

RESEARCH ARTICLE

# The ubiquitin-like modifier FAT10 decorates autophagy-targeted *Salmonella* and contributes to *Salmonella* resistance in mice

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## ABSTRACT

Bacterial invasion of eukaryotic cells is counteracted by cell-autonomous innate immune mechanisms including xenophagy. The decoration of cytosolic bacteria by ubiquitylation and binding of galectin-8 leads to recruitment of autophagy adaptors like p62 (also known as SQSTM1), NDP52 (also known as CALCOCO2) and optineurin, which initiate the destruction of bacteria by xenophagy. Here, we show that the functionally barely characterized IFN $\gamma$ - and TNF $\alpha$ -inducible ubiquitin-like modifier FAT10 (also known as ubiquitin D, UBD), which binds to the autophagy adaptor p62, but has not been shown to associate with pathogens before, is recruited to cytosolic *Salmonella* Typhimurium in human cells. FAT10-decorated *S. Typhimurium* were simultaneously decorated with ubiquitin, p62, NDP52 and the autophagy marker LC3B (MAP1LC3B). FAT10 colocalized with p62-positive microdomains on *S. Typhimurium*, whereas colocalization with NDP52 was only partial. A kinetic analysis revealed an early, but only transient, decoration of bacteria by FAT10, which resembled that of p62. Although bacterial replication was not detectably altered in FAT10-depleted or overexpressing cells *in vitro*, survival experiments revealed that NRAMP1-transgenic mice that were FAT10-deficient had a higher susceptibility to orally inoculated *S. Typhimurium* bacteria than NRAMP1-transgenic mice that were wild-type for FAT10. Taken together, our data suggest a role for FAT10 in the intracellular defense against bacteria.

**KEY WORDS:** Ubiquitin, FAT10, p62, *Salmonella* Typhimurium, Autophagy, Xenophagy

## INTRODUCTION

Pathogens can invade eukaryotic cells and replicate within the cytosol. As a consequence eukaryotes developed cell autonomous mechanisms to counteract pathogenic invasion. Effector mechanisms, which are often cytokine inducible, defend the host cell against infection and rely on oxidative, nitrosative and protonative chemistries. The restriction of cytosolic nutrients and the compartmentalization of pathogens are also effective defense mechanisms. A catabolic process called macroautophagy (here

referred to as autophagy) has been shown to be another defense mechanism (Birmingham et al., 2006; Gutierrez et al., 2004; Nakagawa et al., 2004; Ogawa et al., 2005). Autophagy was originally considered to degrade bulk cytosolic material in a non-specific manner, especially to restore energy homeostasis and amino acid levels during nutritional deprivation. In principle, three stages are involved: (i) initiation (a crescent membrane, called a phagophore is formed), (ii) elongation and closure (where a double-membraned autophagosome encloses sequestered cargo), and (iii) maturation (where the autophagosome fuses with late endosomal and lysosomal organelles to form the degradative autolysosome). The autophagosomal pathway includes recognition, capture and elimination of intracellular components unrestricted by their size or complexity. These processes in turn regulate the elimination of depolarized mitochondria (mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy), aggregated proteins (aggrephagy) or cytosol-colonizing microbes (xenophagy) (Kirkin et al., 2009). In addition, xenophagy, the delivery of antimicrobial products to intracellular niches of pathogens can be allocated to the specialized immune processes that are performed by the autophagy machinery at the cellular level (Ponpuak et al., 2010).

For autophagosomal capturing of cargo, ubiquitin plays a crucial role in mediating substrate specificity. Ubiquitin thereby constitutes a universal degradation signal for both major proteolytic systems in the cytosol: the proteasome and autophagosome. It is still a matter of debate whether pathogens are either directly ubiquitylated or whether ubiquitylated host proteins accumulate on their surface to initiate xenophagy (Huett et al., 2012; Perrin et al., 2004). However, ubiquitin-independent mechanisms for autophagosomal targeting, through diacylglycerol or  $\beta$ -galactose-binding lectins, have also been reported (Shahnazari et al., 2010; Thurston et al., 2012). One lectin, galectin-8, serves as a danger receptor for vesicle-damaging pathogens in general, as it surveys the integrity of the endolysosomal compartment. In a second step, the autophagy adaptors p62 (also known as SQSTM1), NDP52 (also known as CALCOCO2) and optineurin, which have similar but not redundant functions, are recruited to the cytosol-invading pathogen (Thurston et al., 2009; Wild et al., 2011; Zheng et al., 2009). All known autophagy adaptors share the LC3-interacting region (LIR). This binding domain initiates the engulfment of cargo by the nascent autophagosome that comprises LC3 and its paralogs conjugated to phosphatidylethanolamine. During the subsequent maturation process, the closed autophagosome fuses with late endosomal and lysosomal organelles to form the degradative autolysosome.

Escape mechanisms, like avoidance of autophagic capture, suppression of autophagy or even the usage of components of the autophagy pathway for self-serving purposes have been evolved

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by pathogens (Deretic and Levine, 2009). The number of escape mechanisms strengthens the importance of autophagy as a host defense mechanism in immunity. During infection with the facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), ~25–35% of the invading bacteria reach the cytoplasm (Birmingham et al., 2006). A higher percentage replicates within the modified phagosome, the so-called *Salmonella*-containing vacuole (SCV). Cytosolic *S. Typhimurium* is efficiently targeted by autophagy and is frequently used in *in vitro* studies to study mechanistic aspects of xenophagy. Surprisingly, a supportive effect of autophagy on intracellular replication of *Salmonella* has also been recently proposed (Yu et al., 2014). However, it is not yet known whether ubiquitin-based modes of target recognition together with galectin-8-mediated danger-associated molecular pattern (DAMP) signaling remain the only cytosolic pathogen detection mechanisms. Alternative adaptor molecules, as well as targeting mechanisms, await potential identification.

FAT10 (HLA-F adjacent transcript 10; also known as ubiquitin D, UBD) represents the youngest member of the family of ubiquitin-like modifiers (ULMs) (Fan et al., 1996). Although it has not yet been crystallized, the protein is assumed to have two ubiquitin-like domains in a head-to-tail formation connected with a short linker (Schmidtke et al., 2014). The lysine residues corresponding to K27, K33, K48 and K63 in ubiquitin are conserved in both FAT10 UBL-domains as well as the C-terminal glycine carboxylate as a site of substrate conjugation (Bates et al., 1997). Constitutive FAT10 expression has been reported in thymus and secondary lymphatic organs like spleen, fetal liver and lymph nodes (Canaan et al., 2006; Lee et al., 2003; Lukasiak et al., 2008). In addition, FAT10 transcription is induced during maturation of activated dendritic cells (Bates et al., 1997; Lukasiak et al., 2008). In most other tissues, FAT10 is not expressed unless the pro-inflammatory cytokines IFN $\gamma$  and TNF $\alpha$  are present (Liu et al., 1999; Raasi et al., 1999). FAT10 needs no processing, but is activated and conjugated by the constitutively expressed E1 and E2 enzymes UBA6 and USE1, respectively (Aichem et al., 2010; Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007). Potential E3 ligases and deconjugating enzymes have not yet been identified. Recently, our group has identified hundreds of FAT10-interacting proteins by mass spectrometry, including putative FAT10 substrates (Aichem et al., 2012). Among the ULMs, FAT10 is the only one capable of directly targeting covalently modified substrates for proteasomal degradation (Hipp et al., 2005; Schmidtke et al., 2009). The interaction of FAT10 with the long isoform of the UBL-UBA domain protein NEDD8 ultimate buster 1 (Nub1L) accelerates the degradation rate of FAT10 and its conjugates through binding of the 19S regulator subunits Rpn10 and Rpn1, respectively (Hipp et al., 2004; Rani et al., 2012). Since its discovery, FAT10 has been reported to be involved in many partially divergent processes. FAT10 expression is supposed to have proapoptotic functions (Liu et al., 1999; Raasi et al., 2001; Ross et al., 2006), an influence on chromosomal stability and cell cycle regulation (Liu et al., 1999; Ren et al., 2011), NF- $\kappa$ B signaling (Gong et al., 2010) and aggresome formation (Kalveram et al., 2008). In addition, FAT10 has been suggested to have antiapoptotic functions and is implicated in carcinogenesis because it is expressed in various cancer types (Gao et al., 2014; Lee et al., 2003; Lukasiak et al., 2008; Zhang et al., 2006).

