Autophagy and Lc3-associated phagocytosis in host defense against Salmonella

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Citation

Masud, S. (2017, October 12). *Autophagy and Lc3-associated phagocytosis in host defense against Salmonella*. Retrieved from https://hdl.handle.net/1887/58773

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Title: Autophagy and Lc3-associated phagocytosis in host defense against Salmonella
Issue Date: 2017-10-12

Chapter 4

Increased virulence of *Salmonella* mutants in Rubicon-deficient zebrafish

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(Manuscript in preparation)

Abstract

Intracellular pathogens such as *Salmonella* depend on their molecular virulence factors to evade host defense responses like autophagy. Using a zebrafish systemic infection model, we have previously shown that macrophages target *Salmonella* Typhimurium by an autophagy-related pathway known as Lc3-associated phagocytosis (LAP), which is dependent on the host protein Rubicon. Here, we explore the influence of *Salmonella* virulence factors on pathogenicity in the zebrafish model and induction of LAP as a defense response. We investigated five mutant strains, all of which triggered GFP-Lc3 recruitment as puncta or rings around single bacteria or bacterial clusters in a Rubicon-dependent manner. We found that *S.* Typhimurium strains carrying mutations in PhoP or PurA, responsible for adaptation to the intracellular environment and efficient metabolism of purines respectively, displayed attenuated virulence in the zebrafish model. However, both strains showed increased virulence when LAP was inhibited by knockdown of Rubicon. Mutations in type III secretion systems 1 and 2 factors, SipB and SsrB, which are important for invasion and replication in non-phagocytic cells, did not affect the ability to establish successful infection in the zebrafish model. This observation is in line with our previous characterisation of this infection model revealing that macrophages actively phagocytose the majority of *S*. Typhimurium. In contrast to SipB mutants, SsrB mutants were unable to become more virulent in Rubicon-deficient hosts, suggesting that type III system 2 effectors are important for intracellular replication of *Salmonella* in the absence of LAP. Finally, we found that mutation of FlhD, required for production of flagella, rendered *S.* Typhimurium hypervirulent both in wild type zebrafish embryos and in Rubicon-deficient hosts. FlhD mutation also led to lower levels of GFP-Lc3 recruitment compared with the wild type strain, indicating that recognition of flagellin by the host innate immune system promotes the LAP response. Together, our results provide new evidence that the Rubicon-dependent LAP process is an important defense mechanism of macrophages against *S*. Typhimurium.

Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is one of the most common causes of foodborne gastroenteritis in humans, claiming more than 150,000 lives each year (Majowicz et al., 2010). Although *S.* Typhimurium infections generally resolve without treatment, immunocompromised patients can develop serious complications, such as bacteremia and other forms of systemic infection. *S.* Typhimurium also causes a systemic infection in mice that resembles typhoid fever, as caused in humans by another *S.* enterica serovar, *S.* Typhi. *S.* Typhimurium and *S.* Typhi are facultative intracellular pathogens that are able to replicate in both phagocytic and non-phagocytic cells. To this end, these pathogens employ a broad range of virulence strategies that mediate host cell invasion, growth in the intracellular environment, and subversion of the host cell's microbicidal mechanisms. These virulence strategies depend for a major part on effector proteins translocated by two type III secretion systems (T3SSs), T3SS1 and T3SS2, encoded by *Salmonella* pathogenicity island (SPI), SPI1 and SPI2 respectively (Ibarra & Steele-Mortimer, 2009). Other factors, such as flagellar motility and the ability to make structural and metabolic adaptations to its environment, also play important roles in *Salmonella* virulence.

S. Typhimurium accomplishes active invasion into non-phagocytic cells via the T3SS1 secretion system, which translocates a limited number of tightly regulated effector proteins (Myeni et al., 2013) that collectively induce dramatic changes to the host cell cytoskeleton leading to membrane ruffling and ultimately resulting in bacterial internalization (McGhie et al., 2009). The delivery of these SPI1 effectors is dependent on translocator proteins like SipB and SipC, which are also encoded in the SPI1 region (Myeni et al., 2013). In contrast, professional phagocytes such as macrophages recognize *S*. Typhimurium-related pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), and utilize their phagocytic abilities for direct uptake of the bacterial cells. This recognition and phagocytosis initiates a number of host signalling responses in phagocytes including a bactericidal oxidative burst, cytokine production, and the activation of autophagy (Dupre-Crochet et al., 2013; Huang et al., 2009; Levine et al., 2011).

Once internalized, *S.* Typhimurium survives by avoiding anti-bacterial host responses and replicates intracellularly inside a modified phagosome, the *Salmonella*-containing vacuole (SCV). The function of the T3SS2 secretion system, translocating SPI2 effector proteins such as SifA, SseF and SseG, is important for the maturation and maintenance of this intracellular niche (Hensel et al., 1998; Kuhle & Hensel, 2002). Expression of the T3SS2 is activated by the two-component system SsrA-SsrB (Walthers et al., 2007). Other factors that play a role in *S.* Typhimurium pathogenesis include flagellar-based motility, which increases invasiveness of *Salmonella* (Schmitt et al., 2001). On the other hand, recognition of flagellin by host PPRs induces the innate immune response (Franchi et al., 2006; Miao et al., 2006). *S.* Typhimurium highly depends on a number of metabolic products to survive intracellularly and cause invasiveness. One such factor is PurA (succinyl AMP synthase), which functions in purine metabolism by converting IMP to AMP (Benson & Gots, 1976; McFarland & Stocker, 1987). PurA mutants grow poorly in vivo and are non-virulent in mice (O'Callaghan et al., 1998). Additionally, *S.* Typhimurium is highly dependent on the PhoP/PhoQ two-component system to survive inside professional phagocytes (Miller et al., 1989; Bijlsma & Groisman, 2005). The PhoP/PhoQ system detects acidification of the phagosome and presence of cationic antimicrobial peptides, leading to essential adaptations of the protein and lipid contents of the outer membrane (Dalebroux & Miller, 2014).

Recently, autophagy has emerged as an important host defense mechanism against *S.* Typhimurium and other intracellular pathogens. Autophagy is a cellular degradative pathway that can target bacterial invaders in a similar manner as it destructs defective organelles or protein aggregates (Huang & Brumell, 2014; Levine et al., 2011). A number of studies in epithelial cells have shown that *Salmonella* bacteria escaping from the SCV into the cytosol are ubiquitinated, recognized by ubiquitin receptors, enclosed in a double membrane autophagosome, and degraded following the fusion with lysosomes (Birmingham et al., 2006 ; Cemma et al., 2011; Huang et al., 2009; Thurston et al., 2016; Thurston et al., 2009; Thurston et al., 2012; Wild et al., 2011; Zheng et al., 2009). This selective, ubiquitin-dependent, autophagy mechanism, can also detect damage to the SCV membrane, likely functioning as a repair mechanism to delay cytosolic escape. Alternatively, components of the autophagy

machinery can be recruited directly to phagosomes, in a process that has been termed Lc3-associated phagocytosis (LAP) (Huang & Brumell, 2014; Sanjuan et al., 2007). LAP is initiated by PRRs, including Toll-like receptors (TLRs), and characterized by recruitment of the autophagy component LC3 to the phagosomal membrane and subsequent phagosome fusion with lysosomes, leading to rapid acidification and enhanced bacterial killing (Sanjuan et al., 2007).

