this strategy, we found that macrophage-depleted embryos with increased neutrophil numbers were unable to curtail *S*. Typhimurium infections and could not survive more than 1 dpi, indicating that macrophages are essential for the zebrafish host to cope with *S*. Typhimurium infection **(Chapter 3)**. In agreement, a previous study using a zebrafish hindbrain infection model demonstrated an important role in host defense against *S.* Typhimurium for immunoresponsive gene 1 (*irg1*), which is specifically expressed in macrophages (Hall et al., 2013). Irg1-deficient macrophages are impaired in the production of the antimicrobial compound itaconic acid and also fail in the effective utilization of fatty acids as an energy source for mitochondrial reactive oxygen species (ROS) production (Hall et al., 2013).

A

Host factor	Function	Infection phenotype in factor-deficient host
wt	-	
irf8	Transcription factor responsible for macrophage fate determination	
atg5	Membrane elongation during autophagy	
atg13	Member of ULK1 complex	
rubcn	Induces LAP and suppresses xenophagy	
cyba	Component of NOX2-complex	
dram1	Promotes autophagy	

B

Bacterial factor	Function	Infection phenotype in wt host	Infection phenotype in *rubcn*-deficient host
wt	-		
Δ*purA*	Purine metabolism		
Δ*phoP*	Defense against anti-microbial peptides		
Δ*flhD*	Flagellin formation		
Δ*sipB*	SPI1 translocator		
Δ*ssrB*	SPI2 regulator		

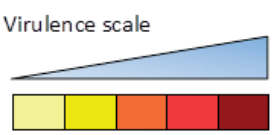

Figure 1: Heat map representations of infection phenotypes caused by deficiencies in host or bacterial factors. A: Summary of the loss-of-function effects of the zebrafish host factors studied in this thesis. The severity of *S.* Typhimurium infection in zebrafish embryos deficient in the indicated host genes is shown in comparison to wild type embryos. **B:** Summary of the infection phenotypes induced by different *S.* Typhimurium strains in wild type hosts and Rubicon-deficient hosts. The severity of infections caused by the indicated mutant strains is shown in comparison with the wild type strain. The virulence scale below shows the color coding for increasing severity of the infection phenotype, based on host survival rates and bacterial burden.

In another zebrafish-*Salmonella* infection model a role for a neutrophil-specific gene (*gbp4)* has been shown (Tyrkalska et al., 2016). This gene encodes guanylate-binding protein 4a, a CARD-domain containing GTPase that has been identified as a mediator of inflammasome activation (Tyrkalska et al., 2016). In contrast to our work, this study used the yolk of the zebrafish embryo as the site of injection with *S.* Typhimurium. In our experience with yolk infection models for other pathogens (*Staphylococcus epidermidis* and *Mycobacterium marinum*), bacteria injected via this route initially replicate extracellularly as the yolk is not accessible to phagocytes, and only at later stages the yolk infection spreads systemically into the host (Veneman et al., 2013). Therefore, the role of *gbp4* in this model may be associated with the inflammatory response of neutrophils attracted to the tissue surrounding the intra-yolk infection. We consider the intravenous and subcutaneous infection routes used in our work more suitable to study the direct interaction of phagocytes with *S.* Typhimurium bacteria. The fact that macrophages are the predominant cell type containing *Salmonella* under these infection conditions makes the zebrafish embryo a highly useful model to study systemic Salmonellosis, since macrophages also constitute the major replication site of *Salmonella* during systemic infections in mammalian hosts (Watson & Holden, 2010).