Our group has recently investigated functional aspects of FAT10 being conjugated to the autophagy adaptor p62 (Aichem

et al., 2012). Like other autophagy adaptors, p62 contains a LIR domain that determines its fate as a selective autophagy substrate, being continuously degraded by autophagy (Bjørkøy et al., 2005; Pankiv et al., 2007). In combination with its C-terminal UBA domain, p62 is an efficient autophagosomal adaptor for ubiquitylated substrates (Pankiv et al., 2007). We have described a UBA-independent covalent interaction between FAT10 and p62, as well as a strong non-covalent interaction (Aichem et al., 2012). This finding encouraged us to further analyze prospective consequences of this interaction for the p62 adaptor function. As both FAT10 and p62 are similarly upregulated by pro-inflammatory cytokines we hypothesized that FAT10 might, analogous to ubiquitin, target pathogens for autophagosomal degradation. Indeed, we found that FAT10 decorated cytosolic *S. Typhimurium* which were targeted for xenophagy. Survival experiments in NRAMP1-transgenic mice revealed a higher susceptibility for *S. Typhimurium* when the FAT10 gene was deleted suggesting a role for FAT10 in the intracellular defense against bacteria.

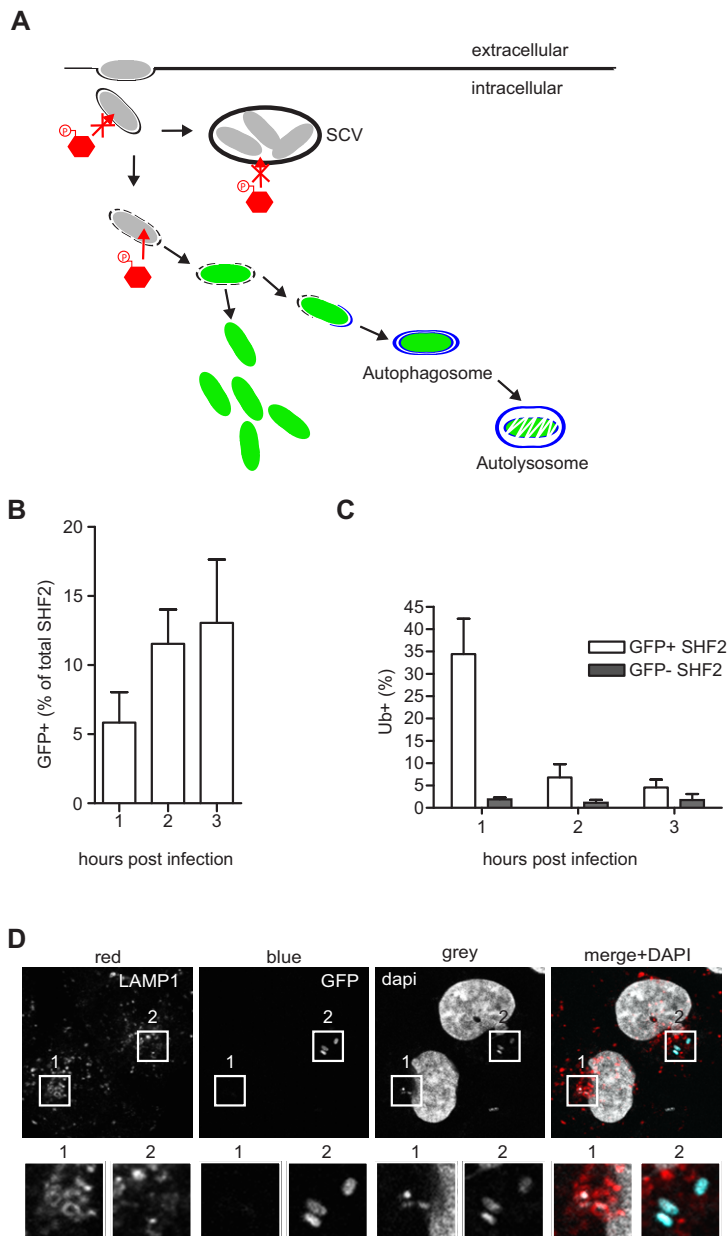
## RESULTS

### *Salmonella* SHF2 expresses GFP in the cytosol

In a previous study, we demonstrated a noncovalent interaction between the autophagy adaptor p62 and FAT10 (Aichem et al., 2012). This interaction can be considered as a direct interaction, demonstrated by the co-precipitation of recombinant p62 with recombinant GST–FAT10 (supplementary material Fig. S1). First, we hypothesized that a parallel between ubiquitin and FAT10 with regard to autophagosomal targeting might exist. However, we failed to demonstrate autophagosomal targeting of an mCherry–EGFP–FAT10 fusion protein (Aichem et al., 2012). Nevertheless, we further investigated the hypothesis as to whether the cytokine-inducible modifier FAT10 might be involved in autophagosomal degradation of cytosolic pathogens. We therefore generated a modified *Salmonella* SL1344 strain (SHF2) that expresses the GFP-tagged hexose phosphate transport protein (GFP–UhpT) under the control of a glucose 6-phosphate-responsive promoter. This glucose metabolite is exclusively present in the cytoplasm and excluded from the lumen of endomembranes, including the SCV (Fig. 1A). Expression of GFP in SHF2 *Salmonella* is indicative of an exposure to glucose 6-phosphate. We tested whether green fluorescence in SHF2 *Salmonella* faithfully reports cytosolic bacteria. To this end, human umbilical vein endothelial cells (HUVECs) were infected with SHF2, and GFP-positive bacteria were enumerated during the first 3 h of infection (Fig. 1B). Up to 20% of the total SHF2 *Salmonella* had induced GFP expression within that time period. In addition, ubiquitin decoration of *Salmonella* strongly correlated with induced GFP expression (~35%). In contrast, GFP-negative SHF2 *Salmonella* showed only a minor decoration with ubiquitin (Fig. 1C). In addition, HeLa cells were infected with SHF2 and stained for Lamp1, a marker for SCVs (Fig. 1D). *Salmonella* which did not express GFP were clearly surrounded by Lamp1, indicating their presence in intact SCVs. Furthermore, GFP expression correlated with *Salmonella* that did either not exhibit any appreciable association to Lamp1 staining or at least no continuous decoration with Lamp1. We therefore conclude that green fluorescence in cells infected with SHF2 indicates exposure of these bacteria to the cytosol.

### FAT10 decorates autophagy-targeted *S. Typhimurium*

Functioning as an autophagosomal adaptor, p62 has been implicated in the capture of cytosolic bacteria or their remnants (Dupont et al., 2009; Zheng et al., 2009). We speculated that the