A key player in LAP is RUN and cysteine rich domain containing Beclin1 interacting protein (Rubicon). Rubicon is found to regulate autophagy by modulating the activity of the PI3 kinase VPS34 (Zhong et al., 2009). Under normal conditions, Rubicon suppresses autophagy by interacting with the Beclin1-VPS34 complex (Matsunaga et al., 2009). However, upon TLR stimulation, Rubicon interacts with the NADPH oxidase complex on phagosomes. It then stabilizes the $p22^{phox}$ and $gp91^{phox}$ components of the NADPH-oxidase, inducing the production of bactericidal reactive oxygen species (ROS) (Yang et al., 2012; Martinez et al., 2015). Thus, Rubicon functions as a molecular switch between canonical autophagy and LAP.

We have recently shown that both Rubicon and NADPH oxidase are required for Lc3 recruitment to *S*. Typhimurium and for control of infection in a systemically challenged zebrafish host **(Chapter 3)**. This led us to conclude that LAP is the major pathway involved in the anti-*Salmonella* autophagy response under conditions where macrophages dominate the defense response. In the present study we investigated which of the *S*. Typhimurium virulence factors, PhoP, PurA, SipB, SsrB, and FlhD, are required for pathogenicity in this systemic infection model, and how these factors affect the LAP response. Our study revealed that PhoP and PurA facilitate *S.* Typhimurium replication in the zebrafish host, while FlhD-regulated expression of the flagellar apparatus benefits host defense. We found LAP to occur as a general response to *S.* Typhimurium internalization, since Rubicon-dependent recruitment of Lc3 was observed in infections with both wild type and mutant strains. With the exception of Δ*ssrB* mutants, all tested strains displayed increased virulence under conditions of Rubicon knockdown, indicating that LAP functions to restrict *S.* Typhimurium growth and suggesting that SPI2 effectors promote *S.* Typhimurium virulence in the absence of LAP.

Results

Virulence of *S.* Typhimurium in the zebrafish model relies on PhoP and PurA factors

S. Typhimurium requires a number of effector proteins for infecting a wide range of cell types, including macrophages (Buckner et al., 2011; LaRock et all., 2015). Mutant strains of *S.* Typhimurium that cannot survive inside macrophages are avirulent (Fields et al., 1986), but the interaction of the macrophage defense machinery with *S.* Typhimurium is not well understood (Gog et al., 2012). PhoP, which is part of the PhoP/Q two component regulatory system, has been shown to be an essential virulence factor as bacteria containing an inactive PhoP are defective in intracellular survival in murine macrophages (Miller et al., 1989). We previously showed that *S.* Typhimurium infection in zebrafish embryos/larvae predominantly resides inside macrophages **(Chapter 3)**, providing an in vivo context to determine the importance of PhoP for establishing systemic infection. To this end, we challenged 2 days post fertilization (2 dpf) embryos either with a PhoP-deficient *S.* Typhimurium mutant strain or its wild type counterpart (SL1344) **(Figure 1A)**. We evaluated the relative virulence of both strains on the basis of survival of infected embryos following intravenous injection of 200-400 colony forming units (CFU) and monitored bacterial burdens in infected hosts. We observed that Δ*phoP*-infected larvae showed higher survival rates as compared to individuals infected with the wild type strain **(Figure 1B)**. Similarly, the retrieved bacterial counts for hosts infected with the Δ*phoP* strain were significantly lower at 24 hours post infection (24 hpi) as compared to wild type bacteria **(Figure 1C)**. These results confirm that PhoP is required for *S.* Typhimurium infection establishment and virulence in the zebrafish host.

Another important factor for *S.* Typhimurium virulence in *in vitro* (Grant et al., 2012) and in *in vivo* models (Benson & Gots, 1976; O'Callaghan et al., 1988) is PurA, which is required by *S*. Typhimurium for metabolic adaptation to the host environment (McFarland & Stocker, 1987). We found that a PurA-deficient *S.* Typhimurium (Δ*purA*) strain was avirulent to zebrafish larvae and failed to cause mortalities in comparison to the wild type strain **(Figure 1D)**. Similarly, the Δ*purA* strain could not establish a successful infection as depicted by significantly lower numbers of CFU counts at 24

hpi in Δ*purA* infected larvae when compared to larvae infected with the wild type strain **(Figure 1 E)**.