Association of the autophagy marker Lc3 with intracellular *Salmonella*

In order to study the autophagy response to *S.* Typhimurium we took advantage of a transgenic reporter line constitutively expressing a GFP fusion of Lc3 (He et al., 2009). Under basal conditions this reporter displays a diffused signal of cytosolic GFP-Lc3, but when autophagy is activated, Lc3 is lipidated at the C-terminus and recruited to autophagosomal membranes. We studied the dynamics of GFP-Lc3 association over the first hours of *S.* Typhimurium infection and observed bacteria enclosed in different types of GFP-Lc3 positive vesicles as well as the presence of small GFP-Lc3 vesicles (puncta) in close vicinity of the bacterial cells **(Chapter 3)**. GFP-Lc3 puncta in association with bacterial cells are also frequently observed during *M. marinum* infection; however, the presence of *M. marinum* bacteria inside GFP-Lc3 positive vesicles has only been detected at later stages of infection from 1 dpi and not during the first hours (Hosseini et al., 2014; van der Vaart et al., 2014). In contrast, the level of GFP-

Lc3 recruitment to *S.* Typhimurium peaked at 4 hpi and several different types of Lc3-decorated vesicles containing bacterial cells were observed. These structures appeared as tight or spacious GFP-Lc3 rings enclosing single bacterial cells, but also as spacious GFP-Lc3 rings surrounding larger bacterial clusters. In some cases the *mCherry* fluorescent label of the bacterial cells was diffused, suggesting degradation of the bacteria inside the larger GFP-Lc3 vesicles. GFP-Lc3 association with *S.* Typhimurium at 4 hpi was observed in approximately 60% of infected phagocytes and the response was not triggered by heat-killed bacteria. Although the different types of GFP-Lc3 positive rings or puncta were detected both in macrophages and neutrophils, their presence was at least twice more frequent in macrophages. Five *S.* Typhimurium mutant strains tested in our study **(Figure 1B)** triggered the formation of similar diverse GFP-Lc3-decorated vesicles with different degrees of this response **(Chapter 4)**.

Correlative light and electron microscopy would be required to define the nature of the GFP-Lc3 positive vesicles in which *Salmonella* resides. In our initial non-correlative electron microscopy analysis we observed macrophages containing many bacteria inside a large phagosome, while other cells had smaller phagosomes with one or few bacteria. In addition, we observed bacteria freely in the cytoplasm, in multivesicular compartments, and in compartments that also contained cytoplasmic material suggestive of an autophagic origin **(Chapter 3)**. However, we did not detect *Salmonella* inside the typical double membrane vesicles that are characteristic for the early stages of canonical autophagy.

Lc3-associated phagocytosis as a host-protective mechanism

The observation that GFP-Lc3 recruitment to *Salmonella* follows rapidly after ingestion by phagocytes suggested that a recently discovered autophagy-related pathway, named Lc3-associated phagocytosis (LAP), might be involved. To discriminate between canonical autophagy and LAP we performed knockdown studies of host factors specific for the two processes **(Figure 2A)**. In LAP, Lc3 is recruited directly to the single membrane of the phagosome by a mechanism that relies on components of the autophagy conjugation systems, including ATG5, but does not require the ULK1 preinitiation complex, including ATG13 (Martinez et al., 2011; Martinez et al., 2015). Furthermore, LAP induction is dependent on

phagosomal production of ROS and this requires stabilization of NADPH oxidase on the phagosomal membrane by the RUN and cysteine-rich domain containing Beclin1 interacting protein, Rubicon (Huang et al., 2009; Martinez et al., 2015; Matsunaga et al., 2009), **(Figure 2A)**. We found that knockdown of Atg5, Rubicon, or the $p22^{phox}$ component (Cyba) of NADPH oxidase reduced GFP-Lc3 association with *Salmonella* **(Chapter 3)**. In contrast, Atg13 knockdown had no effect on GFP-Lc3 recruitment. These results are consistent with the hypothesis that *Salmonella* is targeted by LAP. To corroborate this view, we infected embryos with a *Salmonella* biosensor strain that emits green fluorescence when bacteria are exposed to ROS (Burton et al., 2014). In wild type embryos we observed activation of the ROS biosensor in a subpopulation of bacteria contained by phagocytes. This activation was completely abolished not only in the absence of NADPH oxidase function, but also by deficiency of Rubicon. The dual requirement of NADPH oxidase and Rubicon for the ROS response and GFP-Lc3 recruitment provides strong evidence for the involvement of LAP. Furthermore, we conclude that LAP functions as a host-protective response towards *Salmonella*, as hosts deficient in Atg5, Rubicon, or NADPH oxidase, but not Atg13-deficient hosts, are hypersusceptible to infection **(Chapter 3 & Figure 1A)**.