**Fig. 1. *Salmonella* SHF2 expresses GFP in the cytosol.**

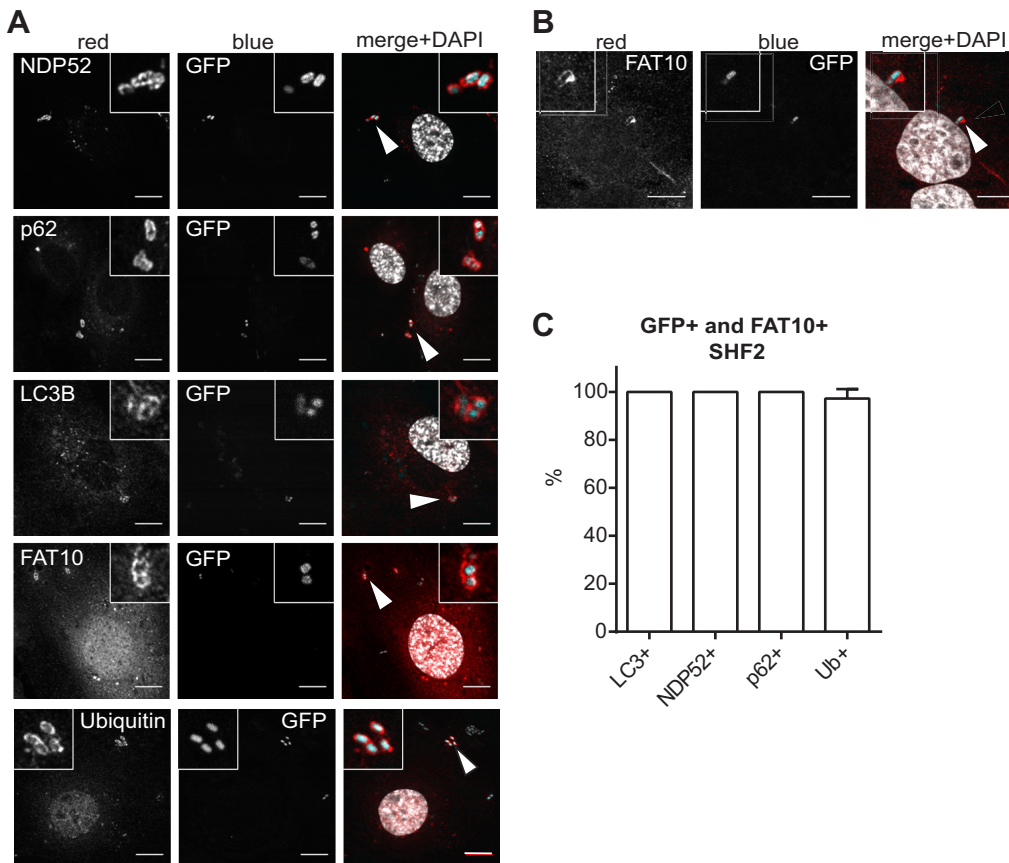
(A) Schematic of the *Salmonella* strain (SHF2) that expresses GFP–UhpT under the control of glucose 6-phosphate. This glucose metabolite is exclusively present in the cytoplasm and excluded from the lumen endomembranes, including the *Salmonella*-containing vacuole (SCV). Expression of GFP in SHF2 is indicative of an exposure to glucose 6-phosphate in the cytosol or within damaged SCVs. (B) HUVECs were infected with SHF2 (MOI 100) for up to 3 h, fixed and stained for total SHF2 (anti-LPS antibody). The fraction of GFP-positive (GFP+) *Salmonella* SHF2 was quantified by fluorescence microscopy. The mean  $\pm$  s.d. is shown for at least 1000 total bacteria analyzed for each condition in two independent experiments. (C) HUVECs were infected as in B and cells were fixed and stained for total SHF2 (anti-LPS antibody) and ubiquitin (FK2 antibody). The percentage of ubiquitin-positive (Ub+) SHF2 *Salmonella* that were GFP+ and GFP– was enumerated by fluorescence microscopy. The mean  $\pm$  s.d. is shown for a total of at least 1000 bacteria analyzed for each condition in two independent experiments. (D) HeLa cells were infected with SHF2 (MOI 100) for 2 h and cells were fixed and stained for Lamp1. Enlarged images of the indicated area are shown below each main image.

upregulation and interaction of FAT10 with p62 during inflammation might be of functional interest. As both proteins are induced by proinflammatory cytokines, it is tempting to speculate that FAT10 can function as a xenophagy marker. To assess this possibility, we performed *in vitro* infection experiments to analyze whether FAT10 is recruited to cytosolic bacteria using confocal microscopy. We could indeed observe FAT10 decoration of cytosolic SHF2 at 1 h after infection and 24 h after cytokine treatment in HUVECs (Fig. 2A) and HeLa cells (Fig. 2B). Importantly, we observed differences in the quantity of bacterial FAT10 decoration between these two cell types. Cancer cell lines in general can display abnormal cytokine signaling. For HeLa cells, it has previously been shown that the induction of FAT10 is not as strong as for primary cells, like HUVECs (Liu et al., 1999; Raasi et al., 1999). Therefore, we performed the subsequent infection experiments primarily in HUVECs. Co-stainings revealed that FAT10-decorated SHF2 *Salmonella* were additionally decorated with NDP52, p62, LC3 and ubiquitin (Fig. 2C). Interestingly, we

could not find FAT10-decorated bacteria without an autophagy adaptor or LC3B (MAP1LC3B) present. Only a very small fraction of autophagy-targeted bacteria were positive for FAT10 but not positive for ubiquitin (Fig. 2C). Xenophagy induction takes place very rapidly after bacterial entry into the cytosol (Tattoli et al., 2012a; Zheng et al., 2009). In particular, the timeline of cytosolic recognition, adaptor recruitment and LC3B association, at least for Effectene-coated latex beads, spans only a few minutes. The decoration with ubiquitin and p62 also takes place almost simultaneously (Fujita et al., 2013). Therefore, we assume that as soon as a defined FAT10 decoration is detectable by confocal microscopy, adaptors are already present on the bacterial surface. In summary, we conclude that a certain pool of cytosolic SHF2 is decorated with FAT10. Remarkably, these bacteria are at the same time targeted for autophagy.

Some xenophagy-targeted pathogens have evolved strategies to escape this cellular defense mechanism, especially the detection through ubiquitylation. Therefore FAT10 decoration might





**Fig. 2. FAT10 decorates autophagy-targeted *S. Typhimurium*.**

(A) Confocal micrographs of IFN $\gamma$ - and TNF $\alpha$ -induced HUVECs and (B) HeLa cells infected with *S. Typhimurium* (SHF2) for 1 h, fixed and immunostained for endogenous NDP52, p62, LC3B, ubiquitin and FAT10. DAPI, 4,6-diamidino-2-phenylindole. Arrowheads indicate cytosolic bacteria expressing GFP, which are shown with higher magnification in the insets. Scale bars: 10  $\mu$ m. (C) HUVECs were infected with SHF2 for 1 h, fixed, and co-immunostained for NDP52, LC3B, p62 or ubiquitin together with FAT10. The percentage of FAT10+ and GFP+ SHF2 *Salmonella* also decorated with p62, NDP52, LC3B or ubiquitin was enumerated by fluorescence microscopy. The mean  $\pm$  s.d. is shown for at least 190 FAT10-decorated GFP+ bacteria analyzed for each condition in two independent experiments.

represent a backup mechanism for ubiquitin to target these pathogens in particular. However, we found no FAT10 decoration on Gram-positive *L. monocytogenes* (supplementary material Fig. S2A,B), a pathogen that is known to evade autophagy (Deretic and Levine, 2009).

#### FAT10 colocalizes with p62-positive but significantly less with NDP52-positive microdomains

Cemma et al. have suggested that the decoration of cytosolic bacteria with p62 and NDP52 is not homogeneous but instead is present in patches. Moreover, they report that, in immunostainings, p62 and NDP52-specific fluorescent signals are rather segregated and that the recruitment of the two adaptors to cytosolic bacteria is independent of each other (Cemma et al., 2011). We therefore wanted to investigate whether the FAT10-specific fluorescent signal also correlates with certain microdomains. To address this question, we performed co-immunostainings of SHF2-infected, and IFN $\gamma$ - and TNF $\alpha$ -treated HUVECs (Fig. 3A). With confocal microscopy, we could indeed observe microdomains representing the bacterial coat. The degree of colocalization of FAT10 with p62, NDP52 ubiquitin or LC3B showed marked differences (Fig. 3B). Therefore we quantified colocalizing fluorescent signals by calculating the Pearson's correlation coefficient. The FAT10-specific fluorescent signal colocalized best with p62 and LC3B but significantly less with NDP52 (Fig. 3C). Colocalization with FAT10 and ubiquitin revealed a correlation factor lower than p62 but higher than NDP52.