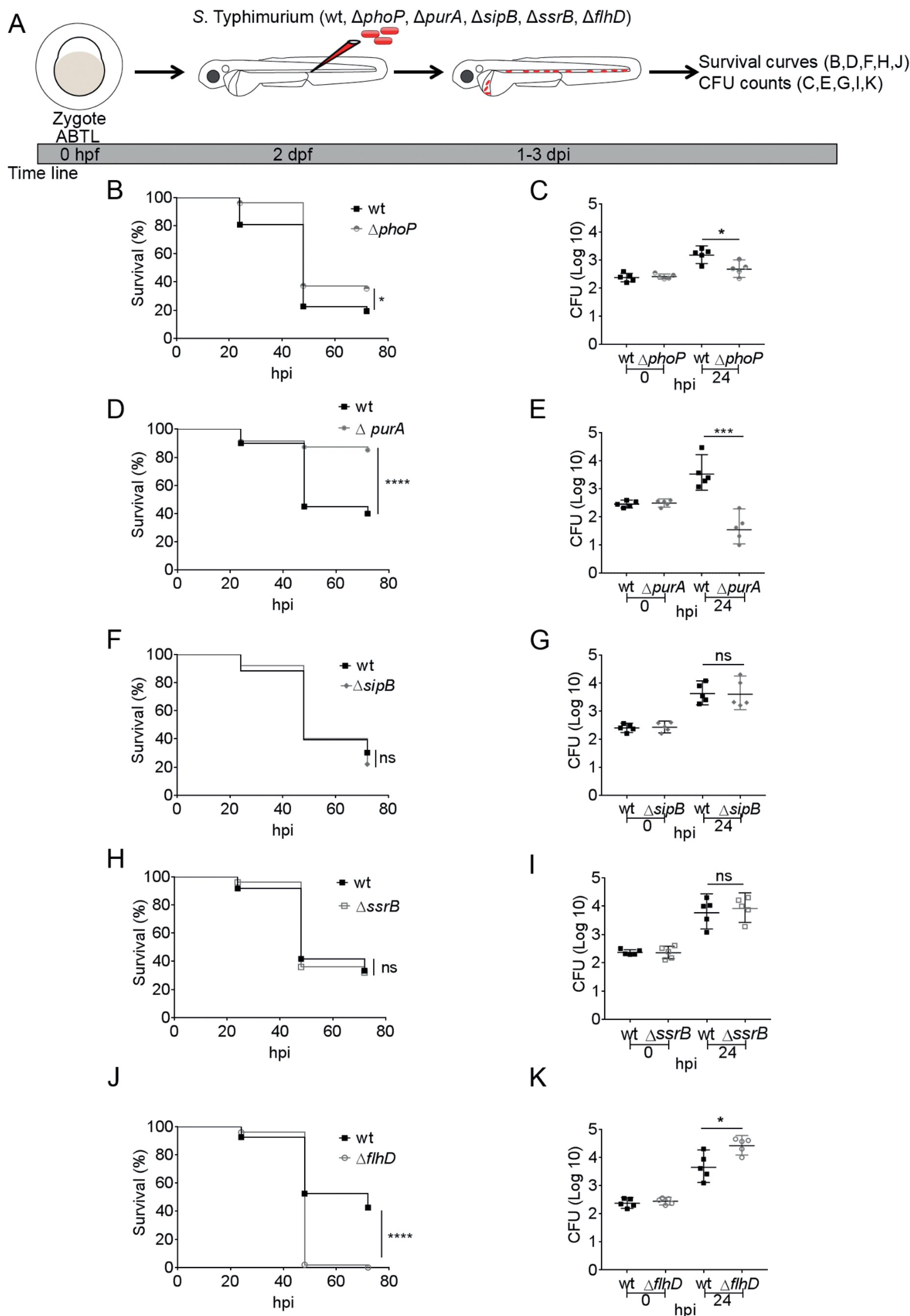

Figure 1: Comparison of virulence and infection progression of *S.* Typhimurium mutants. *(legend on next page).*

Figure 1: Comparison of virulence and infection progression of *S*. Typhimurium mutants. *(figure on previous page).* **A:** Work flow for experiments followed in B-K, displayed along the time line of zebrafish development. **B,D,F,H,J:** Survival curves for systemically challenged zebrafish with *S*. Typhimurium mutant strains, Δ*phoP* (B), Δ*purA* (D), Δ*sipB* (F), Δ*ssrB* (H) and Δ*flhD* (J) with their respective control groups injected with wild type strain SL1344. One representative of three replicates is shown (n= 50 embryos per group). **C,E,G,I,K:** Representative CFU counts of the infections with *S*. Typhimurium mutant strains, Δ*phoP* (C), Δ*purA* (E), Δ*sipB* (G), Δ*ssrB* (I) and Δ*flhD* (K) with their respective control groups injected with wild type strain SL1344 at 24 hpi. One representative of three replicates is shown, where five embryos per time point were used and the log transformed CFU data are shown with the geometric mean per time point. Error bars represent SD. ****P< 0.0001, ***P<0.001, *P<0.05, ns= non-significant.

SPI1 and SPI2 factors, SipB and SsrB, are dispensible for establishing systemic infection in zebrafish embryos

S. Typhimurium depends on active invasion and formation of the SCV to successfully infect and survive inside non-phagocytic host cells (LaRock et al., 2015). However, in our model, active invasion by *S*. Typhimurium is not required due to swift phagocytosis of the bacteria by macrophages and neutrophils following injection into the bloodstream of the zebrafish host. We therefore investigated the hypothesis that SPI1 effectors, required for active invasion, would be dispensable for virulence in this model. To this end, we infected zebrafish embryos with a SipB-deficient *S*. Typhimurium strain, which fails to produce the translocation apparatus for delivery of SPI1 effectors, resulting in severe inability to cause infections in non-phagocytic cells (Kaniga et al., 1995; Myeni et al., 2013). The Δ*sipB* strain and its isogenic wild type strain caused similar mortalities to zebrafish larvae **(Figure 1F)**. Moreover, the *sipB* mutant *S.* Typhimurium was equally virulent as the wild type strain, as CFU counts at 24 hpi for Δ*sipB* and wild type did not differ from each other **(Figure 1G)**.

In order to determine the requirement for SPI2 effectors in our model, we used an SsrB-deficient strain. SsrB is a part of the two component regulatory system SsrA/B and is required for expression of most of the SPI2 proteins, required for maintenance of the SCV (Walthers et al., 2007). We found no significant difference in survival rates **(Figure 1H)** and in CFU

counts **(Figure 1I)** between the groups injected with Δ*ssrB* or wild type *S.* Typhimurium strains. Collectively, our results suggest that both SipB and SsrB are less relevant factors for pathogenicity of *S*. Typhimurium in the zebrafish-*Salmonella* infection model, presumably due to no or little requirement of active bacterial invasion into macrophages.

Mutation of the flagellin transcriptional regulator FlhD increases virulence of *S*. Typhimurium in the systemically infected zebrafish host

FlhD is a part of the *flhDC* master operon responsible for initiating the flagellum production. Mutations in FlhD factor render *S*. Typhimurium immotile due to loss of flagellin (a main component of the bacterial flagellum) synthesis. Flagellin positively and negatively affects *Salmonella* virulence as *Salmonella* requires flagellum-based motility for optimal invasion of the host cells (Ibarra et al., 2010), but on the other hand flagellin is a ligand for TLR pathway detection by the host, activating the innate immune response (Hayashi et al., 2001). In fact, flagellin has already been shown to initiate an immune cascade via TLR5 recognition in zebrafish (Stockhammer et al., 2009). In order to address the role of the flagellum as either a host or bacterial beneficial factor, we infected zebrafish larvae with the Δ*flhD* strain or its wild type counterpart and determined the virulence in terms of CFU counts and survival curves, as above. This revealed that infection with the Δ*flhD* strain leads to reduced host survival **(Figure 1J)** and increased bacterial proliferation **(Figure 1K)** when compared to infection with the wild type strain. These results suggest that flagellin detection by the innate immune system restricts *S.* Typhimurium growth in the zebrafish infection model.