Our results do not exclude that canonical autophagy might also contribute to host defense in our model. In our electron microscopy analysis we observed that a fraction of *Salmonella* bacteria were localized in the cytosol of infected phagocytes. Although we have not investigated ubiquitination or co-localization with selective autophagy receptors, it is likely that these cytosolic bacteria are targeted by xenophagy, as demonstrated by many studies in cultured epithelial cells but also macrophages (Birmingham et al., 2006; Cemma et al., 2011; Hubber et al., 2017; Thurston et al., 2016; Thurston et al., 2009; Thurston et al., 2012; Watson & Holden, 2010; Wild et al., 2011; Zheng et al., 2009). A recent study suggest that xenophagy also targets *Salmonella* in zebrafish, since knockdown of the ubiquitin receptor Optineurin was found to increase susceptibility of zebrafish embryos to infection (Chew et al., 2015). However, in our model, LAP appears to be the initial response of the zebrafish phagocytes, predominantly macrophages but also neutrophils, that take up *Salmonella*. It is likely that mammalian phagocytes also target *Salmonella* by LAP, as suggested by a study showing that GFP-Lc3 recruitment to *Salmonella*-

containing phagosomes in mouse neutrophils was dependent on NADPH oxidase (Huang et al., 2009). Demonstration of Rubicon dependency and independency of the ULK1 complex would be required to formally prove the role of LAP in other systems.

Role of Lc3-associated phagocytosis during infections with virulence mutants

Similar to wild type *S.* Typhimurium, several mutant bacterial strains also elicit GFP-Lc3 recruitment in phagocytes of the zebrafish host in a Rubicon-dependent manner **(Chapter 4)**. The level of GFP-Lc3 recruitment was slightly but significantly enhanced in response to strains carrying mutations in the PhoP regulator of outer membrane composition or in the SipB translocator of the SPI1 T3SS. This suggests that these virulence factors could be involved in evasion of LAP. In contrast, a recent study of *Legionella dumoffi* showed that the recruitment of Lc3 to Legionella-containing vacuoles in cultured macrophages requires the Dot/ICM type IV secretion system (T4SS) of this pathogen (Hubber et al., 2017). This T4SS-dependent process was defined as LAP, based on requirement for Rubicon and NADPH-oxidase and independency from ULK1, similar as we have shown for the response to *S.* Typhimurium in the zebrafish host. Furthermore, the LAP response to *L. dumoffi* was also shown to serve a host-protective role (Hubber et al., 2017). The T4SS of *L. dumoffi* is also required for inhibiting endosome maturation and establishing a replication niche in an endoplasmic reticulum (ER)-like compartment. Therefore, expression of the T4SS involves a trade-off between intracellular pathways promoting (maturation of the LCV) or limiting (LAP) the intracellular survival of this pathogen. In the case of *S.* Typhimurium, we found that LAP is significantly reduced by mutations in genes required for purine metabolism (Δ*purA*) and for the expression of flagella (Δ*flhD*). The Δ*purA* mutant is rapidly cleared by the zebrafish host and therefore might fail to induce signals for LAP. In contrast, the Δ*flhD* mutant is hypervirulent. As LAP has been shown to occur downstream of TLR signaling (Sanjuan et al., 2007), we hypothesize that Tlr5-Myd88-mediated detection of *Salmonella* flagellin contributes to the induction of LAP and other host defense mechanisms that restrict growth of flagellated strains. In future work, it would be of interest to test this hypothesis using previously established

genetic tools and zebrafish mutants for probing TLR signaling (Stockhammer et al., 2009; van der Vaart et al., 2014; Yang et al., 2015).