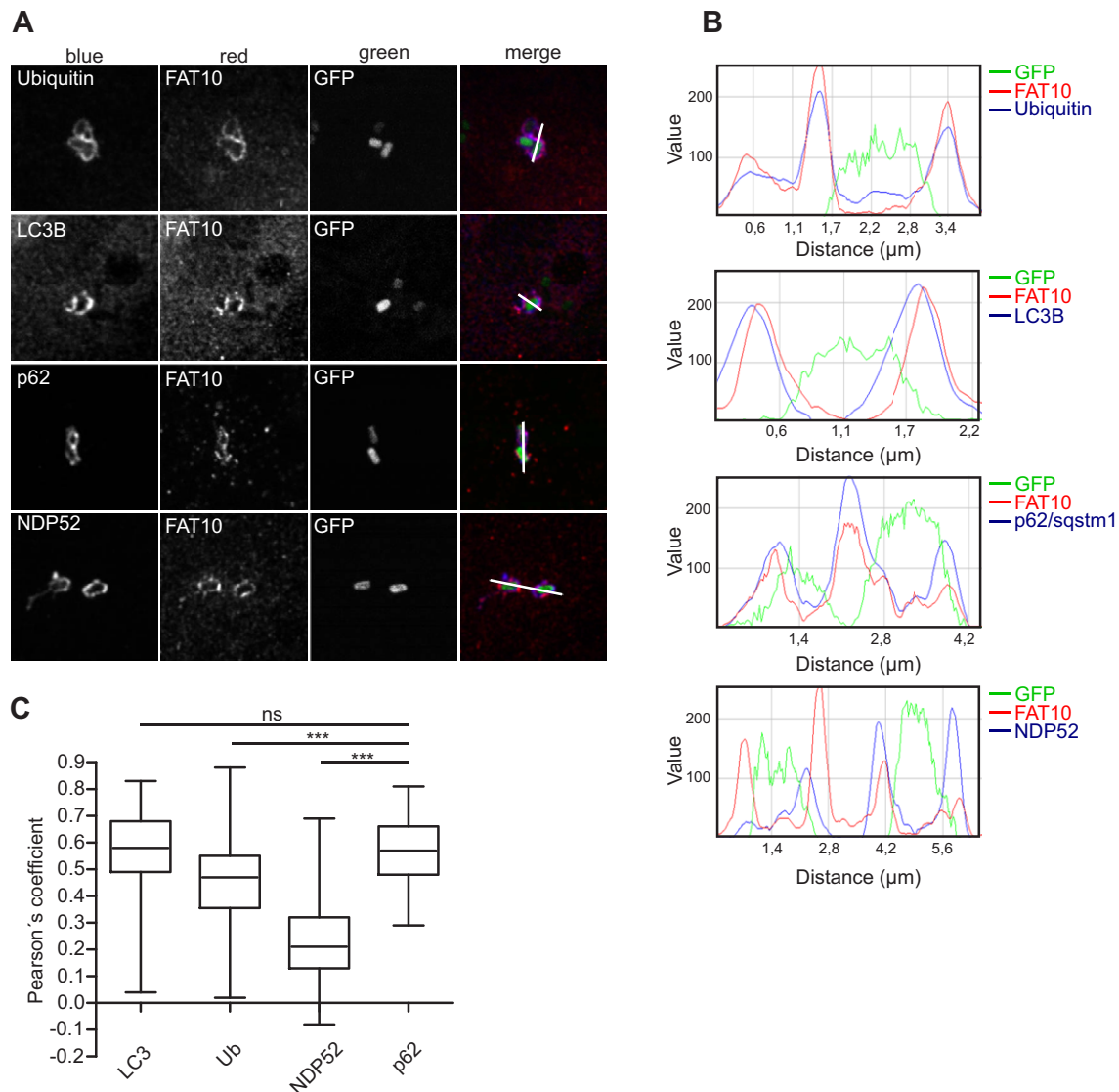
#### FAT10 decoration and autophagosomal targeting of *S. Typhimurium* occur with the same kinetics

Ubiquitin decoration is an early event in autophagosomal targeting of *Salmonella* (Fujita et al., 2013; Huett et al., 2012;

Perrin et al., 2004; Zheng et al., 2009) and the subsequent process of xenophagy occurs with little delay (Birmingham et al., 2006; Tattoli et al., 2012b; Zheng et al., 2009). Given that FAT10 and ubiquitin decorate the same fraction of cytosolic bacteria (Fig. 3A), we asked whether the two modifiers are recruited to cytosolic bacteria with the same kinetics. To this aim, we infected cytokine-stimulated and untreated HUVECs for 3 h and quantified the percentage of FAT10- or ubiquitin-positive cytosolic bacteria. We found extensive ubiquitin decoration ( $50.6\% \pm 3.6$  of bacteria, mean  $\pm$  s.d.) but no FAT10 decoration of SHF2 bacteria in unstimulated control cells (Fig. 4A), consistent with the absence of FAT10 expression in unstimulated HUVECs. Ubiquitin decoration was maximal at 1 h after infection and declined over 3 h. In IFN $\gamma$ - and TNF $\alpha$ -treated cells ubiquitin decoration ( $51.1\% \pm 0.6$ ) and the cytokine-induced decoration with FAT10 ( $11.7\% \pm 8.5$ ) occurred as early as 1 h after infection and waned with the same kinetics as that for p62, NDP52 and LC3B (Fig. 4A,B). We can therefore conclude that FAT10 and ubiquitin associate not only with the same fraction of cytosolic *S. Typhimurium* but also with similar kinetics. In addition, FAT10 induction did not reduce p62 decoration of *S. Typhimurium*. Instead, xenophagy of cytosolic bacteria was protracted in cytokine-treated HUVECs (Fig. 4B).

#### FAT10 deficiency and overexpression do not significantly change bacterial replication *in vitro*

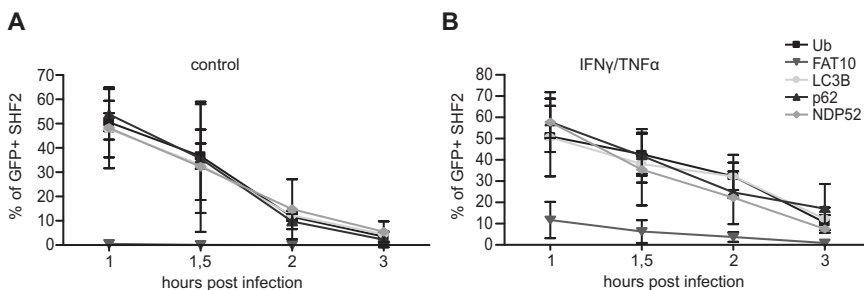
Although the percentage of FAT10-decorated SHF2 is lower than for ubiquitin, we wondered whether it had an impact on intracellular replication of SHF2 *Salmonella*. In HUVECs, we observed FAT10 decoration with the highest percentages. Hence, as a first approach, we knocked down FAT10 in cytokine-treated



**Fig. 3. FAT10 localization correlates with p62-positive but less with NDP52-positive microdomains.** (A) Confocal micrographs of IFN $\gamma$ - and TNF $\alpha$ -induced HUVECs infected with SHF2 *Salmonella* for 1 h, fixed and co-immunostained for NDP52, LC3B, p62 or ubiquitin together with FAT10. (B) Fluorescence line-scan along the white line in the merge picture of corresponding micrographs in A, as recorded with ImageJ software. The 'value' axis shows the relative intensity of fluorescent signal; the distance axis is along white line in merged pictures in A. (C) Quantification of colocalization of immunostained NDP52, p62, LC3B and ubiquitin together with FAT10. The coloc2 plugin of ImageJ software was used to measure the Pearson's correlation above threshold. The box plot shows median, box (25th to 75th percentiles) and whiskers (max and min) for at least 190 FAT10 decorated bacteria analyzed for each condition in at least two independent experiments. \*\*\* $P < 0.001$  (one-way ANOVA); ns, not significant.

HUVECs and quantified bacterial replication by using gentamicin protection assays. We observed rapid replication of SHF2 *Salmonella* in uninduced control cells within 6 h of infection.

In contrast, cells that were stimulated with IFN $\gamma$  and TNF $\alpha$ , which induces FAT10, could control and almost completely prevent bacterial replication, whether they were depleted of