S. Typhimurium virulence factor mutants elicit GFP-Lc3 recruitment in similar patterns as the wild type strain

We recently identified a host protective role of an autophagy-related process known as Lc3-associated phagocytosis (LAP) during infection of zebrafish embryos with *S.* Typhimurium **(Chapter 3)**. In this work, we determined whether LAP also targets *S.* Typhimurium with non-functional PhoP, PurA, SipB, SsrB, and FlhD factors. To visualize the autophagy response we injected a high dose (2000-4000 CFU) of each bacterial mutant strain or the wild type strain into the *Tg(CMV:GFP-map1lc3b)* transgenic zebrafish

line, which expresses a GFP-fusion of the autophagy marker Lc3 **(Figure 2A)**. Evaluation of the autophagy response at 4 hpi showed that GFP-Lc3 associated with *S*. Typhimurium independent of the different virulence factors tested **(Figure 2B-G)**. The GFP-Lc3 localization patterns observed in response to infections with the mutant *S.* Typhimurium strains were similar to those observed with the wild type strain **(Chapter 3 and Figure $\mathbf{2B_{1\text{-}4}}$)**, as in all cases the autophagy signal appeared either in the form of GFP-Lc3 puncta associated with single bacterial cells or bacterial clusters, or as GFP-Lc3 rings around the bacterial cells or clusters **(Figure 2B-G)**. However, differences were observed in the level of GFP-Lc3 recruitment between the strains **(Figure 2H)**. Infection with Δ*phoP* and Δ*SipB* strains elicited slightly higher levels of GFP-Lc3 recruitment, whereas GFP-Lc3 recruitment was reduced in infections with the Δ*FlhD* and Δ*purA* strains **(Figure 2H)**. These differences in GFP-Lc3 recruitment did not correlate with the virulence of the mutants, since Δ*phoP* and Δ*purA* were attenuated and Δ*flhD* was more virulent in the zebrafish model **(Figure 1)**.

Figure 2: Host LAP response to *S.* Typhimurium mutants. *(figure on next page).* **A:** Work flow and time line of experiments followed in B-H. **B-G:** Representative confocal micrographs of GFP-Lc3 positive infected phagocytes with *mCherry* labelled *S.* Typhimurium mutant strains wild type (B), Δ*phoP* (C), Δ*purA* (D), Δ*sipB* (E), Δ*ssrB* (F) and Δ*flhD* (G). Yellow arrows indicate GFP-Lc3 associations with bacterial cells while numbers 1-4 represent four different examples of Lc3 association patterns for each *S.* Typhimurium mutant strain. At the top of each column of images is a symbolic representation of the pattern of GFP-Lc3 and bacterial cell association as reported earlier **(Chapter 3)**. **H:** Quantification of GFP-Lc3-*Salmonella* association for *S.* Typhimurium mutant strains Δ*phoP*, Δ*purA*, Δ*sipB,* Δ*ssrB* and Δ*flhD* along with wild type SL1344 at 4 hpi. Numbers of infected phagocytes positive or negative for GFP-Lc3-*Salmonella* associations were counted from confocal images and the percentages of Lc3-positive (Lc3+ve) over the total were averaged from five embryos per group. Error bars represent the SD. One of the two replicates is shown. Scale bars B-G= 5 μm, ****P< 0.0001, **P< 0.01, *P<0.05, ns= non-significant.

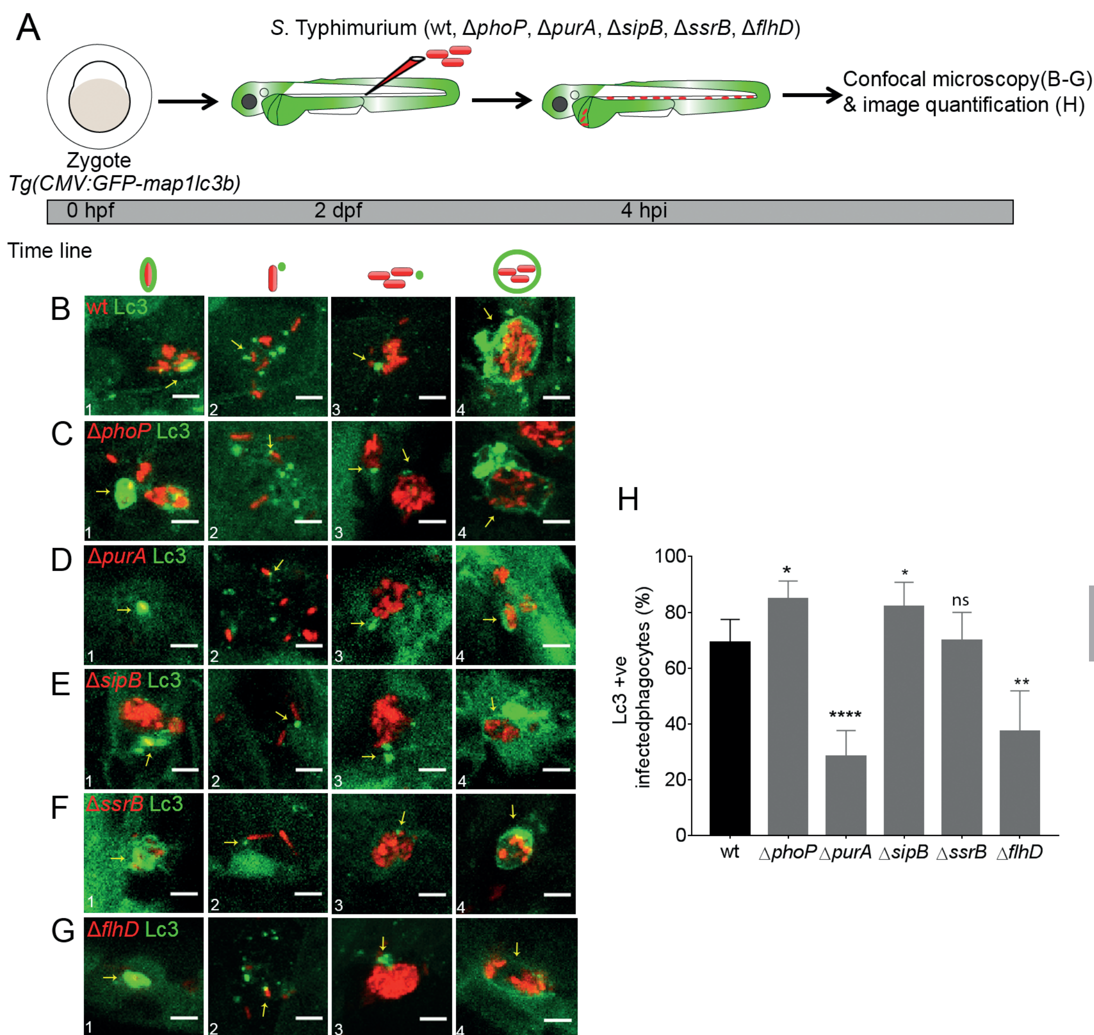

Figure 2: Host LAP response to *S.* Typhimurium mutants. *(figure legend on previous page).*

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S. Typhimurium virulence factor mutants are targeted by the Rubicon-dependent LAP pathway