The hypervirulence of the Δ*flhD* mutant was further increased when LAP was inhibited by Rubicon knockdown. Moreover, Rubicon knockdown also increased virulence of the Δ*sipB* mutant, and mutants with attenuated infections (Δ*purA*, Δ*PhoP*) became virulent in LAP-deficient embryos **(Chapter 4 & Figure 1B)**. Interestingly, virulence of Δ*ssrB*, a strain impaired in function of the SPI2 T3SS, remained unchanged in LAP-deficient embryos **(Chapter 4 & Figure 1B)**. This result, together with the fact that all other SsrB-competent strains tested showed increased virulence under Rubicon knockdown conditions, suggests that SPI2 function is required for intracellular replication and/or spreading from infected cells in the absence of LAP. When LAP fails to restrict *Salmonella* growth, SPI2 function could mediate maturation of the SCV as a replication niche and/or contribute to escape from the SCV resulting in cytosolic replication, subsequently causing macrophage cell death and extracellular spreading. This hypothesis is in line with a study in mice suggesting that SPI2 function mediates the exit from infected cells and bacterial spreading into the host tissues (Grant et al., 2012). It was beyond the scope of the present study to investigate the possible effects of SsrB mutation by electron microscopy, but in case of wild type *S*. Typhimurium we could record an example of an SCV forming tubular extensions into the cytoplasm, which might represent SPI2-dependent formation of the *Salmonella* induced filaments (Sifs) that are characteristic for SCV maturation **(Chapter 3)**. Although SsrB mutation did not detectably alter virulence of *S.* Typhimurium, this possible example of Sif formation is consistent with the expression of SPI2 effectors during infection in the zebrafish host and with the increased virulence of SsrB-competent strains in the absence of LAP **(Figure 1B)**. *Salmonella* possesses a number of other virulence factors that would be relevant to study in connection with LAP. For example, the ADP-ribosyltransferase spvB has been shown to inhibit autophagosome formation through depolymerization of actin filaments (Chu et al., 2016; Li et al., 2016; Wu et al., 2016). Therefore, it is an interesting question whether or not spvB can also promote *S.* Typhimurium pathogenity by the avoidance of LAP.