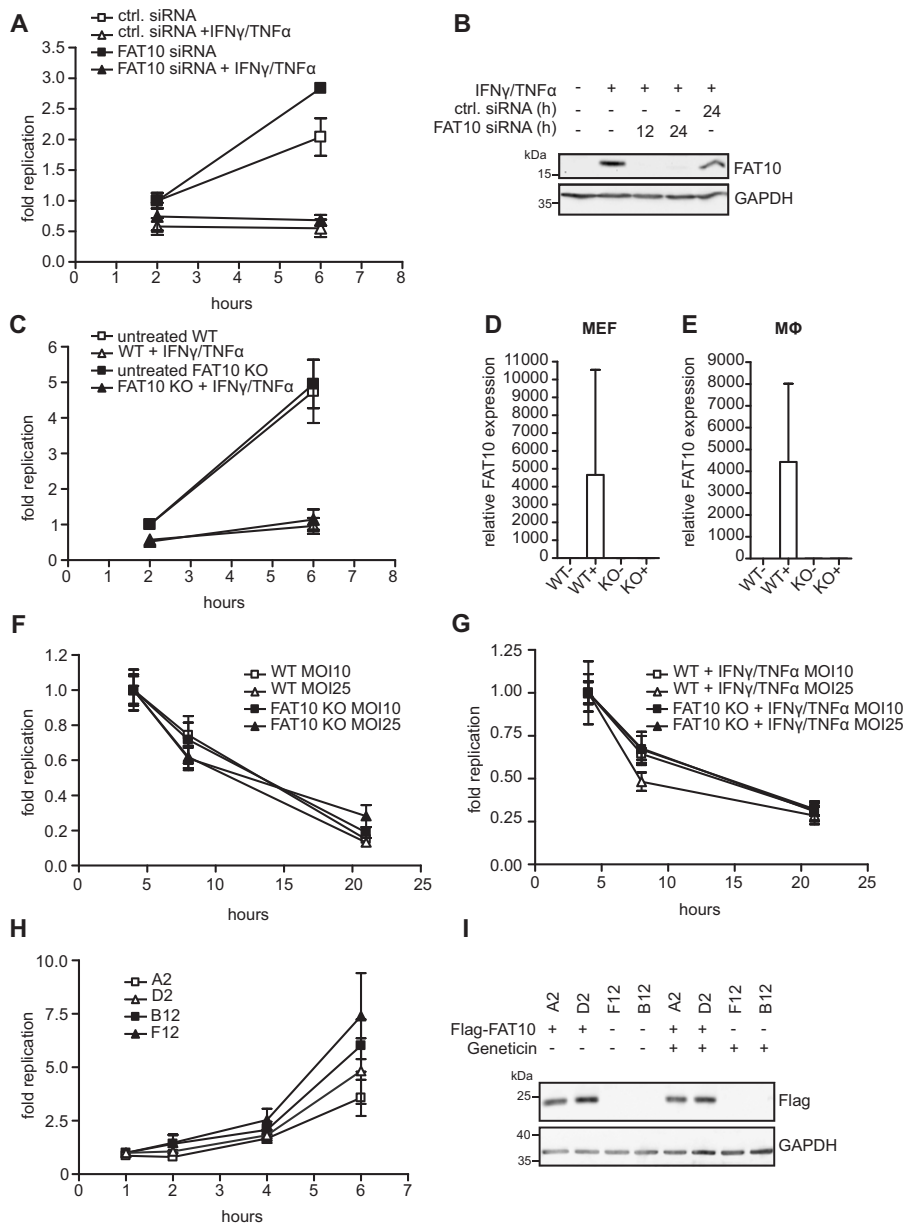


**Fig. 4. FAT10 and ubiquitin decoration of *S. Typhimurium* follow the same kinetics.**

Quantification of SHF2 decoration in (A) untreated and (B) IFN $\gamma$ - and TNF $\alpha$ -induced HUVECs infected with SHF2 *Salmonella* for 1 h. Cells were fixed at the indicated time points and immunostained for NDP52, LC3B, p62, FAT10 or ubiquitin. The percentage of decorated bacteria was enumerated for each immunostaining by fluorescence microscopy. 200 GFP-positive bacteria were counted for each time point. The mean  $\pm$  s.d. is shown for at least two independent experiments.

FAT10 expression or not (Fig. 5A). Fig. 5B shows that IFN $\gamma$  and TNF $\alpha$  mediated FAT10 induction and that the knockdown of FAT10 was successful at protein level. Hence, the potential effect of FAT10 depletion on bacterial replication could be dwarfed by other potent cytokine effects in HUVECs. Next, we turned to mouse embryonic fibroblasts (MEFs) from C57BL/6 wild-type and FAT10 $^{-/-}$  mice. However, we faced the same problem as with HUVECs when performing gentamicin protection assays with MEFs (Fig. 5C) that were stimulated with cytokines to induce FAT10 expression (Fig. 5D). Primary fibroblasts and epithelial cell lines are commonly used for *in vitro* infection assays, although they represent tissues with low physiological relevance. For systemic dissemination, more than 80% of detectable *Salmonella* have been described to be associated with monocytes, macrophages and neutrophils (Tam et al., 2008). Macrophages are considered to be the principal cell type involved in the activation of the immune system by *Salmonella*, which can invade murine macrophages and survive within them (Schwan

et al., 2000). Therefore, we additionally investigated the expression of FAT10 (Fig. 5D) and its potential impact on bacterial replication in peritoneal macrophages (Fig. 5E). In comparison to in MEFs and HUVECs, bacterial replication was rapidly blocked and macrophages effectively reduced the bacterial load over time, an observation also described by Schwan et al. (Schwan et al., 2000). There was only a minor difference between cytokine-primed and untreated peritoneal macrophages, but again no significant difference in bacterial colony forming units (CFU) could be observed between FAT10-deficient and wild-type macrophages (Fig. 5F,G). Finally, we addressed the same question with the help of stable HEK293 cells overexpressing FLAG-tagged FAT10. Because these cells were under geneticin selection pressure and prokaryotes are much more sensitive to this drug than eukaryotic cells, we withdrew geneticin 3 days prior to infection. To have suitable control cells for infections, we generated stable geneticin-resistant HEK293 clones. Gentamicin protection assays to monitor the intracellular



**Fig. 5. Neither FAT10 deficiency nor overexpression significantly changes bacterial replication *in vitro*.** Gentamicin protection assay performed with HUVECs (A), mouse embryonic fibroblasts (MEFs) (C), murine peritoneal macrophages (F,G), and HEK293 cells (H). SHF2-infected cells were incubated with gentamicin and subsequently lysed at the indicated time points. For CFU enumerations, dilutions of lysates were plated on agar plates. The mean  $\pm$  s.e.m. is shown for at least two independent experiments and triplicate colony counts. WT, wild-type; KO, knockout. (B) Immunoblot of total lysate of IFN $\gamma$ - and TNF $\alpha$ -stimulated and untreated HUVECs, treated with FAT10 or control siRNA for the indicated time periods. GAPDH served as a loading control. FAT10 expression in MEFs (D) and peritoneal macrophages (M $\phi$ ) (E) analyzed by quantitative real-time RT-PCR relative to HPRT. (I) Immunoblot of total lysates of HEK293 clones stably expressing FLAG-FAT10 (D2, A2) or control clones (B12, F12). FLAG-FAT10 expression was analyzed after 3 days of culture with or without geneticin selection. GAPDH served as a loading control.



numbers of *S. Typhimurium* with two FLAG–FAT10-expressing and two control clones revealed a tendency for reduced bacterial replication in the FAT10 overexpressing clones A2 and D2 6 h after infection, but the evaluation of three independent experiments did not reveal a statistically significant difference (Fig. 5H). The FLAG–FAT10 expression was monitored by immune blot to confirm persistent FAT10 expression after 3 days without selection pressure (Fig. 5I). In summary, we analyzed primary cells of different origin by either knocking down or knocking out FAT10, as well as in FAT10-overexpressing cells. However, confounded by the bactericidal action of IFN $\gamma$  and TNF $\alpha$  that are needed for the induction of FAT10, we were not able to show a significant effect of FAT10 expression on bacterial replication.

### FAT10 deficiency in NRAMP1<sup>tr</sup> mice revealed a higher susceptibility to *S. Typhimurium*

In humans with typhoid fever or in mouse strains susceptible to *S. Typhimurium* infection, bacteria gain access to extraintestinal tissues, causing severe systemic disease. Our *in vitro* experimental setup could so far not show a functional effect of FAT10 expression on intracellular *Salmonella* replication. To test our hypothesis *in vivo* we generated NRAMP1-transgenic C57BL/6 mice that were wild-type for FAT10 or had a FAT10 knockout, and orally infected them with *Salmonella* SL1344. The extreme susceptibility of inbred C57BL/6 mice to different intracellular pathogens could mask any effect of FAT10 deficiency because infected mice die within a few days. To monitor bacterial load, body weight and survival over a longer period of time we used the more-resistant NRAMP1 transgenic C57BL/6 mouse model. At 4 and 14 days after infection we analyzed the bacterial load in spleen, liver, mesenteric lymph nodes and cecum (Fig. 6A–D). The bacterial burden increased from 4 to 14 days in all tested organs (Fig. 6A–D). After 14 days, we could detect a significantly enhanced bacterial load in mesenteric lymph nodes from FAT10<sup>-/-</sup> as compared to wild-type mice (Fig. 6B). However, *in vivo* infections revealed no influence of FAT10 on bacterial replication of *L. monocytogenes* in liver (supplementary material Fig. S2C). In addition, no difference of viral titers in ovaries following Vaccinia virus infection were detectable comparing wild-type and FAT10<sup>-/-</sup> C57BL/6 mice (supplementary material Fig. S2D).