In order to determine if the GFP-Lc3 response to the different *S.* Typhimurium mutant strains can be classified as LAP, we tested for dependency on the host protein Rubicon, which is essential for LAP but not for

canonical autophagy (Martinez et al., 2015). Depletion of Rubicon using a previously described splice blocking morpholino oligonucleotide **(Chapter 3)** resulted in significantly reduced levels of GFP-Lc3 recruitment in response to the Δ*phoP,* Δ*purA,* Δ*sipB* Δ*ssrB,* and Δ*flhD* strains **(Figure 3)**, in agreement with previous evidence for the role of LAP during infection with wild type *S.* Typhimurium **(Chapter 3)**. The results of this experiment confirmed that the *S.* Typhimurium mutants elicited GFP-Lc3 recruitment to different extents. In case of the Δ*purA,* ΔssrB and Δ*flhD* mutants, which elicited lower levels of GFP-Lc3 recruitment **(Figure 2H)**, GFP-Lc3 association was almost completely abolished under conditions of Rubicon knockdown **(Figure 3D,E, H,I and J,K)**. In contrast, during infections with the Δ*phoP* and Δ*sipB* strains, which elicited higher levels of GFP-Lc3-recruitment **(Figure 2H)**, Rubicon-deficient hosts displayed residual levels of GFP-Lc3-*Salmonella* association **(Figure 3 B,C and F,G)**, suggesting that canonical autophagy might target these strains when LAP is inhibited. Together, these results extend our previous findings and strengthen our conclusion that LAP is the main autophagy-related host defense response of macrophages targeting *S.* Typhimurium during systemic infection in zebrafish larvae.

Figure 3: Host LAP response to *S.* Typhimurium mutants in Rubicon-depleted embryos. *(figure on next page)*. **A:** Work flow and time line of experiments in B-K. **B,D,F,H,J:** Representative confocal micrographs of GFP-Lc3 positive infected phagocytes with *mCherry*-labelled *S*. Typhimurium mutant strains Δ*phoP* (B), Δ*purA* (D), Δ*sipB* (F), Δ*ssrB* (H), and Δ*flhD* (J) in Rubicon-depleted hosts (B',D',F',H',J') along with respective controls (B,D,F,H,J). **C,E,G,I,K:** Quantification of GFP-Lc3-*Salmonella* association for *S.* Typhimurium mutant strains Δ*phoP* (C), Δ*purA* (E), Δ*sipB* (G), Δ*ssrB* (I), and Δ*flhD* (K) in Rubicon-depleted larvae and their respective controls. Numbers of infected phagocytes positive or negative for GFP-Lc3-*Salmonella* associations were counted from confocal images and the percentages of Lc3-positive over the total were averaged from five embryos per group. Error bars represent the SD. One of the two replicates is shown. Scale bars (B,D,F,H,J)= 10 µm, ****P< 0.0001, ***P< 0.001, **P< 0.01, *P<0.05.

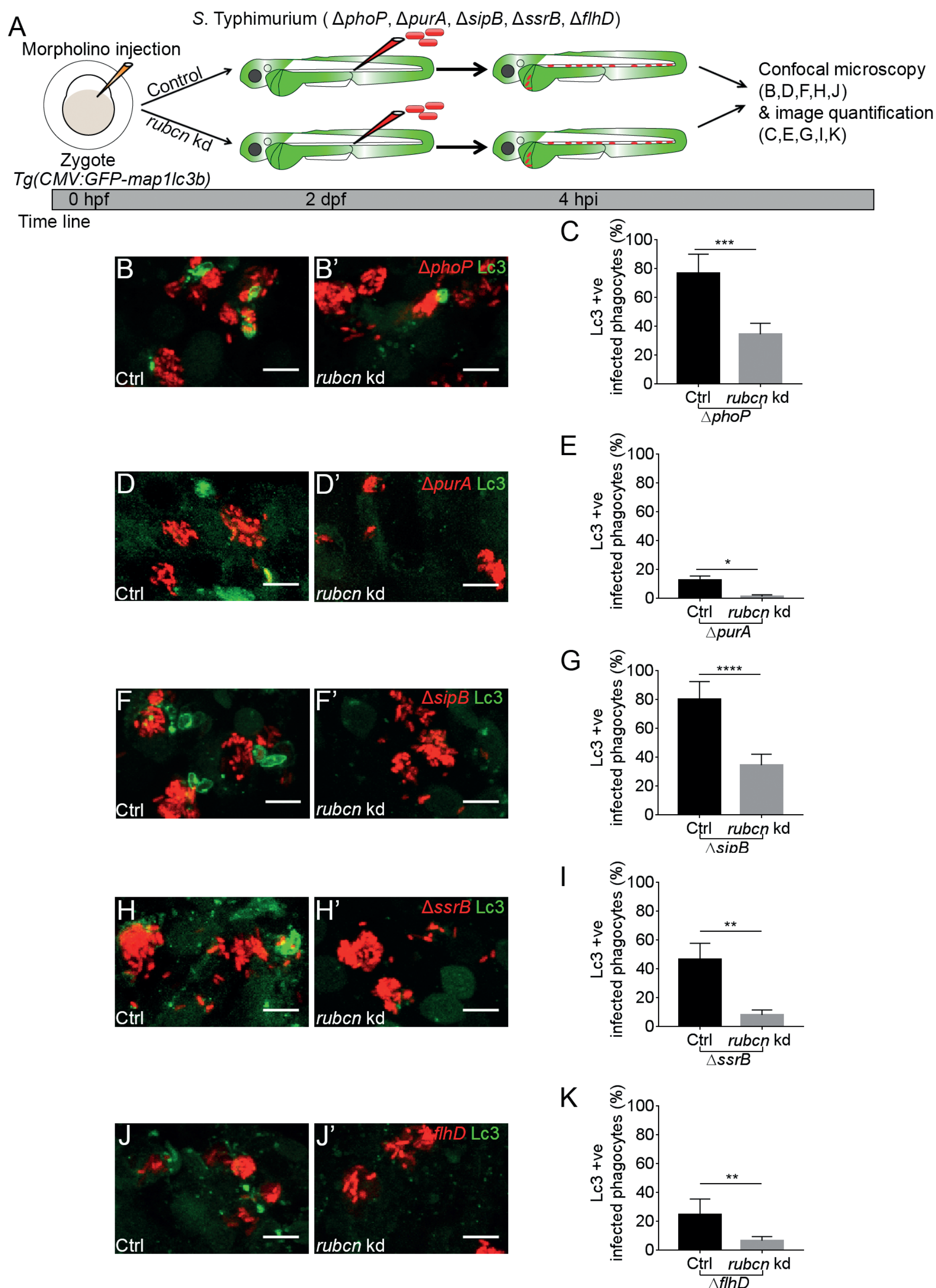

Figure 3: Host LAP response to *S.* Typhimurium mutants in Rubicon-depleted host. *(legend on previous page).*

S. Typhimurium mutants show increased virulence in a LAP-deficient host with the exception of Δ*ssrB*

Our previous results showed that LAP-deficient zebrafish embryos are more susceptible to wild type *S*. Typhimurium infection **(Chapter 3)**. To expand on this finding, we investigated the importance of LAP in defense against the *S*. Typhimurium mutant strains. In order to inhibit LAP we knocked down host Rubicon with the splice blocking morpholino approach and assessed the effect on host survival and bacterial burden **(Figure 4A)**. We found that infections with Δ*phoP*, Δ*purA*, Δ*sipB*, and Δ*flhD* **(Figure 4B,D,F,J)** resulted in significantly increased mortality of Rubicon-deficient hosts as compared to control embryos. In agreement, the CFU counts for these strains were significantly increased in Rubicon-deficient embryos **(Figure 4C,E,G,K,)**. In contrast, the virulence of the Δ*ssrB* strain and its infection establishment were independent of Rubicon levels of the host, as there were no significant differences between the survival curves of Rubicon-deficient and control larvae **(Figure 4H)** and the CFU counts **(Figure 4I)**. Therefore, we conclude that the Rubicon-dependent LAP response is required to restrict growth of *S.* Typhimurium strains carrying mutations in critical virulence factors and that only a strain deficient in expression of SPI2 factors (Δ*ssrB*) is unable to display increased virulence in a LAP-deficient host.