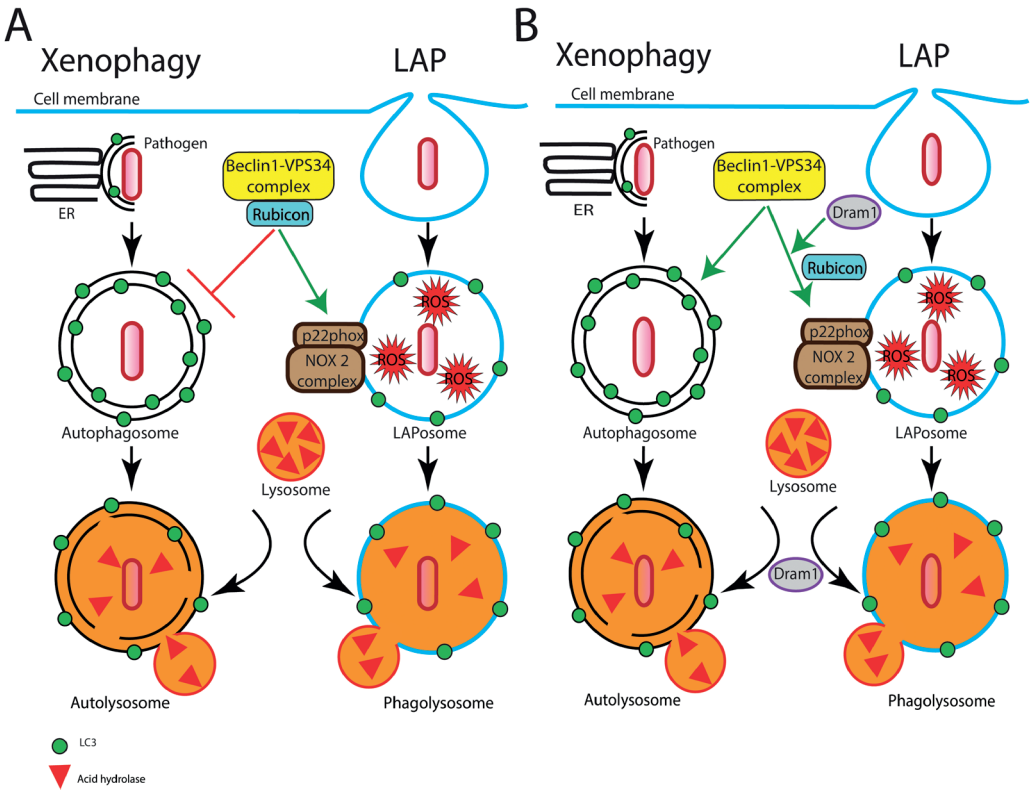

Figure 2: Working model for the role of Dram1 in promoting host defense responses dependent on the autophagy machinery. A: Graphical comparison of xenophagy and LC3-associated phagocytosis (LAP). Rubicon acts as a molecular switch between the two processes, where it negatively regulates xenophagy by binding to the Beclin 1-VPS34 PI3 kinase complex, which also includes the UV resistance associated gene protein UVRAG. On the other hand, Rubicon induces LAP by promoting NADPH oxidase assembly on phagosomes. In turn, NADPH oxidase generates reactive oxygen species (ROS) responsible for Lc3 recruitment resulting in the formation of LAPosomes. Both xenophagy and LAP ultimately result in lysosomal-mediated killing of microbial invaders. **B:** Graphical presentation of the possible roles of Dram1 during xenophagy and LAP. Dram1 might act at different steps during both processes. First, it might promote the release of Rubicon from the Beclin1-VPS34-UVRAG complex on autophagosomal membranes, thereby facilitating the progression of xenophagy and the induction of LAP at the same time. Second, Dram1 might promote both processes by increasing fusion of lysosomes to autophagosomes or LAPosomes. The signaling processes mediated by the Beclin1-VPS34 complex, Rubicon, and Dram1 are localized to membranes of the different organelles in the figure, which is not indicated for simplicity.

Host-protective function of the autophagy modulator Dram1

Previous work in our laboratory had shown that *S.* Typhimurium infection leads to transcriptional induction of *dram1*, which codes for DNA-damage regulated autophagy modulator 1 (Stockhammer et al., 2010). Dram1 belongs to an evolutionary conserved family of six-transmembrane proteins, which includes DRAM1, DRAM2, TMEM150A, TMEM150B/DRAM3, and TMEM150C in human and Sfk1 in yeast (Chung et al., 2015; Crighton et al., 2006; O'Prey et al., 2009). Currently, three of the human DRAM proteins have been identified as positive regulators of autophagy, and the corresponding genes are induced under different stress conditions, being the p53-mediated DNA damage response in case of *DRAM1*, amino acid starvation in case of *DRAM2*, and glucose restriction in case of *DRAM3* (Crighton et al., 2006; Mrschtik et al., 2015; O'Prey et al., 2009; Yoon et al., 2012). In addition, *DRAM1* expression is induced during infections with HIV and *Mycobacterium tuberculosis* (Laforge et al., 2013; van der Vaart et al., 2014). In contrast, *M. tuberculosis* infection has also been shown to result in induction of a microRNA (miR144*) that down-regulates *DRAM2* expression and thereby contributes to evasion of autophagy (Kim et al., 2017). In zebrafish embryos, *dram1* protects against *M. marinum* infection by promoting the Sting- and p62-dependent autophagic mechanism against bacteria following their escape into the cytosol, which is mediated by secreted virulence factors (van der Vaart et al., 2014). Since *S.* Typhimurium infection triggers LAP rather than xenophagy in zebrafish embryos **(Chapter 3)**, it was of interest to investigate if Dram1 is also involved in this autophagy-related process.

To establish a possible functional link between Dram1 and the induction of LAP, we performed loss-of-function analyses by morpholino-mediated knockdown and CRISPR/Cas9 mutation as well as gain-of-function analysis by mRNA injection **(Chapter 5)**. The results led to the conclusion that Dram1 is required for host defense against *S.* Typhimurium infection, since increased bacterial burden and host mortality was observed under loss-of-function conditions, while gain-of-function enabled embryos to restrict *S.* Typhimurium replication and improved survival rates. This host-protective function of Dram1 correlated with a reduction of GFP-Lc3-*Salmonella* association in *dram1*-deficient embryos and an increase under overexpression conditions of *dram1*. Furthermore, activation of a

Salmonella ROS biosensor construct was largely abolished in *dram1* mutants, indicating that the presence of Dram1 is required for activation for NADPH oxidase, which is an essential step in LAP. Therefore, it can be concluded that Dram1 function is required to promote the targeting of *S.* Typhimurium bacteria by LAP.