Furthermore, we performed three independent survival experiments following *S. Typhimurium* infection with NRAMP1 transgenic wild-type and FAT10<sup>-/-</sup> mice. This resulted in a higher rate of death events for FAT10<sup>-/-</sup> mice than for wild-type mice (Fig. 6E). When combining all three experiments comprising 23 NRAMP1-transgenic C57BL/6 mice and 23 NRAMP1-transgenic FAT10<sup>-/-</sup> mice, a lower survival of the FAT10<sup>-/-</sup> mice was recorded (Fig. 6F). Moreover, FAT10<sup>-/-</sup> mice showed a tendency to lose more body weight than their FAT10-proficient wild-type controls (Fig. 6G). Taken together, it appears that FAT10 decorates *Salmonella Typhimurium* in cells and contributes to the protection of mice against this pathogen.

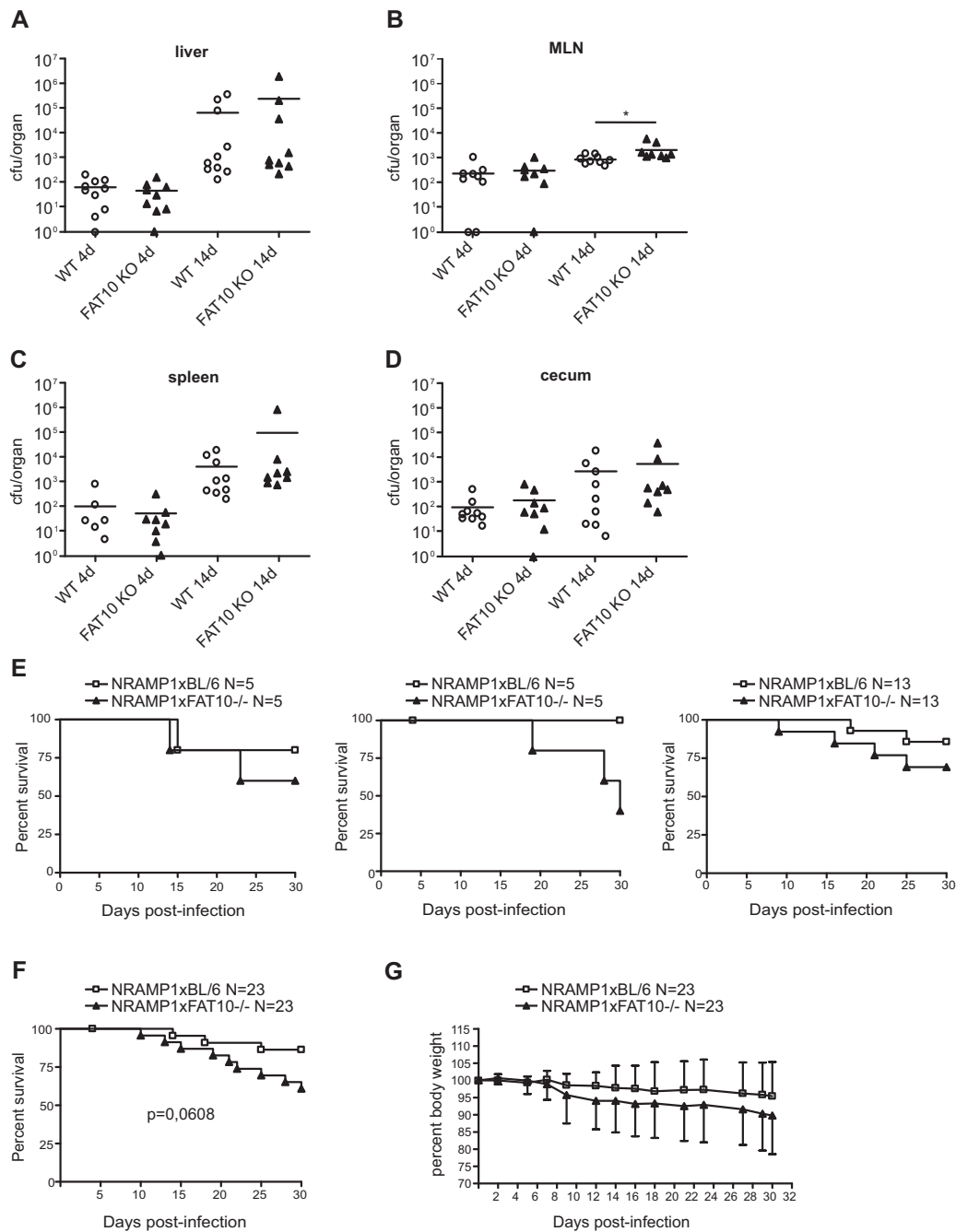
### DISCUSSION

The ubiquitin-like modifier FAT10 is in many aspects similar to ubiquitin. FAT10 is the only ubiquitin-like modifier known to date, which, like polyubiquitin chains, can directly target several substrates for degradation by the 26S proteasome (Schmidtke et al., 2014). If the proteasome is overwhelmed or inhibited, both ubiquitin and FAT10 accumulate in aggresomes. For both

modifiers, this localization is mediated through binding to histone deacetylase (HDAC) 6 which shuttles them through linkage to dynein motors along microtubules to the microtubule-organizing center (Kalveram et al., 2008). Another striking parallel is that both modifiers bind to p62 and colocalize in p62-containing bodies (Aichem et al., 2012). In this study, we add another parallel to the list of common properties and that is that the *Salmonella enterica* serovar Typhimurium is coated by ubiquitin and FAT10 upon release from its vacuole into the cytoplasm of infected cells (Figs 3 and 4).

A major, essentially unanswered, question in FAT10 biology is why two modifiers with such similar characteristics are needed. In particular, the results of this study pose the question as to how the intracellular antibacterial defense might benefit from coating with two ubiquitin family proteins that both bind to p62. To discuss this issue, it is important to appreciate the striking differences between ubiquitin and FAT10, which also exist. Ubiquitin, as its name reveals, is expressed ubiquitously, whereas FAT10 is only expressed in IFN $\gamma$ - and TNF $\alpha$ -stimulated cells and in mature dendritic cells (Bates et al., 1997; Lukasiak et al., 2008). The FAT10 system is therefore selectively switched on in tissues that have been invaded by bacteria and that are under the influence of pro-inflammatory cytokines. Ubiquitin needs to assemble into chains in order to target for proteasomal degradation, whereas FAT10 with its two ubiquitin-like domains serves as a degradation signal with no need for chain formation (Rani et al., 2012). As no FAT10 chains need to be formed, bacteria cannot interfere with FAT10 action by suppressing chain formation as they could with ubiquitin. Likewise, several pathogens encode deubiquitylating enzymes (Jiang and Chen, 2012) that counteract ubiquitylation, whereas FAT10-deconjugating enzymes have not been described to date in spite of considerable efforts to identify such proteins (Hipp et al., 2005). Consequently, ubiquitin is cleaved off and recycled prior to substrate degradation at the proteasome and is therefore long lived, whereas FAT10 is probably degraded along with its substrate and is hence short lived (Hipp et al., 2005). This would render ‘FAT10ylation’ as an irreversible degradation signal, whereas polyubiquitylation is reversible and therefore amenable for intervention by pathogens. Strikingly, a recent comprehensive protein microarray analysis of the conjugation substrates of ubiquitin and the ULMs SUMO1, SUMO2 and SUMO3, NEDD8, UFM1, ISG15 and FAT10 revealed that FAT10 substrates differed the most from ubiquitin substrates when compared to the other five ULMs (Merbl et al., 2013). If FAT10 modifies proteins on the surface of *S. Typhimurium*, it is quite possible that different proteins are modified by ubiquitin than by FAT10. Finally, the ubiquitin system is evolutionarily much older than the FAT10 system, which only exists in mammals (i.e. FAT10 evolved even later than the adaptive immune system). An attractive hypothesis would therefore be that FAT10 has evolved as a defense mechanism against certain pathogens of mammals.