Figure 4: Virulence and infection progression of *S.* Typhimurium mutants in Rubicon-depleted hosts. *(figure on next page)*. **A:** Work flow and time line of experiments followed in B-K. **B,D,F,H,J:** Survival curves for systemically injected *S*. Typhimurium mutant strains, Δ*phoP* (B), Δ*purA* (D), Δ*sipB* (F), Δ*ssrB* (H), and Δ*flhD* (J) in Rubicon-depleted larvae with their respective control groups. One representative of two replicates is shown (n= 50 embryos per group). **C,E,G,I,K:** Representative CFU counts of the infections with *S*. Typhimurium mutant strains, Δ*phoP* (C), Δ*purA* (E), Δ*sipB* (G), Δ*ssrB* (I), and Δ*flhD* (K) in Rubicon-depleted zebrafish with their respective control groups at 24 hpi. One representative of two replicates is shown, where five embryos per time point were used and the log transformed CFU data are shown with the geometric mean per time point. Error bars represent SD. ****P< 0.0001, **P< 0.01, *P<0.05, ns= non-significant.

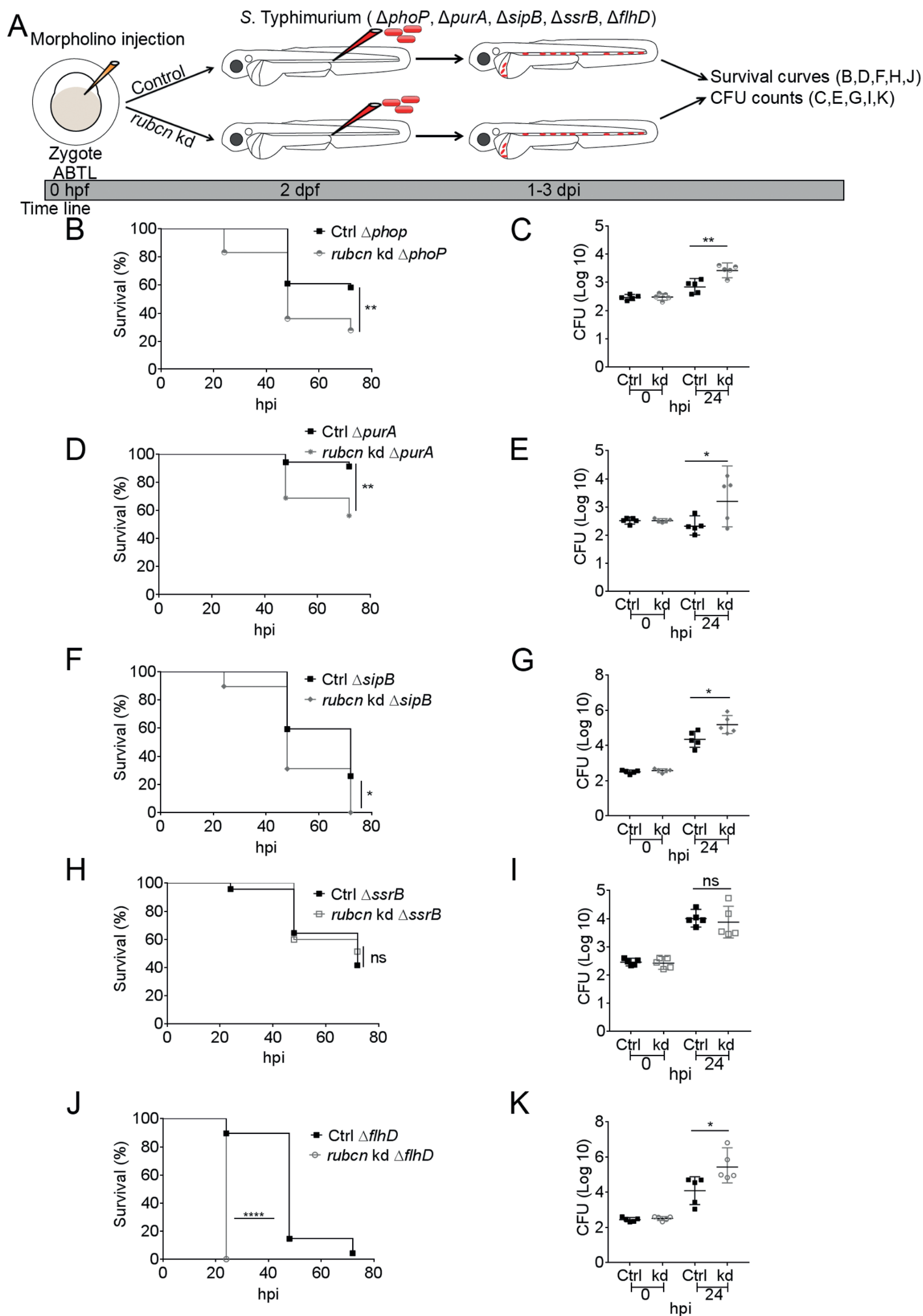

Figure 4: Virulence and infection progression of *S.* Typhimurium mutants in Rubicon-depleted hosts. *(legend on previous page).*

Discussion

The functions of *S.* Typhimurium virulence factors in invasion of host cells and intracellular replication are well described as a result of many years of studies in various cell culture and mouse models (Ibarra & Steele-Mortimer, 2009; van der Heijden & Finlay, 2012). However, how these factors induce or inhibit the host autophagy machinery is only beginning to be understood (Mesquita et al., 2012; Owen et al., 2014; Tattoli et al., 2012a; Tattoli et al., 2012b). Recently, we exploited GFP-Lc3 transgenic zebrafish to study the dynamics of activation of the autophagy machinery *in vivo*. Following intravenous infection in zebrafish embryos, *S.* Typhimurium is mainly contained by macrophages and as such this model mimics the advanced stages of systemic infections in humans. We found that internalization of live *S.* Typhimurium by macrophages in the zebrafish host is associated with rapid induction of an autophagy-related response known as LAP, whereby Lc3 associates with phagosomes in a Rubicon and NADPH oxidase dependent manner and independent of the ULK1 pre-initiation complex **(Chapter 3)**. Here we provide new evidence for the host-protective function of LAP, showing that *S.* Typhimurium strains carrying mutations in virulence factors PhoP, PurA, SipB, SsrB, and FlhD are all able to trigger LAP and that all mutants except the SsrB-deficient strain become more virulent in a LAP-deficient host.