The question remains how Dram1 can stimulate different anti-bacterial autophagy responses, including xenophagy and LAP. The answer could lie in an interaction with components of the Beclin1-VPS34 complex, in agreement with a recent study on DRAM2 (Kim et al., 2017). Beclin1 forms a complex with the class III phosphatidylinositol 3-kinase, VPS34, and this complex can take different compositions by including regulatory proteins. The Beclin1-VPS34-Atg14L complex functions in early steps of autophagosome formation, whereas the Beclin1-VPS34-UVRAG complex promotes the later steps of the maturation process. Rubicon, which functions as a negative regulator, associates specifically with a subset of Beclin1-VpPS34-UVRAG complexes (Matsunaga et al., 2009). The interaction between Rubicon and UVRAG has not only been shown to block autophagosome maturation, but also endosome maturation, which is due to inhibition of Rab7 activation (Sun et al., 2010). This agrees with the predominant localization of Rubicon on endosomes/lysosomes and with accumulating evidence that autophagosome maturation involves fusion with endocytic vesicles (Sun et al., 2010). Like Rubicon, human DRAM1 and zebrafish Dram1 have also been shown to localize to lysosomes (Crighton et al., 2006; van der Vaart et al., 2014). Therefore, similar to what has been proposed for DRAM2, interactions of Dram1 with the Beclin1-VPS34-UVRAG complex might facilitate the dissociation of Rubicon and thereby promote autophagosome maturation **(Figure 2B)**. This is consistent with other studies that have provided evidence for a role of DRAM1 in stimulating autophagic flux (Guan et al., 2015; Zhang et al., 2013). The Beclin1-VPS34-UVRAG complex but not the Beclin1-VPS34-Atg14L complex is involved In LAP, where Rubicon acts as a positive regulator (Martinez et al., 2015). Therefore, an interaction of Dram1 with the Beclin1-VPS34-UVRAG complex might also be the mechanism underlying the stimulation of LAP **(Figure 2B)**. Possibly, Dram1-mediated dissociation of Rubicon from the Beclin1-VPS34-UVRAG complex is required for Rubicon to stabilize the NADPH oxidase, which precedes LC3 recruitment. However, molecular interactions of Dram1 with other proteins involved in

endosomal maturation and lysosomal function can also be envisaged and further research will be required to elucidate the precise function of this autophagy modulator. An important step in this research will be to study the co-localization of Dram1 with its proposed interaction partners under different stress and infection conditions that rely on Dram1-mediated responses. The zebrafish embryo provides a useful model to study the dynamics of these responses *in vivo*. To this end, a zebrafish line is under construction in our laboratory, in which the endogenous *dram1* is tagged with GFP using CRISPR/Cas9 technology.

Concluding remarks

The work described in this thesis has provided new insights into the role of the autophagy machinery in host defense against *Salmonella* that are particularly relevant for the systemic stages of infection when the pathogen parasitizes macrophages. We demonstrate that an autophagy-related process, named Lc3-associated phagocytosis or LAP, targets *Salmonella* bacteria when they are engulfed by phagocytes, predominantly macrophages, of the zebrafish embryo. Rubicon and NADPH oxidase are essential host factors for this response. Depletion of these factors leads to increased virulence of both wild type and attenuated strains, showing that LAP functions as a host-protective mechanism against *Salmonella* infection. Our studies also revealed a novel functional link between the autophagy modulator Dram1 and induction of LAP. The results in this thesis provide evidence that host resistance to systemic *Salmonella* infection can be promoted by genetically increasing Dram1 activity, which encourages further studies aimed at the identification of autophagy modulating drugs for host-directed therapy of antibiotic-resistant *Salmonella* infections.

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