Because we had previously found that FAT10 is localized in aggresomes, which probably can be degraded in autolysosomes, and that FAT10 binds covalently and non-covalently to p62, we hypothesized that FAT10, like ubiquitin, is degraded by autophagy. However, a mCherry–EGFP–FAT10 fusion protein, in spite of binding non-covalently to p62, did not localize to acidifying autolysosomes when the fusion proteins mCherry–EGFP–p62 and mCherry–EGFP–ubiquitin did (Aichem et al., 2012). However, we did find that FAT10 and p62 associated on xenophagy-targeted *S. Typhimurium* (this work). When we



**Fig. 6. NRAMP1 transgenic FAT10-knockout mice are more sensitive to orally inoculated *S. Typhimurium*.** Groups of 8–12-week-old male and female NRAMP1 transgenic mice were orally inoculated with  $1.6 \times 10^8$  CFUs *S. Typhimurium* (SL1344) in 100  $\mu$ l PBS. (A–D) Four and 14 days post-infection organs were harvested and homogenized for colony enumeration. Data represent two independent experiments; with the mean indicated. \* $P < 0.05$  (Student's *t*-test). WT, wild-type NRAMP1 transgenic mice; KO, knockout. (E) Survival plots of three independent and (F) combined experiments with indicated group size. Death events also represent mice with body weight loss of more than 20%. \* $P < 0.05$  by log-rank curve comparison test. Data represents three independent experiments. (G) Bodyweight curve of  $n = 23$  mice during the 30 day survival experiment in E. BL/6, wild-type non-transgenic C57BL/6 mice.

compared the decoration of *S. Typhimurium* in HUVECs by FAT10 in co-stainings with ubiquitin, LC3B, p62, and NDP52 (Fig. 3), it emerged that the overlap was least prominent with NDP52. The staining of *S. Typhimurium* with p62 and NDP52 was not homogeneous but was confined to patches that were segregated. This is consistent with the observation that these two autophagy adaptors are recruited to bacteria independently of each other (Cemma et al., 2011). Given that FAT10 colocalizes in

patches with p62 but not NDP52 it seems that FAT10 is not involved in sensing damaged-bacteria-containing endosomes through the cytosolic lectin galectin-8 because this recruits NDP52 (Thurston et al., 2012). It is noteworthy that almost all FAT10-decorated bacteria were also positive for all four markers, ubiquitin, p62, NDP52 and LC3B (Fig. 2C). Therefore, we propose that FAT10 decoration is indeed associated with the process of xenophagy. However, the ultimate proof for



autophagosomal targeting would be electron microscopy to exclude LC3-associated phagocytosis (LAP). But given that FAT10 targets almost exclusively GFP-positive SHF2 bacteria, and they are simultaneously decorated with p62 and NDP52, we reason that FAT10 most likely decorates autophagy-targeted cytosolic bacteria. Obviously, it would be important to know whether FAT10 binds directly to bacterial structures or to cellular structures surrounding them. We have tried to investigate in HUVECs whether the decoration of *S. Typhimurium* relies on covalent or non-covalent binding to FAT10 by transfecting the cells with wild-type FAT10 or the conjugation deficient  $\Delta$ GG mutant of FAT10 (Raasi et al., 2001). However, although we could successfully transfect these cells for GFP expression, this was not possible with FAT10, most likely because FAT10 overexpression induced apoptosis in HUVECs (our unpublished observation). As an alternative strategy, we tried knocking down the E1 (UBA6) and/or the E2 (USE1) enzymes in charge of FAT10 conjugation in these cells. However, the knockdown lowered monomeric FAT10 protein levels, as observed previously (Rani et al., 2012) (supplementary material Fig. S3A). Initial quantification experiments of FAT10 decoration in these samples resulted in high variances (data not shown), probably because differences in total FAT10 protein levels affect quantification of FAT10 decoration on SHF2 *Salmonella*. Addressing the question as to whether FAT10 decoration on SHF2 *Salmonella* might be dependent on p62 recruitment and therefore on non-covalent interaction, we encountered the same experimental drawback of varying FAT10 levels in p62-knockdown samples (supplementary material Fig. S3B). Therefore, quantification of FAT10 decoration in cells depleted of p62, USE1 or UBA6 cannot provide insight into the mechanism of FAT10 recruitment to SHF2 *Salmonella*. Ultimately, it will be pertinent to recover FAT10-decorated *Salmonella* from cells and determine potential FAT10 linked proteins by mass spectrometry, as we have done with cellular FAT10 conjugation substrates previously (Aichem et al., 2012).

A nagging question concerns the functional redundancy of ubiquitin and FAT10 decoration for the intracellular defense against *S. Typhimurium*. In this respect, it is noteworthy that only ~10% of GFP<sup>+</sup> cytosolic SHF2 bacteria were decorated with FAT10 whereas ~50% of them were decorated with ubiquitin in IFN $\gamma$ - and TNF $\alpha$ -stimulated HUVECs (Fig. 4). This result suggests in itself that the FAT10-targeted structures are not the same as the ubiquitylated ones. It implies that either FAT10 recruitment to *S. Typhimurium* is of a transient nature or, alternatively, that FAT10 localizes only to a fraction of cytosolic bacteria with certain, unknown characteristics. To address whether FAT10 is essential for optimal suppression of *Salmonella* replication, we performed gentamicin protection assays in MEFs and peritoneal macrophages from C57BL/6 wild-type and FAT10<sup>-/-</sup> mice (Canaan et al., 2006) (Fig. 5). In both experimental *in vitro* systems, we had to induce FAT10 with IFN $\gamma$  and TNF $\alpha$  because neither MEFs nor peritoneal macrophages express significant amounts of FAT10. However, when we stimulated both cell types with these two pro-inflammatory cytokines, the growth of *Salmonella* was repressed. We faced the same problem when we used these cytokines to induce FAT10 in HUVECs treated with control or FAT10-specific siRNAs. Finally, we resorted to assessing two stable clones of HEK293 cells strongly overexpressing FLAG-tagged FAT10. In these experiments, we could observe that both FAT10 transfectants had a tendency for lower bacterial replication. Ideally, one would

perform such experiments in the absence of the ubiquitin system to avoid redundancy, but as ubiquitin is absolutely essential for cell survival this is not possible. Nevertheless, such negative data need to be interpreted with caution because a human embryonic kidney cell line is not a typical host cell for *Salmonella* replication. Although HEK293 cells do express UBA6 and USE1, and are permissive for bulk FAT10 conjugation in general (Aichem et al., 2010), we cannot exclude that a putative E3 ligase needed for FAT10 decoration of *S. Typhimurium* is not expressed in HEK293 cells. Therefore, we investigated whether the deficiency of FAT10 would affect *S. Typhimurium in vivo*. Because the FAT10<sup>-/-</sup> mice are on the C57BL/6 background, we had to use this mouse strain for oral infection experiments. C57BL/6 mice are hypersusceptible for *Salmonella* infection and die rapidly after infection because they have gained loss-of-function mutations in the chromosome 1 locus *Bcg/Ity/Lsh*. This locus encodes for the divalent cation transporter *Slc11a1*, formerly known as natural resistance-associated macrophage protein 1 (NRAMP1). It is believed that this protein functions as a pH-dependent divalent cation efflux pump within the phagosomal membrane, thereby restricting the access of pathogens to iron and manganese. Therefore, we bred an NRAMP1 transgene into FAT10<sup>-/-</sup> mice and compared their bacterial load, body weight and survival to that of NRAMP1 transgenic C57BL/6 wild type mice over a period of 30 days. We found that on day 14 of infection FAT10<sup>-/-</sup> mice contained significantly more *S. Typhimurium* in the mesenteric lymph nodes (Fig. 6B). Moreover, fewer NRAMP1 transgenic FAT10<sup>-/-</sup> mice survived the oral infection with these Gram-negative bacteria as compared to NRAMP1 transgenic C57BL/6 wild-type mice in three independent experiments (Fig. 6E). This finding cannot be explained by a higher sensitivity of FAT10<sup>-/-</sup> mice to low doses of lipopolysaccharide (LPS) as reported by Canaan et al. because it is known that *S. Typhimurium* resides intracellularly during infection, preferably in macrophages (Canaan et al., 2006). Furthermore, the late effect of FAT10 deficiency on *Salmonella* infection in mice would be consistent with a role in xenophagy, although it could also indicate a role in adaptive immunity. We conclude that FAT10 decorates *Salmonella* in infected human cells and plays a role in the defense of mice against *S. Typhimurium* that is apparently not completely redundant to that of ubiquitin. These results lend first support to our hypothesized function of FAT10 in the defense against microbial pathogens.