It has previously been shown that lipopolysaccharide mutants are less virulent than wild type *S.* Typhimurium in the zebrafish model (van der Sar et al., 2003). However, the functions of other critical virulence factors had not yet been well characterized. In this study we show the importance of two virulence factors, PhoP and PurA, for *S.* Typhimurium infection in the zebrafish host. The PhoP/Q regulon, which controls outer membrane composition, has been reported to inhibit phagolysosomal fusion and also to enable adaptation to other intramacrophage stresses (Garvis et al., 2001; Thompson et al., 2011). In addition, it has been suggested to decrease immune responses, by reducing TLR activation (Dalebroux & Miller, 2014). Infection with a PhoP-deficient strain led to attenuated infection in the zebrafish model, which is in agreement with studies in murine macrophages and *in vivo* infection in mice (Miller et al., 1989; Miller & Mekalanos, 1990; Thompson et al., 2011), and with the fact that PhoP-deficient *S.* Typhi has been found safe for human vaccination (Hohmann

et al., 1996). We observed an even stronger attenuation in the case of the PurA-deficient strain, which was largely avirulent in the zebrafish host. This observation is consistent with avirulence of PurA-deficient *S.* Typhimurium *in vivo* in mice (O'Callaghan et al., 1988). These results support the validity of the zebrafish model for studying *S.* Typhimurium infection.

When LAP was inhibited by knockdown of Rubicon, both the PhoP-deficient and the PurA-deficient strains induced more severe infections in the zebrafish host. That virulence of attenuated strains can be (partially) restored in the absence of LAP extends our previous finding that LAP is a host-protective response against *S*. Typhimurium **(Chapter 3)**. While both attenuated strains triggered recruitment of GFP-Lc3 in a Rubicon-dependent manner, they had opposite effects on the level of GFP-Lc3 recruitment, which was increased in the infection with PhoP-deficient bacteria and reduced in response to PurA-deficient bacteria. Since we examined only a single time point, this difference might be due to different kinetics in the clearance of bacteria. We have previously found that no GFP-Lc3 response to heat-killed bacteria is present at this time point **(Chapter 3)**. Therefore, the lower levels of GFP-Lc3 in response to Δ*purA S.* Typhimurium could be due to rapid clearance of this largely avirulent strain and failure to induce signals for LAP. The higher levels of GFP-Lc3 targeting the Δ*phoP* mutant could point towards a role for PhoP in LAP evasion, which adds to its other reported functions in escaping host defense mechanisms (Dalebroux & Miller, 2014; Garvis et al., 2001; Miller & Mekalanos, 1990; Thompson et al., 2011). Therefore, the PhoP deficiency might enhance intraphagosomal killing of *S.* Typhimurium in zebrafish macrophages possibly due to higher LAP activity.

SipB-deficient bacteria, defective in the translocation of SPI1 effectors, showed similar virulence as wild type *S.* Typhimurium in the zebrafish model. SPI1 effectors are essential for gaining access to non-phagocytic target cells such as those in the gut epithelium (Watson & Holden 2010), but this invasion step is bypassed in the intravenous infection route used for infecting zebrafish embryos. Therefore, the non-attenuated phenotype of Δ*sipB* infection in zebrafish is consistent with studies in mice showing that virulence of SPI1 mutants is unaffected when the bacteria are delivered intraperitoneally, whilst administration by the oral route affects the ability to establish systemic infection (Galan & Curtiss, 1989).

In macrophages, SPI1 effectors can trigger caspase-1 dependent pyroptosis and SPI1 mutants, including Δ*sipB*, lack the ability to induce this response (Fink & Cookson et al., 2007). We cannot exclude that SipB deficiency might affect macrophage cell death in the zebrafish host. When *Salmonella* bacteria are released from macrophages, they can continue to replicate extracellularly by forming microcolonies on the endothelial cells of the blood vessels (van der Sar et al., 2003). Therefore, it is possible that SipB mutation could have altered the ratio between intra- and extracellular bacteria, without alteration in overall bacterial burden or effect on host survival. Our observation of a small but significant increase in GFP-Lc3 recruitment by Δ*sipB* bacteria compared with the wild strain suggests increased targeting of the mutant strain by LAP, but apparently insufficient to impact on the overall infection development. However, as in the case of Δ*phoP* and Δ*purA* mutants, depletion of Rubicon revealed the host-protective function of LAP during infection with the Δ*sipB* strain.

Impaired SPI2 function, by mutation of SsrB, did not significantly affect GFP-Lc3 recruitment and had no detectable effect on virulence in the zebrafish host. The lack of a virulence phenotype was unexpected, since Δ*ssrB Salmonella* bacteria are attenuated in several *in vitro* and *in vivo* models, including human epithelial cells, murine and porcine macrophages, orally and intravenously infected mice, and intravenously infected pigs (Cirillo et al., 1998; Boyen et al., 2008; Grant et al., 2012). However, a study in mice reported that the main cause of the attenuated phenotype of SPI2 mutants is their inability to leave infected cells, whereas growth of SPI2 mutants inside phagocytes exceeds that of wild type *Salmonella* (Grant et al., 2012). Therefore, the lack of an attenuated phenotype of Δ*ssrB Salmonella* in zebrafish embryos might be explained by the fact that phagocytes are the main replication site in this model. Interestingly, a function for SsrB in the zebrafish model was revealed when we inhibited LAP. We found that Δ*ssrB* bacteria, unlike all other mutants tested, were unable to display increased virulence under conditions of Rubicon knockdown. That only SsrB-competent strains become more virulent in a Rubicon-deficient host might be an indication that SPI2 effector proteins controlled by the SsrA/B system promote *S.* Typhimurium virulence in the absence of LAP.

We found that loss of the FlhD regulator, which deprives *S.* Typhimurium from the formation of flagella, leads to increased virulence in both wild type and Rubicon-deficient zebrafish hosts. These results are in agreement with studies in mice showing that non-flagellated mutants of *Salmonella*, including Δ*flhD*, are equally or more virulent than the wild type strains, despite that these mutants lack the ability to invade epithelial cells (Lockman & Curtiss, 1990; Schmitt et al., 2001). The hypervirulence of Δ*flhD* bacteria can be explained by escape from recognition by TLR5, which is required for activation of the innate immune response to flagellin in mice as well as zebrafish (Fournier et al., 2009; Stockhammer et al., 2009). An additional explanation is the escape from inflammasome activation and interleukin 1 beta secretion (Franchi et al., 2006; Lai et al., 2013; Winter et al., 2009). In agreement with the fact that LAP induction has been shown to depend on TLR signaling (Sanjuan et al., 2007), we observed significantly lower GFP-Lc3 association with Δ*flhD* than with wild type bacteria. Considering the evidence for a host-protective function of LAP provided by our study, we propose that the increased virulence of Δ*flhD S.* Typhimurium resides at least partly in lower activation of LAP through TLR5-mediated signalling.