## MATERIALS AND METHODS

### Cells and tissue culture

HeLa cells and HEK293 cells stably expressing FLAG-FAT10 were grown in IMDM glutamax supplemented with 10% FCS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Mouse embryonic fibroblasts (MEFs) were prepared from C57BL/6 mice and FAT10<sup>-/-</sup> mice (Canaan et al., 2006). Mouse primary macrophages, prepared from C57BL/6 mice and FAT10<sup>-/-</sup> mice, were obtained by peritoneal lavage. Both primary mouse cells were cultivated under standard conditions and kept in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Primary human umbilical vein endothelial cells (HUVECs, single donor) (Promocell, Heidelberg, Germany) were kept in antibiotic-free endothelial cell medium (Promocell, Heidelberg, Germany) in tissue culture flasks coated with 0.2% porcine skin gelatin (Sigma, Taufkirchen, Germany).

### Confocal microscopy

Cells were grown on glass coverslips and stimulated with 200 U/ml IFN $\gamma$  and 400 U/ml TNF $\alpha$  for 24 h to induce FAT10 expression. Cells were fixed for 15 min with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 10 min at room temperature and blocked with 0.2%

gelatin. Immunostaining was performed for FAT10 with anti-FAT10 4F1 mouse monoclonal antibody (2 µg/ml) (Aichem et al., 2010), for *Salmonella* with anti-LPS-*Salmonella* rabbit polyclonal antibody (TS 1624, Sifin, Berlin, Germany), for p62 with anti-p62 rabbit monoclonal antibody (D10E10, Cell Signaling Technology), for NDP52 with polyclonal rabbit antibody (Calcoco2, Abcam), for ubiquitin with anti-ubiquitin mouse (FK2, ENZO life science) or rabbit (Invitrogen, Heidelberg, Germany) monoclonal antibody, for LAMP1 with monoclonal mouse LAMP1 antibody (H4A3; DSHB, Iowa, IA) and for LC3B with anti-LC3B polyclonal antibody (PM036, MBL, Woburn, MA). Cells were first labeled with primary antibodies, followed by washing and incubation with the respective Alexa Fluor™-labeled secondary antibodies [F(ab)<sup>2</sup>] (dilution 1:400, Invitrogen). All antibodies were diluted in 0.2% gelatin. All incubations were carried out for 1 h at room temperature. Coverslips were mounted on glass slides with mounting medium (DAPI Fluoromount-G, SouthernBiotech, Birmingham, USA). Images were acquired and analyzed with a LSM 510 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany) using a 63× plan-apochromat, oil-immersion objective (NA=1.4). To determine the Pearson's coefficient a region of interest (ROI) of decorated bacteria was set in images of 0.8 µm thickness and ImageJ software (coloc2 plugin) was used to measure the Pearson's correlation above threshold. Statistical analysis was performed with the GraphPad InStat program.

### Immunoblotting

Cells were lysed in Ripa lysis buffer [50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% NP40, 1× Complete Mini EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany), 10 µM MG 132, 10 mM NEM and 0.5% Na-deoxycholate], incubated on ice for 30 min and centrifuged for 15 min at 20,000 g. Protein quantification was performed using protein quantification reagent (Bio-Rad). Equal amounts of protein extracts were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Endogenous and untagged FAT10 was analyzed by immunoblotting using an anti-human-FAT10 mouse monoclonal antibody 4F1 (Aichem et al., 2010) combined with a secondary polyclonal goat-anti-mouse-IgG antibody conjugated to horseradish peroxidase (HRP) (Dako, Hamburg, Germany). FLAG-tagged FAT10 was detected with a direct HRP-labeled anti-FLAG-M2-HRP antibody (Sigma, Hamburg, Germany). Anti-GAPDH antibody (Sigma, Hamburg, Germany) was used as loading control.

### siRNA knockdown

2×10<sup>5</sup> HUVECs were treated with 200 U/ml IFNγ and 400 U/ml TNFα (both from Peprotech, Hamburg, Germany) for 24 h and then transfected with a pool of four predesigned human-FAT10-specific siRNAs (Hs\_UBD\_1, Hs\_UBD\_2, Hs\_UBD\_3, Hs\_UBD\_5, Qiagen, Hilden, Germany) or with control siRNA (AllStars Negative Control siRNA, Qiagen, Hilden, Germany). Cells were transfected with a total amount of 0.55 µg siRNA (30 nM) using GeneTrans II Transfection Reagent (Mo Bi Tec, Goettingen, Germany) and new cytokines were added after transfection. After 12 and 24 h, cells were either harvested and lysed for immunoblot analysis or infected with *S. Typhimurium* to perform gentamicin-protection assays.

### Quantitative real-time RT-PCR

Total RNA was extracted (RNeasy mini kit, Qiagen) from cells followed by reverse transcription (Promega reverse transcription kit; Promega, Mannheim, Germany). The cDNA was used for PCR amplification with the light cycler instrument (Roche) and a LightCycler Fast Start DNA Master SYBR Green I Kit (Roche) with the following primers: FAT10 mouse (5'-GCTTCTGTCCGACCTGTGTTGT-3' and 5'-TGGGGCT-TGAGGATTTGGAGTCT-3'). The quantitative value of each sample was normalized to hypoxanthine phosphoribosyltransferase (5'-CCAGC-AGGTCAGCAAAGAACTTA-3' and 5'-TGGACAGGACTGAAAGA-CTTG-3') which was used as reference gene.

### Bacterial strains and gentamicin protection assay

For infections we used wild-type *S. Typhimurium* (strain SL1344) and *Salmonella* expressing GFP-UhpT under the control of glucose-6

phosphate (strain SHF2). To generate SHF2 *Salmonella*, SL1344 was transformed with the GFP-UhpT Plasmid (a kind gift from Cecile Arrieumerlou and Christoph Kasper, University of Basel, Switzerland). *S. Typhimurium* (strain SL1344 and SHF2) were grown overnight in LB-LS (low-salt conditions, 5 g/l NaCl) containing 90 µg/ml streptomycin (SL1344 and SHF2) and 100 µg/ml ampicillin (SHF2) and sub-cultured (1:33) in fresh LB-LS for 3 h [to achieve an optical density at 600nm (OD<sub>600</sub>)>1] prior to infection. SHF2 bacteria cultures were washed twice with PBS, and cells in 24-well plates were infected with the respective multiplicity of infection (MOI), centrifuged for 5 min at 1000 g and incubated for 30 min (HEK293, MEFs) or 60 min (HUVECs, peritoneal macrophages) at 37°C. Following two washes with PBS and incubation with 100 µg/ml gentamicin (HEK293, MEFs, peritoneal macrophages) or 25 µg/ml gentamicin (HUVECs) for 30 min cells were cultured in 25 µg/ml gentamicin. To enumerate intracellular bacteria, cells were lysed in 0.5 ml cold PBS containing 1% Triton X-100. Serial dilutions were plated in triplicates on streptomycin LB-LS agar plates. Statistical analysis was performed with the GraphPad InStat program.

### Mice and *in vivo* infections

All mice were purchased from the animal facility of the University of Konstanz. C57BL/6 wild-type (Charles River Laboratories) and FAT10<sup>-/-</sup> mice (Canaan et al., 2006) were intercrossed with C57BL/6 NRAMP1 transgenic mice (Forschungszentrum Borstel) to generate NRAMP1 transgenic C57BL/6 wild type and FAT10<sup>-/-</sup> mice. For survival and colony-forming unit (CFU) enumeration experiments, sex and age-matched mice were fasted for 4 h followed by oral gavage with 1.6×10<sup>8</sup> CFU *S. Typhimurium* (SL1344) in 100 µL PBS. The body weight of all mice was monitored and mice were killed when body weight loss exceeded 20%. For CFU enumeration, organs were harvested, homogenized in PBS using a Polytron PT2100 homogenizer (Kinematica, Luzern, Switzerland), diluted and plated on streptomycin LB-LS agar plates. Statistical analysis was performed with the GraphPad InStat program. All animal experiments were performed according to protocols approved by the Review Board of Regierungspräsidium Freiburg.

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### Competing interests

The authors declare no competing interests.

### Author contributions

V.S. designed, performed and interpreted the experiments and wrote the manuscript, H.F. generated SHF2 bacteria, M.B. performed mouse infection experiments, A.A. performed p62-FAT10 interaction studies, A.C. generated FAT10<sup>-/-</sup> mice, and M.G. supervised the project, designed and interpreted the experiments, and refined the manuscript.

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### Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.152371/-DC1>

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