In conclusion, we have demonstrated attenuated virulence of Δ*phoP* and Δ*purA S.* Typhimurium mutants in the zebrafish systemic infection model in contrast to hypervirulence of the non-flagellated Δ*flhD* mutant. Furthermore, our data support that the Rubicon-dependent LAP pathway plays an important role in host defense, since all tested mutant strains were able to cause more severe infections when LAP was inhibited, except for one strain deficient in the expression of SPI2 effectors. While LAP is the predominant autophagy-related response during the macrophage-dominated infection in zebrafish, selective autophagy mediated by ubiquitin receptors (xenophagy) has been found to restrict growth of *S.* Typhimurium in epithelial cells (Huang & Brumell, 2014). Therefore, there is accumulating evidence for the function of autophagy proteins in host defense against *S*. Typhimurium, encouraging further exploration of autophagy modulating drugs for host-directed therapy of antibiotic resistant *Salmonella* infections.

Materials and methods

Zebrafish lines and maintenance

Zebrafish were handled in compliance with local animal welfare regulations and maintained according to standard protocols (zfin.org). Breeding of zebrafish was approved by the local animal welfare committee (DEC) of the University of the Leiden, under license number 10612. All experiments were performed on embryos/larvae before the free feeding stage and did not fall under animal experimentation law in line with the EU Animal Protection Directive 2010/63/EU.

Fish lines used for the present work were the wild type (wt) strain AB/TL, and the transgenic line *Tg(CMV:GFP-map1lc3b)* (He et al., 2009). Embryos from adult fish were attained by natural spawning and were kept at 28.5°C in egg water (60 µg/ml sea salt, Sera Marin, Heinsberg, Germany). PTU (1-phenyl-2thiourea; Sigma Aldrich) was added to egg water at 0.003% to prevent melanization of embryos. For infection delivery and live imaging experiments embryos were anaesthetized in egg water with 0.02% of buffered Tricane (3-aminobanzoic acid ethyl ester; Sigma Aldrich).

Bacterial cultures and infection experiments

Salmonella Typhimurium strains used in this study are listed in **supplementary Table 1** along with their respective mutations and their description. The bacterial strains were plated fresh from -80°C stocks over LB agar plates with respective selection markers and were incubated overnight to grow at 37°C. Before the start of infection experiments colonies from LB agar plates were suspended in phosphate buffered saline (PBS) supplemented with 2% polyvinylpyrrolidone-40 (Sigma Aldrich) to obtain the low dose (200-400 CFU, for survival curves and CFU counts experiments) or the high dose (2000-4000 CFU, for imaging experiments). Bacterial inoculum was injected systemically into the caudal vein of the anaesthetized embryos at 2 dpf. To check the inoculum size, the same dose was spotted onto agar plates, and bacterial counts determined following overnight incubation. After infection, embryos were kept individually in egg water in 48-well plates to score survival during larval development up to 5 dpf and to collect individuals for CFU counts at 24 h intervals.

Determination of *in vivo* bacterial (CFU) counts

Five larvae per time point were sacrificed and homogenized in PBS using the Bullet Blender Tissue Homogenizer (Next Advance Inc.). Homogenates were then serially diluted, and three technical replicates for each embryo/larva were plated on LB solid media with the appropriate antibiotics for *S*. Typhimurium. To determine the CFUs, the resulting colonies were counted manually after 24 h incubation at 37°C.

Morpholino knockdown

Morpholino oligonucleotides (Gene Tools) used for Rubicon (*rubcn*) knockdown and control were diluted in Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES; pH 7.6) to obtain the required concentrations. *rubcn* knockdown was achieved by injecting 1 nl volume of 0. 25 mM of previously described splice morpholino MO2-rubcn **(Chapter 3)***,* similarly 1 nl of standard control morpholino was injected into the yolk of 0 hpf zebrafish embryos with microneedles and a Femtojet injector (Eppendorf) paired with a stereo-microscope.

Imaging and Image analysis

Confocal laser scanning images were acquired using at 63x water immersion objective (NA 1.2) with a Leica TCS SPE system, Samples were fixed at 4 hpi in 4% PFA and washed with PBS before image acquisitions. For Lc3-*Salmonella* associations, images acquired were analyzed through Z-stacks in Leica LAS AF Lite software and bacterial clusters were observed and manually counted in the overlay channel. Max projections in the overlay channels were used for representative images.

For quantification of the autophagic response within infected phagocytes, for each embryo, a total number of observable phagocytes were manually counted through the Z-stacks of the acquired confocal image. Phagocytes were identified in the yolk sac circulation valley by bacterial clusters in the *mCherry* channel and cellular boundaries of phagocytes were determined in the light transmission channel. Among these total observable infected phagocytes, the numbers of cells with GFP-Lc3 signal in association with *Salmonella* bacteria were counted and the percentage of Lc3-positive

phagocytes over the total observable phagocytes was determined for each embryo.

Statistical Analyses

All data sets were analyzed with Prism 7 software. Survival curves were analyzed with Log rank (Mantel-Cox) test. For CFU counts, one way ANOVA was performed on Log transformed data and was corrected for multiple comparisons using Sidak's multiple comparisons test when required. Percentage Lc3-positive phagocyte quantifications was analyzed for significance with unpaired parametric t-test between two groups, and for multiple groups the one way ANOVA test was performed and corrected for multiple comparisons.

Acknowledgements

We thank Dirk Bumann (University of Basel) for sharing of the *Salmonella* strains used in this study, and Daniel Klionsky (University of Michigan) for the zebrafish transgenic line. We are also grateful to all members of the fish facility team for zebrafish care. S.M. was supported by a fellowship from the Higher Education Commission of Pakistan and the Bahaudin Zakriya University, Multan. T.K.P. was supported by an individual Marie Curie fellowship (PIEF-GA-2013-625975).

Supplementary material

Supplementary Table S1: List of *Salmonella* strains used for study.

Serial no.	Name of strain[1]	Mutation
1	SL1344	None (wild type)
2	Δ*phoP*	Mutation in PhoP/Q two-component sensor of the host intracellular environment
3	Δ*ssrB*	Mutation in SsrB regulator of SPI2-proteins
4	Δ*flhD*	Mutation in flagellar transcription regulator
5	Δ*sipB*	Mutation in SPI1 translocator protein SipB
6	Δ*purA*	Mutation in adenylosuccinate synthetase (purine metabolism)

[1] All strains were provided by Dirk Bumann (University of Basel). All mutant strains are in SL1344 background.

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